LUCIANNE LEIGUE DOS SANTOS

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Tese apresentada ao Programa de Pós-graduação em Microbiologia do Instituto de Ciências Biomédicas da Universiade de São Paulo, para obtenção do Título de Doutor em Ciências.

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Orientador: Prof. Dr. Nilton Erbert Lincopan Huenuman

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CERTIFICADO DE ISENÇÃO

Certificamos que o Protocolo CEP-ICB Nº **533/12** referente ao projeto intitulado: "*Mecanismos de resistência a antibacterianos de amplo espectro em Escherichia coli isolada da microbiota fecal de equinos: aspectos clínicos e epidemiológicos*" sob a responsabilidade de **Lucianne Leigue dos Santos,** foi analisado na presente data pela CEUA - COMISSÃO DE ÉTICA NO USO DE ANIMAIS e pela CEPSH- COMISSÃO DE ÉTICA EM PESQUISA COM SERES HUMANOS, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da lei 11.794 de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº196 de 1996.

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PROF. DR. PAOLO M.A ZANOTTO
Coordenador da CEPsh - ICB/USP

I dedicate this thesis…

To my parents, *Iramar* and *Gladdys*, for their unconditional love, example and guidance.

To my little brother, *Ismar*, who has been fighting against so many “resistant bacteria” all his life.

To my beloved husband, *Guilherme*, who has been a constant source of support and love.

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“If you climb in the saddle be ready for the ride”.

Jessica Kinney

ABSTRACT

Leigue dos Santos, L. Characterization of Multidrug-Resistant Gram-negative Bacteria producing Extended-Spectrum β-Lactamase (ESBL) in healthy and infected horses. [Ph.D. Dissertation (Microbiology)] – São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016.

The emergence of multidrug-resistant (MDR) bacteria in animals is a public health issue worldwide. In this regard, although MDR bacteria have been increasingly reported in food-producing animals and pets, few studies have addressed the resistance to antimicrobial agents in bacteria of equine origin. The aim of this study was to characterize MDR bacteria isolated from healthy and infected horses in Brazil and France. From March 2012 to December 2014, 102 different samples collected from healthy (*n*= 96) and infected horses (*n*= 6), in Brazil, were screened for the presence of MDR bacteria. French isolates (*n*= 45) were restricted to *E. coli* strains recovered from clinical samples collected between 2014 and 2015. Antimicrobial resistance profiles were investigated by Kirby-Bauer with further MIC determination by agar dilution, E-test or VITEK® method. Presence of extended-spectrum beta-lactamases (ESBLs), plasmid-mediated AmpC (pAmpC), quinolone resistance (PMQR), or/and aminoglycoside resistance determinants were investigated by PCR and sequencing. *E. coli* phylogroups were characterized by PCR and clonality and ancestrality were evaluated by ERIC-PCR, PFGE or/and MLST analysis. For $bla_{CTX\text{-}M}$-type genes, the genetic environment was studied and plasmid location was assessed by southern hybridization with plasmid sizes determined by S1 digestion, followed by PFGE. In Brazilian horses, the analysis of fecal samples from healthy animals revealed the presence of clonally unrelated A, D or B2 phylogroups of *E. coli* strains carrying $bla_{CTX\text{-}M\text{-}1}$ (*n*= 1), $bla_{CMY\text{-}2}$ (*n*= 4), *qnr*- and/or aminoglycoside adenyl transferase (*aad*)-type genes, and Inc HI1, I1, P, F_{REP}, FIA or FIB plasmid types; whereas in the infected horses, nine MDR isolates (3 *E. coli*, 1 *Proteus mirabilis*, 1 *Klebsiella pneumoniae*, 3 *Pseudomonas aeruginosa* and 1 *Serratia marcescens*) carrying $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}1}$, *rmtD* 16S rRNA methylase, *qnr*-type, *aac(6´)-Ib-cr* and *aad*-type genes were identified. In this regard, $bla_{CTX\text{-}M}$-type genes were carried by >90-kb IncFIA, HI2 and L/M plasmids, where $bla_{CTX\text{-}M\text{-}1}$ genes were associated with differents arrays of IS*Ecp1* and/or IS*26*. Finally, In French infected horses, most MDR *E. coli* isolates were positive for CTX-M-1-, followed by CTX-M-2- and CTX-M-9-type ESBLs. In summary, we report the presence of MDR Gram-negative bacteria carrying clinically important genes encoding resistance to broad-spectrum beta-lactam, fluoroquinolone and aminoglycoside antibiotics in horses, highlighting the presence of CTX-M-type producing bacteria, even in countries from different continents, underlying the importance of horses as a new reservoir of MDR bacteria.

Keywords: Equine. CTX-M. RmtD. ESBL. Fluoroquinolones. Aminoglycosides. Antibiotic resistance.

RESUMO

Leigue dos Santos, L. Caracterização de Bactérias Gram-Negativas Multirresistentes produtoras de β-Lactamase-de-espectro-extendido (ESBL) em cavalos saudáveis e doentes. [Tese de Doutorado (Microbiologia)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016.

O aparecimento de bactérias multirresistentes (MR) em animais é uma questão de saúde pública global. Embora as bactérias MR sejam cada vez mais relatadas em animais produtores de alimentos e animais de estimação, poucos estudos têm abordado a resistência aos agentes antimicrobianos em bactérias de origem equina. O objetivo deste estudo foi caracterizar bactérias MDR isoladas de cavalos saudáveis e infectados no Brasil e na França. De março de 2012 a dezembro de 2014, 102 amostras foram coletadas de cavalos saudáveis (*n*=96, fezes) e doentes (*n*=6, amostras clínicas), no Brasil. Entre as amostras francesas (*n*=45), os isolados foram restritos a *E. coli* e recuperados a partir de amostras clínicas coletadas entre 2014 e 2015. O perfil de resistência antimicrobiana foi investigado por Kirby –Bauer e a determinação da CIM por diluição em ágar, E- test ou método VITEK®. A presença de espectro estendido beta-lactamases (ESBL), AmpC plasmidial (pAmpC), resistência às quinolonas mediada por plasmídeos (PMQR), e resistência aos aminoglicosídeos foram investigados por PCR e sequenciados. Filogroupos de *E. coli* foram caracterizados por PCR e a clonalidade e ancestralidade foram avaliadas por ERIC-PCR , PFGE e/ou análise MLST. Para os genes $bla_{\text{CTX-M-1}}$, o ambiente genético foi estudado e determinado por PCR; e a localização do plasmídeo e o tamanho do plasmídeo carreador, foram determinados por digestão com enzima S1, seguido por PFGE e hibridaçao. As amostras fecais de animais saudáveis, revelaram a preseça de *E.coli* clonalmente não relacionadas pertencentes ao filogrupos A, D ou B2 careadoras de genes $bla_{\text{CTX-M-1}}$ (*n*= 1), $bla_{\text{CMY-2}}$ (*n*= 4), *qnr*- e aminoglicosideo adenil transferase (*aad*)-tipo, e plasmideos do tipo Inc HI1, I1, P, F_{REP}, FIA ou FIB; enquanto nas amostras clínicas, nove isolados MDR (3 *E. coli*, 1 *Proteus mirabilis*, 1 *Klebsiella pneumoniae*, 3 *Pseudomonas aeruginosa* e 1 *Serratia marcescens*) carreadoras de genes $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-1}}$, *rmtD* 16S rRNA metilase, *qnr*-type, *aac(6´)-Ib-cr* e *aad*-tipo foram identificados. As amostras carreadoras grupo $bla_{\text{CTX-M-1}}$ tinham genes de tamanhos >90-kb e eram do tipo IncFIA, HI2 ou L/M plasmids, nos quais os genes $bla_{\text{CTX-M-1}}$ estavam associados com sequências de inserção (IS) do tipo IS*Ecp1* e/ou IS*26*. Finalmente, dentre as amostras francesas, a maioria dos isolados de *E. coli* MDR foi positivo para CTX-M-1grupo, seguido por CTX-M-2 e CTX-M-9. Assim, este trabalho relata a presença de bactérias Gram-negativas MDR carreadoras de genes clinicamente importantes que codificam resistência aos antibioticos beta-lactâmicos de amplo espectro, fluoroquinolonas e aminoglicosídeos oriundas de cavalos. Destaca-se também a presença de bactérias produtoras do gene CTX-M, mesmo em países de diferentes continentes, este trabalho aponta a importância de cavalos como um novo reservatório de bactérias MR.

Palavra-chaves: Equino. CTX-M. RmtD. ESBL. Fluorquinolonas. Aminoglicosídeos. Resistência, Antibiótco.

LIST OF ABBREVIATIONS

AGs - aminoglycosides
AST - antibiotic susceptibility testing
ATB - antibiotic
ATM - antimicrobial
BLAST basic local alignment tool
Bp – base pairs
CLSI - clinical laboratory standards institute
CMY - cephamycins
CTX-M - cefotaxime hydrolyzing capabilities
DDST - Double Disc Synergy Test
DNA - deoxyribonucleic acid
EDTA - Ethylenediamine tetraacetic acid
FQs – fluoroquinolones
GNB – Gram negative bacteria
EUCAST - European Committee on Antimicrobial Susceptibility Testing
ESBL extended-spectrum β-lactamase
ExPEC - extraintestinal pathogenic *E. coli*
IM – intramuscular
IS - insertion sequence
IV – intravenous
MALDI-TOF Matrix-assisted Laser Desorption/Ionization Time of Flight
MDR - Multidrug resistant
MIC – Minimum inhibitory concentration
MLST - multi locus sequence typing
ORF - Open reading frame
PBP - penicillin binding protein
PBRT - polymerase chain reaction-based replicon typing
PFGE - Pulsed Field Gel Electrophoresis
PCR - polymerase chain reaction
PMQR - Plasmid-mediated quinolone resistance
SHV - sulfhydryl variable
ST - sequence type
TEM - Temoneira
Tn – transposon
UK – United Kingdom
USA – United States of America
UTI – Urinary tract infection
VO – oral administration

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1 INTRODUCTION

The overuse and misuse of antibiotics in human medicine, as well as in farm animals or even in other agribusiness activities have resulted in the selection of pathogenic bacteria exhibiting multidrug-resistant profile. Multidrug resistance in bacteria may be generated by different mechanisms, such as the accumulation of genes coding for resistance to multiple drugs, which can be mobilized on resistance plasmids, or by genes that code for the overexpression of multidrug efflux pumps, which extrude a wide range of antimicrobial agents (Haenni et al., 2016, Trott, 2013).

The multidrug resistance (MDR) in clinically important bacteria has been defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magioarakos et al., 2011).

In the last years, the production of extended-spectrum beta-lactamase (ESBL) among Gram-negative bacteria has been pointed out as being a worldwide public health issue. In veterinary medicine, most likely the long-term use or misuse of β-lactam drugs has contributed to the selection of ESBL-producing Enterobacteriaceae among commensal microbiota (ACSQH, 2014). In particular, ESBL and pAmpC-producing Enterobacteriaceae bacteria have been recently observed in companion animals, livestock, wildlife and zoo animals (Aizawa et al., 2014; Bae et al., 2013, Carvalho et al., 2016). Different types of ESBL variants such as CTX-M-, SHV-, TEM- and OXA-type have been reported in Gram-negative bacteria isolated from both healthy and diseased animals (Ahmed et al., 2010; Dolejska et al., 2010).

In this study, we investigated the presence of MDR bacteria, with emphasis on the production of ESBL/pAmpC, in healthy or diseaded horses in Brazil and France, in order to contribute with epidemiological information about this important topic. The results can become a support for the development of therapeutic schedules directed to the treatment of related infections in Veterinary Medicine. On the other hand, the identification of new reservoir of ESBL/pAmpC-producing bacteria is essential for the management and epidemiological control of zoonotic MDR bacteria.

2 REVIEW OF LITERATURE

2.1 Antimicrobial agents used in the equine medicine

Several antibiotics can be used in both veterinary and human medicine. Some examples are: cephalosporins, penicillins, aminoglycosides, quinolones, tetracyclines, phenicols, lincosamides, macrolides, nitrofuranes, sulfonamides (plus trimethoprim), polymyxins and nitroimidazole antibiotics (Prescott, 2000). In this regard, antibiotics are used in pets (cats, dogs and uncommon pets such as rabbits, ferrets, reptiles and birds), farm/zoo animals (cattle, swine, poultry, and sheep) and also aquaculture. Horses, in particular, can be considered both, companion animals or farm-food animals. For food-farm animals and aquaculture, antibiotics are used for therapy or prophylaxis, and, in some cases, as growth promoters and in feed efficiency. Companion animals can receive different kinds of antibiotics; even under serious resistant infection, they can receive the so-called “last-resort” antibiotics, which are forbidden for food animals (ACSQH, 2014).

In horses, a restricted number of antibiotics are approved for clinical use. Antibiotic therapy in these animals has always been challenging because many drugs are not practical to administer to horses. In fact, oral drugs are a problem, considering that horses can be extremely sensitive and they may not absorb some oral medications or, even worse, they can suffer enteritis as an adverse reaction (Papich, 2001). On the other hand, only a limited number of injectable antibiotic compounds are available (Damborg et al., 2012). Another point is the high cost: antibiotics are expensive and, considering the fact that the dosage is usually the same for humans (by body weight) and also considering that horses weigh 10 times more then people, the antibiotic will cost 10 times more. Probably this is the reason why penicillin, cephalosporins and gentamicin are the most commonly used drug in equine practice.

Choosing the appropriate drugs depends on many important factors being considered, such as the site of infection, appropriate via of administration of antibiotics, host condition (age, health status, pregnancy), the length of treatment

required, geographic location, veterinary experience, type of facility where the horses live and, finally, potential risks related to the antibiotic administration. As reported by Baggot (1987), the success of the antimicrobial therapy depends on administration of an agent to which the bacteria are susceptible at the concentrations reached at the site of infection. The route of administration, size of the dose and dosing interval must be appropriate for the drug preparation selected.

Veterinarians usually treat horses empirically with antibiotics, since in most cases sample collection is not viable in order to get a proper diagnosis from culture and antibiogram results. The empiric therapy is based on prior experience with similar infections and, in horses, usually consists of systemic broad-spectrum antibiotics, mainly cephalosporins, fluoroquinolones and/or aminoglycosides (Papich, 2001).

In studies conducted in the UK, the penicillins are the most common class used in horses, followed by cephalosporins, aminoglycosides, trimethoprim-sulphonamides, and oxytetracyclines (Anon 2010; Heuer et al., 2005). In the USA, commonly used systemic antibiotics in equine veterinary practice include trimethoprim/sulfamethoxazole, procaine penicillin, gentamicin and ceftiofur. Less commonly used antibiotics include metronidazole, enrofloxacin, amikacin and chloramphenicol (Ta, 2015). In Brazil, aminoglycosides, fluoroquinolones or cephalosporins are frequently administered to horses (Dra. Carolina Dunin, personal communication, April, 2016).

Unlike in veterinary medicine, several studies in human medicine have evaluated antimicrobial prescription practices, whereas in animals there are still limited data available. On the other hand, whereas for human antibiotic resistance patterns are often published and updated frequently in the humam medicine literature by local health departments or hospitals, in veterinary medicine this information has been mostly neglected (Papich, 2001). Due to that, to achieve the principles of responsible use of antibiotics in animals and to achieve a veterinary antimicrobial decision, clinical sample is collected and laboratory analysis is necessary for the identification and antimicrobial susceptibility profile of the bacterial agent.

According to Hughes et al., (2013), prescription of antimicrobials at improper doses is common practice even when provided the weight of the horse.

Antimicrobials are often misused and overused, or administered by owners without consulting a veterinarian. It is essential that horses with serious infections receive adequate treatment to prevent a chronic or life-threatening condition (Papich, 2001). It is unusual for equine veterinary practices to consult frequently guidelines for antimicrobial use. Therefore, the introduction of such guidelines could lead to more prudent use of antimicrobials (Hughes et al., 2013) and it will also help the rational use of these drugs, since it is essential to avoid the increase of bacterial resistance.

2.2 Beta-lactam antibiotics

Beta-lactams represent one of the most important antibiotics used in both humans and animals and are amongst the most commonly prescribed drugs worldwide. All drugs in this group share a common structural feature, the beta-lactam ring (Calderwood et al., 2012). The mode of action is by inhibiting the last phase of bacterial cell wall synthesis (Marin, Gudiol, 2003) more specifically, inhibiting the growth of bacteria by enzymes, which can inactivate the bacterial cell wall synthesis (Calderwood et al., 2012). These drugs target the penicillin-binding proteins (the “PBPs”) which are protein anchored in the cell membrane and involved in the cross-linking of the bacteria cell wall process. The beta-lactam ring structure is essential for their antibacterial activity, because it binds the PBPs and consequently interferes with the biosynthesis of bacterial cell wall causing death and lysis of bacteria (Tomasz, 1979).

The beta-lactams are classified as penicillins (penems), cephalosporins (cephens), cephamycins (cephens), carbapenems, monobactams and beta-lactamase inhibitors. A diversity of beta-lactams is currently licensed for use in animals and these are commonly prescribed in veterinary medicine due to their extensive margin of safety, good pharmacokinetic properties and broad spectrum of activity against most animal pathogens (Trott, 2013). Ampicillin, amoxicillin, benzylpenicllin, cloxacillin, hetacillin, nafcillin, and penethamate hydroiodide are

examples of penicillins used in veterinary medicine, and they are normally useful for treating gram-positive infections; whereas cefadroxil, cefapirin cephalexin (first generation), cefovecin, cefpodoxime, ceftiofur (third generation) and cefquinome (fourth generation) are cephalosporins used in companion animals or food-animals and the spectrum of the specific cephalosporin depends on the drug. The first-generation drugs are effective mainly against gram-positive organisms while higher generations normally have wide spectrum, mainly against aerobic gram-negative bacilli (Li et al., 2007).

2.2.1 Cephalosporins used in Veterinary Medicine

The range of cephalosporins available for use in food-producing animals and companion animals is limited when compared to humans. This group of drugs can be given either orally or by injection, depending on the target species. Normally, cattle, horses and pigs are treated by daily injections (NOAH, 2016).

Cephalexine and cefadroxil (first generation) are important cephalosporins used in humans and companion animals, the third-generation cephalosporin, ceftiofur, and cefovecin; and the fourth-generation cephalosporin, cefquinome, have been developed strictly for veterinary use (Hornish, Kotarski, 2002). Cefpodoxime (with veterinary formulation), ceftriaxone and ceftazidime are 3rd generation used in veterinary practice to cover serious infections (Viana, 2007). Cephalosporins are licensed for use in dogs, cats, horses, cattle and pigs whereas the fourth generation cephalosporin (cefquinome) specifically are only licensed in cattle, horse and pigs. Normally, cephalosporins are used to treat individual animals and not as cover therapy for large groups (NOAH, 2016).

Cefadroxil is a first-generation cephalosporin used in dogs and cats, indicated for treatment of susceptible strains of *Escherichia coli, Proteus mirabilis* and *Staphylococcus aureus* isolated from genitourinary tract infections or soft tissue infections (AAVPT$_a$, 2007). In the USA, Canada and Brazil, this drug is presented as oral suspension and some commercial names are: Cefa-Drop®, Duricef®, Cefa-cure®, and Cefa-drops®. It is not recommended to use in adult horses (AAVPT$_a$, 2007; Viana, 2007) but can be used in foals (Giguere et al.,

2010). Cephalexin is another oral first-generation cephalosporin available as veterinary compound (Desflexv, Lexin®, and Rilexine®) and indicated for therapy of gram-positives infections in companion animals, reptiles and swine, but not recommended for use in horses (Viana, 2007).

Cefovecin sodium (Convenia®, Zoetis$_b$, USA) is an injectable third-generation cephalosporin indicated for the treatment of skin infections associated with *Staphylococcus pseudintermedius* and *Streptococcus canis* (Group G) in dogs and *Pasteurella multocida* in cats (Six et al., 2008; Stegman et al., 2006). Like other β-lactam antimicrobials, cefovecin exerts its inhibitory effect by interfering with bacterial cell wall synthesis. This interference is primarily due to its covalent binding to the penicillin-binding proteins (PBPs). For *E. coli*, the in vitro activity of cefovecin is comparable to other cephalosporins, but due to the high-afinity protein- binding, the in vivo free concentration of cefovecin does not reach the MIC90 for *E. coli* and *Pseudomonas aeruginosa* and enterococci (Zoetis$_b$, 2013). This drug has an interesting characteristic, as it is formulated for a single subcutaneous dose administration and has an extended half-life persisting for 14-day dosing intervals in companion animals (Six et al., 2008). In monkeys (Bakker et al., 2011) and alpacas (Cox et al., 2015) the use of this drug has already been described, but in horses there is still no data available.

Two important third-generation cephalosporins that are described with veterinary formulation are ceftiofur and cefpodoxime. For horses and other farm animals, the main injectable third-generation cephalosporin indicated is ceftiofur (commercial names: Excede® for horses, Zoetis$_c$, USA; Excenel® for cows, Zoetis$_c$, USA; Naxcel® for swine, Zoetis$_c$, USA; and Bioxell®, Valéé, Brazil) (AAVP, 2007; Viana, 2007). Ceftiofur can be administered as either ceftiofur sodium (NAXCEL® Sterile Powder) or ceftiofur crystalline free acid (EXCEDE® Sterile Suspension) (Zoetis$_c$, 2009) and is the main product used in veterinary medicine for a variety of mixed infections. It is often combined with an aminoglycoside when greatest gram-negative coverage is needed. In horses it is indicated mainly for treatment of lower respiratory tract infections associated with *Streptococcus equi* subsp. *zooepidemicus* but it has a good spectrum, and this cephalosporin can be used in other equine infections (AAVPT$_a$, 2007; Viana,

2007; Zoetis$_c$, 2009). Nevertheless, ceftiofur is not active against *Pseudomonas* spp. and enterococci (Zoetis$_c$, 2009).

Ceftiofur may be used to provide coverage against nearly 80% of isolates recovered from blood cultures from equine neonates (Guigere et al., 2010) and in foals with bacteria infections. According to recent studies (Hall et al., 2011; Meyer et al., 2009), no adverse effects attributed to this drug administration were observed. Ceftiofur can reach sufficient plasma concentrations to effectively treat common bacterial pathogens isolated from septicemic foals.

Cefpodoxime (Simplicef®, Pfizer Animal Health, USA or the generic Putney Cefpodoxime Proxetil Tablets®, Putney, Austria) is another 3rd-generation cephalosporin (oral administration) indicated initially for treating skin infections (wounds and abscesses) in dogs caused by susceptible strains of *Staphylococcus intermedius, S. aureus, Streptococcus canis* (group G, ß hemolytic), *Escherichia coli, Pasteurella multocida,* and *Proteus mirabilis* (AAVP, 2007; Putney, 2012). Similarly to other 3rd-generetion cephalosporins, it is ineffective against *Pseudomonas* spp., *Enterococcus* spp. and *Rhodococcus equi*. Although the safety and efficacy have not been well established in horses, based on the results of one study, oral administration at a dosage of 10 mg/kg every 6 to 12 hours would appear appropriate for the treatment of equine neonates with bacterial infections (Carrilo et al., 2005). However, adult horses can develop adverse signs of colic (Magdesia, 2015).

Finally, the last cephalosporin used for veterinary purposes is Cefquinome (Cobactan®, MSD Animal Healthy, Netherlands; Virbactan, Virbac, Germany). This drug is a fourth-generation cephalosporins, initially described to be used only in cattle by parenteral route, for individual treatment or treatment of bovine respiratory disease (BRD) (FDA, 2014). Since 2003, the European Committee for Veterinary Medicinal Products recommended the inclusion of cequinome in Equidae treatment protocols (EMEA, 2003). In the UK, this drug is licensed for use in foals with septicemia and adult horses with respiratory tract infection (Magdesia, 2015). Cefquinome has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria including *Pseudomonas aeruginosa*

and activity against Enterobacteriaceae producing AmpC-type beta-lactamase (FDA, 2014).

2.3 Aminoglycosides

Aminoglycosides (AGs) are bactericidal (dose dependent) drugs. The mechanism of action is by binding the highly conserved A-site of the 16S rRNA of the bacterium (30S ribosomal subunits) and by disrupting the protein resulting in bacterial death (Doi et al., 2004; EMA/ESVAC, 2014; Garneau-Tsodikova, Labby, 2015).

Aminoglycosides have broad-spectrum and are used to treat aerobic gram-negative infections (*Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae*, others enteobacteria, and *Pseudomonas aeruginosa),* some *Mycoplasma* spp, *Staphylococcus* spp and as second-line of defense treatment for multidrug-resistant tuberculosis (*Mycobacterium tuberculosis*) (Garneau-Tsodikova, Labby, 2016; Giguere et al., 2010). They are not suggested for anaerobes or *Streptococcus* infections (AAVPT$_b$, 2007; Wilson, 2001).

Aminoglycosides are extensively used in veterinary medicine (EMA/ESVAC, 2014), where they are frequently associated with other antibiotics to treat serious infections. The most common AGs used in veterinary medicine are: Amikacin; Dihydrostreptomycin; Gentamicin; Neomycin; Kanamycin, Streptomycin and the aminocyclitol, Apramycin (with a chemical structure very similar to that of aminoglycosides) (O'Neil, 2012).

Apramycin is an exclusive veterinary drug, which is used in the treatment of colibacillosis and salmonellosis in calves, pigs, lambs, rabbits and poultry. It is usually administered via drinking water or food or via parenteral (IV or IM) (EMA, 2009; Viana, 2007). The therapeutic spectrum of Apramycin includes: *Bordetella bronchiseptica, Brucella* spp., *Escherichia coli, Salmonella* spp., *Pasteurella multocida, Pseudomonas* spp., *Klebsiella aerogenes* as well as some Gram-positive microorganisms such as *Staphylococcus* spp. and α-haemolytic Streptococci (EMA, 2009).

In Equine Medicine, gentamicin sulphate is the most commonly AG administered to mature equine patients, followed by amikacin, streptomycin,

tobramycin, or neomycin (Briyene, 2014; Pinto et la., 2010). In general, AGs are not well absorbed orally, thus administration by parenteral infusions (intramuscular, intravenous, intra-articular or uterine) is recommended (AVVPT$_b$, 2007).

Kanamycin and Streptomycin have been reported as commonly resistant and considered much less useful than gentamicin and amikacin (Wilson, 2001). Also, the use of neomycin is limited, since there is relatively a high risk of toxicity with systemic use and it is not available for parenteral administration. Neomycin sulfate (oral solution and powder for oral solution) is indicated in the control and treatment of bacterial enteritis caused by susceptible *Escherichia coli* (AAVPT$_b$, 2007).

Amikacin and gentamicin are highly recommended for the treatment of intrauterine infections (endometritis, metritis and pyometra) in mares, neonatal infections (AAVPT$_b$, 2007; Wilson, 2001), ocular infections (Moore, Naisse, 1999) or intra-articular infections (Sanchez et al., 2012). Amikacin is recommended as first choice when the infection is caused by *Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella* sp*.,* and *E. coli* (Wilson, 2001).

In cases of peritonitis, acute pleuropneumonia, arthritis, cellulitis and urinary infection, AGs are frequently combined with beta-lactams to provide a broad spectrum coverage. It is suggested to use amikacin or gentamicin associated with penicillin or ampicillin or cefazolin (Wilson, 2001).

Some concerns are applied to aminoglycosides because of their relatively narrow margin of safety limits the amount of drug that can be administered (Bagott, 1991) and some adverse effects such as nephrotoxicity, neuromuscular blockade and ototoxicity are reported (Magdesia, 2015; Wilson, 2001). Neomycin is the most nephrotoxic, followed by, in this order, gentamicin, kanamycin and amikacin, streptomycin and finally tobramycin (Wilson, 2001), especially when AG is administered orally (Papich, 2001). For a more efficient therapy, which is also more economical and less nephrotoxic, it has been suggested to use a high dosage once a day (Pinto et al., 2010).

2. 4 Quinolones/Fluoroquinolones

The quinolone class of antimicrobial (ATMs) agents has generated considerable interest since their discovery about 50 years ago. Different from other common ATMs, this class of antimicrobial agents was not originally isolated from living organisms but, rather, was synthesized in laboratory (Adriole, 2005). Most, quinolones are broad-spectrum synthetic antibiotics first obtained from chloroquine and represent a group of ATMs which is the most important in the treatment of severe and invasive infections in animal and humans, representing a special interest in public health (EMEA, 2009).

The first prototype of quinolones was the nalidixic acid which was initially used mainly for urinary tract infections caused by *E. coli* and other pathogenic Gram-negative bacteria (Rice, 2012), but now it is rarely used (Wilson, 2001). Usually, the variation at the carbon positions (C-6 and C-8) gives more effective drugs, and produce wahts is called fluoroquinolones (FQs) (Naeem et al., 2016; Rice, 2012). So, the fluoroquinolones are known as the modern bactericidal group of synthetic antimicrobials, active against a variety of clinically important organisms (AAVPT$_c$, 2007).

The FQ mechanism of action is by binding sites in the DNA gyrase and topoisomerase IV (DNA gyrase-DNA complex) and then interrupting the supercoiling of the bacterial chromosome. This disruption leads to defects in the necessary supercoiling, and render the bacteria unable to multiply and survive (AAVPT$_c$, 2007, Rice, 2012). FQ have an exclusive mechanism of action, not related to usual classes of ATB; in this manner, their efficacy should be preserved as long as possible (EMEA, 2009).

These drugs are classified according to their spectrum of activity into four generations. The first and second generation have a limited spectrum and are being used mainly for gram-negative bacteria, with the exception of some *Staphylococcus* spp. strains (AAVPTc, 2007; Viana, 2007). Most strains of *Rhodococcus equi* and anaerobic bacteria are resistant to these drugs (Wilson et al., 2001). Among these compounds, ciprofloxacin and ofloxacin are the most widely used today, with ciprofloxacin being one of the most active against *Pseudomonas aeruginosa* (Van-Bambeke et al., 2005). The third and fourth

generation have an expanded spectrum of activity against gram-positive bacteria, anaerobic bacteria, mycobacteria, and species of *Chlamydia*, *Chlamydophila, Rickettsia* sp., *Erlichia* sp., *Mycoplasma*, and *Ureaplasma* (Kowalski et al., 2003; Ledbetter et al., 2007; Moore et al., 1995; Wilson, 2001). The new fluoroquinolones are rarely first-line agents and should be employed prudently. Incorrect use of these agents will likely worsen current problems with antibiotic resistance (Oliphant, Green, 2002).

FQs have high bioavailability, an excellent lipid solubility, a relative low toxicity, few adverse effects favorable pharmacokinetics (Naeem et al., 2016), and are also well distributed into most tissues, including low levels in the central nervous system and eye (AAVPTc, 2007). For humans, most quinolones have excellent oral bioavailability, with serum drug concentrations equivalent to intravenous administration (Oliphant, Green, 2002). Oral absorption of fluoroquinolones is high for most animal species studied and the FQs are well tolerated by animals, and usually can be administered by a variety of routes (VO, IM, IV) (AAVPTc, 2007).

Some examples of FQs currently available and widely used in Europe in humans are norfoxacin, ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin (Van-Bambeke et al., 2005) while the 2nd – generation FQ: Danofloxacin (Advocin®, A180®), Difloxacin (Dicural®), Enrofloxacin (Baytril®, Duotril®, Kinolox®), Marbofloxacin (Zenequim®) and Orbifloxacin (Orbax®) are used strictly at the veterinary practice (Viana, 2007). Ibafloxacin and Pradofloxacin are two veterinary fluoroquinolones available in Europe and are sold as oral compounds for use in dog and cat infections (Walker, Dowling, 2010).

Even though Ciprofloxacin is a widespread prescription medication used in veterinary practice, it is not an FDA-approved drug for veterinary use in the USA (AAVPTc, 2007) and it is used in an extra-label manner (Walker, Dowling, 2010). Ciprofloxacin is administered to treat cats and dogs for lower respiratory tract infections, skin infections and urinary tract infections (Viana, 2007) and should not be used in horses (Wilson, 2001). The bioavailability of orally administered ciprofloxacin is very low, and numerous adverse effects like colitis

(mild to severe), endotoxemia and laminitis occurred following oral or IV administration (Cole, 2014).

FQ labeled for use in animals have the same quinolone structure, each with variations that account for pharmacokinetic differences in the medications but do not significantly change the antibacterial spectrum of activity (AAVPTc, 2007). Most part of these FQ is approved for use in small animals and cattle in the USA and is sporadically used in an extra-label way to treat diseases in horses by IV or oral route (Wilson, 2001).

In general, fluoroquinolone drugs are not the first option suggested for treatment of infections in horses, the reason being the supposed adverse reactions and they may cause serious gastrointestinal disturbances or tissue irritation in cartilages, or the price of therapy may become extreme (Kaartinen et al., 1997).

Enrofloxacin and marbofloxacin are two veterinary fluoroquinolones used to treat severe bacterial infections in horses even though no specific FQ product for equine is available (Peyrou et al., 2006). Therefore, they remain only as extra-label drugs for specific indications in valuable individuals (Kaartinen et al., 1997).

According to the American Academy of Veterinary Pharmacology and Therapeutics ($AAVPT_c$, 2007), Enrofloxacin (Baytril®) was the primary member of this class used in horses. It should be prescribed only after culture confirmation for gram-negative infections. As a routine first choice antibiotic, it is not appropriate as a single agent due to its lack of anaerobic bacteria coverage and may be variable against *Streptococcus* infections.

Arthropathies such as articular cartilage damage and tendon abnormalities in neonates and foals from administration of enrofloxacin are some of side effects described ($AAVPT_c$, 2007; Viana, 2007), so this drug should not be administered to young growing horses (animals less than 3 years of age), except in cases as a last resort for severe infections not treatable with other medications ($AAVPT_c$, 2007). In some adult horses, tendonitis and cellulitis of the plantar ligament were already described in some animals after therapy with

high doses of enrofloxacin for a long period of treatment (Cole, 2014). Also, enrofloxacin injections can cause clinically tissue reactions (swelling, tenderness) after IM shots, so injectable enrofloxacin, should be administered via the IV route (Kaartinen et al., 1997). Although the safety and efficacy of enrofloxacin, marbofloxacin, and orbifloxacin in the treatment of susceptible bacterial infections in horses have not been established, pharmacokinetic evidence is available to suggest that they may be effective ($AAVPT_c$, 2007).

2.5 Development of antibiotic resistance in bacteria

The antimicrobial resistance is the expression of the resistance of a microorganism to an antimicrobial drug that was originally efficient for the treatment of infections caused by it (WHO, 2016). Currently, the multidrug-resistant (MDR), extensively-drug resistant (XDR) and pandrug-resistant (PDR) definitions are also being used in the medical literature to characterize the different patterns of resistance found in clinically important bacteria. MDR has been defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories; XDR has been defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and PDR was defined as non-susceptibility to all agents in all antimicrobial categories (Magiorakos et al., 2012).

The extensive use and sometimes misuse of antibiotics in human and agricultural settings accelerate the emergence of drug-resistant bacteria and have resulted in a growing problem of antimicrobial resistance worldwide.

Antimicrobial classes for which resistance has become a major problem include the β-lactams, the aminoglycosides and the fluoroquinolones (Angulo et al., 2004).

The evolution of resistant isolates is a natural phenomenon that occurs when bacteria replicate themselves erroneously or when resistant traits are exchanged between them (WHO, 2016).

Antibiotic resistance can occur in the following main ways: intrinsic resistance (i.e. an organism lacks a transport system for an antibiotic; or an organism lacks the target of the antibiotic molecule) (Todar, 2016); adaptive, as a natural selection through random mutation; or by acquiring resistance such as: a) transferring the genetic DNA information in a horizontal mode by plasmid

exchange between two bacteria (conjugation), b) by transformation, when parts of DNA are taken up by the bacteria from the external environment; or c) by transduction (bacteria-specific bacteriophages transfer DNA to a bacterium) (Trabulsi, 2004; Toleman, Walsh, 2011; Quinn et al., 1994).

2.5.1 Resistance mechanisms of beta-lactams

Bacteria have developed some strategies to escape the activity of beta-lactam antibiotics. The mechanisms of beta-lactam resistance include inaccessibility of the drugs to their target by decrease of drug permeation across the bacterial membrane (e.g. efflux pumps), target alterations (penicillin-binding protein - PBPs) and/or inactivation of the drugs by enzymes, which are known as beta-lactamases (Li et al., 2006; Majiduddin et al., 2002).

Resistance to β-lactams in gram-negative bacteria occurs mainly by expression of these β-lactamases. ESBLs (Extended-Spectrum-Beta-Lactamases) confer resistance to the cephalosporins, penicillins and aztreonam and usually they are inhibited by beta-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam), whereas chromosomal and plasmidial AmpC β-lactamases, also confer resistance to a wide variety of β-lactam drugs including the cephamycins but are not repressed by commercially available beta-lactamase inhibitors (Paterson, Bonomo, 2005; Philippon et al., 2002).

AmpC genes can be inducible by chromosomal beta-lactamase in *Enterobacter cloacae, Serratia marcescens, Citrobacter freundii, Providencia* spp. and *Morganella morganii* (often termed as 'ESCPM' group) (Harris, Ferguson, 2012). For this group, after exposure to β-lactams drugs, AmpC β-lactamases may express these enzymes at a high level, either by induction or selection for derepressed AmpC mutants leading to a clinical failure, even if an isolate was initially in vitro tested as susceptible (Harris, Ferguson, 2012; Wassef et al., 2014). All of the available beta-lactams (with the exception of the carbapenems) can be cleaved by the AmpC and their activity is not inhibited by clavulanate, sulbactam, or tazobactam (Harris, Ferguson, 2012; Paterson, Bonomo, 2005; Philippon et al., 2002;).

ESBLs are most often encoded on plasmids, which can easily be transferred between isolates. ESBL enzymes are plasmid-mediated and the

genes encoding these enzymes are easily transferable among different bacteria. The main mechanism of acquired resistance to extended-spectrum cephalosporins (ESCs) in Enterobacteriaceae is the production of ESBL and/or plasmid-mediated AmpC (pAmpC) β-lactamases, and the major enzymes involved are TEM, SHV, CTX-M or CMY variants (Bradford, 2001; Rawat, Nair, 2010).

Gram-negative bacteria can also show co-production of ESBLs/pAmpC with other resistance determinants, especially to fluoroquinolones, aminoglycosides and sulfonamides, where the treatment of these MDR organisms is a therapeutic challenge (Jacoby, 2005; Rice, 2012).

2.5.2 Resistance mechanisms of Aminoglycosides

Resistant mechanisms for the aminoglycosides can occur in several ways. The most common mechanism of resistance is by mutations of the ribosome or enzymatic modifications of the ribosome, modification of their outer membrane permeability or through efflux pumps (active transport of aminoglycosides out of cells through porins) (Tsodikova, Labby, 2015).

The enzymes responsible for resistance can be acquired through other bacterial species by receiving a plasmid containing this gene and two different types of enzymes are involved: 16S ribosomal RNA methyltransferases, such as RMTases, or by the most common and large group of enzymes N-acetyltransferases/AAC, O-nucleotidyltransferases/ANT or AAD, and O-phosphotransferases/APHs (Doi, Arakawa, 2007; Wachino, Arakawa, 2012).

The 16S rRNA methylase-producing Gram-negative bacteria are highly resistant to all clinically important aminoglycosides. AGs were originally isolated from actinomycetes and the RMTases can naturally occur in this bacterial group (intrinsically resistant) (Doi et al., 2004; Doi, Arakawa, 2007; Wachino, Arakawa, 2012). RMTases are encoded by the rmtase gene, and these genes are mostly located in transferable large plasmids and transposons, which provides them a potential spreading and could in part explain the already worldwide distribution of this novel resistance mechanism (Doi, Arakawa, 2007).

Since 2003, several plasmid-associated 16S rRNA methylase genes, *arm*A, *npm*A, and *rmt* group (*rmt*A, *rmt*B, *rmt*C, *rmt*D, *rmt*E, *rmt*F, *rmt*G, *rmt*H) have been recognized in clinical and veterinary isolates from various geographic areas, including Asia, Europe and the Americas (Doi et al., 2004; Doi et al., 2006; Francisco et al., 2015; Leigue et al., 2015; O'hara et al., 2013; Tada et al., 2013; Wachino, Arakawa, 2012). Some of these organisms have been found to coproduce extended-spectrum beta-lactamases or metallo-beta-lactamases, contributing to their multidrug-resistant phenotype (Doi, Arakawa, 2007).

2.5.3 Resistance mechanisms of fluoroquinolones

Mutations may occur rapidly during fluoroquinolone therapy and may be the most significant factor limiting the use of these antimicrobials (Oliphant, Green, 2002). Bacteria become resistant to quinolones by different mechanisms. The most common is through alteration in the target site (gyrase and/or topoisomerase IV) of quinolones by mutations in the chromosomal genes (called *gyrA, gyrB, parC* and *parE*) (Naeem et al., 2016; Van-Bambeke et al., 2005). Cell respiration and division end, and other processes are interrupted, including membrane integrity leading to bacteria death (AAVPTc, 2007). One mutation in the *gyrA* gene mediates full resistance to 1st quinolone generation (nalidix acid and flumequin) and reduced susceptibility to FQ. When a second mutation occurs in either *gyrA* and *gyrB* genes, it will mediate high resistance to FQs (EMEA, 2006).

Another mechanism of resistance is attributed to an alteration of outer-membranes porins (OmpF) resulting in a reduction in the cellular permeability (influx of the drug into the cell and/or enhance efflux of quinolones) with a decrease in the interaction of the drug with gyrase (topoisomerase IV), normally mediated chromosomally or by gene associated with mobile elements (Naeem et al., 2016; Van-Bambeke et al., 2005).

The last and more recent mechanism described for quinolones can also be mediated by plasmids that produce proteins that protect the quinolone targets from inhibition (Jacoby, 2005). According to Cattoir; Nordmann (2009), the following mechanisms are known until now: Qnr proteins, aminoglycoside acetyltransferase AAC(6')-Ib-cr, and efflux pump QepA. DNA gyrase and type

IV topoisomerase can be protected from quinolone inhibition by Qnr proteins. The plasmid-mediated quinolone resistance (PMQR) gene was named "*qnr*" (Jacoby, 2005).

Plasmid mediated quinolone resistance is due to three group of genes. These genes are *qnr* genes, *aac(6')-Ib-cr* and the third group comprised of efflux pumps, *qep*A, *qep*A1 and *oqx*AB (Naeem et al., 2016).

Until now, different types of Qnr proteins with different variants have been described (QnrA with six variants, QnrB with19 variants, QnrC, QnrD and QnrS with three each variants) (Jacoby, 2005; Naem et al., 2016; Van-Bambeke et al., 2005). Qnr proteins can act in two ways: a) decreasing the attachment of gyrase and topoisomerase IV to DNA, by reducing available enzyme targets on the chromosome and then protecting cells from quinolones or b), binding the gyrase and topoisomerase IV and them inhibiting quinolones from entering cleavage complexes formed (Aldred et al., 2014). The AAC(6')-Ib-cr enzymes decrease the activity of the quinolones by acylation and determinantly acetylates several fluoroquinolones, such as norfloxacin and ciprofloxacin (Jacoby, Naeem et al., 2016). QepA protein is a plasmid-mediated novel efflux pump, involved in the excretion of some FQ from the cytoplasm to the exterior of bacterial cells. It is responsible for the resistance levels elevation of several clinically important FQs (ciprofloxacin, norfloxacin, and enrofloxacin) (Yamane et al., 2007).

2.6. Global status of extended-spectrum-β-lactamase(ESBL)-producing Enterobacteriaceae in companion and food-animals

ESBLs are widely detected in various human medical institutions but they are less frequently reported in the bacterial population circulating in animals. This could indicate that these enzymes are less prevalent in animals than in humans, but it also can indicate that they have not been extensively studied (Carattoli, 2008).

Enterobacteriaceae producing extended-spectrum beta-lactamases or transferable AmpC betalactamases (pAmpC) are an emerging public health issue. These enzymes have been found in a number of different organisms, including *Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli, Proteus mirabilis, Enterobacter cloacae, Morganella morganii, Serratia* sp., *Shigella* sp., *Citrobacter* sp., and *Salmonella* species (Chong et al., 2013; Kiratsin, 2013; Lee et al., 2013; Marchandin, 1999, Pena, 1998; Nedjai et al., 2011; Tofteland et al., 2012). Gram-negative bacteria expressing extended-spectrum β-lactamases have emerged globally as well as increasingly reports in the veterinary context.

Different classes of antimicrobials are used by veterinarians to treat, to control, and to prevent infections caused by a number of bacteria in many animal species. β-lactam antibiotics such as amoxicillin (not vet exclusively), ceftiofur, cefquinome, cefadroxil, cefpodoxime, cefovecin and cefuroxime are the most frequently used cephalosporins, and are approved exclusively for the treatment of infections in veterinary clinic around the world (Carattoli, 2008; Trott 2013). Prescription drugs are not the only source of antibiotics in the environment. In Europe and China for example, antibiotics can be found in beef cattle, poultry and pigs (Schmid et al., 2013; Tian et al., 2009). Antimicrobial use in production animals has been shown to lead to the emergence of resistant bacteria throughout the food chain. According to Trott (2013), the use of small doses of antibiotics by the modern food animal industry as growth-promoting compounds in farm animals to stimulate animal growth and to avoid infections rather than cure infections is responsible for drug-resistant bacteria emerging on farms, which reach the general population through human or animal carriers, and through the food that consumers eat.

These same antibiotics can find their way into public water systems when the runoff from hospitals, farms or housing facilities contaminates rivers and groundwater (Todar, 2016). Aquatic ecosystems (urban surface waters comprise streams, lakes, wetlands, or rivers) are recognized as a reservoir for resistant bacteria and the occurrence of trace levels of antibiotics and antibiotic-resistant bacteria in source water may also affect public health and is an emerging issue for the general public (Fontes et al., 2011; Lee, Tissera, 2013; Lu et al., 2010;). Since the 1940s, ever-increasing amounts of antibiotics designated for human

and veterinary applications have been manufactured, used clinically, released into the environment, and widely disseminated, thus providing constant selection and maintenance pressure for populations of resistant strains in all environments (Davies, Davies, 2010).

Over the past 20 years, different species of enterobacteria (mostly *E. coli, K. pneumoniae, and P. mirabilis*) have revealed acquisition of plasmids encoding ESBL and different resistance rates have been reported. CTX-M type ESBL have been isolated globally and their occurrence has increased intensively, ESBLs reports are coming from all parts of the world in a variety of bacteria, and unfortunately, still growing (Lahlaoui et al., 2014). Interestingly, the antibiotic resistance profile is more common in some countries than others. This happens especially because in some countries the use of antibiotics is less or more strictly controlled. Antibiotic resistance levels in Sweden, Scandinavia, and Denmark, for example, are still comparatively low (Mölstad et al., 2008).

Knowledge about the current epidemiology status with regard to the occurrence, distribution, identification and ecology of ESBL-producing bacteria is an important tool to understand and also help a reduction or even a slowing of the rate of increase in the occurrence of ESBL in Enterobacteriacea, specially from animals.

2.6.1 ESBLs among companion animals

Companion animals represent potential sources of spread of antimicrobial resistance. Meyer et al., (2012) showed that contact with pets increases by almost seven-fold the chance of the owner to be colonized with ESBL. Their close contact with humans offers favorable conditions for the transmission of bacteria by direct contact (petting, licking, physical injuries, etc.) or through the domestic environment (furnishings, contamination of food, etc.) associated with the extensive use of antimicrobial agents in animals can create an opportunity for interspecies transmission of those resistant bacteria (Guardabassi, 2004).

Evaluation of the situation of bacteria antibiotic resistance in companion animals is quite difficult and just a few formal surveillance programs exist,

principally when compared to the data existing for food animal species. Much of the data comes from retrospective studies of clinical isolates. Geographical variation in pathogen species and susceptibility patterns can occur, but some overestimation of resistance rates can happen, because medical specimens could characterize both the extreme serious infections and those that had failed to respond to ATB treatment (Weese, 2008). National monitoring programs on antimicrobial resistance in animals typically focus on food animal, and do not provide data on companion animals. More efforts should be undertaken to obtain reliable data of the antimicrobial susceptibility of bacteria from pet animals.

The first beta-lactamase encoded by mobile genetic element was identified in the early 1960s, isolated from an *E. coli* isolated from a patient named Temoniera, so the enzyme was designated TEM-1(Datta et al., 1965). Likewise, since 1983 also SHV-1 has become widespread worldwide (Knothe, 1983) followed by reports of AmpC β-lactamases (Jacoby, 2009). The first report of an ESBL from an animal was described in Japan in 1988. It was a CTX-M-type enzyme, designated FEC-1 (Fujisawa *E. coli*-1). The cefotaxime-resistant E. coli strain was isolated from the fecal microbiota of a laboratory dog, which was used for pharmacokinetic studies of beta-lactam antimicrobials and had received β -lactams drugs (Matsumoto et al., 1988). Since that time, a number of cases of ESBL-positive isolates from animals have been described.

2.6.1.1 Europe

Most reports of ESBL isolated from pets come from Europe. One of the first reports, dated from 1998 in Madrid, Spain, described a resistant *Escherichia coli* strain which was isolated from a urine specimen from a dog with a recurrent urinary tract infection (UTI). This strain showed resistance to amoxicillin, cephalothin, cefotaxime, ceftazidime, and aztreonam and the ESBL profile was confirmed as SHV-12 type (Teshager et al., 2000).

Subsequent studies followed in Portugal described uropathogenic *Escherichia coli* isolated also from urine samples from dogs appointed resistance to β-lactams and the production of TEM-1, SVH, AmpC and OXA-1 enzymes were confirmed (Féria et al., 2002). Some years later, it was detected the pandemic O25-ST131 human virulent *Escherichia coli* CTX-M-15-producing with

a multidrug resistance region containing the *bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, *aac(6')-Ib-cr*, and *qnrB2* genes from a dog with urinary tract infection (chronic cystitis) that had been treated with multiple classes of antimicrobials. (Pomba et al., 2009)

ESBL-producing bacteria and beta-lactamases are also being reported in the pet's microbiota. Costa et al., (2004) reported the occurrence of *bla*$_{CTX-M-1}$ (25%) and *bla*$_{TEM-2}$ (75%) in fecal samples from healthy dogs in Portugal and a significant extended-spectrum β-lactamase was found in animals coming from a kennel in Italy (CMY-2, SHV-12, and CTX-M-1 in *E. coli* from sick and healthy dogs and cats) (Carattoli et al., 2005).

The first report of CTX-M producing *Escherichia coli* in the United Kingdom was described in 2007. The isolates came from three different samples: two from wound infection, one from preputial discharge and one from urine of the same dog (Steen, Webb, 2007). Followed that, *E.coli* CTX-M-15 ESBL-positive was identified in 2011 from bile sample in dogs with hepatobiliary disease and the authors affirm the concern about this variant, since CTX-M-15 is associated with clinical disease in humans in the United Kingdom (Timofte et al., 2011). In the same year (2011), Wedley et al., confirmed only two isolates carrying *bla*$_{TEM-1}$ and seven $_{CMY-2}$ among 183 healthy dogs fecal samples from a semi-rural area in the UK (Wedley et al., 2011).

The Public Health Agency of Sweden (Swedres-SVARM, 2013) reports, described since 2008 until 2013, eight *E.coli* strains carrying CTX-M-15, three, carrying CTX-M-1, one CTX-M-2, two CTX-M-3, four CTX-M-9, four CTX-M-27 and nineteen strains carrying CMY-2 from dogs. Whereas, from cats, the number is much smaller, and only one *E.coli* harboring CTX-M-15 and two CMY-2 were described. More recently, from Swiss companion animals, ESBL-producers were detected among uropathogenic *E. coli* and the eight CTX-M-15-producing isolates belonged to three important sequence types (ST410, ST533, ST648). The authors state that this is the first report of an *E. coli* of ST533 carrying *bla*$_{CTX-M-15}$ detected in a dog (Huber et al., 2013).

In France, a high considerable colonization rate of 18.5% ESBL/pAmpC (mostly *E.coli*, and one strain of *Salmonella and K.pneumoniae*) was found in

fecal samples from 368 unrelated healthy urban dogs in Paris. The main CTX-M-type were M-1 followed by M-9, M-15 and M-32. The authors consider that the acquisition of CTX-M-1 plasmids from dogs to humans, is plausible (Haenni et al., 2014). For clinical samples, it was found that 3.7% of *E.coli* isolates from pets in France produced ESBL (Dahmen et al., 2013). ESBL-producing *Enterobacter cloacae* from France in animals seems also to carry potential CTX-M-type. A study conducted by the same research group (Haenni et al., 2016), found that almost 70% of isolates belonged to potentially high-risk clones in humans, in particular ST114 (44.4%) and most of them carried CTX-M-15. This data raises questions and potential concerns about the transfer of *E. cloacae* between animals and humans.

In a study conducted by Carattoli et al., (2005), they reported expanded-spectrum cephalosporin resistance in *Escherichia coli* from dogs and cats in Rome, Italy. Three major beta-lactamases (CMY-2, SHV-12, and CTX-M-1) are reported for the first time in *E. coli* from sick and healthy dogs and cats. The authors state the worrying condition of prescribed-use of off-labels expanded-spectrum cephalosporins drugs registered for human use in pet treatment by italian companion animal practitioners. The authors also affirm that: *the use has happened since the early 1990s, even earlier than in farm animal practice, where their administration is still limited to selected cases, for obvious economic reasons. The results of this study are of public health concern because nonjudicious use or misuse of highly valuable antimicrobial drugs can result in selective pressure on bacterial populations of companion animals. This may lead to the spread of pathogens carrying resistance to newer antimicrobials by vertical and horizontal transmission of genes, with the subsequent risk of transfer to humans*. Another report about uropathogenic CTX-M-producing *E.coli* ST131 in cats in Italy was described in 2014. Urine samples were collected from 138 cats with non-complicated cystitis and the following variants were identified in these animals: CTX-M-14> CMY-2=TEM-1>-M-1=M-15 (Nebbia et al., 2014).

In Germany, dogs and cats isolates were confirmed as ESBL producers and the main CTX-M-type found was -M-1 in two different studies (Falgenhauer et al., 2014; Schmiedel et al., 2014).

Another study in Netherlands showed an even higher rate of intestinal carriage of ESBL/AmpC-producing (CTX-M-1, M-14, M-15 and CMY-2) Enterobacteriaceae in healthy dogs (45%), and both diarrheic dogs (55%) and cats (25%) (Hordjik et al., 2013).

In companion animals, *bla*$_{CTX-M-1}$ seems to be the most common ESBL gene reported.

2.6.1.2 Asia and Australia

The presence of CTXM-like genes in the Enterobacteriaceae has also been documented across the globe. In Asia, fecal samples from apparently healthy pups (not above two months of age) from Japanese kennels, with no history of antimicrobial use, described *E. coli* isolates harboring multidrug-resistant genes, including ESBL-producing isolates (Harada et al., 2011). Also in Asia, the emergence of O25b-ST131 clone harboring CTX-M-27 beta-lactamase in extraintestinal pathogenic *E.coli* from dogs and cats was described (Harada et al., 2012). Although most *E.coli* isolates harbored CTX-M beta-lactamases (CTX-M-27, CTX-M-14, CTX-M-15, and CTX-M-55), the isolates carried the CTX-M beta-lactamases/pAmpC in addition to TEM-1, CMY-2 and DHA-1 (Harada et al., 2012).

A study conducted among 240 *E. coli* isolates recovered from healthy and sick pets in South China from 2007 to 2008, confirmed that 96 (40.0%) of them harbored CTX-M. The most common CTX-M types were CTX-M-14 (n = 45) and CTX-M-55 (n = 24), followed by CTX-M-64, -27, -15, -65, -24, -3 and -9 (Sun et al., 2010). Ma et al., (2008) demonstrated a high prevalence of beta-lactamase genes from China dog feces. A total of 68.3% of the isolates have produced ESBLs lactamases (CTX-M-9 and CTX-M-1).

In the Republic of Korea, (Tamang, 2012) ESBL *E. coli* strains isolated from stray dogs in 2006 and 2007 were investigated using molecular methods. ESBL and AmpC β-lactamase phenotypes were identified in all isolates carrying *bla*$_{CTX-M}$ genes. The most common CTX-M types were CTX-M-14 and CTX-M-24 followed CTX-M-3, CTX-M-55, CTX-M-27, and CTX-M-65. Also, AmpC β-

lactamase-producing isolates were found carrying *bla*$_{CMY-2}$ and *bla*$_{DHA-1}$ genes. The occurrence of *bla*$_{CTX-M}$ genes in isolates from dogs underlines the possibility of spreading such strains to humans.

The first report from Australia on the detection of *bla*$_{CMY-7}$ and *bla*$_{TEM}$ plasmid-mediated in animal isolates was reported in 2006. The samples analyzed came from ten clinical cases of opportunistic infection in hospitalized dogs (cystitis, post-surgical and miscellaneous wound infections) (Sidjabat, 2006). A year later, the same research group showed that not only in *E. coli* the ESBLs/AmpC were detected, and the occurrence of beta-lactamase in *Enterobacter* spp. isolated from dogs with clinical opportunistic infections also occurred. Due to the high-level production of chromosomal AmpC by de-repressed mutants in *Enterobacter* spp., the detection of ESBLs complicate the treatment with cephalosporins. Those findings highlight the need to test for ESBL production in *Enterobacter* spp. in order to determine the spread of these resistance mechanisms (Sidjabat, 2007). In a survey (Gibson et al., 2011) in which more clinical extraintestinal infections samples (UTIs, surgical site infections, wounds tissue, joint fluid, tracheal wash, ear swab, bone and peritoneum liquid) were obtained between 1999 and 2007 from hospitalized dogs and cats, the ExPEC isolates contained a plasmid-borne AmpC *bla*$_{CMY-7}$, $_{CMY-2}$ or *bla*$_{OXA-10}$. Interestingly, all isolates that contained a plasmid-borne AmpC *bla*$_{CMY-7}$ gene also showed co-resistance with fluoroquinolones. The authors (Gibson et al., 2011) support that most samples probably came from animals that had intensive care facilities with complicated underlying medical or surgical conditions, where treatment with multiple classes of antimicrobial agents for prolonged periods is not uncommon and may select for antibiotic-resistant bacteria. The emergence of nosocomial infections in small animal clinics is one of the major drawbacks of this development, especially in terms of multidrug-resistance and potentially zoonotic pathogens.

2.6.1.3 Africa

Recently, it was documented in Tunisia, *E. coli* harboring *bla*$_{CTX-M-1}$, *bla*$_{CMY-2}$, and q*nrB19* from healthy pets fecal samples, pointing to a special relevance for

human medicine (Sallem et al., 2012). More recently, Grami et al., (2013) showed that CTX-M-1 is the dominant type of ESBL in pets from Tunisia.

In north Kenya, dogs, cats and their owners were investigated with an emphasis on the presence of ESBL. Rectal swabs of 216 dogs, 50 cats and 23 humans were collected. A total of 47 (22%), 2 (4%), and 4 (17%) ESBL-positive *E. coli* isolates were obtained from dogs, cats, humans, respectively. Three dogs harbored the international clone B2-O25-ST131. Moreover, CTX-M-15 and OXA-1 were detected in all *E.coli* isolates (Albrecthova et al., 2012).

More recently, a study (Albrecthova et al., 2014) conducted with stray dogs from rural Angola, which never received antimicrobial treatment, described that 75% of samples carried ESBL-CTX-M-15-producing *E.coli* co-producing PMQR. According to the authors, although the 3rd-generation and fluorquinolones are unlike ATB administered for dogs in Angola, the colonization in this animals may be an indirect evidence of their occurrence in humans and could pass to animals. They also support the idea that, once ESBL plasmid is introduced in an area, the spread of such plasmid is also perpetuated.

2.6.1.4 America

In South America, the first report of ESBL in pets occurred in 2008 and was from an *E. coli* isolated from fecal samples of dogs and cats treated or untreated with enrofloxacin from veterinary clinics in Chile, and the strains belonged to the CTX- M-1 and CTX-M-14 (Moreno et al., 2008).

In Brazil, ESBL *E.coli* were isolated from pets with UTI and the CTX-M-types described were: CTX-M-15-, CTXM-8-, CTX-M-2, along with CMY-1, CMY-2- and DHA-1. Interestingly, all the *E. coli* strains isolated in this work were not clonally related, suggesting plasmid transfers. Also, the clonally unrelated CTX-M-producing *E. coli* belonged to low-virulence phylogenetic group A and B1, suggesting that commensal *E. coli* can acquire ESBL-encoding genes through horizontal gene transfer (Barbato, 2012). Another study from Brazil analyzed 216 samples (feces and saliva swabs) from 108 pet animals (29 cats and 79 dogs) housed in shelters or at a Zoonosis Control Center; the samples were collected

from the city of São Paulo, whereas 14 strains of ESBL- producing-bacteria were described (*bla*$_{CTX-M,\ TEM}$ and $_{SHV}$). These results showed a first-time epidemiological data in Brazil, which indicate that companion animals are asymptomatic carriers of ESBL-producing strains and PMQR (Melo, 2014). Another study from Brazil also analyzed *E. coli* isolated from fecal samples of heathy dogs and their owners from Rio de Janeiro and similar results were addressed (presence of CTX-M, TEM and SHV) (Carvalho et al., 2016).

In the United States, the major threat from ESBLs has come from *Salmonella* spp. and *Escherichia coli*. In 2007 Frye et al., (2007) found enteric *Salmonella* harboring CTX-M, SHV, TEM and CMY-2 lactamases. From 1999 to 2003, 34,411 *Salmonella* strains were isolated from cattle, avian, horses and dogs and the proportion of ceftiofur-resistant isolates for each *Salmonella* serotype varied widely. *Salmonella* Newport had a significantly high level for ceftiofur resistance and the genotype predominantly was mainly associated with *bla*$_{CMY-2}$ followed by a few strains with *bla*$_{TEM-}$or *bla*$_{SHV}$ from pets. The first report of CTX-M- and SHV-type ESBLs in *E.coli* in the USA was described in 2010 (O´Keefe et al., 2010). Out of 60 canine and feline *Escherichia coli* isolates associated with UTI suspected to be ESBL positive, eleven (n=11) ESBLs were confirmed, including one SHV-12, one CTX-M-14, and nine CTX-M-15. During the 1990s, the most dominant ESBLs in the United States and throughout the world were TEM and SHV types, while CTX-M-producing organisms were rarely isolated. More recent studies have identified the increase in CTX-M genes between human and animal isolates (O´Keefe et al., 2010). According to Shaheen et al., (2011), 6% of clinical *E. coli* isolates from companion animals in the USA have reduced susceptibility to extended-spectrum cephalosporins. A diverse of beta-lactamase families were found in isolates and CTX-M, mainly *bla*$_{CTX-M-1-group}$, appears to be endemic in the United States.

The rapid spread of co-resistant ESBL with other classes of ATB in Enterobacteriacea from pets is a concern among veterinarians who need to find alternative drugs to treat clinical infections caused by these resistant bacteria. The emergence of ESBL resistance can limit the treatment options in the veterinary context.

2.6.2 ESBLs in poultry.

The first reports of ESBL-producing bacteria in poultry were performed in Europe. In this regard, in Spain, *E. coli* strains isolated from fecal samples of healthy and sick poultry were found to harbor *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, or *bla*$_{SHV-12}$ (Briñas, et al., 2003). Genes encoding CTX-M-1 also were detected in *E. coli* and *Salmonella enterica* isolates recovered from sick hen (septicemia) and healthy poultry, in France (Cloeckaert et al., 2010; Girlich et al., 2007; Meunier et al., 2006). In Portugal, cefotaxime-resistant *E. coli* was obtained from fecal samples from broilers at slaughterhouse level and the following ESBLs were detected: TEM-52, CTX-M-14 and CTX-M-32. The authors affirm that the intestinal tract of healthy poultry is a reservoir of ESBL-positive *E. coli* isolates (Costa el al., 2009). Another work, which characterized *E. coli* isolates with reduced susceptibility to cefotaxime or ceftiofur obtained from healthy broilers in Italy farms, confirmed the presence of *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-32}$ and *bla*$_{SHV-12}$ (Bortolaia et al., 2010). The German National *Salmonella* Reference Laboratory (NRL-Salm) reported the production of CTX-M-1 in *S. enterica* ser. Typhimurium and *S. enterica* ser Paratyphi B in chicken meat and chicken, respectively (Rodriguez et al., 20009). In Switzerland, CTX-M-1, SHV-12 and TEM-52 ESBls were detected in *E. coli* strains recovered from fecal samples of healthy chicken (Geser et al., 2012).

In the Henan province, China, presence of ESBL genes *bla*$_{TEM-57}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-24}$ and *bla*$_{CTX-M-65}$ was reported in a chicken farm reaching alarming levels (Yuan et al., 2009). Moreover, genes encoding SHV-12, CTX-M-27, CTX-M-55, CTX-M-24, CTX-M-105, CTX-M-14 and CTX-M-24 were identified in fecal samples of healthy ducks and environmental samples from a duck farm in South China (Ma et al., 2012). In Japan, ESBL-positive *E. coli* isolates from broilers harbored various ESBL genes: *bla*$_{SHV-12}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-44}$ (Hiroi et al., 2012). More recently, in Korea, CTX-M-3 and CTX-M-14 positive *Salmonella* spp. were identified in poultry slaughterhouses (Bae et al., 2013). In Africa, CTX-M-1 was the dominant type of ESBL in chickens from Tunisia (Grami et al., 2013).

Curiously, the identification of ESBL in chickens from Brazil was reported in a study performed in the UK, where chicken breast fillets imported from Brazil

and other countries, such as France, Poland and Netherlands, were positive for CTX-M-2-producing E. *coli* (Warren et al., 2008). Later, in another study, *E. coli* strains isolated from raw chicken meat imported to the UK from Argentina, Chile and Brazil were found to carry *bla*$_{CTX\text{-}M\text{-}2}$ and *bla*$_{CTX\text{-}M\text{-}8}$ genes (Dhanji et al., 2010). Another Brazilian report conducted from 2008 to 2009, isolated 93 *Salmonella* spp. from commercial poultry (e.g. chicken, turkey, and tinamou) and related sources (poultry farm floor, drag swab of the rearing facility, carcasses, and eggs) in farms of five different Brazilian states. The ESBL rate found was 14% and all isolates were CTX-M-2 (Silva et al., 2012).

The emergence of CTX-M-2-producing *Salmonella enterica* serovars Schwarzengrund, Agona and Typhimurium have been reported in Brazilian poultry farms (Fernandes et al.,2009; Silva et al., 2013). More recently, another study from Brazil reported for the first time the isolation of MDR *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis* strains harboring *bla*$_{CTXM\text{-}2}$ or $_{CTXM\text{-}8}$ in chicken meat sold in markets in southeast Brazil (Casella et al., 2015).

All these studies show a widespread diffusion of ESBL-genes in *E. coli* in food-producing poultry in Europe, Asia and South America.

2.6.3 ESBLs in livestock animals.

2.6.3.1 Cattle

Currently, the emergence of ESBL-producing bacteria colonizing livestock animals, mainly bovines, is a public health concern, since the transmission to humans cannot be rejected. Some reasons for this emergence include the off-label and the abusive use of cephalosporins as treatment and/or prophylaxis to mastitis, and the use of antibiotic-contaminated waste milk to feed calves in their first days of life (Carattoli, 2008; Trott, 2013).

Escherichia coli, Salmonella spp. and *Klebsiella pneumoniae* strains isolated in different countries from livestock and food of animal origin (meat and raw milk) have shown ESBL-producing phenotype. The molecular analysis of these bacteria has demonstrated presence of plasmidial ESBL gene families

belonging to the following *bla* genes: CTX-M, SHV, TEM and the plasmidial AmpC-type betalactamase CMY-2. Among these gene families, the most frequent ESBL types found in livestock animals are CTX-M -1, -2, -3, -8, -9, -13, -14, -15, -24, -28, -32; and SHV (-2, -5, -12) (Carattoli, 2008; Coque et al., 2013).

Watson et al., (2012) studied the presence of ESBL-producing bacteria in dairy cattle in the United Kingdom and demonstrated that 82.8% of calves studied were colonized by CTX-M ESBL *E. coli.* These high rates are directly related to the feed provided to calves, waste milk (milk unfit to human consumption) generally contaminated with antibiotic residues, which may select ESBL-producing bacteria in calves' microbiota (Brunton et al., 2012; Hornish, Kotarski, 2002). There is a hypothesis that dairy cows, in which the use of cephalosporins for the treatment of mastitis is intensive, can act as a reservoir of resistant bacteria.

According to the Department for Environment, Food and Rural Affairs (DEFRA) in the UK, since July 2006 different cattle farms have been confirmed as having *E. coli* and other Enterobacteriaceae members carrying ESBLs of the CTX-M type, including CTX-M-1, CTX-M-14 and CTX-M-15 variants. Furthermore, some *E. coli* isolates carrying CTX-M-15 have been identified as serotype O25, a common *E.coli* serotype found as human pathogen (DEFRA, 2008).

Plasmidial $bla_{\text{CTX-M}}$ is currently the most frequent ESBL gene family present in livestock. Studies conducted in the UK (Horton et al., 2011), France (Dahmen et al., 2013; Madec et al., 2012) Germany (Schmid et al., 2013), the USA (Wittum et al., 2010) and Brazil (Aizawa et al., 2014) reinforce this thesis. CTX-M-1 and CTX-M-14-producing *E. coli* and *K. pneumoniae,* for instance, were isolated from dairy cattle with symptomatic or asymptomatic mastitis in France (Dahmen et al., 2013; Madec et al., 2012) and Italy (Locatelli et al., 2010). Interestingly, some E. *coli* clones (ST10, ST23 and ST58) found as ESBL-carrier in humans were found in cattle from France, indicating these animals and animal-food were a source of contamination (Dahmen et al., 2013). Another study conducted in France (Madec et al., 2012) demonstrated that $bla_{\text{CTX-M-15}}$-carrying plasmids from cattle-derived non-ST131 *E. coli* isolates were highly similar to

those found in ST131 *E. coli* reported in humans. It also exemplifies the key role of plasmids versus clonal dissemination in the spread of the *blaI*$_{CTX\text{-}M\text{-}15}$ gene among cattle, and possibly between *E. coli* isolates detected in humans and cattle.

A study conducted in Germany found the presence of ESBL-producing *E. coli* in different dairy cattle, beef cattle and mixed (both dairy and beef) farms. The results showed high prevalence of bacteria (33%) harboring CTX-M-1, CTX-M-2, CTX-M-9 and CMY-2 genes (Schmid et al., 2013).

In America, ESBL-producing bacteria isolated from cattle were reported in some countries. In 2010, *Salmonella* spp. producing *bla*$_{CTX\text{-}M}$ (-1 and -79) were isolated from cattle in the USA. The authors believe this finding is a concern, specially because *Salmonella* spp. transmission is primarily by food and resistant enteric bacteria from livestock can be transferred through the food supply chain to consumers. Furthermore, cephalosporins are the therapy of choice for invasive gram-negative infections, including cases of salmonellosis in children (Wittum et al., 2010). Winokur et al., (2000), analyzing transfers of resistant genes between bovine, porcine and human species in the USA, isolated *Salmonella* spp. from these animals and found multidrug-resistant isolates (bovine, porcine, and human). These isolates presented resistance to cephamycins and extended-spectrum cephalosporins and the molecular analyses showed AmpC (CMY-2) genes.

An epidemiological surveillance study conducted in Brazil reported the spread of multidrug resistance bacteria exhibiting a high resistance profile to veterinary- and human-use β-lactams in buffalos (Aizawa et al., 2014). This study identified the presence of CTX-M-8-type ESBL and CMY-2-type plasmid AmpC-producing *E. coli.* Moreover, 55% β-lactamase producing *E. coli* strains belonged to the low virulence phylogenetic groups A and B1, which reinforces the hypothesis about endogenous selection of commensal microbiota in food-animals, due to the selective pressure caused by broad-spectrum antibiotic use and/or acquired by horizontal gene transfer. The MLST analysis showed diversity among the strains, revealing a common ancestor in Asia, Africa, South America, North America and Europe (Barbato et al., 2012).

Studies conducted in Asia also showed the presence of ESBL-producing bacteria. The first report of ESBL was in Japan in 2000-01, using samples from cattle (fecal and carcasses), and the predominant type found was CTX-M-2 (Shiraki et al., 2004). In China, intestinal *E. coli* obtained from healthy food animals, including dairy and beef cattle, were tested for the presence of ESBL genes. *E. coli* isolates showed reduced susceptibility to cefotaxime and different $bla_{\text{CTX-M}}$ gene types were detected. The most common CTX-M types were CTX-M-14, CTX-M-55 and CTX-M-65, followed by CTX-M-27, -15, -98, -24, -3, -102 and -104 (Zheng et al., 2012).

Another study from Japan (Hiroi et al., 2012), approximately ten years later, evaluated the percentage of ESBL genes among *E.coli* isolates from food-producing animals (cattle, pig and broiler). For cattle, the researchers found 12.5% of beef cattle carrying *E.coli* –ESBL, whereas in pigs it was 3%.

Antimicrobial resistance in commensal Enterobacteriaceae of food animals may play an important role in the ecology of resistance and may serve as an important reservoir for these transmissible resistance genes.

2.6.3.2 Swine

Escudero et al., (2010), from Spain, investigated the prevalence of ESBL gene mediating resistance in *Escherichia coli* from fecal samples of healthy pigs at different farms. They found reduced susceptibility or resistance to extended-spectrum cephalosporins, from which 72% were ESBL and it was detected SHV-12, CTX-M-1, CTX-M-9 and CTX-M-14 genes. Another research conducted in a Spanish province (Briñas et al., 2005) analyzed the presence of genes encoding β-lactamase resistance in *E. coli* strains from sick and healthy pigs. *Escherichia coli* isolates showed genes encoding TEM-1, CTX-M-14, CTX-M-32, SHV-12 β-lactamases. Also in Spain, Blanc et al., (2006) determined the presence of ESBL-producing Enterobacteriaceae in pig farms and most of these samples presented *E. coli* harboring $bla_{\text{CTX-M-1}}$ gene. *E. coli* strains from food-producing animal farms can be an important reservoir of ESBL genes. Geser et al., (2012) studied the dissemination of ESBL in fecal samples from pigs and 15.3% of the porcine

samples were positive for ESBL-producing *E. coli*. CTX-M-1, M-14, TEM-1 and TEM-186 were the most common enzymes identified and those findings show a relatively high rates of ESBL producers in pigs farms from Switzerland. Wu et al., (2008), in a similar study in Denmark, found the same genes, meaning that resistant genes occur all over Europe.

In a study in Western China in a period of five years (Tian et, al 2009) the observations revealed a dramatically increase of ESBL-producing *E. coli* (mostly CTX-M-type) from 2.2% to 10.7% during this period. Among these isolates, the variants detected were: CTX-M-1, CTX-M-15, CTX-M-22, SHV-2 and SVH-12. These findings indicate that this study was the first study to identify $bla_{\text{CTX-M-22}}$ from pigs and also indicate that ESBL genes have been present in farm animals in China since at least 2002.

Recently, in Brazil, it was described for the first time the isolation of CTX-M-15-producing E*scherichia coli* strains belonging to sequence type (ST) 410, ST224 and ST1284 in a commercial swine facility. The $bla_{\text{CTX-M-15}}$ gene was located on IncF-plasmid-type, surrounded by a new genetic context, comprising the IS*26* insertion sequence truncated with the IS*Ecp1* upstream of $bla_{\text{CTX-M-15}}$. These results reveal that commercial swine have become a new reservoir of CTX-M-15-producing bacteria in South America (Silva et al., 2016).

Antimicrobials have been used in swine production at sub-dose levels since the early 1950s to increase feed efficiency and promote growth. Several ATBs are available for use in swine. Thus, the continuous administration of sub-doses to pigs also provides selective pressure for antimicrobial-resistant bacteria. For this reason, subtherapeutic antimicrobial use in livestock remains a source of controversy and concern (Chenier, Holman, 2015).

In the past, most attention was paid to glycopeptide and streptogramin resistance in enterococci, fluoroquinolone resistance in campylobacter and multi-drug resistance in *Escherichia coli* and salmonella. While these are still important classes of antibiotics, the focus has shifted to ESBL-producing organisms selected by the use of ceftiofur and cefquinome in pigs (Barton, 2014).

Pigs treated with ceftazidine and ceftiofur, from twenty Danish farms, showed that two farms presented pigs carrying CTX-M-1 and CTX-M-5 types. This study demonstrated a significant statistic association between the use of ceftiofur and reduced susceptibility to cefotaxime due to production of either AmpC or CTX-M-1 β-lactamase (Jorgensen et al., 2007). The usage of cephalosporins in pigs has been leading to a self-ban of these antibiotics by the Danish pig industry since 2010 (Hansen et al., 2013).

Australia has a very strict national system for registration of antibiotics used in animals, especially for food producing animals. As a result, almost all antibiotics used in animals are prescription-only. Most antimicrobial use in the Australian pig industry is based on drugs of low importance to public health. Ceftiofur is the only third generation cephalosporin currently registered for use in animals in Australia. Although antimicrobial resistance is present in Australian bacterial isolates from animals, the livestock industries have responded to concerns and resistance rates in enteric bacteria from pigs has declined (Barton, 2014; Jordan et al., 2009).

The continued use of antibiotic growth promoters has been questioned and there is a need to ensure that antibiotics important in human medicine are not used therapeutically or prophylactically in animals (Barton, 2014).

The use of antimicrobials in the pig industry has welfare and economic benefits, but it is also related to the increase in ATB resistance rates, leading to a public healty concern. Monitoring programs, national committees, periodic surveillance and reviews underlying reduced use of ATB are urgently needed. It is necessary to control the use, create laws, request prudent use by vet or other animal's practitioners and monitor the ATB rational use and correct dosage.

2.6.4 ESBLs in Horses

Horses can be classified as companion or hobby animals, since they are involved in equestrian sports or as transport animals, or, in some countries, they

are considered food animal and processed for human consumption. These animals have close contact with humans and, like dogs and cats, they could share the bacteria microbiota with humans suggesting an important reservoir of resistant bacteria (Trott, 2013).

Antibiotic resistance data for these animals is sparse. The first report came from Rankin et al., in 2005, in which *Salmonella enterica* serovar Newport MDR-AmpC expressing TEM-1b and SHV-12 was isolated from affected animals during an outbreak of salmonellosis that led to a 3-month closure of one of the largest equine hospitals in the United States. Following that, Frey et al., (2007) also isolated *Salmonella enterica* from horses carrying $bla_{\text{CTX-M, SHV, TEM}}$ and $_{\text{CMY-2}}$ in the USA. After that, an outbreak of food poisoning caused by a multi-resistant *Salmonella enterica* serovar Newport AmpC-producing in France, was associated with imported horse meat from the USA. Several characteristics like sequencing type, PFGE profiles and the presence of $bla_{\text{CMY-2}}$ genes were shared between US and French *Salmonella* isolates. The authors assumed that during 2000–2005 some isolates likely entered in France from North America through imported meat food (Egorova et al., 2008).

Most reports about the ESBL epidemiology in horses come from Europe. In Germany, the most common findings for ESBL *E. coli* are: CTX-M-15, M-1, M-14, M-2 and M-9 (Ewers et al., 2010; Ewers et al., 2014; Schmiedel et al., 2014). Even the highly virulent clone *E.coli* B2-O25b-ST-131 CTX-M-15- producing was already described in a horse from Germany in 2010. The strain, originating from a case of eye inflammation, showed a higher number of resistant genes when compared with other samples analyzed during that study (Ewers et al., 2010).

Similar patterns have been identified in horses from several countries in Europe. In Belgium, studies with clinical isolates showed *E.coli* harboring CTX-M-15 and CTX-M-2 (Smet et al., 2010, Smet et al., 2012); in the Czech Republic, *E.coli* harboring CTX-M-1 (Dolejska et al., 2010); and from the Netherlands, a studied revealed that the isolates of *E.coli* and *K.pneumoniae* from diseased horses (including foals of one month old) carried the $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-1}}$ and/or $bla_{\text{SHV-1}}$ genes (Vo et al., 2007). Another report in the Netherlands, five years later, described *E.coli* carrying mainly CTX-M-type 1 (positive in three *E.coli*

isolates) followed by M-2 (n=1) and M-14 (n=1) (Dierikx et al., 2012). A cross-sectional study of fecal carriage of antimicrobial-resistant in *E. coli* amongst horses in the general equine community was performed in the UK. The prevalence of fecal carriage for *E. coli* with resistance to any antimicrobial was 69.5% and the prevalence of extended-spectrum β-lactamase ESBL-producing *E. coli* was 6.3% (Maddox, 2011). Another British work described the presence of $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$ amongst the horses studied (John et al., 2012). Fecal samples of 90 Lusitano horses from Portugal showed only three isolates carrying ESBL genes (bla_{SHV}, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$) (Moura et al., 2010). In *Enterobacter cloacae* isolated from French horses, CTX-M-15 was also detected (Haenni et al., 2016).

In Brazil, the first description of *Escherichia coli* (ST2179) with CTX-M-15 co-producing *rmtD* and *aac(6)-Ib-cr* genes isolated from a foal was described in 2015 (Leigue et al., 2015). This fact is very alarming, since no reports of *rmtD* genes (aminoglycoside resistance gene) associated with CTXM15 from horses had been described until now in South America, and also highlights the increased ESBL co-resistance strains in veterinary samples.

In Australia, Gibson et al., (2010) showed only pAmpC genes among horse's samples, and the CMY-7 type was prevalent. The CMY-2 was first identified in *Salmonella* isolates (Frye et al., 2007; Rankin et al., 2005) and a few reports of CMY-2 in *E.coli* isolated from fecal horses samples were found (Vo et al., 2007). Another report from Denmark with pAmpC *E.coli* isolated from horses with infections were described CMY-34 type and CMY-53 (Damborg et al., 2012).

Hospitalization seems to be an important risk factor associated with the development of antimicrobial resistance. In a longitudinal study, investigating the occurrence of fecal *E.coli* in horses receiving broad-spectrum antimicrobial during admission in a vet hospital, Dunoswska et al., (2006) showed that the prevalence of antimicrobial resistance differs significantly among the three groups tested (hospitalized horses receiving ATBs, hospitalized horses with no ATB administered and community horses). The highest prevalence was among isolates from the hospitalized receiving ATB group and the lowest rate was noted among isolates from the community group. Isolates recovered from the

hospitalized horses receiving ATBs and hospitalized horses with no ATBs groups were also significantly more likely to be resistant to multiple ATBs.

The Damborg et al., (2012) work investigated the occurrence and genetic background of fecal *Escherichia coli* resistant to cefotaxime in horses receiving broad-spectrum antimicrobial after admission in a vet teaching hospital in Denmark. The genetic characterization of all fecal isolates revealed an abundance of *E. coli* producing either CTX-M-1 or CTX-M-14 and some CTX-M-1 isolates producing additional beta-lactamases (TEM-1, CMY-34 and the novel variant CMY-53). Also, CTX-M-producing *E. coli* appeared intermittent in four horses and persisted two weeks after antimicrobial treatments in five of six patients tested after discharge from hospital. Nosocomial transmission was suggested by finding five identical CTX-M-1-producing *E. coli* pulsotypes in multiple horses. Additionally, the authors believe that the microbiota is a very dynamic environment, and suggested some hypothesis like: a) the strains could be acquired from the hospital environment; b) the *E.coli* were already present in the intestinal tract and proliferated after antimicrobial therapy or c) CTX-M genes could be mobilized from the intestinal microbiota bacteria to *E.coli*, as consequence of antimicrobial treatment or other stress factor associated with the hospitalization.

More similar studies addressing antibiotic-resistant bacteria in feces from hospitalized horses or at antimicrobial treatment were described for horses. A work conducted by Ahmed et al., (2010), analyzed 264 fecal samples collected from 138 horses in hospital and stabled facilities and concluded that the proportion of antibiotic resistant isolates and multidrug resistant isolates was significantly higher in hospital samples compared to not-hospitalized horse samples. The *E. coli* isolates described in the Ahmed et al., (2010) paper were largely associated with TEM β-lactamase genes, with only one isolate being positive for SHV β-lactamase genes.

One study that addressed the impact of hospitalization and antimicrobial drug administration on the prevalence of resistance in commensal fecal *E. coli* of horses from Ireland was lead by Bryan et al., (2010). Fecal samples were collected from ten hospitalized horses treated with ATB, ten hospitalized horses

not treated with ATB and nine non-hospitalized horses over a consecutive period of five days. The presence of *E. coli* cephalosporins resistant appeared recurrent in some horses and could have persisted for a period of weeks after antimicrobial therapy, moreover, the results revealed that hospitalization alone was associated with increased prevalence of antimicrobial resistance and multidrug resistance in commensal *E. coli* of horses.

Two years later a similar study (John et al., 2012) was conducted with fecal *Escherichia coli* isolated from horses treated with antimicrobial drugs in both the hospital and in the community. Different from Bryan et al., (2010) study, the proportion of resistant samples was not significantly different between hospitalized and non-hospitalized treated horses but the odds of a sample containing a resistant isolate increased significantly at day five in treated horses. Table 1 summarizes all reports found on Pubmed homepage and the more common ESBL among Enterobacteriacea and *Acinetobacter* sp. isolated from horses, whereas CTX-M-1 type was the most cited ESBL type.

The widespread use of antibiotics in human and animal has raised the concern about the development of resistant and multi resistant bacteria that possess a potential danger to animals and humans. The use of 3rd or 4th cephalosporins such as ceftiofur (3rd) or cefquinome (4th generation) and some important aminoglycosides or fluoroquinolones are available for systemic use in horses. The use of important classes of ATB must be prudent in veterinary medicine, once the MDR bacteria could have serious implications in the treatment of diseases, not only for animals, but also for humans. Veterinarians need to stay aware of antibiotic resistance patterns in their facilities and a detailed epidemiological data and further studies about ESBL/AmpC isolates from horses are important to clarify the risk factors associated with these animals.

Table 1 - Presence of Extended-spectrum-beta-lactamase-producing gram negative bacteria in horses in chronological order according to the date of publication (modified from Ewers et al., 2011).

Bacteria species	*bla*-type	Year of isolation	Country	Sample origin	Reference
Salmonella sp.	SHV-12 TEM-1 CMY-2	2003-04	USA	Clinical	Rankin et al., 2005
Salmonella enterica	CTX-M-LIKE TEM-1 SHV-LIKE CMY-2	1999-2003	USA	ND	Frye et al., 2007
E.coli *K.pneumoniae*	CTX-M-1 CMY-2 CTX-M-1	2003-05	Netherlands	ND	Vo et al., 2007
E.coli *K.pneumoniae* *Enterobacter* spp. *Serratia odorifera*	CTX-M-1 CTX-M-14 CTX-M-15 SHV-12 CTX-M-15 SHV-12 CTX-M-1 SHV-12 CTX-M-1	2008-2013	Sweden	Clinical	SVARM, 2014
E.coli	TEM-1 SHV-like	ND	England	Fecal	Ahmed et al., 2010
E.coli	CTX-M-15	2008-09	Germany	Clinical	Ewers et al., 2010[a]
Citrobacter freundii	CTX-M-1	2008-10	Europe	Clinical	Ewers et al., 2010b
E.coli	CMY-7	1999-2007	Australia	ND	Gibson et al., 2010
E.coli	CTX-M-15	ND	Belgium	Clinical	Smet et al., 2010
E.coli	CTX-M-1	2007-08	Czech Republic	Clinical	Dolejska et al., 2011
Acinetobacter baumanni	TEM-1 OXA-66 OXA-69	2004-09	Switzerland	Clinical	Endimiani et al., 2011
Acinetobacter spp	OXA-23	2012	Belgium	Feces	Smet er al., 2012[a]
E.coli	CTX-M-1 CTX-M-9	ND	UK	Fecal	Johns et al., 2012
E.coli	CTX-M-1 CTX-M-14 TEM-1 CMY-34	2009	Denmark	Clinical	Damborg et al., 2012
E.coli *Enterobacter cloacae*	CTX-M-1 CTX-M-2 CTX-M-14 TEM-1 TEM-1	2007-08 2009	Netherlands	Clinical	Dierikx et al., 2012
E.coli	CTX-M-2	2008-10	Belgium	Clinical	Smet et al., 2012b
E.coli	TEM-1 CTX-M-1 CTX-M-14 CTX-M-15	2008-11	Germany	Clinical	Ewers et al., 2014
E.coli	CTX-M-1 SVH-12	ND	Germany	Clinical	Walther et al., 2014
Enterobacteriaceae	TEM-1 OXA-1 CTX-M-1 CTX-M-2 CTX-M-9 CTX-M-15	2009-10	Germany	Clinical	Schmiedel et al., 2014
E.coli	CTX-M-15	20112	Brazil	Clinical	Leigue et al., 2015
Enterobacter cloacae	CTX-M-15	2010-13	France	Clinical	Haenni et al., 2016
E.coli	CTX-M-1 CTX-M-2 CTX-M_9 CTX-M-15 CTX-M-32	2014-15	France	Clinical	This study
E.coli	TEM-1 CTX-M-1 CMY-2	2012-13	Brazil	Feces	This study
E.coli *K.pneumoniae* *P. mirabilis* *S. marcescens*	CTX-M-15 CTX-M-15 CTX-M-1 CTX-M-1	2012-2014	Brazil	Clinical	This study

ND: not determined; UK: United Kingdom

2.7 Final comments

The global emergence and spread of *bla*$_{CTX-M}$ genes is the main problem associated with resistance to cephalosporins in *E. coli* and *Salmonella* strains isolated from companion and food-producing animals. In this regard, the identification of CTX-M-15-producing *E. coli* belonging to the international clone O25-ST131 is an epidemiological concern. In food-producing animals, the off-label use of cephalosporins as prophylaxis treatment to prevent bacterial infections has been considered as an important risk factor contributing to the selection and spread of ESBL-producing bacteria. Epidemiological data reports about antimicrobial resistance in animals are important tools to be used in clinical management of infectious diseases leading to the rational use of antimicrobial agents. Thus, food-producing animals and pets can become an important source for the dissemination of ESBL-encoding genes in humans. Nevertheless, further studies are needed to clarify the reason why clinically relevant ESBL-producing Enterobacteriaceae are emerging worldwide.

2.8 Objectives

2.8.1 General Objectives

The aim of this study was to characterize phenotypically and genotypically the resistant mechanins of multidrug-resistant gram-negative bacteria isolated from healthy and infected horses in Brazil and France, giving special attention to cephalosporins resistance genes.

2.8.2 Specific Objectives

- To describe the antimicrobial susceptibility profile of resistance gram-negative bacteria isolated from horses;
- To characterize the plasmidial mechanisms of resistance to β-lactams, quinolones and aminoglycosides by genotypic testing;
- To describe the *Escherichia coli* phylogenetic group;
- To determine the profile of genetic similarity among all *Escherichia coli* isolates producing β- lactamases or AmpC mediated by plasmid;
- To identify global clonal relatedness by "*Multi Locus Sequence Typing*" (MLST);
- To investigate and to describe the genetic environment elements involved with the ESBL CTX-M-1 group;
- To identify and classify plasmids harboring $bla_{\text{CTX-M}}$ genes.

3 MATERIAL AND METHODS

3.1 Bacterial Isolates

A total of 62 GNB (Gram-negative bacteria) were investigated and characterized in this study including:

- *n* = 8 - *E. coli* isolates from healthy horses - fecal samples, Brazil
- *n* = 3 - *E. coli isolates* from infected horses, Brazil
- *n* = 1 - *K. pneumoniae* from a diseased horse, Brazil
- *n* = 1 - *Proteus mirabilis* from a diseased horse, Brazil
- *n* = 1 - *Serratia marcescens* from a diseased horse, Brazil
- *n* = 3 - *P. aeruginosa* from diseased horses, Brazil
- *n* = 45 - *E.coli* isolated from diseased horses, France

3.2 Samples origin

3.2.1 Horses fecal samples from Brazil

From 2011-13, ninety-six (n=96) feces samples from healthy horses were screened. The samples were collected into sterile capped universal bottles with sterile spatula or by swabbing the stool and then immediately transported to the laboratory. The animals belonged to different riding centers in Brazil. This study was undertaken in accordance with the ethical and legal requirements and was approved by the Research Ethic Committee.

3.2.2 Clinical horse samples from Brazil

From 2012-2014, six clinical cases were analyzed. The biological sample or the isolated strain was sent to us by different vet schools from São Paulo State or Paraná State. In all 6 clinical cases, antibiotic therapy had been administered previously, but no response was noted to treatment. Details about clinical history are described in chapter 4.2.

3.2.3. Clinical horse samples from France

Between 2014 and 2015, the isolates were collected from diseased horses from different regions of France. This study is a part of a national surveillance program for antimicrobial resistance in pathogenic bacteria in France (www.resapath.anses.fr). All fourty-five isolates (n=45) were previously identified as *E. coli* and defined as suspected ESBL producers.

3. 3 Isolation and Identification

Isolates were recovered from the samples after culturing on MacConkey agar and incubated for 18–24 hours at 37 °C. Samples from horse feces were streaked on MacConkey agar with ceftriaxon (2 mg/L). The identification was performed by conventional biochemical tests and confirmed using API 20 E multi-test systems (bioMérieux, France), Vitek (bioMérieux, Marcy l'Étoile, France) or by Matrix-assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF, Bruker Daltonics).

3.4 Antimicrobial Susceptibility Tests

3.4.1 Disk-diffusion Antibiotic Susceptibility Profile

The antibiotic susceptibility profile was evaluated by Kirby-Bauer disk-diffusion according to CLSI (CLSI, 2013) and EUCAST (EUCAST, 2015). The following discs (Oxoid, UK) were used: Amoxicillin-clavulanic acid (30 µg); Cefoxitin (30 µg), Cefotaxime (30 µg), ceftadizim (30 µg), aztreonam (30 µg), ceftiofur (30 µg), Imipenem (30 µg), Tetracycline (30 µg), Gentamicin (10 µg), amikacin (30 µg), and Trimethoprim-sulfamethoxazole (25 µg), nalidixic acid (30 µg), Ciprofloxacin (5 µg), enrofloxacin (5 µg), norfloxacin (10 µg), levofloxacin (5 µg), pefloxacin (5 µg), ofloxacin (5 µg), moxifloxacin (5 µg),

For Brazilian clinical samples, besides those ATB already cited, further antibiotics were tested: tobramicina (10 µg), neomicina (30 µg), kanamicina (30

µg), imipenem (10 µg), meropenem (10 µg) ertapenem (10 µg), tigeciclina (15 µg).

For French samples it was used: Amoxicillin (25 µg); amoxicillin-clavulanic acid (30 µg); cephalothin (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), ceftiofur (30 µg), cefoxitin (30 µg), ceftadizime (30 µg), cefepime (30 µg), cefquinome (30 µg), piperacillin (30 µg) Piperacillin/tazobactam (110 µg), Ticarcillin/ clavulanate (75/10 µg), aztreonam (30 µg), Imipenem (10 µg), Tetracycline (30 µg), chloramphenicol (30 µg), florfenicol (30 µg), Gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), streptomycin (10 µg), kanamycin (100 µg) apramycin (30 µg), colistin (50 µg)Trimethoprim-sulfamethoxazole (25 µg), nalidixic acid (30 µg), (5 µg), enrofloxacin (5 µg) and ofloxacin (5 µg) diks.

E.coli ATCC 25922 or *E.coli* CIP 7624 were used as quality control strains.

3.4.2 Minimum Inhibitory Concentrations (MIC)

The MIC was performed according to CLSI (CLSI, 2014) or EUCAST (2015) guidelines and were performed by: E-test (AB Biodisk, Solna, Sweden); or Vitek or the agar dilution method.

Agar dilution method was used only for ceftiofur. A metal multiloop inoculating device which delivered about 1-3 µL samples of bacterial suspensions was pressed on the Mueller-Hinton agar supplemented with ceftiofur (eleven different dilutions were used). Plates were incubated overnight at 36 °C and evaluated for the presence of growth. ATCC 25922 was used as quality control.

3.5 Phenotypic test for ESBL

3.5.1 Double-disc-synergy-test

The detection of ESBL-producing Enterobacteriaceae was perfomerd by the double-disc-synergy-test (DDST). Cephalosporin discs (ceftazidime, cefotaxime, ceftriaxone, cefepime or ceftiofur) were applied surronded by amoxicillin-clavulanic acid or ticarcillin + clavulanic acid disks (Figure 1).

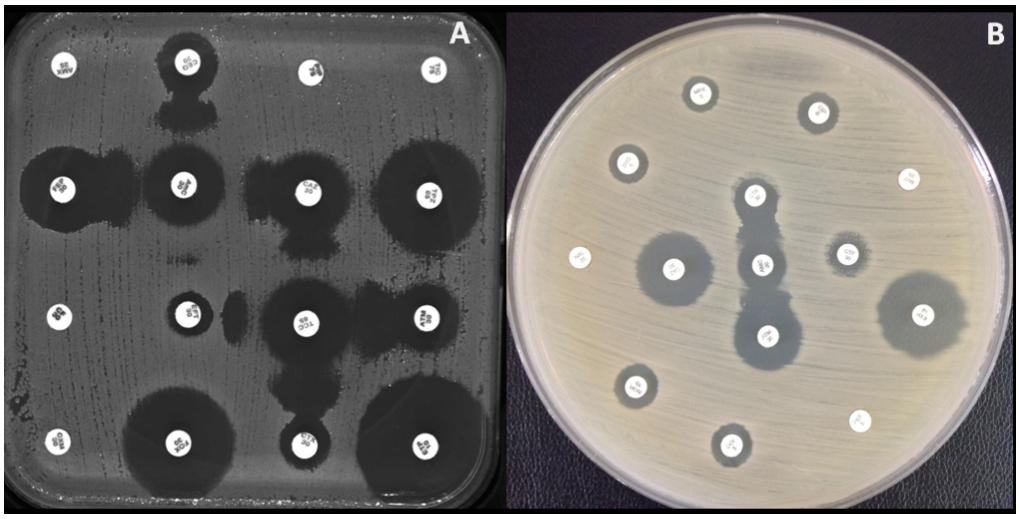

Figura **1 -** Enhance of the inhibition zone of any one of ceftazidime, cefotaxime ceftriaxone, cefepime, cefpodoxime, ceftiofur, or aztreonam when combined with clavulanic acid or ticarcillin + clavulanic acid. Photo A: Antibiogram from a French horse *E.coli* isolate. Photo B: Antibiogram from a Brazilian horse *E.coli* isolate**.**

3.5.2 Evaluation of ESBL presence by Chromogenic medium

Bacteria that were resistant for disc-diffudion tests were also screened on ESBL Chomogenic agar (Probac®, Brazil). The typical appearance of microorganisms on agar were: *E.coli* ESBL was dark pink to reddish, *Klebsiella* was metallic blue and sensitive Gram negative strains were inhibited (Figure 2).

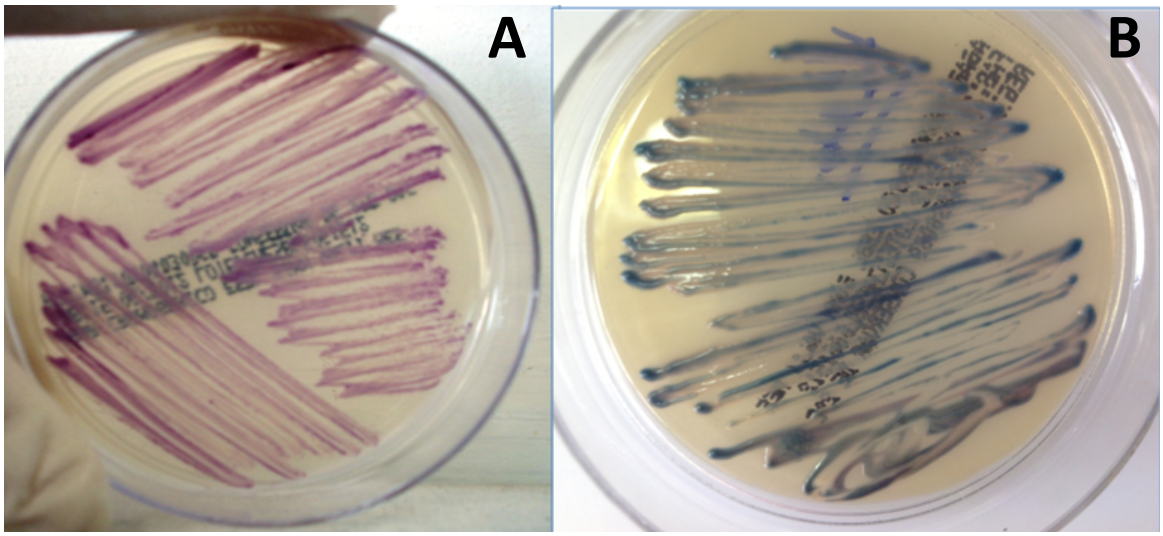

Figure 2 - ESBL-CHROM-agar showing different species of Enterobacteriacea. Photo A: ESBL *E.coli* colony, appears dark pink to reddish and photo B is a *Klebsiella* sp., in which appears metallic blue in this agar.

3.6 Phenotypic test for AmpC

Isolates exhibiting resistance to cefoxitin and 3rd generation cephalosporins, and susceptibility to clavulanate inhibition were screened for AmpC production.

The boronic acid (BA) test was performed by using antibiotic disks and BA+antibiotic disks (in-house) (ANVISA, 2013). In a few words, it was made a solution with: 240 mg of phenylboronic acid dissolved in 3 mL of dimethylsulphoxide and 3 mL of sterile distilled water. Ten microliter of the stock solution was dispensed onto disks containing cefoxitin and cefotan. The BA disk test was performed by inoculating Mueller-Hinton agar by the standard disk diffusion method and placing the disks containing antibiotics with and without BA onto the agar. After overnight incubation at 35° C, an organism with a zone diameter around the cefoxitin disk and BA ≥5 mm than the zone diameter around the disk containing CX alone was considered as an AmpC producer (Song et al., 2007).

For French samples it was used Mueller-Hinton agar supplemented (Oxoid, UK) with 200 µg/mL cloxacillin (cloxacillin is an inhibitor of AmpC enzymes) and cephalosporin antibiotics disks. Then, the susceptibility was evaluated.

Figure 3 describes the phenotypic characterizations and the followed techniques used in this study.

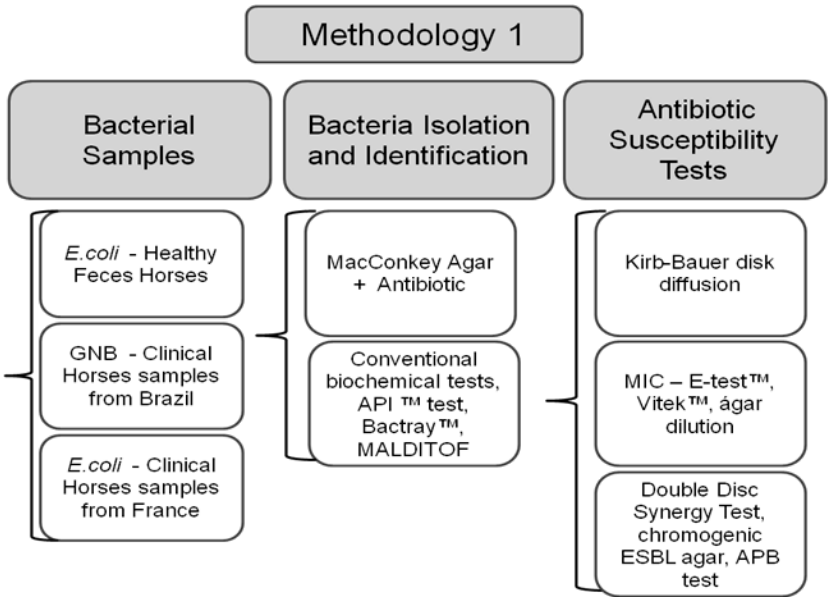

Figure 3 - Diagram with brief description of the phenotypic characterizations and the followed techniques used in this study.

3.7 DNA extraction and PCR

DNA extraction was based on Chapman (2001) protocol. A single colony of each isolate was inoculated from a Mac Conkey agar plate into 5 mL of LB broth (Oxoid, UK) and incubated overnight at 37 °C. From the broth, it was used 1.5 mL in an eppendorf and centrifugated at 3500 rpm for 5 min. After the supernatant was decanted, the pellet was resuspended in 100 µL of distilled water. The cells were lysed by heating at 100 °C for 10 min and quickly replaced in a freezer for 5 min. The cellular debris was removed by centrifugation at 11,000 rpm for 10 min. Supernatant (2 µL) was used as the DNA template source for amplification.

The PCR reactions were performed with a final volume of 25 µL. The primers used for PCR amplification are listed in appendix I. Each reaction contained: DNA (2 µL), MgCl2 (2.5 mM), dNTP (0.5mM – mix of each deoxynucleoside triphosphate), PCR buffer (Fermentas®, USA), Taq DNA polymerase (0.25 U - Fermentas®, USA), Mili-Q water, and 0.5 mM primers (appendix I). The PCR amplification cycle consisted of an initial denaturation step at 95 °C for 2 - 5 min, followed by 30 cycles of DNA denaturation at 95 °C for 45-60 sec, primer annealing at 50 – 62 °C for 45-60 sec, and primer extension at 72

°C for 1 min. After the last cycle, a final extension step at 72°C for 5 min was added. PCR product (5 µL) was analyzed by gel electrophoresis with 1% agarose. Gels were stained with Gel Red™ (Biotium, USA) and visualized by UV transillumination. A 100-bp DNA ladder (Fermentas, USA) was used as molecular ladder. Negative controls were PCR mix with water in place of template DNA. All reactions used positive DNA controls.

3.8 Detection of ESBL and plasmid-mediated AmpC beta-lactamase genes

Genotypic screening (PCR) for the ESBL resistance genes was performed for the following genes: *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$ (including *bla*$_{CTX-M}$ groups: *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2,}$ *bla*$_{CTX-M-8}$ and *bla*$_{CTX-M-15}$). For plasmidial AmpC detection was tested: CMY-2, FOX, MIR, ACT, DHA-1 and DHA-2. The positive samples for *bla* genes were confirmed by sequencing.

For French isolates, it was used a multiplex PCR reaction using primers for CTX-M-1, CTX-M-2 and CTX-M-9 (Dallene et al., 2010). PCR amplicons containing the *bla*$_{CTX-M-1}$ coding regions were sequenced using primers IS*Ecp1* and P2D.

All primers used are described on appendix I.

3.9 Detection of other resistance genes by PCR

3.9.1 Plasmid-mediated quinolone resistance (PMQR)

Isolates that were phenotypically resistant to at least two quinolone antimicrobials were tested for: *qnrA*, *qnrB, qnrC,_qnrD, qnrS, qepA*, *oqxA, oqxB* and *aac*(*6'*)-*Ib-cr*. The primers details are listed on appendix I.

3.9.2 16S ribosomal RNA methyltransferase

The isolates that were phenotypically resistant to amikacin, gentamicin and tobramycin (zone diameter ≤12 mm for gentamicin or ≤14 mm for amikacin) were tested the presence of 16S rRNA methylase gene allele. The PCR primers used were: ArmA, RmtA, RmtB, RmtC and RmtD (Doi, Arakawa, 2017). Other aminoglycoside-resistance genes were also tested, such as: *aad*A gene,

encoding resistance to streptomycin and spectinomycin; *aadB* gene, which confers resistance to kanamycin, gentamicin, and tobramycin and *aad*D, encoding tobramycin resistance.

3.10 *E.coli* Phylogenetic groups

Clermont research group (2000), developed a triplex PCR assay to detect the genes *chu*A, *yja*A, and *TspE4* and classified *E.coli* strains in groups. This study of phylogenetic analysis for *Escherichia coli* was basically composed of four main phylogenetic groups (A, B1, B2, and D) and the high virulent extra-intestinal strains mainly belong to groups B2 and D (Clermont, 2000). More recently, the same research group added an additional gene target, *arp*A, and now eight phylo-groups are described. It is possible to categorize an *E. coli* strain into A, B1, B2, C, D, E and F belonging to *E. coli* sensu stricto, whereas the eighth is the *Escherichia* cryptic clade I. (Clermont et al., 2013).

The distribution of phylogenetic groups amongst *E. coli* isolates from Brazil was determined by a recent method (Clermont et. al, 2013) and for French *E.coli* isolates, it was was used Clermont, 2000.

3.11 Clonal Relatedness of *E.coli*

3.11.1 E.coli Clonal Relatedness by ERIC-PCR – Brazilian isolates

ERIC-PCR method uses a combination of primers designed to find the conserved ERIC region (Enterobacterial repetitive intergenic consensus) in order to generate an electrophoretic banding pattern based on the frequency and orientation of ERIC sequences in a bacterial genome. Usually the sequences are short and highly conserved in Enterobacteriaceae (Meacham, 2003). This method is faster and cheaper than PFGE or multilocus sequencing typing (MLST) for generating information about the genetic relationship of bacterial strains. The primers used for the ERIC-PCR reaction were: Forward: 5'ATGTAAGCTCCTGGGGATTCAC3' and the Reverse: 5'AAGTAAGTGACTGGGGTGAGCG3'. The PCR product was run on a 2%

agarose gel and visualized under UV light. Gel images were captured and converted to file format (TIFF). Images were analyzed by BioNumerics/GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium).

3.11.2 E.coli Clonal Relatedness by PFGE – French isolates

Pulsed-field gel electrophoresis (PFGE) it is an important tool of genetic fingerprinting methods for *E. coli*. French *E.coli* isolates were submitted to genetic fingerprinting by PFGE according to the folling protocol.

Each isolate was grown for 18 h at 37 °C in Columbia Blood Agar. A bacterial suspension (100 uL of NaCL 0.9% - 4.8 McFarland) was prepared and centrifugated. After centrifugation at 13,000 rpm for 5 min at 4 °C, the bacterial pellet was washed with 45uL of TrisEDTA buffer. Equal volume of pre-warmed at 55 °C agarose (Clean Cut Agarose – BioRad, Hercules, CA, USA) was mixed and immediately, 23 uL from the mix, was placed into plug molds. After solidification, agarose plugs were incubated overnight at 37 °C in 1000 uL of the lysis buffer (10 mM Tris-Cl, 100 mM EDTA), and then it was added 1% N-lauroylsarcosine (Sigma) and 1 mg/mL of proteinase K (Sigma). Agarose blocks were then transferred to an eppendorf and washed three times with 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and kept for 1h at room temperature followed by three washes with the same buffer. The agarose plugs were then stored in TE buffer at 4 °C or directly used for digestion with XbaI restriction endonuclease (40–50 U of enzyme at 37 °C, overnight). After digestion, the agarose blocks were placed in an 1.0% agarose (Bio-Rad pulsed field certified agarose) gel. Running parameters for DNA electrophoresis were 24 hours at a constant voltage of 6 V/cm, with a pulse time of 10 seconds (initial switch time) and 60 seconds (final switch time), an electric field angle of 120 °, and a temperature of 14 °C. Completed gels were stained with ethidium bromide. PFGE gel was photographed under UV light using the Gel Doc XR+ documentation system (Bio-Rad). A Lambda Ladder Chef DNA size standard marker (Sigma) was included into each agarose run.

3.11.3 Computer Analysis of PFGE and ERIC Patterns

To perform the phylogenetic analyses of the pulsotypes, gel images were captured and the images were converted to tagged image file format (TIFF). TIFF files were analyzed with BioNumerics/GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium). The Dice correlation coefficients for band matching with a 1.0% position tolerance were grouped and the UPGMA clustering algorithm was used to depict the groups as a dendrogram.

3.11.4 Multilocus sequence typing (MLST)

MLST is a molecular technique that characterizes isolates of microbial species using the DNA sequences of internal fragments of multiple housekeeping genes. It has been considered the "gold standard" of typing, but it is traditionally performed in an expensive and time-consuming manner. This method takes advantage of the nucleotide sequences variations of these gene fragments. All the unique sequences for a given locus are assigned an allelic number (Larsen et al., 2012). The combination of the multiple loci numbers defines a sequence type (ST) number for each strain. All primers used in this section are listed on appendix I.

3.11.4.1 MLST for *E.coli*

E. coli MLST scheme used internal fragments of seven house-keeping genes: *adk* (adenylate kinase), *icd* (isocitrate/isopropylmalate dehydrogenase), *pur*A (adenylosuccinate dehydrogenase) *fum*C (fumarate hydratase), *gyr*B (DNA gyrase), *mdh* (malate dehydrogenase), and *rec*A (ATP/GTP binding motif) (Wirth et al., 2006).

For determination of the allelic profile and the sequence type (ST) number, protocols listed on the followed database were used: http://mlst.warwick.ac.uk/mlst/mlst/dbs/Ecoli/.

3.11.4.2 MLST for *K.pneumoniae*

Multilocus Sequence Typing for *Klebsiella pneumonia* was performed using the protocols listed on the Pasteur Institut website database: http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html .

The *K. pneumoniae* MLST scheme used internal fragments of the following seven housekeeping genes: *rpo*B (beta-subunit of RNA polymerase), *gap*A (glyceraldehyde 3-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *pho*E (phosphorine E), *inf*B (translation initiation factor 2), *ton*B (periplasmic energy transducer) (Brisse et al., 2009; Diancourt et al., 2005).

To obtain an allelic profile, the results of sequencing were blasted in the Pasteur MLST Database and then these tools were used to search for the MLST number.

3.11.4.3 MLST for *Pseudomonas aeruginosa*

The *P. aeruginosa* MLST scheme used seven house-keeping genes fragments: *acs*A (Acetyl coenzyme A synthetase), *aro*E (Shikimate dehydrogenase), *gua*A (GMP synthase), *mut*L (DNA mismatch repair protein), *nuo*D (NADH dehydrogenase I chain C, D), ppsA (Phosphoenolpyruvate synthase) and trpE (Anthralite synthetase component I). PCR amplicons were sequenced using specific primers.

Further details about this technique are available in Curran et al., work (2014). This group described a scheme to characterize a diverse collection of clinical and environmental isolates of *P. aeruginosa*. The sequences were analyzed on: http://pubmlst.org/paeruginosa/ .

3.12 PCR – Based Replicon Typing (PBRT)

It was described by Carattoli et al., (2005) and this method was created for identification and classification of Enterobacteriaceae plasmids in groups, according to replicons, especially those plasmids involved in antibiotic drug resistance.

The method used 3 simplex PCR reactions (Inc F, Inc B/O, and Inc K) and 5 multiplex PCR reaction recognizing A/C, FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N,

P, W, T, X, Y, and FIIA as the main incompatibility groups among Enterobacteriaceae family (Carattoli et al., 2005). The primers are listed in appendix I.

The PCR reaction was performed with: 1 µL of plasmid DNA or genomic DNA, 2.5 µl 10 x reaction buffer (with 15 mM MgCl2), 10mM of each dNTP, 25 µM of each primer pair (see appendix II for the primers list), 0.5U of Taq polymerase and made up to 25 µL with MiliQ water. All PCR amplifications were performed under the following conditions; 94 °C for 5 minutes, 30 cycles of 94 °C for 1 minute, annealing at 60 °C (52 °C for F simplex PCR) for 30 seconds, 72 °C for 1 minute and a final extension step at 72 °C for 5 minutes. Products were run on a 2 % agarose gel and visualized under UV light.

3.13 Plasmidial DNA extraction and conjugation

Plasmidial DNA was extracted using a commercial kit (Wizard Plus SV Minipres, Promega, USA) according to the manufacturer protocol. The conjugation was performed only for one sample (*E.coli* - sample 10E-ICB, from diseased horse) and it was performed in collaboration with Profa. Dra. Rosa Maria Silva (Medicine School, UNIFESP).

3.14 Genetic Environment

The genetic environment context of bla_{ESBL} was investigated by mapping the occurrence and linkage with mobile elements by PCR and then sequencing. The objective was to determine the bla_{ESBL} gene association or not with mobile elements. The primers sequences used in this study were based in previously reports of genes associated with the $bla_{\text{CTX-M group}}$, such as IS*Ecp1*, IS*26* or IS*CR1* (Fig. 4) and the primers are listed in appendix I.

The PCR amplified products were sequenced and the results were analyzed using BioEdit Sequence Aligment Editor.

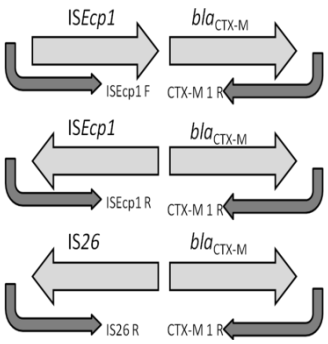

Figure 4 - IS*26* e IS*Ecp1* insertion sequences with *bla*$_{CTX-M-1}$ gene and the possible association with the gene. The dark arrows are the primers position used in this study. (adaptaded from Dropa, 2013).

3.15 Location of *bla*$_{CTX-M-1}$

To confirm location of *bla*$_{CTX-M-1}$ genes, DNA isolates were digested using S1 enzyme by pulsed-field gel electrophoresis (PFGE) and transferred to a membrane followed by hybridization with a specific probe which targeted the *bla*$_{CTX-M-1}$ and Inc group.

3.15.1 PFGE and Southern Blotting

The CTX-M-1 isolates were treated with S1-nuclease and subsequently performed the PFGE to detect plasmids and size.

Each isolate was grown for 18 h at 37 °C in Columbia Blood Agar and a bacterial suspension (1000 uL of NaCL 0.9% - 4.8 McFarland) was prepared and centrifugated. After centrifugation at 13,000 rpm for 5 min at 4 °C, the bacterial pellet was washed with 100 uL of TrisEDTA buffer.

Equal volume of pre-warmed at 55 °C agarose (Clean Cut Agarose – BioRad, Hercules, CA, USA), were mixed and, immediately, 23 uL from the mixer was placed into plug molds. After solidification, agarose plugs were incubated overnight at 37 °C in 1000 uL of the lysis buffer (10 mM Tris-Cl, 100 mM EDTA), 1% N-lauroylsarcosine (Sigma) and 1 mg/mL of proteinase K (Sigma).

Agarose plugs blocks were then transferred to an eppendorf and washed three times with 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and kept for 1h at room temperature followed by three washes with the same buffer. The

agarose plugs were then stored in TE buffer at 4 °C or directly used for digestion with S1 restriction endonuclease (80 U/uL of enzyme, buffer and water at 37 °C, for 30 minutes).

After digestion, the plugs blocks were placed in 1.0% agarose gel (Bio-Rad pulsed field certified agarose). PFGE was performed on a CHEF DRIII apparatus (Bio-Rad Laboratories, Munich, Germany) and running parameters were: 20 hours at a constant voltage of 6 V/cm, with a pulse time of one (1) second (initial switch time) and 30 seconds (final switch time), an electric field angle of 120 ° and a temperature of 14 °C.

Completed gels were stained with ethidium bromide. PFGE gel was photographed under UV light using the Gel Doc XR+ documentation system (Bio-Rad). A Lambda Ladder Chef DNA size standard marker (Sigma) was included into each agarose run. The size of the plasmids was identified by lambda phage PFGE-DNA marker (Sigma).

DNA information carried in the gel was transferred by Southern blot using Pierce™ Power Blotter (Thermo Scientific, USA). For each gel, two sheets of ~0.83mm thick Western blotting filter paper were used and one sheet of nitrocellulose membrane was cut to the same agarose gel size.

The membranes obtained were hybridized with specific probes for Inc L/M, HI1, HI2, I1 and F replicons and *bla*$_{\text{CTX-M-1}}$ gene, which were obtained by PCR amplification with the primers previously described (Carattoli et al., 2005; Dallene et al., 2010). DNA probes were digoxigenin-labeled, using the digoxigenin PCR DIG Probe Synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). Hybridization and detection were performed according to the manufacturer's instructions. The plasmid size was determined by comparison with linear DNA markers.

3.16 DNA purification and Sequencing

It was used a commercial kit for DNA purification (Quick Reference Card – illustra™ GFX and Gel Band Purification kit, GE Healthcare, UK). The purification was necessary for sending a better DNA sample for sequencing.

Sequencing was done by "Centro de Pesquisa sobre o Genoma Humano e Células-Tronco" (http://genoma.ib.usp.br/pt-br) in an ABI 3730 DNA analyzer (Life Technologies – Applied Biosystems). The samples were sent to this laboratory according to the company instructions. All results obtained were blasted in the National Center for Biotechnical Information genome database: (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Figure 5 summarizes all molecular processin this study.

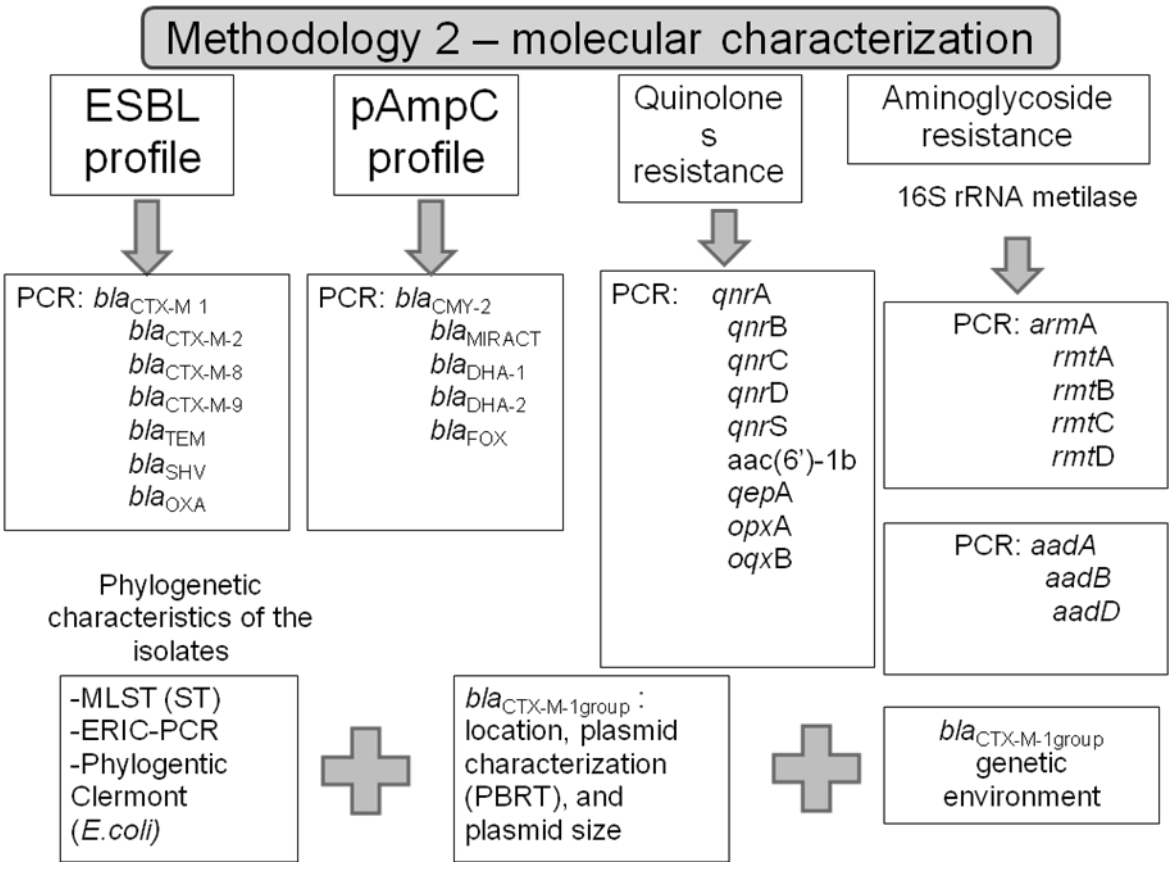

Figure 5 - An overview of molecular methods used in this study for characterization of gram-negative bacteria isolated from horses (Brazil and France).

4 RESULTS

4.1 Cephalosporin-resistant *E.coli* strains isolated from healthy horses, Brazil

During the study period (May, 2012/October, 2013), a total of 96 fecal samples were collected from apparently healthy horses. All animals included in this study have received one or more antibiotic therapies during their lifetime. Cephalosporin-resistant *E.coli* was isolated from 8 fecal samples (8.3% - 8/96) and six of those samples were multi-drug resistant (resistance to 3 or more antibiotic classes). After the cephalosporins, the antimicrobial disk-diffusion test showed high resistance rates among the isolates for sulfamethoxazole+ trimethoprim (87.5% - 7/8), followed by gentamicin (75% - 6/8) and nalidixic acid (37.5% - 3/8). No isolate was resistant either to imipenem or fosfomycin. All isolates were capable of growing in chromogenic ESBL (Fig. 6).

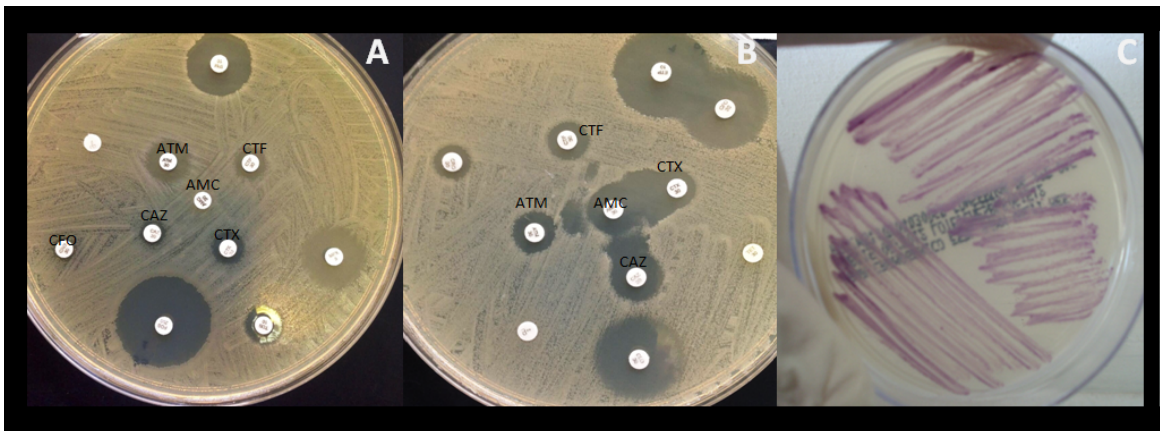

Figure 6 - Representative results of: A) Antibiogram of AmpC producers, enzymes can degrade cephalosporins and cephamycins and are not inhibited by AMC; B) DDSTs Antibiogram of *E.coli* ESBL producer. The phenotypic test was performed by using: CTX, CTF,CAZ and ATM; in combination with AMC. C) Typical colony appearance (violet) of *E.coli* ESBL plated on chromogenic ESBL agar. AMC, amoxicillin+clavulanate; CAZ, ceftazidime; CTX, cefotaxime; CFT, ceftiofur; ATM, aztreonam

The minimum inhibitory concentration was tested for 18 different antibiotics and all results are documented on table 2. No isolate showed resistance either to ertapenem, imipenem, meropenem, amikacin, tigecycline or colistin by Vitek™ method. All samples were resistant to: ceftiofur, by agar dilution; to cefoxitin, cephalotin and cefotaxime by Vitek™; and cefotaxime/clavulanate+cefotaxime by E-test™.

Table 2 - Minimum inhibitory concentration (MIC) of *E.coli* strains isolated from horses fecal samples in this study.

MIC - Minimum Inhibitory Concentration by E-test (µg/mL)																		
Strain	E-test®			Agar diluition	VITEK®													
	ENO	CIP	CT/CTL	CTR	AMP/SUL	PPT	CEF	CFO	CTX	CAZ	CPM	AZT	ERT	IMI	MER	AMI	GEN	TIG	COL
59	0,25	**≥32**	**≥16/0,032**	**2**	8	≥4	**≥64**	≥4	**≥64**	≥1	≥1	≥1	≥0,5	≥1	≥0,25	≥2	≥1	≥0,5	≥0,5
72	0,94	0,47	**12/1,0**	**32**	**≥32**	16	**≥64**	**≥64**	**8**	**16**	≥1	4	≥0,5	≥1	≥0,25	≥2	≥1	≥0,5	≥0,5
75	**2**	0,25	**≥16/1**	**8**	**≥32**	32	**≥64**	**≥64**	**32**	**≥64**	≥1	**16**	≥0,5	≥1	≥0,25	≥2	**≥16**	≥0,5	≥0,5
81	**3**	0,75	**≥16/0,094**	**32**	**≥32**	≥4	**≥64**	≥4	**16**	2	2	**≥64**	≥0,5	≥1	≥0,25	≥2	≥1	≥0,5	≥0,5
85	**1,5**	0,38	**≥16/1**	**8**	**≥32**	8	**≥64**	**≥64**	**8**	**16**	≥1	2	≥0,5	≥1	≥0,25	4	≥1	≥0,5	≥0,5
87	1	0,25	**≥16/0,032**	**2**	**≥32**	8	**≥64**	≥4	**8**	**≥64**	≥1	2	≥0,5	≥1	≥0,25	≥2	≥1	≥0,5	≥0,5
96[a]	**≥32**	**6**	**3/1,0**	**32**	**≥32**	32	**≥64**	**≥64**	**32**	**≥64**	≥1	**16**	≥0,5	≥1	≥0,25	≥2	**≥16**	≥0,5	≥0,5
96B	0,75	0,13	**≥16/1**	**16**	**≥32**	32	**≥64**	**≥64**	**32**	**≥64**	≥1	**16**	≥0,5	≥1	≥0,25	≥2	**≥16**	≥0,5	≥0,5

ENO, enrofloxacin; CIP, ciprofloxacin; CT/CTL: Cefotaxime/cefotaxime +clavulanate, CTR, ceftiofur, AMP/SUL, ampicillin/sulbactan; PPT, piperacillin/tazobactam; CEF, cephalotin; CFO, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; CPM, cefepime; AZT, aztreonam; ERT, ertapenem; IMI, Imipenem; MER, meropenem; AMI, amikacin; GEN, gentamicin; TIG, tigecyclin; COL, colistin. Results for resistant isolates are in boldface type.

The genetic characterization confirmed that all eight isolates were positive for *bla*$_{TEM-1}$, four were positive for pAmpC (*bla*$_{CMY-2}$) and one isolate was positive for *bla*$_{CTX-M-1}$ positive. Furthermore, four CMY-2 isolates carried aminoglycosides resistance genes (2 *aad*A and 2 isolates *aad*B) and only one isolate (sample 96A) showed plasmid-mediated quinolone resistance (gene *qnr*S).

Despite having a small numbers of samples, the phylogenetic group results indicated that the phylogroup B1 was the predominant group (n=3), followed by phylogroup A (n=2) and the other isolates belonged to the phylogroup B2 (n=1), D (n=1) and F (n=1). The only genotype by MLST determined was for *bla*$_{CTX-M-1}$ isolate (sample n°81 = ST1250). The main plasmid replicon type described were Inc HI1 and Inc I1 for CMY-2 isolates. CTX-M-1 isolate was positive for Inc F_{rep}, FIA and FIB. Table 3. shows the phenotypic and genotypic characteristics of all isolates.

Table 3 - Phenotypic and genotypic characteristics of *E.coli* strains isolated from feces of healthy horses from São Paulo.

Strain	Year	Antibiotic resistance profile*	Screening for ESBL and pAmpC		Resistant Genotype			Phylogenetic groups	ERIC-2 Profile	Replicon type
			DDT	APB	bla_{ESBL}	bla_{AmpC}	Other genes	(Clermont)		
59E	2012	AMC, CTX, CTF, CAZ, SUT,	+		TEM-1			B1	A	ND
72E	2012	AMC, CTX, CTF, CAZ, CFO, ATM, SUT	-	+	TEM-1B	CMY-2	*aadA*	A	B	HI1, I1
75E	2012	AMC, CTX, CTF, CAZ, CFO, SUT, GEN	-	+	TEM-1B	CMY-2	*aadA*	D	C	HI1, I1, P
81E	2013	AMC, CTX, CTF, CAZ, CFO, GEN, NAL	+	-	CTX-M-1		*qnr*A	B1	D	F_{rep}, FIA, FIB
85E	2013	AMC, CTX, CTF, CAZ, CFO, SUT, GEN, NAL, PEF	-	-	TEM-1B			F	E	ND
87E	2013	AMC, CTX, CTF, CAZ, SUT, GEN, NAL, PEF	+	-	TEM-1B			B1	F	ND
96EA	2013	AMC, CTX, CTF, CAZ, CFO, ATM, SUT, CIP, LVX, NOR, ERO, MOX, GEN	-	+	TEM-1B	CMY-2	aadB, *qnr*S	B2	G	HI1, I1
96EB	2013	AMC, CTX, CTF, CAZ, CFO, SUT, GEN, NOR	-	+	TEM-1B	CMY-2	aadB	A	H	HI1, I1

AMC,amoxicillin+clavulanate; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; CTF, ceftiofur; CFO, cephoxitin; CIP, ciprofloxacin; ERO, enrofloxacin; NOR, norfloxacin; NAL, nalidixic acid; OFL, ofloxacin; LEV, levofloxacin; MOX, moxifloxaci; SUT, trimethoprim-sulfamethoxazole; GEN, gentamicin. DDT:double-disk test; PB: phenil buronic test; ND: not determined.

The results demonstrated high genetic variability among the *E.coli* strains studied. The eight (n=8) isolates showed unique patterns. The ERIC–PCR profiles allowed separation of the isolates into 8 ERIC-types and the dendrogram of the phylogeny tree had two main cluster (I and II) (Figure 7).

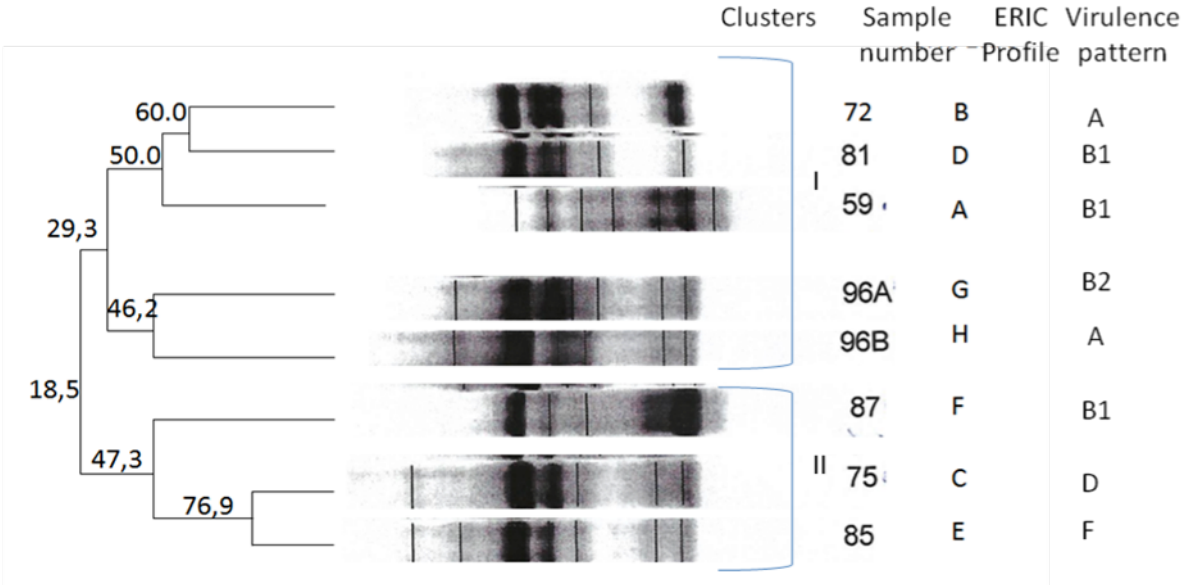

Figure 7 - Dendrogram from ERIC analysis of eight (*n* = 8) *E.coli* isolates from faecal healthy horses samples. The genetic similarity between isolates was determined and analyzed according to presence or absence of band on gel. It is constructed with the unweighted pair group method (UPGMA).

4.2 Multidrug-resistant gram-negative bacteria isolated from diseased horses, Brazil

From six clinical samples, nine multidrug-resistant gram-negative bacteria were phenotypically and genotypically characterized.

Case 1: In March, 2012. A 3-month-old foal English Thoroughbred (Figure 8A) from Curitiba, Parana, was admitted in a private veterinary clinic with signs of watery diarrhea, dehydration, colic and prostration. Treatment was initiated with intense fluids (lactated ringer solution), dexamethasone (0.2 mg/kg, intravenous, once a day), and gentamicin 3 mg/kg. Clinical signs did not improve and the animal died two days after admission. Organ samples were aseptically collected and *E.coli* was isolated from different organs (spleen, liver and lungs). One multi-resistant *E.coli* strain from the liver was sent to us for further microbiological investigation. Sample identification:10E – ICB.

Case 2: In May, 2013. An adult male Zangersheide horse was admitted at a veterinary teaching hospital from São Paulo and underwent surgery to repair an umbilical hernia. One day after the procedure, the animal had colic and underwent abdominal surgery. Postoperative abdominal wound infection was noted and antibiotic treatment with oral Streptomycin and topical antibiotic ointment (200 mg nitrofurazone) was initiated. A wound swab sample was collected and sent to us for culture. Three different multi-resistant bacteria were isolated: *Klebsiella pneumonia* (sample n° 101A), *Proteus mirabilis* (sample n° 101B) and *Pseudomonas aeruginosa* (sample n°101C). The animal survived after treatment.

Case 3: In May, 2013. An adult male Quarter horse was hospitalized at a veterinary teaching hospital from São Paulo with nasal fistula abscess with persistent drainage (Figure 8B). No history of previous treatment was available. Nasal swab sample was collected and sent to us for microbiological studies. It was isolated a multi-resistant *E.coli* (Sample n° 102). After treatment, the animal fully recovered.

Case 4: July, 2013. The case present here is about an adult mixed breed mare with keratitis hospitalized and treated in a teaching veterinary hospital from

Curitiba. A swab sample was collected by veterinarians (Figure 8C) and sent to a veterinary microbiology laboratory in Curitiba. The bacterium isolate in pure culture showed a resistant *P. aeruginosa* (sample n° 103 - ICB). The isolate was sent to us for further investigation. This patient had complications and died in few days.

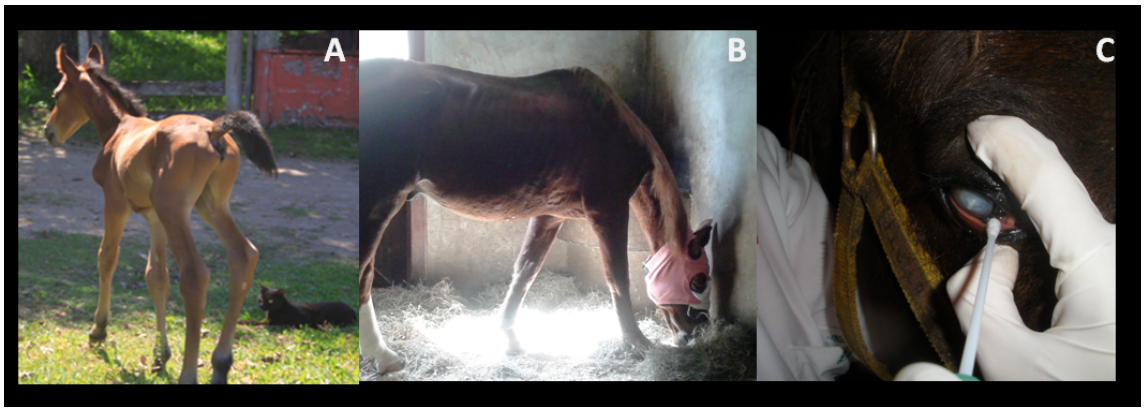

Figure 8 - Representative figure of different horses which participated in this study. A) foal with diarrhea B) Horse using a mask for nasal fistula protection. C) Horse with ulcerative keratitis being submitted to corneal scraping with a swab.

Case 5: October, 2013. A swab sample was collected from an open abscess in the caudal maxillary sinus from an adult male horse. It was a persistent lesion with fungus infection suspicion. The culture for fungus was negative but two gram-negative bacteria were isolated. The bacteria were sent to us and a very resistant *P. aeruginosa* and *Serratia marcescens* were isolated (Samples n° 104A – ICB and 104B – ICB respectively). Animal had a good recovery with antibiotics treatment.

Case 6: January, 2014. Male mix bred foal had limb fracture and was treated surgically. The animal had bone infection and another surgery was performed. The veterinary surgeons performed a curettage of the bone and the bone tissue was sent to us. The animal received a combination of antibiotics (gentamicin and ceftiofur) and had a good recovery. It was isolated *E.coli* and the sample was named 105-ICB.

Table 4 summarized all clinical information, animal characteristics and the bacteria isolated.

Table 4 - Clinical and epidemiological data of multi-resistant Gram -negative strains isolated from horses infections in this study

Clinical case	Strain Identification	Source	Breeds	Outcome	Year/State	Isolated bacteria
1	10E- ICB	Foal/ liver	Thoroughbred	Death	2012/PR[a]	*Escherichia coli*
2	101A- ICB	Horse / abdominal surgicalwound	Zangersheide	Survived	2013/SP[b]	*Klebsiella pneumoniae*
	101B- ICB				2013/SP	*Proteus mirabilis*
	101C- ICB				2013/SP	*Pseudomonas aeruginosa*
3	102- ICB	Horse/ Facial fistula , nasal abscess	Quarter Horse	Survived	2013/SP	*Escherichia coli*
4	103- ICB	Mare / corneal ulcer	mixed breed	Death	2013/PR	*P. aeruginosa*
5	104A- ICB	Horse / abscess in the maxillary sinus	Brasileiro de Hipismo	Survived	2013/SP	*P. aeruginosa*
	104B- ICB				2013/SP	*Serratia marcescens*
6	105– ICB	Foal/bone tissue	mixed breed	Survived	2014/SP	*Escherichia coli*

a: Paraná State, b: São Paulo State.

All isolates were *in vitro* susceptible to fosfomycin, tigecycline, imipenem, meropenem and ertapenem by disk-diffusion. Among nine strains, six isolates (67%) were resistant to all quinolone tested. Only one isolate (101A) was susceptible for choramphenicol and all isolates were resistant to sulphamethoxazole and trimethoprim. ESBL production was detected by DDT in all Enterobacteriaceae tested. All isolates were highly resistant to the cephalosporins tested and grew on ESBL chromogenic agar (Table 5).

Table 5 - Antibiotic susceptibility and resistance patterns for gram-negative bacteria isolated from horses with different diseases in this study .

Strain	Bacteria	Resistance phenotype	Susceptibility phenotype	DDT[#]	ESBL Chromogenic Agar Colony color
10	*Escherichia coli*	AMC, CTX, CAZ, ATM, CTF, CFO, ETP, CIP, ERO, NOR, NAL, GAT, OFL, LEV, MOX, SUT, GEN, TOB, NEO, KAN, AMI, CLO	ERT, TIG, MER, IMI,FOS	Ghost zone	Purple
101A	*Klebsiella pneumonia*	AMC, CTX, CAZ, ATM, CTF, CFO, ETP, CIP, ERO, NOR, NAL, GAT, OFL, LEV, MOX, SUT, GEN, TOB, NEO, KAN, AMI	ERT, TIG, MER, IMI, CLO, FOS	Ghost zone	Blue
101B	*Proteus mirabilis*	AMC,CTX,CAZ,ATM,CTF,CFO,ETP,CIP,ERO,NAL,GAT,MOX,SUT, GEN, TOB,NEO, KAN,AMI,CLO	ERT, TIG, MER, IMI, NOR, LEV, OFL,FOS	Ghost zone	Beige
101C	*Pseudomonas aeruginosa*	AMC,CTX,CAZ,ATM,CTF,CFO,ETP,CIP, ERO, NOR, NAL,GAT,OFL,LEV,MOX,SUT,GEN,TOB,NEO,KAN,AMI,CLO,TIG	PPT, ERT, MER,IMI, FOS	_	Metalic
102	*Escherichia coli*	AMC, CTX, CAZ, ATM, CTF, CFO, ETP, CIP, NAL, MOX, SUT, GEN, TOB, NEO, KAN, AMI, CLO	ERT, TIG, MER, IMI, ERO, NOR, LEV, GAT, STR, FOS	Ghost zone	Purple
103	*Pseudomonas aeruginosa*	AMC, CTX, CAZ, ATM, CTF, CFO, ETP, CIP, ERO, NOR, NAL, GAT, OFL, LEV, MOX, SUT, GEN, TOB, NEO, KAN, AMI, CLO, SUT, TIG	MER,IMI, FOS, POL, TIC, PPT, CPM	_	Metalic
104A	*Pseudomonas aeruginosa*	AMC, CTX, CAZ, ATM, CTF, CFO, ETP, CIP, ERO, NOR, NAL, GAT, OFL, LEV, MOX, SUT, GEN, TOB, NEO, KAN, AMI, CLO, SUT, FOS	PPT, IMI, MER, FOS, CPM	_	Metalic
104B	*Serratia marcescens*	CTF, CAZ, CFO, CPM, AZT, TET, NET, GEN, KAN, TOB, STR, NEO, AMI	CIP, ERO, NOR, LVX, MOX, PFL, PPT, IMI, MER, CPM,FOS, IMI, MER, TIG, ERT, FOS	Ghost zone	Cream
105	*Escherichia coli*	AMC, CTX, CAZ, ATM, CTF, CFO, ETP, CIP, ERO, NOR, NAL, GAT, OFL, LEV, MOX, SUT, GEN, TOB, NEO, KAN, AMI, CLO, SUT	ERT, TIG, MER, IMI,FOS, PPT	Ghost zone	Purple

: Double-disc test , A: MER, meropenen ; IMI , iminpenen ; TIG , tigecycline ; AMC, clavulanate / amoxicillin ; CAZ , ceftazidime ; CTX , cefotaxime ; ATM, aztreonam ; CTF , ceftiofur ; CFO , cefoxitin ; ETP , ertapenen ; CIP , ciprofloxacin ; ERO , enrofloxacin ; NOR , norfloxacin ; NAL , ac nalidixic ; TAO , Gatifloxacin ; OFL , ofloxacin ; LEV, levofloxacin ; MOX , moxifloxacin ; SUT , trimethoprim - sulfamethoxazole ; GEN , gentamicin ; CPM , cefepime ; TOB , tobramycin ; Neo , neomycin ; KAN , kanamycin ; AMI , amikacin ; STR , streptomycin; CLO , chloramphenicol .; PPT pipetazobactam ; TIC , ticarcillin ; ERT , ertapenem ; POL , polymyxin .

Minimum inhibitory concentration was performed by E-test strips for 15 antibiotics and agar dilution method was used for ceftiofur. Table 6 summarizes the MIC (µg/ml) results to sixteen antibiotics tested for all strains of Gram-negative. Full susceptibility was noted for iminpenem, meropenem and ertapenem. Eight isolates presented MIC values that demonstrated resistance to ciprofloxacin and enrofloxacin (sample 104B - *S. marcescens* was susceptible). Sample number 101B (*Proteus mirabilis*) was the only isolate susceptible to aztreonam and ceftazidime. Sample number 101A (*K. pneumoniae*) was the only bacteria resistant to piperacillin/tazobactam and sample number 102 (*E.coli*) was the only one susceptible to streptomycin. Full resistance was noted in all other antibiotics tested.

Table 6 - Minimum inhibitory concentration of Gram-negative multi-resistant bacteria isolated from horses clinical cases in this study.

	Minimum Inhibitory Concentration by E-test (µg/mL)															Agar Dilution
Strain	ENO	CIP	CTX	CPM	AZT	IMI	MER	ERT	PPT	SUL	STR	GEN	POL	TZ/TZL	CT/CTL	CFT
10	**≥32**	**≥32**	**≥32**	2	**≥64**	0,19	0,19	0,01	4	**≥32**	**≥1024**	**≥1024**	0,38	NT	≥16/0.03	**≥64**
101[a]	**≥32**	**≥32**	**≥32**	NT	**≥256**	0,38	0,06	0,19	**256**	**≥32**	**≥1024**	**64**	1,5	**32/4**	≥16/0.06	**≥64**
101B	**≥32**	**≥32**	**≥32**	NT	0,5	3	0,06	0,01	0,19	**≥32**	**≥1024**	**512**	NT	0,75/0,4	≥16/0.05	**32**
101C	**≥32**	**≥32**	NT	**12**	**16**	1	4	NT	16	**≥32**	**≥1024**	**≥1024**	1,5	**32/4**	NT	NT
102	**2**	**2**	**≥32**	NT	**128**	0,38	0,06	0,09	16	**≥32**	3,84	**≥1024**	4	**32/2**	≥16/0.1	**32**
103	**≥32**	**≥32**	NT	**64**	**12**	1,5	4	NT	12	**≥32**	**≥1024**	**≥1024**	8	**2/ ≤4**	NT	NT
104[a]	**≥32**	**≥32**	NT	**12**	**16**	0,75	3	NT	24	**≥32**	**≥1024**	**≥1024**	8	**3/ ≤4**	NT	NT
104B	0,5	0,4	NT	NT	**≥64**	0,5	0,25	0,2	1,5	**≥32**	**≥1024**	**256**	**1024**	**32/4**	NT	**≥64**
105	**≥32**	**≥32**	**≥32**	NT	**≥64**	0,5	0,25	0,2	8	**≥32**	**≥1024**	**256**	4	**32/4**	≥16/0.05	**≥64**

ATB: antibiotic; NT : not tested ; ND : not determined ENO , enrofloxacin ; CIP , ciprofloxacin, CTX , ceftriaxone ; CPM , cefepime ; AZT , aztreonam ; IMI , imipenem ; MER, meropenem ; ERT , ertapenem ; PPT, piperacillin / tazobactam ; SUL, sulfa - trimethoprim ; STR , streptomycin, GEN , gentamicin ; POL , polymyxin ; TZ / TZL , ceftazidime , ceftazidime + tazobactam ; CT / CTL , cefotaxime and clavulanic acid; CFT , ceftiofur . Resistant patterns are in boldface type.

The molecular investigations underlying resistance mechanisms confirmed by resistance phenotypes were based on the following genes (Table 7): cephalosporins (*bla*$_{\text{TEM1-like}}$ and *bla*$_{\text{CTX –M-1-group}}$ genes), aminoglycosides (aadA, *aad*B, *aad*D and *rmt*D genes), and quinolones (*oqxA, oqxB, qnrA* and *aac(6')-Ib-CR* genes).

Further analysis of the *bla*$_{\text{CTX-M}}$ gene sequences indicated that 4 of the 6 Enterobacteriacea isolates detected belonged to the variant CTX-M-15 and 2 (*Proteus mirabilis* – 101B, and *Serratia marcesnces* – 104B) belonged to CTX-M-1. In the case of the *bla*$_{\text{TEM}}$ molecular studies, the analysis indicated that all Enterobacteriacea isolates harbored the *bla*$_{\text{TEM-1}}$ and those results were confirmed by sequencing. The gene *bla*$_{\text{SHV}}$ was detected only in one sample (101A- *K.pneumoniae*).

ERIC-PCR was performed for the 3 *E.coli* and the 3 *P. aeruginosa.* The analysis showed three different clusters for *E.coli* and 2 clusters for *P.aeruginosa* tested. The sequence type numbers (ST) for *E.coli* were: sample10-ICB ST 2179; sample 102-ICB ST 1081; and sample 105-ICB ST 5140. For *K.pneumoniae* (sample 101A) the ST number was ST 101. For *P. aeruginosa* the sequence type numbers were: sample 101C ST 304; sample 103 ST 622; and sample 104A ST 304. *E. coli* Phylogenetic group analysis showed that group A was positive for two *E.coli* sample (n=2) and B1 (n=1) appeared in one sample (105-ICB). The genotypic characterization cited here is shown in Table 7.

Table 7 - Gram-negative bacteria genotypic characteristics isolated from horse clinical cases in this study, Brazil.

Strain	Bacteria	Plasmid-mediated resistance Gene(s)			Others genes	MLST (ST)	*E. coli*	
		Bla$_{\text{-type}}$	16S rRNA methylase	PMQR			O25	Clermont (2013)
10	*Escherichia coli#*	TEM-1A,CTX-M-15 (KC618390)*	*rmtD1* (KC593856)*	*aac(6´)-Ib-CR (KC593857) **	*aadD, aadB*	2179	-	A
101A	*Klebsiella pneumoniae*	TEM -1B,CTX-M-15,SHV		*aac(6´)-Ib-CR, oqxA, oqxB*	*aadA, aadD*	101	NA	NA
101B	*Proteus mirabilis*	TEM-1B, CTX-M-1		*qrnA*	*aadA, aadD*	NA	NA	NA
101C	*Pseudomonas aeruginosa*	ND		*aac(6´)-Ib-CR*	*aadA, aadB*	304	NA	NA
102	*E.coli*	TEM-1B,CTX-M-15		*aac(6´)-Ib-CR, qnrA*	*aadA, aadD*	1081	+	A
103	*P.aeruginosa*	ND		*aac(6´)-Ib-CR*		622	NA	NA
104A	*P.aeruginosa*	ND		*aac(6´)-Ib-CR*	*aadB*	304	NA	NA
104B	*Serratia marscens*	TEM-1B, CTX-M-1				NA	NA	NA
105	*E.coli*	TEM-1B, CTX-M-15		*aac(6´)-Ib-CR, qnr*A	*aadA*	5140	-	B1

ND: not determined, NA: not aplicable. MLST: *multilocus sequence type.**: GenBank number. *Ec: Escherichia coli*, *Pa: Pseudmonas aeruginosa.#Case already published: Leigue et al., 2015.* PMQR, Plasmid-Mediated Quinolone Resistance ; MLST, *multi-locus sequence type*.

The plasmid replicon typing of isolates revealed significant plasmid diversity. Amongst Enterobacteriaceae isolates, the most common plasmid incompatibility group detected was FII (n=3) and L/M (n=3) followed by HI2 (n=2) , FIA, FIB, R, A/C and Y (n=1). Each isolate contained between three and five distinct large plasmids. Sample 101B (*Proteus mirabilis*) had no visible plasmid using S1 restriction enzyme. Plasmids of variable sizes were detected and the PFGE/S1 procedure indicated plasmids with an estimate size (by means of plasmid size standards) between 96–330 kb. Southern blot was used to link CTX-M-1 or CTX-M-15 gene and replicon type with specific plasmids on the S1/PFGE gel. *E.coli* CTX-M-15 location was detected on a Inc FIA with 96kb and Inc HI2 (one sample 330kb and other with 291kb). *Klebisella pneumoniae* CTX-M-15 was harbored into a 194kb plasmid but, after several attempts, the Inc group was not determined. CTX-M-1 *Serratia marcescens* was located on a 97kb Inc L/M plasmid. Table 8 summarizes the plasmid characteristics studied.

Table 8 - Plasmid characteristics and content of ESBL isolates.

Strain	Bacteria	Number of plasmid detected by S1-PFGE and range sizes	Replicon types detected (Inc)	*Bla*$_{\text{CTX-M-1/15}}$ Plasmid Size (Kb)	*Bla*$_{\text{CTX-M-1/15}}$ Plasmid location
10-ICB	*E.coli*	3 plasmids ranged from ≈150kb-97	Frep, FIA, FIB, L/M	≈96	Inc FIA
101A-ICB	*K.pneumoniae*	3 plasmids ranged from ≈194kb - 50kb	L/M, R, FII	≈194	ND
101B-ICB	*P.mirabilis*	None visible plasmids on PFGE analysis using S1 enzyme	ND	ND	ND
102-ICB	*E.coli*	5 plasmids ranged from ≈300kb -70kb	FII, HI2	≈330	Inc HI2
104B-ICB	*Serratia marcescens*	3 plasmids ranged from ≈338kb - 96kb	L/M, A/C	≈97	Inc L/M
105-ICB	*E.coli*	3 plasmids ranged from ≈291kb - 97kb	FII, Y, HI2	≈291	Inc HI2

ND: not determined; Inc: incompatibility group; IS: insertion sequence; kb: kilobases; ≈approximately

Different elements can be involved in the mobilization and expression of *bla*$_{CTX-M}$ genes. In this study, for all CTX-M-15 isolates, the insertion sequence IS*Ecp1* was located upstream of the end of the *bla*$_{CTX-M-15}$ gene (Figure 9). In one isolate (sample 10-ICB) the *bla*$_{CTX-M-15}$ gene was flanked upstream by two complete insertion sequences [IS*Ecp1* (1177bp) and IS*26* (820pb)]. Sample 105-ICB had two sequences of IS*Ecp*1 elements, but they were located in opposite orientations.

The *bla*$_{CTX-M-1}$ genes were upstream associated with the common insertion sequences IS26. Upstream of the *bla*$_{CTX-M-1}$ gene, 101B strain, it was harbored a CR from the complex Class 1 integron. All isolates presented ORF 477 downstream of the *bla*$_{CTX-M}$ gene.

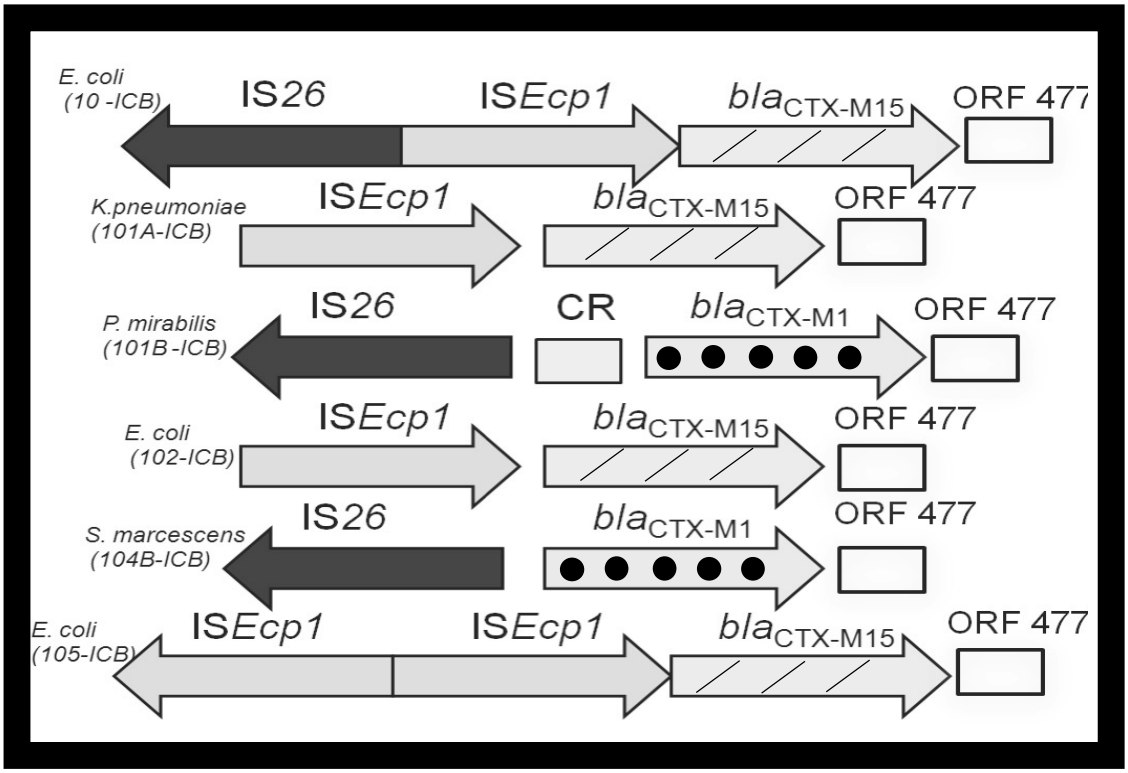

Figure 9 - Schematic presentation of the flanking gene regions of the *bla*$_{CTX-M}$ gene. The genetic location of IS*Ecp1* is upstream of *bla*$_{CTX-M-15}$ and IS*26* is upstream of *bla*$_{CTX-M-1}$. The arrows indicate the direction of transcription.

4.3 Cephalosporin-resistant *E.coli* strains isolated from diseased horses, France

A total of forty-five (n = 45) *E.coli* strains isolated from horses with clinical infections were assessed for antibiotic resistance investigations. These isolates belong to a routine monitoring program on antimicrobial resistance in animals (Resapath), from France. The isolates were previously identified as *E.coli,* collected between 2014/2015 and had a previous high cephalosporin resistance tests. The double-disk test (screening test), 36 isolates had ESBL profile, six had ampC suspect profile, and three samples were negative for growth. The clinical origin of isolates was diverse but endometrium (n=14), skin/mucous (n=6), respiratory (n=6) and renal (n=3) samples were the most common (Table 9).

The phylogenetic analyses showed that isolates belonging to phylogenetic group B1 were found to be the most abundant in the collection (n = 28, 68.3%), followed by group A (n = 10, 24.2%). Five percent (n = 2) of the isolates were grouped in the B2 group, associated with extraintestinal virulence, and only one isolate (2.4%) was identified in D, which is also associated with a pathogenic group.

Genomic DNA from each isolate was analyzed by PFGE after XbaI digestion and it showed patterns of 36 isolates, indicating 35 distinct *E.coli,* comprised in twelve distinct PFGE profiles, designated C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, and C12 (Figure 10 and Table 9). Two isolates had an indistinguishable PFGE pattern (40317 and 40321) and therefore were considered to represent the same strain; and two isolates (40319 and 40334) were considered to be closely related, as these PFGE patterns differed by only two different bands. The isolate 38645 was shown to be genotypically unrelated to any of the other strains, as its PFGE pattern differs considerably from the PFGE patterns of other strains. Some isolates (ID: 39578, 39581, 39582, and 40314) could not be typed by PFGE despite repeated attempts.

Table 9 - Characteristics of 45 cephalosporin resistant *E.coli* recovered from clinical horse samples between 2014/2015, France.

Sample ID	Isolation year	Disease	MH Phenotype	Phylogroup	PFGE XbaI
38645	2014	Kidney	ESBL	B1	C12
38646	2014	Kidney	ESBL	B1	C11
38647	2014	Kidney	ampC	B1	NT
39574	2014	Unknow	ESBL	A	C3
39576	2014	Respiratory	ESBL	B1	C6
39577	2014	Skin/Mucous	ESBL	B1	C9
39578	2015	Unknow	ESBL	B1	ND
39580	2015	Unknow	ESBL	B1	C6
39581	2015	Respiratory	ESBL	B1	ND
39582	2015	Endometrium	ESBL	B1	ND
39583	2015	Endometrium	NEG	NT	NT
39775	2015	Endometrium	ESBL	B1	C5
39785	2015	Endometrium	ampC	B1	C10
39795	2015	Endometrium	ESBL	A	C8
39798	2015	Ceccum	ESBL	A	C7
39799	2015	Placenta	NEG	NT	NT
39807	2015	Endometrium	NEG	NT	NT
40066	2015	Sinus	ESBL	B1	C5
40067	2015	Unknow	ampC	NT	NT
40314	2015	Respiratory	ESBL	A	ND
40315	2015	Endometrium	ESBL	B1	C7
40316	2015	Endometrium	ESBL	B1	C11
40317	2015	Endometrium	ampC	B1	C5
40319	2015	Umbilical	ESBL	B1	C6
40320	2015	Feces	ampC	B1	C7
40321	2015	Endometrium	ampC	B1	C5
40322	2015	Endometrium	ESBL	B1	C5
40323	2015	Endometrium	ESBL	A	C1
40327	2015	Endometrium	ESBL	B2	C9
40328	2015	Endometrium	ESBL	A	C11
40329	2015	Skin/Mucous	ESBL	A	C3
40330	2015	Urine	ESBL	A	C8
40331	2015	Respiratory	ESBL	D	C7
40334	2015	Unknow	ESBL	B1	C6
40335	2015	Urine	ESBL	B1	C9
40893	2015	Unknow	ESBL	B1	C3
40894	2015	Digestive	ESBL	B1	C1
40895	2015	Unknow	ESBL	A	C11
40896	2015	Digestive	ESBL	B1	C2
40897	2015	Unknow	ESBL	B1	C4
40898	2015	Skin/Mucous	ESBL	B2	C6
40899	2015	Skin/Mucous	ESBL	B1	C5
40900	2015	Skin/Mucous	ESBL	B1	C2
40901	2015	Respiratory	ESBL	B1	C4
40902	2015	Skin/Mucous	ESBL	A	C1

MH, Mueller Hinton agar; ampC, CMY-type β-lactamase; ESBL, extended-spectrum beta-lactamse; NT, not tested; ND, not determined; NEG, negative; C, cluster; PFGE: plused-field

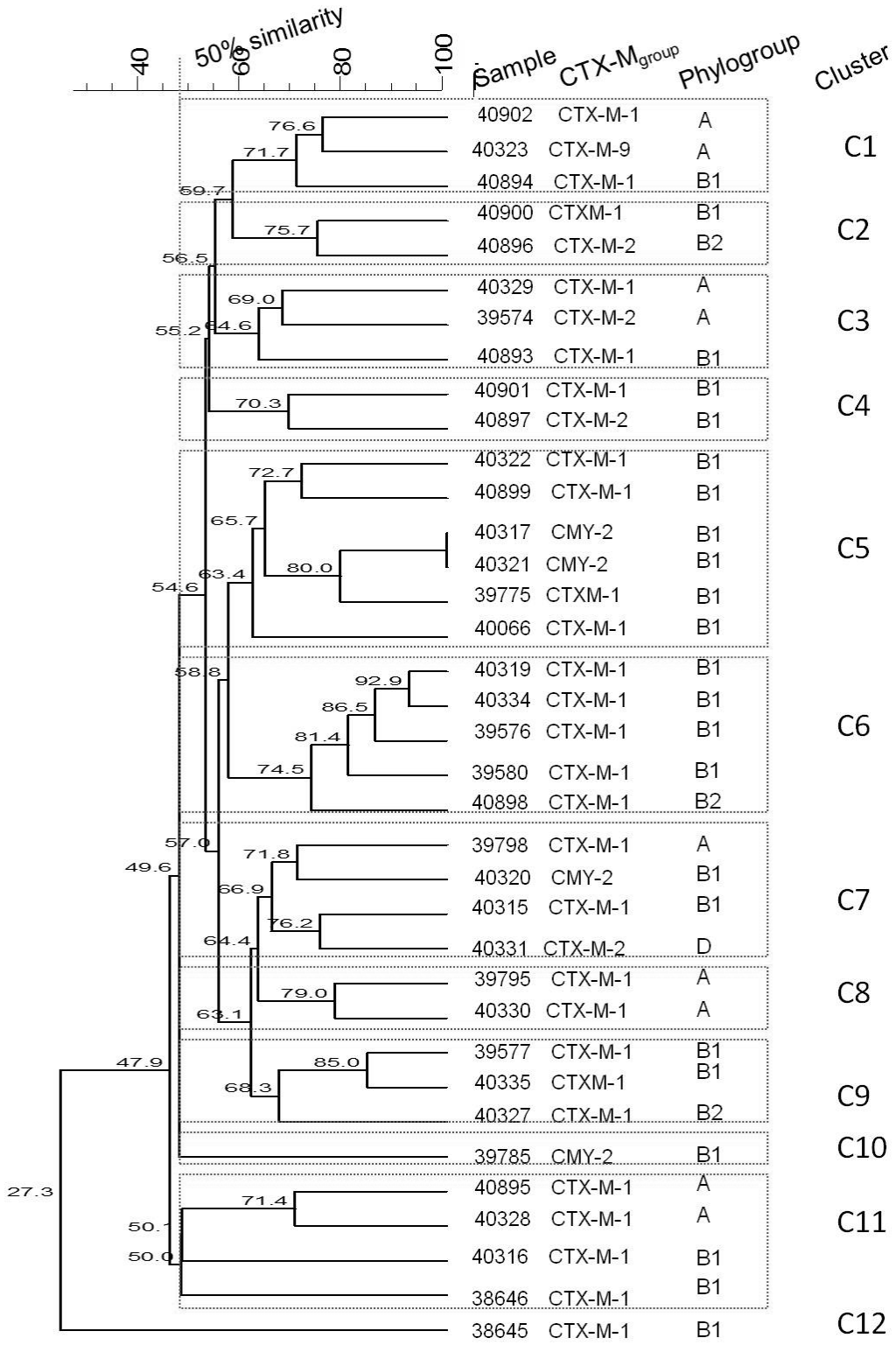

Figure 10 - PFGE – Dendrogram of 36 cephalosporins-resistant equine clinical *E. coli* isolates from France based on XbaI-generated PFGE profiles. According to a similarity index of > 50%, *E.coli* isolates were separated into 12 PFGE clusters (C1-C12).

The antibiotic susceptibility profiles to a panel of thirty-two antibiotics showed that all isolates tested were in vitro susceptible to: imipenem, florfenicol, amikacin, apramycin, pippetacillin/tazobactam and colistine. The highest frequency of resistance (90% or more) was recorded for amoxicillin (97.8%), ticarcillin (95.6%), trimethoprim-sulfamethoxazole (95.5%), cephalotin (93.3%), cefuroxime (93.3%), ceftiofur (91.1%), and piperacillin (91.1%) (Table 10). Forty-one isolates (41/45) were considered multidrug-resistant (resistance to three or more different classes of antibiotics). Zone diameter for each isolate and the individual susceptibility/resistant pattern are displayed in Table 11.

Table 10 – Susceptibility to different antimicrobial drugs of cephalosporins-resistant E.coli from equine clinical samples, France

Antimicrobial tested	Number of resistant isolates (n)	Percentage of resistant isolates (%)
AM	44	97.8
TIC	43	95.6
SU	43	95.6
CFL	42	93.3
CFU	42	93.3
CTF	41	91.1
PI	41	91.1
ST	38	84.4
TT	34	75.6
CEQ	33	73.3
GE	33	73.3
CTX	32	71.1
TCL	29	64.4
AZ	27	60.0
CL	25	55.6
CPM	23	51.1
KA	23	51.1
NA	21	46.7
ER	20	44.4
OF	20	44.4
CAZ	15	33.3
TO	15	33.3
AC	12	26.7
CFO	7	15.6
NE	1	2.2
PIT	0	0.0
IM	0	0.0
AM	0	0.0
AP	0	0.0
FL	0	0.0
C	0	0.0

AM, Amoxicillin; AC, amoxicillin-clavulanate; CFL,cephalothin; CFU, cefuroxime; CTX, cefotaxime; CTF,ceftiofur; CFO, cefoxitin; CAZ, ceftadizime; CPM,cefepime; CEQ, cefquinome; PP, piperacillin; TIC, ticarcillin; PT, Piperacillin/tazobactam;TCL, Ticarcillin/ clavulanate, AZ, aztreonam; IM, Imipenem;TT, Tetracycline;CL chloramphenicol;FL florfenicol;GE Gentamicin;AM amikacin; TO,tobramycin;ST streptomycin ;KA kanamycin; AP apramycin; CO colistin, SU/TR,Trimethoprim-sulfamethoxazole; NA nalidixic acid ; ER enrofloxacin; OF ofloxacin.

Table 11 - Zone diameter (mm) and antimicrobial susceptibility profile in *E. coli* isolated from clinical horse samples, France.

Sample	AM	AC	CFL	CF	CTX	CTF	PP	TC	PT	TCL	IM	CAZ	CFO	CPM	AZ	CEQ	ST	KA	AM	AP	GE	TO	NE	CL	FL	TT	CO	SU	TR	NA	ER	OF
38645	6	19	6	6	12	9	6	6	27	20	33	23	24	16	17	14	6	6	26	21	6	10	16	6	26	6	19	6	6	6	6	6
38646	6	27	6	6	18	15	12	6	32	26	36	30	27	22	27	19	6	6	25	20	8	13	18	6	30	6	19	6	6	24	38	37
38647	6	9	6	9	17	14	12	6	21	11	28	12	6	30	19	26	6	22	24	19	24	23	29	6	27	6	19	6	6	6	6	6
39574	6	19	6	6	12	6	6	6	27	15	34	21	26	16	13	12	9	21	26	21	11	17	22	25	26	6	20	6	6	22	33	31
39576	6	8	6	10	16	15	13	6	23	15	32	13	6	32	21	30	14	18	24	18	6	12	22	10	22	25	20	6	6	6	6	6
39577	6	16	6	6	14	13	8	6	30	12	35	28	25	19	19	15	6	6	28	21	10	16	22	9	31	6	20	6	6	6	6	10
39578	6	23	6	6	13	12	8	6	29	22	35	22	26	24	22	19	17	21	27	20	10	16	21	9	22	22	19	6	6	6	6	6
39580	6	23	6	6	18	15	10	6	29	23	36	28	26	24	22	20	6	6	26	20	6	12	21	25	27	6	20	6	6	6	6	6
39581	6	18	6	6	9	10	6	6	25	19	30	16	22	18	12	14	8	6	27	21	10	15	21	6	30	6	20	6	6	25	34	32
39582	6	25	6	6	10	9	6	6	28	24	31	17	25	17	12	13	6	22	25	20	25	23	29	26	28	6	19	11	6	15	18	18
39583	27	29	23	26	33	32	31	36	34	29	30	30	27	27	38	25	26	26	29	24	29	19	33	29	29	6	21	24	26	25	37	35
39775	6	18	6	6	12	11	8	6	28	22	31	21	29	20	14	16	6	6	26	23	11	18	24	26	26	27	22	6	6	14	32	31
39785	6	8	6	10	18	14	13	6	24	17	31	14	9	31	18	29	6	24	28	22	28	25	30	24	25	25	21	6	6	23	28	28
39795	6	21	6	6	19	14	9	6	29	23	34	28	27	22	27	18	7	24	26	22	27	24	30	23	25	6	20	6	6	19	33	33
39798	6	23	6	6	14	11	11	6	32	23	34	28	28	19	17	9	8	6	26	20	9	12	15	6	30	6	21	6	6	23	34	34
39799	11	28	24	29	40	34	21	6	34	33	40	38	31	40	40	33	20	17	29	22	20	19	33	27	28	25	21	6	6	27	39	37
39807	8	13	6	12	26	24	21	24	24	21	32	21	13	34	24	32	23	24	27	22	28	25	31	26	25	24	21	24	32	6	22	21
40066	6	22	6	6	16	13	25	6	25	22	30	26	26	22	21	18	6	19	26	20	9	15	20	6	27	6	20	6	6	22	32	32
40067	6	22	19	24	34	29	15	6	29	26	40	33	27	30	36	26	6	6	22	20	24	18	27	6	21	6	20	6	6	6	6	6
40314	6	20	6	6	13	9	6	6	26	21	30	15	24	17	11	13	10	6	26	22	11	13	18	6	25	6	21	6	6	6	6	6
40315	6	21	6	6	15	14	11	6	29	24	33	28	25	20	24	18	6	6	25	21	9	15	19	6	30	6	21	6	6	24	34	34
40316	6	18	6	6	13	12	8	6	23	20	32	19	24	20	15	16	14	19	26	20	10	17	21	25	24	6	20	6	28	23	30	30
40317	6	9	6	9	15	12	12	6	21	12	28	11	8	30	18	27	6	6	28	21	25	24	31	29	30	24	21	6	6	23	35	33
40319	6	20	6	6	11	11	6	6	27	22	32	17	24	19	15	13	17	20	27	21	9	16	22	10	19	24	22	6	6	6	6	6
40320	6	10	6	9	17	16	11	6	22	16	29	16	6	34	19	28	6	24	25	23	25	24	27	6	25	6	23	6	6	6	6	6
40321	6	9	6	9	16	12	12	6	22	12	30	12	6	30	21	27	6	6	24	21	24	21	28	22	23	25	21	6	6	24	28	27
40322	6	26	6	6	22	17	12	6	28	25	34	26	27	23	25	19	6	6	26	21	9	14	19	6	29	6	21	6	6	23	35	35
40323	6	20	6	6	16	13	9	6	26	21	31	25	25	22	24	17	6	6	26	21	27	24	30	25	26	6	20	6	6	6	13	13
40327	6	27	6	6	14	12	9	6	26	23	30	19	26	20	13	16	6	20	26	20	11	16	21	6	30	6	21	6	6	23	34	33
40328	6	25	6	6	23	15	14	6	32	26	32	29	28	23	24	18	6	19	27	20	10	15	21	6	31	6	21	6	6	28	37	38
40329	6	28	6	6	18	14	11	6	30	25	36	29	28	24	33	20	6	19	25	20	9	14	20	6	29	6	21	6	6	22	31	30
40330	6	21	6	6	16	13	12	6	30	25	31	27	28	21	22	16	6	6	26	21	8	14	22	26	27	6	21	6	6	6	18	18
40331	6	15	6	6	11	10	6	6	28	18	31	26	22	17	16	11	6	6	25	19	9	15	20	24	28	6	21	6	6	26	35	34
40334	6	20	6	6	12	11	8	6	28	22	32	19	24	19	15	15	16	13	26	17	8	11	20	11	24	6	20	6	6	6	6	6

Table 11. (Continued)

Sample	AM	AC	CFL	CF	CTX	CTF	PP	TC	PT	TCL	IM	CAZ	CFO	CPM	AZ	CEQ	ST	KA	AM	AP	GE	TO	NE	CL	FL	TT	CO	SU	TR	NA	ER	OF
40335	6	24	6	6	16	12	10	6	30	24	30	30	30	19	18	16	6	6	25	20	6	13	18	6	26	6	21	6	6	6	6	6
40893	6	24	6	6	16	13	9	6	30	25	32	27	24	20	23	17	7	6	25	20	9	15	22	6	27	6	19	6	6	20	32	32
40894	6	21	6	6	17	14	10	6	28	24	23	23	26	23	19	16	6	6	26	22	11	18	30	6	27	6	20	6	6	6	6	6
40895	6	28	6	6	18	15	12	6	33	29	34	33	28	23	25	19	6	20	26	20	10	13	20	6	29	6	20	6	6	28	37	36
40896	6	19	6	6	13	11	6	6	28	20	32	26	26	21	20	16	8	6	26	21	10	15	23	27	29	6	21	6	6	23	31	33
40897	6	22	6	6	14	11	6	6	29	23	33	28	26	21	23	16	8	6	26	22	11	16	23	26	28	6	21	6	6	23	33	31
40898	6	19	6	6	12	11	6	6	27	22	34	18	24	19	15	15	16	20	27	21	9	15	21	10	23	24	20	6	6	6	6	6
40899	6	24	6	6	18	16	11	6	32	28	35	30	27	24	29	18	6	21	28	21	10	15	19	6	27	6	21	6	6	26	36	35
40900	6	21	6	6	9	8	6	6	27	23	31	15	23	16	9	11	6	16	26	20	6	12	10	26	27	6	20	6	6	6	6	6
40901	6	20	6	6	13	11	6	6	27	22	34	20	27	20	19	15	6	20	26	20	9	16	21	25	26	6	20	6	6	23	35	33
40902	6	21	6	6	17	13	6	6	28	25	35	28	27	21	24	16	6	18	25	20	6	11	17	28	30	25	21	6	6	16	17	18

AM, Amoxicillin; AC, amoxicillin-clavulanate; CFL,cephalothin; CFU, cefuroxime; CTX, cefotaxime; CTF,ceftiofur; CFO, cefoxitin; CAZ, ceftadizime; CPM,cefepime; CEQ, cefquinome; PP, piperacillin; PT, Piperacillin/tazobactam;TC, Ticarcillin/clavulanate, AZ, aztreonam; IM, Imipenem; ST streptomycin ;KA kanamycin; AP apramycin; GE Gentamicin;AM amikacin; TO,tobramycin; NE, netilmicin, CL chloramphenicol;FL florfenicol; TT, Tetracycline; CO colistin, SU/TR,Trimethoprim-sulfamethoxazole; NA nalidixic acid ; ER enrofloxacin; OF ofloxacin. Pink boxes denote intermediate or resistant breakpoint and green boxes denote susceptible breakpoint (according NCLS or EUCAST).

The PCR array confirmed the presence of *bla*$_{CTX-M}$ genes in 36 isolates, five were CMY-2, one had ampC phenotype but was negative for CMY-2 PCR and three were negative during phenotype screening and were not molecularly tested. Regarding CTX-M groups, 28 isolates belonged to CTX-M-1 group, four were CTX-M-2 and four were CTX-M-9. CTX-M-1 isolates were tested using primers ISEcp1$_F$/P2D$_R$ for amplification of the entire blaCTX-M-1group coding regions genes. All isolates were positive for this reaction and the nucleotide sequence analysis showed products of size of ~1 kb and the sequencing revealed 18 isolates positive for CTX-M-1, eight isolates CTX-M-15 and two CTX-M-32 (Table 12).

Forty-one isolates were tested for the main common incompatibility group in France (Inc: F$_{rep}$, I1 and HI1). The I1 replicon was present in six isolates, F$_{rep}$ replicon in 23 and HI1 replicon was present in 24 isolates. Twenty isolates carried only single replicons (ten carrying Inc HI1, eight carrying IncF$_{rep}$ and two IncI1). A total of 14 isolates carried two different replicons (HI1 was present in 13 isolates and one with IncI1 plus IncF$_{rep}$) and one isolate carrying three different replicons (Inc I, HI1, F$_{rep}$). Among the plasmid replicons tested in this study, five of the isolates could not be identified (Table 12).

According to PFGE-S1 images, the isolates contained between one and three distinct large plasmids with estimated sizes ranged from ≈ 48 to 330 kb. Southern blot was used to link CTX-M-1 gene and the replicon type with specific plasmids on the S1/PFGE gel. CTX-M-1 was located in different plasmids sizes and varied from 48.5kb to over 240kb and the average size was 154kb. In eight isolates, *bla*$_{CTX-M-1}$ gene was located on IncHI1 plasmids, two in Inc I1, and in 18 samples the location could not be determined. The details are described in table 12.

Table 12 - Molecular characterization of equine clinical isolates harboring *bla* genes.

Sample number	CTX_{group} or CMY-2 by PCR	IS*Ecp1* P2D	CTX-M-1 group sequenced	Inc group	Number of plasmid detected by S1-PFGE and sizes	CTX-M-1 ≈size (S1)	Inc plasmid harboring *bla*CTX-M-1
38645	CTX-M-1	positive	CTX-M-1	IncFrep	1/≈97 kb	100 kb	ND
38646	CTX-M-1	positive	CTX-M-1	IncHI1	1/ ≈242 kb	242 kb	HI1
38647	CMY-2	NT	NT	IncI1	NT	NT	NT
39574	CTX-M-2	NT	NT	IncHI1	NT	NT	NT
39576	CTX-M-1	positive	CTX-M-15	ND	1 /≈97 kb	97 kb	ND
39577	CTX-M-1	positive	CTX-M-1	IncHI1	ND	48.5 kb	ND
39578	CTX-M-9	NT	NT	NT	NT	NT	NT
39580	CTX-M-1	positive	CTX-M-1	ND	2 / ≈ 80, 145 kb	48.5 kb	ND
39581	CTX-M-9	NT	NT	IncHI1	NT	NT	NT
39582	CTX-M-9	NT	NT	ND	NT	NT	NT
39775	CTX-M-1	positive	CTX-M-15	IncHI1,IncFrep	2 /≈ 120, 194 kb	97 kb	ND
39785	CMY-2	NT	NT	IncHI1	NT	NT	NT
39795	CTX-M-1	positive	CTX-M-1	Incs: I1,HI1, Frep	2 /≈ 130, 194 kb	100 kb	ND
39798	CTX-M-1	positive	CTX-M-1	IncHI1	2 /≈60, 330 kb	242 kb	HI1
40066	CTX-M-1	positive	CTX-M-1	IncHI1, IncFrep	2 / ≈60, 330 kb	242 kb	HI1
40067	NEG	NT	NT	NT	NT	NT	NT
40314	CTX-M-1	positive	CTX-M-15	IncHI1, IncFrep	ND	100 kb	ND
40315	CTX-M-1	positive	CTX-M-1	IncHI1, IncFrep	2 /≈60, 330 kb	242 kb	HI1
40316	CTX-M-1	positive	CTX-M-15	IncFrep	2 /≈48, 140 kb	97 kb	ND
40317	CMY-2	NT	NT	IncFrep	NT	NT	NT
40319	CTX-M-1	positive	CTX-M-15	IncFrep	1 /≈110 kb	100 kb	ND
40320	CMY-2	NT	NT	NT	NT	NT	NT
40321	CMY-2	NT	NT	IncHI1	NT	NT	NT
40322	CTX-M-1	positive	CTX-M-1	IncHI1,IncFrep	2 /≈ 110, 338 kb	242 kb	ND
40323	CTX-M-9	NT	NT	ND	NT	NT	NT
40327	CTX-M-1	positive	CTX-M-32	IncHI1,IncFrep	3 /≈100, 130, 291 kb	100 kb	ND
40328	CTX-M-1	positive	CTX-M-1	IncHI1, IncFrep	2 /≈130, 338 kb	242 kb	HI1
40329	CTX-M-1	positive	CTX-M-1	IncHI1, IncFrep	2/≈100, 338 kb	242 kb	HI1
40330	CTX-M-1	positive	CTX-M-1	IncHI1, IncFrep	2/≈97, 338 kb	ND	ND
40331	CTX-M-2	NT	NT	IncHI1	NT	NT	NT
40334	CTX-M-1	positive	CTX-M-15	IncFrep	2 /≈110, 130 kb	100 kb	ND
40335	CTX-M-1	positive	CTX-M-1	IncFrep	1/ ≈290 kb	242 kb	ND
40893	CTX-M-1	positive	CTX-M-1	IncHI1	1 /≈245 kb	242 kb	ND
40894	CTX-M-1	positive	CTX-M-1	IncI1, IncFrep	3/≈ 97, 130, 291 kb	95 kb	I1
40895	CTX-M-1	positive	CTX-M-1	IncFrep, IncHI1	2 /≈97, 242 kb	242 kb	HI1
40896	CTX-M-2	NT	NT	IncFrep, IncHI1	NT	NT	NT
40897	CTX-M-2	NT	NT	IncFrep,IncHI1	NT	NT	NT
40898	CTX-M-1	positive	CTX-M-1	IncHI1	1/≈95 kb	95 kb	ND
40899	CTX-M-1	positive	CTX-M-15	IncHI1, IncFrep	1/≈245 kb	242 kb	HI1
40900	CTX-M-1	positive	CTX-M-32	IncFrep	2/≈97, 145 kb	130 kb	ND
40901	CTX-M-1	positive	CTX-M-15	Inc I1	3/ ≈97, 130, 158 kb	95 kb	I1
40902	CTX-M-1	positive	CTX-M-1	IncFrep	2 /≈30, 90 kb	90 kb	ND

NT, not tested; ND, not determined; kb, kilobase; ≈, approximately; Inc groups tested by PCR: I1, HI1, HI2 and F.

5 DISCUSSION

5.1 Cephalosporin-resistant *E.coli* strains isolated from feces of healthy horses, Brazil

The results in this study highlight the presence of ESBL *E.coli* or AmpC-producing multidrug resistant (MDR) among equine fecal microbiota from apparently healthy horses from Brazil.

5.1.1 Cephalosporin-resistant E.coli (CRE) patterns

Several studies have shown the occurrence of ESBL/AmpC genes among different domestic animals (Corté et al., 2010; Ewers et al., 2010; Harada et al., 2012; Moreno et al., 2008; Poeta et al., 2009); however, there is still little information about horses regarding the presence of CRE from fecal samples. In Brazil, the presence of CRE in buffaloes (Aizawa et al., 2014), in dogs (Carvalho et al., 2016), in pigs (Silva et al., 2012) and in poultry (Ferreira et al., 2014; Silva et al., 2013) were already published but no data is available for horses was found.

Cephalosporin-resistant E.coli colonization was detected in 8.3% of the horses studied. The fecal colonization percentage rate of CRE can vary among different animals. In Bangladesh, CRE was isolated from pigeons and the percentage result was 5% (Hasan et al., 2013), in swine samples from China, the presence was higher, and the study showed 32% of healthy pigs carrying ESBL-bacteria in their feces (Hu et al., 2013); and for humans, the same work showed that 17.5% of human fecal samples carrying ESBL isolates (Hu et al., 2013). A study carried out by Costa et al., (2012) in Portugal, verified that no *Escherichia coli* DNA sequences were present in feces of any of the six healthy horses studied; however, the organism was found in eight of the ten horses with colitis. This could explain why the number of animals which were CRE positives was low. Apparently all the horses from which feces were collected were healthy.

Maddox et al., (2012_a), estimated the prevalence of fecal carriage of antimicrobial-resistant *E.coli* in 692 horses from the mainland of the United Kingdom, and their results (6.3%) were quite similar to our results (8.3%) of ESBL-producing *E. coli* detection from horse feces. This group affirms that this number is a high prevalence, since they compare the *E.coli* results rate with *Staphylococcus aureus* methicillin-resistant presence, which is another important resistant bacterium, and it was rare in their study. The group stated that they could not compare their *E.coli* rate results in horses because there are only few other reports to compare those results with. The research group affirms that *E. coli* recovered in their study was not a cause of disease and probably they were commensal isolates, as it occured with our results. However, under certain circumstances, such isolates may be able to cause disease and those findings can have important implications for the horse population. In other studies, from the same research group (Maddox et al., 2012_b), the authors believe that the place where a horse is stabled and how they are managed can be associated with several outcomes. If the horse is stabled at a farm, they can have contact with other animal species, and it can be a risk factor to increase the possibility of acquisition of antimicrobial-resistant *E. coli*. If the horse is stabled at a racing yard, it may be undergoing intensive training, travelling and mixing with other horses and the use of different antimicrobials can also be even a higher risk factor for MDR acquisition. All animals studied herein were from riding centers and probably had an intense routine of training, travelling and we can suppose that they had received antimicrobial therapy at least once during their life time. The animals also had a close contact with humans (owners, groomers, keepers, coaching riders, trainers and veterinarians) and animals as cats, dogs, birds or rats, which facilitates the exchange of antibiotic resistant genes between different sources.

It has been suggested that Enterobacteriaceae antimicrobial resistance genes can be acquired from bacteria from soil. Srinivasan et al., (2008) showed the presence of multiple resistance genes in Enterobacteriaceae members from a dairy farm soil. The authors believe that the soil bacteria may act as a reservoir of antimicrobial resistance genes and could play a role in the dissemination of these antimicrobial resistance genes to other commensal and indigenous

microbial communities in soil, especially when the animal has a close contact with the soil, as cows, and in the case of our study, horses. Saenz et al., (2001) demonstrated a wide variety of resistance genes in multi-resistant nonpathogenic *E. coli* strains from humans, animals, and food products. Consequently, this normal microbiota plays an important role as an acceptor and donor of transmissible antimicrobial resistant genes. The inclusion of some resistance genes inside integrons constitutes an effective mean to spread antibiotic resistance among bacteria from different ecosystems. We also agree with this theory, since horses have a close contact with soil (pasture).

5.1.2 Dominating ESBL genes

The majority part of our CRE isolates carried bla_{TEM-1}(7/8) genes followed by bla_{CMY-2} (4/8) and $bla_{CTX-M-1}$ (1/8) genes. Our findings corroborate Ahmed et al., (2010) study, which was conducted in North England, and identified 91% of CRE isolates from fecal horse samples were positive only for TEM beta-lactamase.

The bla_{CMY-2} genes seem to be more commonly isolated from pets, being observed in cats with urinary tract infections in Australia, Netherlands and Italy (Dierikx et al., 2012; Gibson et al., 2010; Nebia et al., 2014), in dogs with infections in Europe (Dierikx et al., 2012; Wagner et al., 2014) and Australia (Sidjabar et al., 2006) and in healthy stray dogs from Korea (Tamang et al., 2012) and Canada (Murphy et al., 2009). In horses, CMY-2 was first identified in *Salmonella* isolates (Frye et al., 2007; Rankin et al., 2005) and only one report of CMY-2 in *E.coli* isolated from fecal horses samples was found (Vo et al., 2007). Another report with MDR *E.coli* isolated from diseased horses with infections, showed CMY-7 type (Gibson et al., 2010) or CMY-34 type or CMY-53 (Damborg et al., 34). According to our findings, we can suggest that CMY-2 enzyme type seems to be common in *E.coli* isolated from feces samples of healthy horses, and not only in dogs and cats.

The CTX-M-1 ESBL group is one of the most prominent enzyme found in human *E.coli* strains (Schmiedel et al., 2014; Silva et al., 2012) and also seems to be very common for equine clinical isolates (Dolejska et al., 2010; Dolejska A et al., 2014; Ewers et al., 2010; Leigue et al., 2015; Vo et al., 2007; Walther et al., 2014). In a longitudinal study (Damborg et al., 2012), the occurrence of cephalosporins-resistant *E.coli* isolated from horses that received antimicrobial drugs before and after admission on a vet hospital was investigated. All animals were negative for CTX-resistant coliforms at admission but became positive during the first three days of cefquinomone antibiotic therapy. The genetic characterizations revealed most part of *E.coli* harbored CTX-M-1, CTX-M-14, TEM-1 and CMY-34. Probably the microbiota is a very dynamic environment, and the authors suggested three hypothesis: a) the strains could be acquired from the hospital environment; b) the *E.coli* was already present in the intestinal tract and proliferated after antimicrobial therapy, or c) CTX-M genes could be mobilized from the intestinal microbiota bacteria to *E.coli*, as consequence of antimicrobial treatment or other stress factor associated with the hospitalization. We found only one sample carrying CTX-M-1 enzyme, and it came from an apparently healthy horse. Unfortunately, in this Brazilian study, it was not possible to get information about the medical history and make any comparisons with those authors.

5.1.3 Antimicrobial resistance patterns

Cephalosporins (ceftiofur, cefovecina and cefquinomone) for animals in Brazil are routinely prescribed to treat different diseases in different animals such as: urinary tract infection and piodermatitis in dogs (Barbosa et al., 2011); respiratory diseases and pododermatitis in bovines and swines, bovine mastitis and metritis (Vo et al., 2007); and equine respiratory and genitourinary infections (Folz et al., 1992). Together with the cephalosporins, amikacin and gentamicin are important drugs used as empiric antibiotic therapy in the equine clinic. In this study, after the cephalosporins, resistance to aminoglycosides was the most common drug found among the horse isolates. Wilson (2001) suggests using amikacin, as first choice, when the isolate is an *E.coli*, especially because this

drug is safe to use in horses. However, the Australia's Antimicrobial Resistance Standing Committee (2014) recommends to use this drug as the "last-aminoglycoside-resort" to use in horses. According to Giguere (2010), it is recommended to initiate the therapy in GNB infections in horses with gentamicin. Antibiotic therapy in horses with oral drugs is a problem, in view of the fact that those animals can be extremely sensitive and only a limited number of the injectable antibiotic compounds are available (Damborg et al., 2012), and probably this is the reason why cephalosporins and gentamicin are used frequently in equine practice. In Brazil, it is also frequent to use ceftiofur or gentamicin or streptomycin antibiotics as first choice in equine veterinary practice. Among our disk-diffusion and MIC results, amikacin is still a good choice when bacteria are resistant to cephalosporin, whereas gentamicin has already shown some resistant isolates and consequently can not be a good choice when the therapy is empiric. As expected, all the carbapenems, tigecycline and colistin were susceptible, since those groups of antibiotics are not routinely used at equine veterinary practice and are not licensed to use in horses.

5.1.4 Other antibiotic resistant genes

Multidrug-resistance was noted in almost all samples and the molecular panel was also positive for resistance-related genes such as *aad*A, *aad*B and *qnr*S (aminoglycosides and fluoroquinolones). The production of ESBL or AmpC enzymes combined with the co-expression of different classes of resistance-related genes is commonly reported among GNB ESBL producers (Leigue et al., 2015; Shmiedel et al., 2014). This can be due to the antibiotic widespread use or even the overuse or misuse of antibiotics. Also, it is a quite common practice in veterinary clinics not to finish the total courses of antibiotic treatment or to initiate a treatment without veterinary assistance, since it is easy to buy veterinary antibiotics without a vet prescription.

5.1.5 Phylogenetic groups

Genotyping by ERIC-PCR showed all *E. coli* isolates were genetically diverse and heterogeneous. This was expected since the isolates were randomly selected from different riding centers and probably suggesting that each isolate came from a unique horse microbiota.

The phylogenetic group results for fecal samples from Brazilian horses indicated that phylogroup B1 (n=3) was the predominant followed by phylogroup A (n=2). A and B1 groups are well known to be frequently associated with commensal microbiota and isolates belonged to group B2 or D are often associated with extra-intestinal infections (Clermont et al., 2002). Similar observations had been reported in dogs fecal and oral samples from Brazil (Melo, 2014) and from animal samples in Germany (Schmiedel et al., 2014). Another study (Carlos et al., 2010) showed that strains from group B1 were present in all hosts analyzed (animals and humans) but were more prevalent in cow, goat and sheep samples (herbivorous) followed by group A. Subgroup B2 was only found in human clinical and fecal samples. The results showed a similarity between the *E. coli* population structure of humans and pigs (omnivorous mammals) and of cows, goats and sheep (herbivorous mammals) (Carlos et al., 2010). Horses are herbivorous animals and maybe this factor can affect the phylo-group diversity type.

5.1.6 Plasmid replicon types (PBRT)

The main plasmid replicon type described were Inc HI1 and Inc I1 for CMY-2 isolates and Inc F_{rep}, FIA and FIB for CTX-M-1 isolate. Plasmids may be horizontally mobilized between bacteria via conjugation, transformation or transduction. Several studies about CMY β-lactamase-encoding plasmids harbored by *E. coli* strains from human and animal origin from Tunisia (Salle et al., 2014), from Norway (Nasser et al., 2010), from Indonesia (Sidjabat, et al., 2014), and from Italy (Accogli et al., 2013) revealed that IncI1 plasmid carrying bla_{CMY-2} were predominant and present in different clones around the

world. Even for CMY-2 *Salmonella* isolates from the USA (Foster et al., 2007), Spain (Gonzalez et al., 2001) and Japan (Sugawa et al., 2011) were the most common replicon type identified. Among samples from human and companion animals in the USA, *bla*$_{CMY-2}$ PBRT were: IncI1 52%, Inc A/C 13%, IncFII 10%, IncI2 5%, L/M 3% and Inc B/O 2% (Bortolaia et al., 2014). We presume that CMY-2 found in this study is harbored by IncI1 plasmids, but further analyses are necessary to prove that. However, for *bla*$_{CTX-M-1}$ gene, the location can be very diverse. Probably the gene found in this work is on IncF plasmid, but several reports described this gene present in: IncN in poultry from France (Girlich et al., 2007) or Inc FII in dogs from Kenya, France, and Korea (Albrechtova et al., 2012; Dahmen et al., 2013; So et al., 2012; Tamang et al., 2012). In horses, the *bla*$_{CTX-M-1}$ in *E.coli* isolates was carried by IncN (Dolejska et al., 2010;) IncI1 (Dolejska et al., 2010; Smet et al., 2010), Inc HI1 (Dolejska et al., 2014) and more abundant Inc FIA/FIB in Germany (Ewers et al., 2014) and Brazil (Leigue et al., 2015).

5.1.7 Considerations

The complexity of equine fecal microbiota is wide and remains unknown, since this environment is very dynamic and transient. There is a continuous interaction between the microbiota and the host. This microbiota can be affected by several factors such as: changes in nutritional and metabolic status, during diseases, periods with antibiotic therapy or use of another drug, age, sex, environment and other factors. Probably if the same study was performed with the same animals in a different moment, the results would be different.

5.2 Multi-resistant Gram-negative bacteria isolated from diseased horses, Brazil

The clinical findings were very heterogeneous with different clinical presentations, different breeds and various species of bacteria were isolated (*E.coli, P. aeruginosa, K. pneumoniae, P.mirabilis* and *S.marcescens*) with higher levels of resistance to β-lactams, quinolone and aminoglycoside antibiotics.

5.2.1 Antimicrobial resistance patterns

According to the MIC and disk-diffusion results, all isolates were susceptible to carbapenems (meropenem, ertapenem and imipenem), colistin, tigecycline and fosfomycin. In Europe, Australia, Asia and North America (EFSA 2013; Shaheen et al., 2013), carbapenems are not licensed for treatment of animals but they are commonly used in human multi-resistant pseudomonas and enterobacteria infections. The non-appearance of carbapenem resistant isolates in this study should be highlighted, especially when compared to those findings obtained from human medical isolates (Bonnin et al., 2012; Walsh et al., 2011). Although this drug is rarely used in veterinary medicine and is not approved for use in animals by the Food and Drug Administration (USA), it is prescribed by veterinarians as an off-label drug (Gibson et al., 2010). Regarding tigecycline, according to the European Medicines Agency (2013a), there are no tigecycline-containing products authorized for veterinary use in the Europe. In Finland, the use of tigecycline for animals was banned by national legislation. In Brazil, we have no official data about the use of those drugs, but we assume that there is no use for equine veterinary treatment, which is probably the reason why we did not find any bacteria carrying genes associated with resistance to these drugs. Colistin, polymyxin B and fosfomycin are authorized nationally in Europe (European Medicines Agency, 2013b) and Brazil (MAPA, 2014), and they can be used in horses, companion animals and farm animals. In our study, though, resistant phenotypes profiles were not detected.

5.2.2 Other antibiotic resistant genes

Antimicrobial susceptibility patterns revealed a high rate of quinolones, aminoglycosides and beta-lactamic resistance and exhibited a MDR pheno and genotype. Several studies have indicated similar results with bacteria carrying CTX-M genes and also harboring resistance genes for different antibiotic classes, especially for fluorquinolones and aminoglycosides (Nebbia et al., 2014; Schmiedel et al., 2014; Smet et al., 2010). For Morosini et al., (2006) the coresistance can frequently be linked to resistance genes found in *bla* gene surroundings and it is also important for maintenance and dispersion of ESBL-producing organisms. For fluoroquinolones, a general downward trend is observed in France. Resistance is declining in cattle and dogs, and is stable in other species. The rate is low among laying hens and chickens, turkeys and Equidae (ANSES, 2015) but no data about horses fluorquinolone resistance rates is available in Brazil. Almost all enterobacteria isolated in this study were linked with *aac(6')-Ib*-CR and/or *qnr* and *aad* genes. Moreover, one sample (isolate n°10-ICB) carried *rmtD* gene plus $bla_{\text{CTX-M-15}}$, *aac(6')-Ib-cr*, *sul1*, *tetA*, *aadB* and *aadD* (all genes were confirmed by PCR and sequencing and they are deposited on GenBank - accession numbers: KC618390, KC593856, KC593857).

This isolate (10E-ICB) is a cause of clinical concern, since the use of beta-lactams combined with aminoglycosides or fluoroquinolones play an important role in horse antimicrobial therapy. In this regard, the emergence and dissemination of ESBL-type genes encoding resistance to extended-spectrum cephalosporins among *Enterobacteriaceae* isolates from horses has been a recent and alarming event, which has gained worldwide attention after identification of clonally related group of CTX-M-15-producing *E. coli* (ST131 and ST648) and *K. pneumoniae* (ST15) in equine from Germany (Ewers et al., 2010, Ewers et al., 2014; Schmiedel et al., 2014) and Belgium (Smet et al., 2010). On the other hand, although the 16S rRNA methylase genes *rmtB* and *armA* have been detected in isolates from food-producing animals and pets (Deng et al., 2011; Liu et al., 2013), they have not been identified in bacterial pathogens of horses, so far. In fact, the methyltransferase RmtD was described initially in a pan-resistant *P. aeruginosa* strain isolated in 2005 from an inpatient in Brazil

(Bonelli et al., 2014) and from a Brazilian river (Fontes et al., 2011), and, shortly after, surveillance programs demonstrated that *rmt*-like genes were also present in enterobacteria from Argentina, Brazil and Chile, showing a predominance of these related gene families over other ones encoding 16S rRNA methyltransferases in South America (Bonelli et al., 2014; Tijet et al., 2011; Yamane et al., 2008). Regarding the possible origin of the MDR CTX-M-15-producing *E. coli* in the foal (sample 10E-ICB), most likely the extraintestinal infection caused by the ESBL-producing *E. coli* are endogenous, probably resulting in infection with a strain of commensal origin. In this regard, previous studies have revealed that commensal bacteria from animals can play a key role as reservoirs of $bla_{\text{CTX-M}}$-type genes, and the increasing use of broad-spectrum cephalosporins has contributed to the selection and dissemination of ESBL-producing strains. Specifically in horses, it has been demonstrated that both hospitalization and antimicrobial drug administration are associated with the prevalence of antimicrobial resistance among commensal *E. coli* (Berg, 1999; Srinivasan et al., 2008). Additionally, a recent study has showed that sugar metabolic elements could contribute to the successful dissemination and maintenance of multidrug-resistant plasmids, harboring CTX-M-type genes, in the intestinal microflora of horses (Dolejska et al., 2014). Finally, the acquisition of ESBL-producing isolates in horses may also result in the contamination of the animal keeper, other animals around and any caretakers who are in contact with the animal.

Actinomycetes such as *Streptomyces* spp. and *Micromonospora* usually are bacteria isolated from soil (Sripreechasak et al., 2016) and are natural producers of aminoglycosides. These aminoglycoside-producing actinomycetes are inherently resistant to aminoglycosides, because they harbor intrinsic 16S rRNA methyltransferase (16S-RMTase) genes (Wachino, Arakawa, 2012). The natural feeding behavior of horses is characterized by continuous grazing and this practice provides a close contact with pasture/grass/soil. We can speculate that this horse´s habits could facilitate the exchange between bacteria genes in the soil and the microbiota from the horse's gut and this may have facilitated the appearance of methytranferase gene in a *E.coli* isolated from a horse. This case was reported in an important scientific journal (Leigue et al., 2015).

5.2.3 Dominating ESBL genes

The most common ESBL genes detected in this study was *bla*$_{TEM}$ and *bla*$_{CTX\text{-}M\text{-}1group}$. All *E.coli* and *K.pneumoniae* harbored CTX-M-15 and TEM-1 and *P. mirabilis* and *S. marcescens* were positive for CTX-M-1, also co-producing TEM-1. CTX-M-1 is one of the most common ESBL type among companion animal, humans and livestock (Bush, Fish, 2011; Ewers et la., 2014; Mathers et al., 2015). Our findings corroborate past studies. Among *E.coli* isolated from horses with soft tissue infections or metritis in Germany, CTX-M-15 was the most prevalent followed by CTX-M-1 and CTX-M-14, and all isolates harbored TEM-1 (Ewers et al., 2014). Walther et al., (2014) identified only CTX-M-1 amongst horses with nosocomial infections and, in another study in Germany (Schmiedel et al., 2014), *bla*$_{CTX\text{-}M\text{-}1}$ was the dominant resistance subtype in horses (37%), but it was less frequently isolated in dogs (16.4%). In contrast, *bla*$_{CTX\text{-}M\text{-}15}$ occurred more often in dog isolates (59.7%) than in isolates from horses (38%) or cats (36.4%). In the southeast United Kingdom, the most common CTX-M type was also CTX-M-1 and some isolates also carried TEM-1 (Johns et al., 2012) and, in northwest England, all isolates were positive for TEM beta-lactamase genes (Ahmed et al., 2010). Similar patterns have been identified in horses from Copenhagen, Denmark, in which CTX-M-1 was positive in 28 isolates and only one carried CTX-M-14, and four isolates co-produced TEM-1 (Damborg et al., 2012); and from the Netherlands, a study revealed that the isolates of *E.coli* and *K.pneumoniae* carried the *bla*$_{CTX\text{-}M\text{-}1}$, *bla*$_{TEM\text{-}1}$ and/or *bla*$_{SHV\text{-}1}$ genes (Vo et al., 2007) and more recently (Dierikx et al., 2012), CTX-M-1 was positive in three *E.coli* isolates and one for CTX-M-14 and one CTX-M-2. In *Enterobacter cloacae* isolated from French horses, CTX-M-15 was also detected (Haenni et al., 2016). No reports were found in South America to compare our results with, but we can assume that our findings are somewhat similar from those of a previous study.

All *P. aeruginosa* isolated in this study presented TEM-1 enzyme. TEM-1 is commonly spread worldwide and are also found in many different species of the Enterobacteriaceae family and *Pseudomonas aeruginosa* (Rawati, Nair, 2010). Penicillins and narrow spectrum cephalosporins (cephalothin or cefazolin) are hydrolyzed by TEM-1 and TEM-2. On the other hand, they are not valuable against higher generation cephalosporins (Paterson, Bonomo, 2005). The

P.aeruginosa from those horses were resistant to higher generation of cephalosporins (cefepime and ceftazidime) and all isolates were tested for VEB, GES, PER and SHV genes but they were negative. Further studies are necessary to clarify which ESBL is involved.

5.2.4 Phylogenetic groups and Sequence Types (ST)

The Clermont *Escherichia coli* phylo-typing method detected two *E.coli* isolates yielding a Clermont A phylo-type and one B1. The source of the three isolates were spleen, bone tissue and facial fistulae. According the classification, A and B1 groups are associated with commensal microbiota and isolates belonging to group B2 or D are often associated with extra-intestinal infections (Clermont et al., 2002). A recent report described 60.2% of *E.coli* isolates from diseased dogs, cats and horses belonging to group A followed by 17.4% group D and 11.5% group B1 (Ewers et al., 2014). According to Schmiedel et al., 2014, the frequencies of the phylogenetic group differed among humans and animals. Amongst humans isolates, *E.coli* were evenly distributed (A, 30.5%; B1, 27.7% and B2, 26.2%). In contrast, the phylogenetic group B2 (2.4%) was strongly underrepresented among the ESBL-*E.coli* isolates from diseased animals, and most part belonged to group A (43.7%) and B1 (35.7%). There is a concern that some *E.coli* resident of equine microbiota (host microbiota) are assigned as “opportunistic pathogens” and can overcome the normal body barriers and invade the body, and may cause severe infections (Walther et al., 2014).

ERIC-PCR was applied to analyze three *E.coli* isolates and they presented three distinguished patterns. The MLST was performed and the following ST numbers were detected: 10E -ICB, ST2179; 102-ICB, ST1081; 105-ICB, ST5140. No isolates belonging to important clonal complexes as ST648 Cplx or ST131 Cplx were detected.

MLST was also performed to *K.pneumoniae* sample and the ST101 was found (Clonal complex 11). Several occurrence of *K.pneumoniae* ST101 carrying both ESBLs and carbapenemases in dogs and cats from Italy (Donati et al., 2014), and mainly in humans, have been increasingly reported in Europe

(Hashimoto et al., 2014; Marcade et al., 2013; Melegh et al., 2015; Mezzatesta et al., 2013; Potron et al., 2012; Seiffert et al., 2014) and Africa (Potron et al., 2012). For Seiffert et al. (2014), it is an emerging clone with high potencial for dissemination of important antibiotic resistance traits (e.g. ESBL and/or carbapenemases). In Brazil, this ST was already described for human samples (Seike et al., 2011) but, to our knowledge, this is the first description of the CTX-M-15 producing *K. pneumoniae* clone ST101 from an animal sample in Brazil. This finding underlines the dissemination of this genotype.

P. aeruginosa MLST of one isolate (103-ICB) showed that the allelic profile corresponded to ST622, which belongs to the important international clonal complex CC235. This clone was already identified in Germany (Pournaras et al., 2013) and the clonal complex is worldwide distributed (Lee et al., 2013; Maatallah et al., 2011). The other two *P.aeruginosa* isolates (101C-ICB and 104A-ICB) belonged to ST304. This clone seems to be a Brazilian *Pseudomonas aeruginosa* clone, according to PubMLST internet page; three isolates ST304 had already been deposited and the source origin was from human clinical isolates (sputum, blood and soft tissue infection) from Brazil, in 2007.

5.2.5 CTX-M gene location and plasmids characteristics

The plasmid replicon typing analysis demonstrated that there is a great variability in the plasmids circulating among horses (Inc FII, FIA, FIB, HI2, L/M, A/C, R, Y). This variability of plasmids can enhance the ESBL genes dissemination among *Enterobacteriaceae* and the epidemiology of ESBLs in horses may be complex.

The PFGE images showed that isolates $bla_{\text{CTX-M-1}}$ group positive contained three or five plasmids. This phenomenon seems to be common among enterobacteria and the number of plasmids can vary. Plasmid analysis of *E.coli* isolated from Turkey indicated that, in all cases, the strains possessed more than one plasmid, with some strains possessing up to five (Gonullu et al., 2008). In Germany, 20 CTX-M-1 *E.coli* were isolated from dogs, cats and humans and

most part of them harbored at least two plasmids and only two isolates carried four plasmids (Falgenhauer et al., 2014).

After several attempts, the PFGE tests were unsuccessful and one sample (*P.mirabilis* – 101B-ICB) could not be evaluated using S1 enzyme. According to reports, the location of $bla_{CTX\text{-}M\text{-}15}$ in *Proteus mirabilis* tests confirmed chromosomal location in some isolates (Mahrouki et al., 2012; Navon-Venezia et al., 2008; Song et al., 2011). This *P. mirabilis* isolate suggests a chromosomal $bla_{CTX\text{-}M\text{-}15}$ location and further analysis, with different enzymes, must be performed.

IncF plasmids are often associated with CTX-M-15 in human clinical *E. coli* isolates in most European countries (Caratolli, 2009) and this incompatibility group has contributed significantly to the success of these genes (Livermoore et al., 2007; Matthers et al., 2015). In Tunisia (Mnif et al., 2013) *E.coli* CTX-M-15 isolates were related to IncF plasmids (72/88) and rarely to IncL/M, IncI1, IncN and IncHI2. The $bla_{CTX\text{-}M\text{-}15}$-carrying plasmid studied here showed only one *E.coli* CTX-M-15 associated with IncF (sample 10E-ICB) and two *E.coli* CTX-M-15 isolates had IncHI2 (size 330 kb and another with 291 kb). IncHI2 plasmids have usually >250 kb sizing and are frequently reported in clinical enterobacterial isolates and associated with the dissemination of important antimicrobial resistance genes (Garcia-Fernandez, Caratolli, 2010). This is also supported by a study in Norway, in which *Enterobacter* CTX-M-15 isolates were associated with IncHI2 of similar size and were successfully transferred to *E.coli*. In addition, the authors affirm that IncHI2 plasmids seem able to harbor a variety of genes encoding resistance to both β-lactams, quinolones and aminoglycosides (Nilsen et al., 2013). Similar findings were reported in *E.coli* and *Klebsiella pneumoniae* from Spain (Briales et al., 2010; Coelho et al., 2012).

Serratia marcescens CTX-M-1 isolated in this study carried two different Inc types: A/C and L/M. The ESBL gene was located on a 97 kb IncL/M plasmid and these results corroborate authors from different parts of the world. The prevalence of *S. marcescens* from Korean hospitals pointed that all isolates carried IncL/M type plasmid and some of them also carried IncA/C type plasmid (Park et al., 2009). A work from Bulgaria (Markovska et al., 2014) showed that *S.*

marcescens plasmids harboring *bla*$_{CTX-M-3}$ belonged to IncL/M type. A co-occurrence of *armA* methyltransferase with ESBL genes in S. *marcescens* outbreak in Algeria demonstrated the transferability of a conjugative plasmid of incompatibility group incL/M type (Batah et al., 2015). Even for *E.coli* harboring CTX-M, Inc L/M was already described in Tunisia (Mnif et al., 2013).

The hybridization with CTX-M-1 probe test signaled a fragment of ~194 kb found in *K.pneumoniae* (101A-ICB), suggesting that this gene is located on the same element. Unfortunately, even after several attempts, Inc group was not determined in this sample.

Even though our data has limitations and as we received a small number of isolates, the bacteria which were isolated showed different plasmids size and Inc group. For us, it seems reasonable to assume that these isolates have evolved independently.

5.2.6 Genetic environment of bla$_{CTX-M}$

The investigation of genetic environments of *bla*$_{CTX-M}$ genes showed the presence of the IS*Ecp1*-*bla*$_{CTX-M}$-15-orf477 or IS*26*-*bla*$_{CTX-M-1}$-orf477 structure in the horses isolates from Brazil. The *bla*$_{CTX-M}$ genes can be mobilized by different genetic elements (Lartigue et al., 2004). IS*Ecp1* insertion sequences are the most frequent element reported in the mobilization of *bla*$_{CTX-M-1/15}$ genes, followed by IS*26*, which has been observed upstream of *bla*$_{CTX-M-1/15}$ as well (Canton, Coque, 2006; Lartigue et al., 2004; Saladin et al., 2002; Toleman, Walsh, 2011).

Genetic analysis revealed that IS*Ecp1* insertion sequences have been observed upstream of genes belonging to CTX-M-15 *E.coli* (10E-ICB, 102-ICB, 105-ICB) and *K. pneumoniae* (101A-ICB) isolates. In a study conducted by Smet et al., (2010), one plasmid (92,970bp) from an *E. coli* strain, which carried two resistance genes (*bla*$_{TEM-1}$ and *bla*$_{CTX-M-15}$) and belonged to the IncI1 group, was isolated from the joint of a horse with arthritis and also showed IS*Ecp1* linked with *bla*$_{CTX-M-15}$.

Despite the samples have presented the IS*Ecp1*-*bla*$_{CTX-M-15}$-orf477 structure, it was identified three distinct genetic environments associated with

IS*Ecp1*. Two samples harbored CTX-M-15 downstream with an intact copy of IS*Ecp1*. This structure had been previously reported in Isolates from the UK (Amos et al., 2014; Dhanji et al., 2011). Sample 10E-ICB had the *bla*$_{\text{CTX-M-15}}$ gene flanked upstream by IS*Ecp1* (1,177bp) followed by IS*26* (820pb). This plasmid possesses interesting features, since the *bla*$_{\text{CTX-M-15}}$ gene was flanked by entire sequences of IS*26* and IS*Ecp1*. A similar finding was described in *E.coli* isolated from feces of travelers returning to the United Kingdom (Dhanji et al., 2011). The last IS*Ecp1* structure found was an insertion sequence IS*Ecp1* upstream of *bla*$_{\text{CTX-M}}$-15 and a similar sequence inverted to the left of IS*Ecp1* upstream of IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$. This particular structure appears not to be so common and more studies are needed to understand this type of mobilization.

Two isolates (101B, *P. mirabilis* and 104B, *S.marcescens*) CTX-M-1 were upstream linked with IS*26*. This structure had already been reported but seems to be less common (Dhaji et al., 2011).

The CTX-M genetic environment patterns described here indicate that plasmids carrying CTX-M-1 or M-15 genes are very diverse in terms of genetic structures.

5.2.7 Considerations

Reports about isolation of ESBL-producing bacteria from equine clinical samples are quite rare and this data might be underestimated, since bacterium isolation and antimicrobial test susceptibilities are not performed in most cases, and a routine testing for ESBL production is not a normal veterinary practice in Brazil.

In equine medicine, beta-lactams are one of the most important antibiotic class and are widely used. The identification of MDR gram-negative bacteria carrying clinically important resistance determinants is a public health issue and a challenge for veterinary medicine, since the therapeutic options are reduced. Thus, the resistance profiles of the Gram-negative bacteria reported here is a subject of concern. Even though the current study was limited by the small number of isolates, these findings represent a significant issue for animal health and could play an important role in potential zoonotic transmission and, therefore, public health. Prospective studies are urgently needed to address the management of infections caused by ESBL producers in equine medicine and urgent actions are required to stop the dissemination of MDR bacteria with zoonotic potential.

5.3 Cephalosporin-resistant *E.coli* strains isolated from diseased horses, France

Escherichia coli producing extended spectrum beta-lactamase (ESBL) has emerged as a global problem for both humans and animals. This study aimed to investigate forty-five cephalosporin resistant *E.coli* strains recovered from French horses with different infections (urogenital, skin/mucous, respiratory and renal diseases).

5.3.1 Antimicrobial resistance/susceptible patterns

The highest frequency of resistance (90% or more) was recorded for amoxicillin (97.8%), ticarcillin (95.6%), trimethoprim-sulfamethoxazole (95.5%), cephalotin (93.3%), cefuroxime (93.3%), ceftiofur (91.1%) and piperacillin (91.1%). Forty-one isolates (41/45 – 91.1%) were considered multi-resistant (resistance to three or more different classes of antibiotics).

For cephalosporins this data was already expected, since those isolates were sent to the lab for further analyses because they were ESBL suspect. According to ANSES data (ANSES, 2015), the 3rd and 4th generation cephalosporins resistance rates in France have been declining in recent years among different bird species, pigs, domestic carnivores, and is stable in Equidae. In contrast, increase resistance has been observed in calves.

High resistant rate for three significant drugs used in horses is alarming. Ceftiofur is one of the most common injectable drug used worldwide approved to use in horses, whereas the main antibiotic approved for oral use is trimethoprim/sulfadiazine and ticarcillin. It is also one of the three antibiotics approved for intrauterine infections (Wilson, 2001).

E.coli isolated from horse's feces (this study, chapter 5.1) also had a high MDR level. For Morosini et al., (2006) the co-resistance can frequently be linked to resistant genes found in the *bla* gene surroundings and it is also important for maintenance and dispersion of ESBL-producing organisms. Levels of multi-

resistance nonetheless vary according to the animal species: it is notably higher in cattle, pigs, horses and dogs than in the poultry French sectors (ANSES, 2015). In 2014 in France there was a downward trend of multi-resistance phenomena (resistance to at least three classes of antibiotics) (ANSES, 2015).

The antibiotic susceptibility profiles showed all isolates tested in vitro were susceptible to: imipenen, colistin, amikacin, apramycin, piperacillin/tazobactam and florfenicol. This data is very important since those drugs are important antibiotics used in humans or animal therapy.

Imipenem, colistin, piperacillin/tazobactam and florefenicol are not common drugs prescribed to horses. Carbapenems such as imipenen, meropenen or ertapenem are widely used in humans with several conditions such as burns, immunocompromised or multidrug resistant bacteria (Sotgiu et al., 2016). The lack of apparent resistance of isolates in this study to imipenem should be highlighted, especially when compared to those findings obtained from cases in humans (Hu et al., 2016). In veterinary practice, imipenem is rarely used and is prescribed by veterinarians as an off-label drug, considered as the “last resort” to use in dogs, cats and horses with multidrug-resistant infections (Orsini et al., 2005). The high cost, concerns about the development of resistance in humans and instability of imipenem solutions (after reconstitution it should be used within 24 hours and kept under refrigeration, stored in a tightly sealed container, protected from light and administered in slowly intravenous doses) are reasons for limited use in veterinary practice (Papich, 2002).

Even with the large usage of colistin in veterinary medicine and the recent reports describing colistin resistance plasmid-mediated by *mcr*-1 gene (Haenni et al., 2016; Liu et al., 2015; Poirel et al., 2016) in animals and humans, no report of detection was described in horses and our findings corroborate this data (Fernandes et al., 2016).

Aminoglycosides are commonly used in equine practice alone or in combination with penicillin for broad-spectrum cover (Briyne, 2014). Amikacin and apramycin are important aminoglycosides used in veterinary practice. Apramycin (technically known as aminocyclitol, a product of *Streptomyces tenebrans*) is an antibiotic that is used exclusively in veterinary medicine

especially for the treatment of colibacilosis and salmonelosis in cattle, bacterial enteritis in swine, colibacilosis in sheep, septicemia in poultry or rabbits (EMEA, 1999; Febler et al., 2011). Amikacin is considered a critically important antimicrobial used in humans and animals, and should be used in exceptional circumstances for individual horses treatment (Australia's Antimicrobial Resistance Standing Committee, 2014). Nevertheless, in the USA, the suggested choice of antibiotic for *E.coli* infections are: first choice should be amikacin, and as alternate choices, it could be ceftiofur, gentamicin, enrofloxacin,, chloramphenicol, or ticarcillin/clavulanic acid (Wilson, 2001). Piperacillin/tazobactam is a penicillin approved to use only in human patients and the use in animals is rare (Greene, 2002, Nemmetz; Lenox, 2004). Florfenicol is a veterinary antibiotic drug for use in swine and cattle. The use of florfenicol in equids is limited and it has been reported to be a cause of diarrhea or acute colitis (Robisson et al., 2011).

When these results are compared with the Brazilian *E.coli* isolates, the resistance patterns were different for the aminoglycosides results, but they were the same for some antibiotics such as carbapenems and colistin. This can reflect of the extended use of aminoglycosides (especially, gentamicin, streptomycin and amikacin) in the veterinary practice in Brazil (C. Dunin, personal communication, April, 2016) and we can assume the use of aminoglycosides, especially amikacin, in France is more controlled.

Another point that must be evaluated is the origin of the isolates. The *E.coli* isolates in Brazil were from animals that were hospitalized and only two hospitals had sent us the samples while, for French samples, they were collected from different regions of France and the animals may not have necessarily been under hospitalization. These results can reflect the French national profile, whereas the Brazilian samples could be a punctual profile of these two veterinary hospitals.

5.3.2 Phylogenetic groups

The phylogenetic analyses showed that isolates belonging to phylogenetic group B1 were found to be the most abundant in the collection (n = 28, 68.3%) followed by group A (n = 10, 24.2%). Five percent (n = 2) of the isolates were grouped in the B2 group, associated with extraintestinal virulence and only one isolate (2.4%) was identified in D, which is also associated with the pathogenic group. The phylogenetic group results for fecal samples from Brazilian horses also indicated that the phylogroup B1 (n=3) was the predominant, followed by the phylogroup A (this study, chapter 5.1.5). Brazilian clinical *E.coli* isolates also presented phylogroups B1 and A as predominat among horses (this study, chapter 5.2.4). Similar observations had been reported for horses in Germany, which had a strong presence of phylogenetic group A/B1 and almost complete lack of group B2 or D (Schmiedel et al., 2014; Walther et al., 2014).

Genotyping by PFGE-Xba-I showed that all PFGE profiles were unique except for two pairs of indistinguishable profiles. This was a predicatable result, since the isolates came from different regions of France and it is expected to have found such a genetic diversity and heterogeneous profile.

Two isolates (40319 and 40334) were considered to be closely related, as these PFGE patterns differed by only two different bands; and two isolates had an indistinguishable PFGE pattern (40317 and 40321). They were from the same origin, and there is a possibility that they came from the same animal and therefore, were considered to represent the same strain.

5.3.3 Dominating ESBL genes

High phenotypic frequency of CTX-M-producing isolates was detected among *E.coli*. On the double-disk test, 80% (36) of isolates had phenotypic ESBL profile, 13.3% (6) AmpC suspect profile. The PCR array confirmed the presence of *bla*$_{\text{CTX-M}}$ gene in the 36 isolates and five were CMY-2.

Among the CTX-M group, 28 isolates belonged CTX-M-1 group, four were CTX-M-2 and four CTX-M-9. CTX-M-1 sequencing revealed 18 isolates positive for CTX-M-1, eight isolates were CTX-M-15 and two were CTX-M-32. These findings are consistent with the data reported in the literature, whereas CTX-M-1 group is by far the dominant type of ESBL enzyme found in horses from different countries of Europe (Dolejska et al., 2010; Dolejska et al., 2014; Ewers et al., 2010; Vo et al., 2007; Walther et al., 2014) and also in Brazil (this study, chapter 5.1 and 5.2), followed by CTX-M-2 and CTX-M-9.

In general, horses in America and Europe are not regarded as food animals but usually they are considered as large companions or athletes. Horse husbandry happens in a totally different way than food animals such as beef cattle, sheep or pigs. Despite horses being usually raised in an agricultural settings, close to livestock animals, they normally have close contact with humans and companion animals (dogs and cats), and they can receive different classes of antibiotics, even aggressive therapy if needed, which is forbidden for food animals. These factors could help to explicain why CTX-M types for these animals share similar patterns with humans and companion animals. The CTX-M-1 ESBL group is the dominant type of ESBL enzyme found in human *E.coli* strains (Schmiedel et al., 2014; Silva et al., 2012). In France, CTX-M-1 is the predominant CTX-M variant, followed by CTX-M-9 (Birgy et al., 2012; Coque et al., 2008). The CTX-M group 1 (including the CTX-M-15 type) is the most widespread throughout Europe (Canton et al., 2008; Coque et al., 2008).

All the *bla*$_{\text{CTX-M-1}}$ group genes were associated with IS*Ecp1*, which could be implicated in their spread and/or expression. IS*Ecp1* insertion sequences are the most frequent element reported in the mobilization of *bla*$_{\text{CTX-M-1}}$ gene (Canton, Coque, 2006; Lartigue et al., 2004; Saladin et al., 2002; Toleman, Walsh, 2011),

and are often located in multidrug resistance regions containing different transposons and ISs linked with *bla*$_{CTX-M}$ genes (Canton, Coque, 2006).

The CTX-M-9 group (CTX-M-9 and CTX-M-14) has been linked directly or indirectly with animals in different countries (Coque et al., 2008). It is the second most predominant CTX-M-group amongst horse isolates samples worldwide (Damborg et al., 2012; Dierikx et al., 2012; Ewers et al., 2014; Johns et al., 2012; Schmiedel et al., 2014; SVARM, 2013). In France, CTX-M-9-producing has been identified in *S. enterica* isolated from human feces, poultry and poultry product (Weil et al., 2004) and from sporadic *E. coli* isolates (Bonnet, 2004).

Four samples were positive for CTX-M-2. The CTX-M-2 group is frequently reported in Brazil (Dropa et al., 2015; Rocha et al., 2015) and other Latin American countries (Radice et al., 2002; Valenzuela De Silva et al., 2006). In Europe, a study with raw chicken imported into the UK from South America showed *E. coli* isolates carrying the CTX-M-2 type (Dhanji et al., 2010). The authors affirm that this ESBL type had not yet become widely disseminated among clinical human isolates in the UK. In France, CTX-M-2 was firstly described in *Salmonella enterica* in poultry and humans (Bertrand et al., 2006) and Saladin et al., (2002) identified CTX-M-2-producing *Proteus mirabilis* in a parisian hospital. The first report of *bla*$_{CTX-M-2}$-producing *E. coli* isolated from diseased horses came from Belgium and was published in 2012 (Smet et al., 2012); after that, a few reports described CTX-M-2 in horses (Dierikx et al., 2012 in Netherlands and Schmiedel et al., 2014 in Germany).

The finding of *bla*$_{CMY-2}$ is not as common as CTX-M-1 but had already been described in horses (Vo et al., 2007) and are in accordance with the small positive number of CMY-2 found in the French isolates (this study).

5.3.4 CTX-M gene location and plasmids characteristics

The plasmids play a key role in the horizontal spread of *bla*$_{CTX-M}$ multidrug resistance genes (Coque et al., 2006). Forty-one isolates were tested for the main common incompatibility group in France (Inc: F_{rep}, I1 and HI1). The most common identified Inc detected was HI1 replicon (24 isolates), followed by IncF_{rep} (n=23) and IncI1 was present in six isolates. According to Carattoli (2009), the

predominant plasmid replicon types found in antibiotic resistant Enterobacteriaceae isolated from animals and humans are: (Inc) groups F, A/C, L/M, I1, HI2 and N. The epidemiological study about the plasmid types circulating in bacterial populations is necessary for understanding the dynamics and for establishing intervention strategies to prevent further propagation of particular plasmids.

According to the PFGE-S1 results, the isolates contained between one and three distinct large plasmids, whereas the Brazilian samples showed isolates *bla*$_{\text{CTX-M-1}}$group–containing three or five plasmids. In a study conducted by the ANSES group (Luana Melo, personal communication, Jan, 2016), isolates from dogs and cats from Brazil also present a high number of plasmids when visualized through the same technique (PFGE-S1). Falgenhauer et al., (2014) described that most part of CTX-M-1 *E.coli* isolated from dogs, cats and humans in Germany harbored at least two plasmids (18 samples) and only two isolates carried four plasmids. One hypothesis that we suggest is that higher temperature, as happen in Brazil, could increase the acquisition frequencies of plasmids. Further studies are necessary to elucidate if the number of plasmids can influence the plasmid spread and why Brazilian isolates seem to have more plasmids.

The plasmid type carrying the *bla*$_{\text{CTX-M-1}}$ gene can vary among humans, animals and the environment. In eight French isolates carrying the *bla*$_{\text{CTX-M-1}}$, the gene was located on IncHI1 plasmids, two in IncI1 and in 18 samples the location could not be determined. In a study in Germany, the incompatibility groups detected among the *bla*$_{\text{CTX-M-1}}$-harboring plasmids included: IncI1, IncN, IncHI1B, IncF, IncFIIS, IncFIB and IncB/O, with plasmid IncI1 predominating in isolates from chicken and from humans (Zurfluh et al., 2014). The IncN was detected mainly in isolates from pigs, and according to this work, for the first time *bla*$_{\text{CTX-M-1}}$ genes encoded on IncHI1 plasmids were detected in isolates from cattle and from water bodies (Zurfluh et al., 2014). In horses, our results corroborate other studies in Europe. The *bla*$_{\text{CTX-M-1}}$ in *E.coli* isolates was already described in IncN (Dolejska et al., 2010;) IncI1 (Dolejska et al., 2010; SMET et al., 2010), Inc HI1 (Dolejska et al., 2014), and more abundantly Inc FIA/FIB in Germany (Ewers et al., 2014).

5.3.5 Considerations

In this study, the number of *E.coli* isolates was much higher in France than in Brazil. In France, a national surveillance monitoring program of antimicrobial resistance in animal pathogens (Resapath; http://www.resapath.anses.fr) receives bacteria from different regions of France. Laboratories are advised to submit such isolates to ANSES - Lyon for further phenotypic and genotypic typing. Unfortunately, Brazil doesn´t have such type of monitoring program, which could help to decrease or stabilize the prudent use of antimicrobials and to monitor the resistance alarming rates among humans and different species of animals.

Several times, in veterinary practice, monitoring ESBL resistance in clinical isolates is mostly passive as it is based on observations at diagnostic laboratories of phenotypes indicating ESBL resistance; in addition, it is not so common to send samples to a microbiology lab, due to financial contingencies or difficulties in sendind the sample to the lab. Even if a sample is sent, there is usually no further study about ESBL or other important resistant enzymes.

It is well known that antimicrobial use in animals contributes to the selection and spread of resistance. Veterinarians must help to protect existing antibiotics and to combat the serious public health threat of antimicrobial resistance. They must carefully consider how they prescribe antibiotics, especially those that are critical in human medicine, so as to help preserve these lifesaving drugs for the future. According to the Swedish National Surveillance (SVARM, 2013), it is urgently necessary to reduce the antimicrobials use. Some practices are strictly necessary such as: if an animal is healthy, the antimicrobial drugs are not necessary; a prudent use would be to prescribe antibiotics only when necessary; and a rational use would be to do so with the correct dosage, correct period of treatment, and, if possible, to choose to use the narrow spectrum ones. The carriage rate of ESBL-producing Enterobacteriacae in horses is noteworthy, underlining the importance of this population as a reservoir.

6 CONCLUSIONS

- Cephalosporin-resistant *E.coli* colonization was detected in 8.3% of fecal samples from healthy horses, Brazilian samples.
- Healthy Brazilian horses can harbor bla_{TEM-1} followed by bla_{CMY-2} genes and $bla_{CTX-M-1}$ in their fecal microbiota.
- In the fecal sample resistance results, following the cephalosporins, the aminoglycosides were the most resistant drug found among healthy horses isolates.
- Multidrug-Resistance was noted in almost all CRE isolates and the molecular panel was also positive for resistant genes such as *aad*A, *aad*B and *qnr*S (aminoglycosides and fluorquinolones).
- The main plasmids replicon types described in CMY-2 *E.coli* (fecal origin) were: IncHI1 and IncI1; whereas, for the CTX-M-1 isolate were: $IncF_{rep}$, FIA and FIB.
- From Brazilian clinical horses isolates, various species of bacteria were identified (*E.coli, P. aeruginosa, K. pneumoniae, P.mirabilis* and *S.marcescens*) with higher levels of resistance to β-lactam, quinolones and aminoglycosides antibiotics.
- All Brazilian clinical isolates were susceptible to carbapenems, tigecycline, colistin, polymyxin B and fosfomycin.
- Antimicrobial susceptibility patterns revealed a high rate of quinolones, aminoglycosides and beta-lactamic resistance and all isolates exhibited a MDR pheno and genotype.
- Almost all clinical enterobacteria from Brazil isolated in this study were linked to *aac(6')-Ib*-CR and/or *qnr* and *aad* genes.
- Genes $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$ can be found in different enterobacteria isolated from diseased horses from Brazil.
- It is the first time that *rmt*D gene is described in *E.coli*. The loss of efficacy of aminoglycosides could have a serious negative impact on human and animal health.
- None of Brazilian *E.coli* isolates were identified as belonging to any important clonal complex as ST648 Cplx or ST131 Cplx.

- The unique *K.pneumoniae* strain in this study belonged to the emergent clone ST101 (Clonal complex 11).
- *P. aeruginosa* MLST profile corresponded to ST622, which belongs to the important international clonal complex CC235; and the other two *P. aeruginosa* isolates belong to ST304.
- The plasmid replicon typing analysis demonstrated that there is a variability in the plasmids circulating among horses (Inc FII, FIA, FIB, HI2, L/M, A/C, R, Y).
- The PFGE images showed that Enterobacteriaceae isolates $bla_{\text{CTX-M-1}}$group–carrying gene had three or five plasmids.
- *E.coli* CTX-M-15 isolates were harbored in IncF and IncHI2.
- IS*Ecp1* and IS*26* were linked to CTX-M-15 and CTX-M-1 respectively.
- In general, horse bacteria isolates (clinical or fecal origin) are still susceptible to imipenen, colistine, amikacin, apramycin, piperacillin/tazobactam.
- The most commom *E.coli* Clermont Phylogroups in this study were B1 and group A (fecal and clinical samples, Brazil and France).
- PFGE-Xba-I showed that French isolates were not-clonally related.
- Amongst the CTX-M group (French samples), the most predominant was CTX-M-1 group, followed by CTX-M-2 or by CTX-M-9;
- And the most common CTX-M-1 type was CTX-M-1, CTX-M-15 and, less frequently, CTX-M-32.
- The $bla_{\text{CMY-2}}$ can also be present among clinical horse isolates from France.
- The Inc groups detected were: F_{rep}, I1 and HI1.
- French *E.coli* isolates seem to have less plasmids (n = 1-3) than Brazilian isolates (n = 3-5).
- In French strains the $bla_{\text{CTX-M-1}}$ gene was located mainly in IncF_{rep} and IncI1.

REFERENCES*

Accogli M, Fortini D, Giufrè M, et al. IncI1 plasmids associated with the spread of CMY-2, CTX-M-1 and SHV-12 in *Escherichia coli* of animal and human origin. Clin Microbiol Infect. 2013 May;19(5): E238-40.

Aguilar MAP. Caracterização molecular da resistência aos carbapenêmicos em Enterobactérias isoladas em hospitais brasileiros [Tese de Doutorado]. São Paulo: Faculdade de Farmácia da USP; 2009.

Ahmed MO, Clegg PD, Williams NJ, et al. Antimicrobial resistance in equine faecal *Escherichia coli* isolates from North West England. An Clinic Microbiol Antimicrob 2010, 9:12.

Ahmed MO, Williams NJ, Clegg PD, et al. Antibiotic Resistance Patterns in Faecal *E. coli:* A Longitudinal Cohort-Control Study of Hospitalized Horses, Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium, Dr. Marina Pana (Ed.), ISBN:978-953-51-0472-8, InTech,2012. [Last access: April, 16, 2016]. Available in: http://www.intechopen.com/books/antibiotic-resistant-bacteria-acontinuous-challenge-in-the-new-millennium/antibiotic-resistance-patterns-in-faecal-e-coli-a-cohort-controlstudy-of-hospitalized-horses

Aizawa J, Neuwirt N, Barbato L, et al. Identification of fluoroquinolone-resistant and extended-spectrum β-lactamase (CTX-M-8)-producing *Escherichia coli* ST224, ST2179 and ST2308 in buffalo (*Bubalus bubalis*). J Antimicrob Chemother. 2014 Oct;69(10):2866-9.

Albrechtova K, Dolejska M, Cizek A, et al. Dogs of Nomadic Pastoralists in Northern Kenya Are Reservoirs of Plasmid-Mediated Cephalosporin- and Quinolone-Resistant *Escherichia coli,* Including Pandemic Clone B2-O25-ST131. Antimicrob Agents Chemother. 2012, July; 56(7): 4013–4017.

Albrechtova K, Kubelova M, Mazancova J, et al. High prevalence and variability of CTX-M-15-producing and fluoroquinolone-resistant *Escherichia coli* observed in stray dogs in rural Angola. Microb Drug Resist. 2014 Aug;20(4):372-5.

Aldred KJ, Kerns RJ, Osheroff N. Mechanism of quinolone action and resistance. Biochemistry. 2014 Mar 18;53(10):1565-74.

American Academy of Veterinary Pharmacology and Therapeutics, AAVPT$_a$. American Academy of Veterinary Pharmacology and Therapeutics. 2007. [Internet]. USA: The United States Pharmacopeial Convention. Cephalosporins. Last access in April, 16, 2016. Available from: http://c.ymcdn.com/sites/www.aavpt.org/resource/resmgr/imported/cephalosporins.pdf

American Academy of Veterinary Pharmacology and Therapeutics, AAVPT$_b$. American Academy of Veterinary Pharmacology and Therapeutics. 2007. [Internet]. USA: The United States Pharmacopeial Convention. Aminoglycosides. Last access in April, 16, 2016. Available from: http://c.ymcdn.com/sites/www.aavpt.org/resource/resmgr/imported/aminoglycosides.pd

*According to: International Committee of Medical Journal Editors. [Internet]. Uniform requirements for manuscripts submitted to biomedical journals. [2011 Jul 15]. Available from: http:/www.nlm.nih.gov/bsd/uniform_requirements.html.

American Academy of Veterinary Pharmacology and Therapeutics, AAVPT$_{c.}$ American Academy of Veterinary Pharmacology and Therapeutics. 2007. [Internet]. USA: The United States Pharmacopeial Convention. Fluoroquinolones. Last access in April, 16, 2016. Available from: http://c.ymcdn.com/sites/www.aavpt.org/resource/resmgr/imported/fluoroquinolones.pdf

Amos GC, Hawkey PM, Gaze WH, et al. Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment. J Antimicrob Chemother. 2014 Jul;69(7):1785-91.

Andriole VT. The quinolones: past, present, and future. Clin Infect Dis. 2005 Jul 15;41 Suppl 2:S113-9.

Angulo FJ, Nunnery JA, Bair HD. Antimicrobial resistance in zoonotic enteric pathogens. Rev Sci Tech 2004, 23(2):485-496.

Anon (2010) VMD. Veterinary Medicines Directorate Product Information Database,

DEFRA (Department for Environment Food ; Rural Affairs) . [Internet homepage]. Last access: April, 02, 2016. Available in: (http://www.vmd.defra.gov.uk/ProductInformationDatabase/)

ANSES (French Agency for Food, Environmental and Occupational Health ; Safety). 2015. [Internet homepage]. Antibiotics for veterinary use: exposure of animals difficult to assess for 2014; levels of resistance that continue to fall. 2015. [Internet]. Last access: April, 25, 2016. Available from: https://www.anses.fr/en/content/antibiotics-veterinary-use-exposure-animals-difficult-assess-2014-levels-resistance-continue

Australian Commission on Safety and Quality in Healthcare. (ACSQH) Importance Ratings and Summary of Antibacterial Uses in Humans in Australia, Veterinary use of antibiotics critical to human health. Sydney. ACSQHC, 2014. [Internet homepage]. Last access: April, 01, 2016. Available in: http://www.ava.com.au/sites/default/files/AVA_website/pdfs/Veterinary%20use%20of%20antibiotics%20critical%20to%20human%20health.pdf

Bae DH, Dessie HK, Baek HJ, et al. Prevalence and characteristics of *Salmonella* spp. isolated from poultry slaughterhouses in Korea. J Vet Med Sci. 2013;75(9):1193-200.

Baggot JD, Prescott JF. Antimicrobial selection and dosage in the treatment of equine bacterial infections. Equine Vet J. 1987 Mar;19(2):92-6.

Bakker J, Thuesen LR, Braskamp G, Skaanild MT, Ouwerling B, Langermans JA,Bertelsen MF. Single subcutaneous dosing of cefovecin in rhesus monkeys (*Macaca mulatta):* a pharmacokinetic study. J Vet Pharmacol Ther. 2011 Oct;34(5):464-8.

Baraniak A, Fiett J, Mrówka A, et al. Evolution of TEM-type extended-spectrum beta-lactamases in clinical Enterobacteriaceae strains in Poland. Antimicrob Agents Chemother. 2005 May;49(5):1872-80.

Barbato L, Melo LC, Abreu J, et al. Extended-Spectrum Beta-Lactamase (ESBL)-Producing Gram-negative Bacteria from Pets, Buffalos and Psittacines, Brazil. IN: 52nd INTERSCiENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY (ICAAC), San Francisco – USA, 2012, September 9-12.

Barbosa DC, Leigue L, Warth JF, et al. Dermatopatias piogênicas em cães de abrigo e padroes de sensibilidade aos antimicrobianos de cepas de *Staphylococcus pseudintermedius.* Clin Vet (São Paulo), v. XVI, p. 72-78, 2011.

Barton MD. Impact of antibiotic use in the swine industry. Curr Opin Microbiol. 2014 Jun;19:9-15.

Batah R, Loucif L, Olaitan AO, et al. Outbreak of Serratia marcescens Coproducing ArmA and CTX-M-15 Mediated High Levels of Resistance to Aminoglycoside and Extended-Spectrum Beta-Lactamases, Algeria.Microb Drug Resist. 2015 Aug;21(4):470-6.

Ben Sallem R, Ben Slama K, Rojo-Bezares B, et al. IncI1 plasmids carrying bla(CTX-M-1) or bla(CMY-2) genes in Escherichia coli from healthy humans and animals in Tunisia. Microb Drug Resist. 2014 Oct;20(5):495-500

Bertrand S, FX Weill, A Cloeckaert M, et al. Clonal emergence of extended-spectrum β-lactamase (CTX-M-2)-producing Salmonella enterica serovar Virchow isolates with reduced susceptibilities to ciprofloxacin among poultry and humans in Belgium and France (2000 to 2003). J. Clin. Microbiol. 44:2897-2903, 2006.

Birgy A, Cohen R, Levy C, et al. Community faecal carriage of extended-spectrum beta-lactamase-producing Enterobacteriaceae in French children. BMC Infect Dis. 2012 Nov 21;12:315.

Blanc V, Mesa R, Saco M. ESBL- and plasmidic class C β-lactamase producing *E. coli* strains isolated from poultry, pig and rabbit farms. Vet Microbiol. 2006; 20;118(3-4):299-304.

Blanco M, Alonso, M. P.; Nicolas-Chanoine et al. Molecular epidemiology of *Escherichia coli* producing extended-spectrum betalactamases in Lugo (Spain): dissemination of clone O25b:H4- ST131 producing CTX-M-15. J Antimicrob Chemother, v. 63, p. 1135-1141, 2009.

Bonelli RR, Moreira BM, Picão RC. Antimicrobial resistance among Enterobacteriaceae in South America: history, current dissemination status and associated socioeconomic factors. Drug Resist Update 2014; 17: 24-36.

Bonnet R. Growing group of extended-spectrum b-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother 2004; 48: 1–14.

Bonnin RA, Poirel L, Carattoli A, et al. Characterization of an IncFII plasmid encoding NDM-1 from *Escherichia coli* ST131. PLoS One. 2012;7(4):e34752.

Bortolaia V, Guardabassi L, Trevisani M, Bisgaard M, Venturi L, Bojesen AM. High diversity of extended-spectrum beta-lactamases in *Escherichia coli* isolates from Italian broiler flocks. J Antimicrob Chemother. 2010; 54:1623-1626

Bortolaia V, Hansen KH, Nielsen CA, et al. High diversity of plasmids harbouring bla_{CMY2} among clinical *Escherichia coli* isolates from humans and companion animals in the upper Midwestern USA. J Antimicrob Chemother. 2014 Jun;69(6):1492-6.

Bradford PA. Extended-Spectrum β-Lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Threat. Clinic Microbiol Rev. 2001;14(4):933-951.

Briales A, Rodríguez-Martínez JM, Velasco C, et al. Prevalence of plasmid-mediated quinolone resistance determinants *qnr* and *aac(6')-Ib-cr* in *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum β-lactamases in Spain. Int J Antimicrob Agents. 2012 May;39(5):431-4.

Briñas L, Moreno MA, Teshager T, et al. Monitoring and Characterization of extended-spectrum β-lactamases in *Escherichia coli* strains from healthy and sick animals in spain in 2003. Antimicrob Agents Chemother. 2005;49(3):1262–1264.

Briñas L, Moreno MA, Zarazaga M, et al. Detection of CMY-2, CTX-M-14, and SHV-12 beta-lactamases in *Escherichia coli* fecal sample isolates from healthy chickens. Antimicrob Agents Chemother, 2003;47:2056-2058.

Brisse S, Fevre C, Passet V, et al. Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization. PLoS One 2009, 4(3):e4982. PMID 19319196

Briyne N, Atkinson J, Pokludová L, Borriello SP. Antibiotics used most commonly to treat animals in Europe. Vet Rec. 2014 Oct 4;175(13):325.

Brumbaugh GW. Rational selection of antimicrobial drugs for treatment of infections of horses. Vet Clin North Am Equine Pract. 1987 Apr;3(1):191-220. Review. PubMed PMID: 3555728.

Brun-Buisson C, Legrand P, Philippon A, et al. Transferable enzymatic resistance to third-generation cephalosporins during nosocomial outbreak of multiresistant *Klebsiella pneumoniae*. Lancet. 1987 Aug 8;2(8554):302-6.

Brunton LA, Duncan D, Coldham NG, et al. A survey of antimicrobial usage on dairy farms and waste milk feeding practices in England and Wales. Vet Record. 2012; 22;171(12):296.

Bryan J, Leonard N, Fanning S, et al. Antimicrobial resistance in commensal faecal *Escherichia coli* of hospitalised horses. Irish Vet J. 2010;63(6):373-379.

Bush K, Fisher JF. Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from gram-negative bacteria. Annu Rev Microbiol. 2011;65:455-78.

Calderwood SB, Hooper DC, Bloom A. Beta-lactam antibiotics: Mechanisms of action and resistance and adverse effects, 2012. [Internet homepage]. Last access: April, 05, 2016. Available in: http://androsma.5gbfree.com/contents/mobipreview.htm?26/2/26663?source=see_link

Carattoli A. Animal reservoirs for extended spectrum beta-lactamase producers. Clinic Microbiol Infect. 2008;14 Suppl 1:117-123.

Carattoli A, Bertini A, Villa L, et al. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods. 2005 Dec;63(3):219-28.

Carattoli A. Resistance plasmid families in Enterobacteriaceae. Antimicrob. Agents Chemother. 2009, 53, 2227–2238.

Carattoli A, Lovari S, Franco A, et al. Extended-spectrum betalactamases in *Escherichia coli* isolated from dogs and cats in Rome, Italy, from 2001 to 2003. Antimicrob Agents Chemother. 2005; 49: 833-5.

Carrillo NA, Giguère S, Gronwall RR, et al. Disposition of orally administered cefpodoxime proxetil in foals and adult horses and minimum inhibitory concentration of the drug against common bacterial pathogens of horses. Am J Vet Res. 2005 Jan;66(1):30-5.

Carvalho AC, Barbosa AV, Arais LR, et al. Resistance patterns, ESBL genes, and genetic relatedness of *Escherichia coli* from dogs and owners. Braz. J. Microbiol. 2016, 47(1): 150-158.

Casella T, Rodríguez MM, Takahashi JT, et al. Detection of blaCTX-M-type genes in complex class 1 integrons carried by Enterobacteriaceae isolated from retail chicken meat in Brazil. Int J Food Microbiol. 2015 Mar 16;197:88-91.

Cattoir V, Nordmann P. Plasmid-mediated quinolone resistance in gram-negative bacterial species: an update. Curr Med Chem. 2009;16(8):1028-46

Chapman PA, Ellin M, Ashton R, et al. Comparison of culture, PCR and immunoassays for detecting *Escherichia coli* O157 following enrichment culture and immunomagnetic separation performed on naturally contaminated rae meat products. Int. J. Food Microbiol. V. 68, p. 11-20, 2001.

Chong Y, Shimoda S, Yakushiji H, et al. Community spread of extended-spectrum β-lactamase-producing *Escherichia coli, Klebsiella pneumoniae*, and *Proteus mirabilis*: A long-term study in Japan. J Med Microbiol. 2013 Jul;62(7):1038-43.

Clermont O, Johnson JR, Menard M, et al. Determination of *Escherichia coli* O types by allele-specific polymerase chain reaction: application to the O types involved in human septicemia. Diagn Microbiol Infect Dis. 2006 Feb;57(2):129-36.

Clermont O, Lavollay M, Vimont S, et al. The CTX-M-15- 17 producing *Escherichia coli* diffusing clone belongs to a highly virulent B2 phylogenetic subgroup. J Antimicrob Chemother, 2008, v. 61, p. 1024- 1028.

Clermont, O; Bonacorsi, S and E. Bingen. Rapid and simple determination of the Escherichia coli phylogenetic group. Appl Environ Microbiol, 2000, vol. 66, no. 10, pp. 4555–4558.

Clermont O, Christenson JK, Denamur E et al. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Reports, 2013, vol. 5, no. 1, pp. 58–65.

Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals—Third Edition: Approved Standard M31-A3. CLSI, Wayne, PA, USA, 2008.

Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-third Informational Supplement M100-S23. CLSI, Wayne, PA, USA, 2013.

Cloeckaert A, Praud K, Lefevre M, et al. IncI1 plasmid carrying extended-spectrum-beta-lactamase gene $bla_{CTX-M-1}$ in *Salmonella enterica* isolates from poultry and humans in France, 2003 to 2008. Antimicrob Agents Chemother. 2010 Oct;54(10):4484-6.

Coelho A, Piedra-Carrasco N, Bartolomé R, et al. Role of IncHI2 plasmids harbouring *bla*$_{VIM-1}$, *bla*$_{CTX-M-9,}$ *aac(6')-Ib* and *qnr*A genes in the spread of multiresistant *Enterobacter cloacae* and *Klebsiella pneumoniae* strains in different units at Hospital Vall d'Hebron, Barcelona,Spain. Int J Antimicrob Agents. 2012 Jun;39(6):514-7.

Cole, C. Basic of antimicrobial therapy for the horse. In Equine Pharmacology. USA, WilleyBlack, 2ed. 2014.

Cortés P, Blanco V, Mora A, et al. Isolation and characterization of potentially pathogenic antimicrobial-resistant *Escherichia coli* strains from chicken and pig farms in Spain. Appl Environ Microbiol. 2010 May;76(9):2799-805.

Coque TM, Baquero F, Canton R. Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. Euro Surveill. 2008 Nov 20;13(47).

Costa D, Poeta P, Brinas L, et al. Detection of CTX-M-1 and TEM-52 beta-lactamases in *Escherichia coli* strains from healthy pets in Portugal. J Antimicrobial Chemother. 2004;54(5):960-961.

Cox S, Sommardahl C, Seddighi R, Videla R, Hayes J, Pistole N, Hamill M, Doherty T. Pharmacokinetics of intravenous and subcutaneous cefovecin in alpacas. J Vet Pharmacol Ther. 2015 Aug;38(4):344-9.

Cromwell GL. Why and how antibiotics are used in swine production. Anim Biotechnol. 2002 May;13(1):7-27. Review.

Curran B, Jonas D, Grundmann H, et al. Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. J Clin Microbiol. 2004 Dec;42(12):5644-9.

Dahmen S, Haenni M, Châtre P, et al. Characterization of *bla*$_{CTX-M}$ IncFII plasmids and clones of *Escherichia coli* from pets in France. J Antimicrob Chemother. 2013 Dec;68(12):2797-801.

Dahmen S, Metayer V, Gay E, et al. Characterization of extended-spectrum beta-lactamase (ESBL)-carrying plasmids and clones of Enterobacteriaceae causing cattle mastitis in France. Vet Microbiol. 2013;23;162(2-4):793-799.

Damborg P, Marskar P, Baptiste KE, et al. Faecal shedding of CTX-M-producing *Escherichia coli* in horses receiving broad-spectrum antimicrobial prophylaxis after hospital admission. Vet Microbiol. 2012, Jan 27;154(3-4):298-304.

Datta N, Kontomichalou P. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nature. 1965;208(5007):239-41

Davies J, Davies D. Origins and Evolution of Antibiotic Resistance. Microbiol Mol Biol Rev. 2010;74(3):417-433.

Department for Environment, Food and Rural Affairs – United Kingdom (DEFRA). Extended-Spectrum Beta-Lactamases (ESBL) in bacteria associated with animals. 2013. [Internet Homepage]. Accessed May 10, 2013. Available from: http://archive.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/zoonoses/esbl.htm .

Dhanji H, Murphy NM, Doumith M, et al. Cephalosporin resistance mechanisms in *Escherichia coli* isolated from raw chicken imported into the UK. Journal of Antimicrobial Chemotherapy. 2010; 65(12):2534-7.

Dhanji H, Patel R, Wall R, et al. Variation in the genetic environments of bla(CTX-M-15) in *Escherichia coli* from the faeces of travellers returning to the United Kingdom. J Antimicrob Chemother. 2011 May;66(5):1005-12

Diancourt L, Passet V, Verhoef J, et al. Multilocus Sequence Typing of *Klebsiella pneumoniae* nosocomial isolates. J Clin Microbiol 2005, 43:4178-82.

Dimitris A, Kafetzis D, Craig B, et al. Clinical Pharmacology of Cefotaxime in Pediatric Patients. Antimicrob Agents Chemother. 1981 October; 20(4): 487–490.

Doi Y, Arakawa Y. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. Clin Infect Dis. 2007 Jul 1;45(1):88-94.

Doi Y, de Oliveira Garcia D, Adams J, et al. Co-production of novel 16S rRNA methylase RmtD and metallo-β-lactamase SPM-1 in Panresistant *Pseudomonas aeruginosa* isolate from Brazil. Antimicrob Agents Chemother 2006;51:852-6

Doi, Y., Yokoyama, K., Yamane, K., et al. Plasmid-Mediated 16S rRNA Methylase in Serratia marcescens Conferring High-Level Resistance to Aminoglycosides. Antimicrobial Agents and Chemotherapy, 2004. 48(2), 491–496.

Dolejska M, Duskova E, Rybarikova J, et al. Plasmids carrying CTX-M-1 and qnr genes in *Escherichia coli* isolates from an equine clinic and a horseback riding centre. J Antimicrob Chemother. 2011 Apr;66(4):757-64.

Dolejska M, Villa L, Minoia M, et al. Complete sequences of IncHI1 plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ and *qnrS*1 in equine *Escherichia coli* provide new insights into plasmid evolution. J Antimicrob Chemother. 2014 Sep;69(9):2388-93.

Dropa M, Balsalobre LC, Lincopan N, et al. Complex class 1 integrons harboring CTX-M-2-encoding genes in clinical Enterobacteriaceae from a hospital in Brazil. J Infect Dev Ctries. 2015 Aug 29;9(8):890-7.

Dunowska M, Morley PS, Traub-Dargatz JL et al. Impact of hospitalization and antimicrobial drug administration on antimicrobial susceptibility patterns of commensal *Escherichia coli* isolated from the feces of horses. J Am Vet Med Assoc 2006, 228(12):1909-1917

Edelstein M, Pimkin M, Palagin I, et al. Prevalence and molecular epidemiology of CTX-M extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals. Antimicrob Agents Chemother. 2003 Dec;47(12):3724-32.

Egorova S, Timinouni M, Demartin M, et al. Ceftriaxone-Resistant *Salmonella* enterica Serotype Newport, France. Emerging Infectious Diseases, 2008, 14(6), 954–957.

European Agency for the Evaluation of Medical Products (Veterinary Medicines Evaluation Unit). EMEA,1999. January, 1999. [Internet Homepage] Last Access: April, 04, 2016. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500010684.pdf.

European Agency for the Evaluation of Medical Products (Veterinary Medicines Evaluation Unit). EMEA, 2014, 10 July 2014. Committee for Medicinal Products for Veterinary Use (CVMP) 4 Concept paper on use of aminoglycosides in animals in 5 the European Union: development of resistance and 6 impact on human and animal health. [Internet Homepge]. Last Access: April, 02, 2016. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/07/WC500170029.pdf

European Agency for the Evaluation of Medical Products (Veterinary Medicines Evaluation Unit). EMEA, 2013a. Use of glycylcyclines in animals in the European Union: development of resistance and possible impact on human and animal health EMA/291760/2013. [Internet Homepage] Last Access: April, 04, 2016. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Report/2013/07/WC500146814.pdf

European Agency for the Evaluation of Medical Products (Veterinary Medicines Evaluation Unit). EMEA, 2013b. Use of colistin products in animals within the European Union: development of resistance and possible impact on human and animal health. [Internet Homepage] Last Access: April, 04, 2016. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Report/2013/07/WC500146813.pdf

European Committee on Antimicrobial Susceptibility Testing (EUCAST). Clinical Breakpoints. [Internet]. 2015. Last access: December, 20, 2015. Available from: http://www.eucast.org/clinical_breakpoints.

European Food Safety Authority (EFSA): Scientific Opinion on Carbapenem resistance in food animal ecosystems EFSA Panel on Biological Hazards (BIOHAZ). EFSA Journal 2013;11(12):3501 Parma, Italy. [Internet Homepage] Last Access: April, 04, 2016. Available from: http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3501.pdf

Escudero E, Vinue L, Teshager T, et al. Resistance mechanisms and farm-level distribution of fecal Es*cherichia coli* isolates resistant to extended-spectrum cephalosporins in pigs in Spain. Research Vet Sci. 2010; 88:83-87.

Ewers C, Bethe A, Stamm I et al. CTX-M-15-D-ST648 *Escherichia coli* from companion animals and horses: another pandemic clone combining multiresistance and extraintestinal virulence? J Antimicrob Chemother 2014a; 69: 1224-1230.

Ewers C, Bethe A, Semmler T, et al. Extended-spectrum-β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect. 2012 Jul;18(7):646-55.

Ewers C, Bethe A, Wieler LH, et al. Companion animals: a relevant source of extended-spectrum β-lactamase-producing fluoroquinolone-resistant *Citrobacter freundii*. Int J Antimicrob Agents. 2011a; Jan;37(1):86-7.

Ewers C, Grobbel M, Bethe A, et al. Extended-spectrum-beta-lactamases-producing gram-negative bacteria in companion animals: action is clearly warranted! Berl Munch Tierarztl Wochenschr. 2011b; Mar-Apr;124(3-4):94-101.

Ewers C, Grobbel M, Stamm I, et al. Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-beta-lactamase-producing *Escherichia coli* among companion animals. J Antimicrob Chemother. 2010b; Apr;65(4):651-60.

Ewers C, Stamm I, Pfeifer Y et al. Clonal spread of highly successful ST15-CTX-M-15 *Klebsiella pneumoniae* in companion animals and horses. J Antimicrob Chemother 2014b; Jun 27. pii: dku217.

Falbo V, Famigliatti M, Caprioli A. Gene block encoding production of cytotoxic necrotizing factor 1 and hemolysin in *Escherichia coli* isolates from extraintestinal infections. Infect. Immun., v. 60, p. 2182-2187, 1992.

Falgenhauer L, Schmiedel J, Ghosh H, et al. Resistance plasmids in ESBL-encoding *Escherichia coli* isolates from humans, dogs and cats. Berl Munch Tierarztl Wochenschr. 2014 Nov-Dec;127(11-12):458-63.

Feßler AT, Kadlec K, Schwarz S. Novel Apramycin Resistance Gene apmA in Bovine and Porcine Methicillin-Resistant *Staphylococcus aureus* ST398 Isolates. Antimicrob. Agents Chemother. January 2011 vol. 55 no. 1 373-375

Féria C, Ferreira E, Correia JD, et al. Patterns and mechanisms of resistance to beta-lactams and beta-lactamase inhibitors in uropathogenic *Escherichia coli* isolated from dogs in Portugal. J Antimicrob Chemother. 2002 Jan;49(1):77-85.

Fernandes SA, Paterson DL, Ghilardi-Rodrigues AC, et al. CTX-M-2-producing *Salmonella* Typhimurium isolated from pediatric patients and poultry in Brazil. Microbial Drug Resist. 2009;15(4):317-21.

Fernandes MR, Moura Q, Sartori L, et al. Silent Dissemination of Colistin-Resistant *Escherichia coli* In South America Could Contribute to the Global Spread of the *Mcr*-1 Gene. Euro Surveill. 2016 Apr 28;21(17).

Folster JP, Pecic G, McCullough A, et al. Characterization of bla_{CMY}-encoding plasmids among *Salmonella* isolated in the united states in 2007. Foodborne Pathog Dis. 2007;8:1289–1294.

Folz SD, Hanson BJ, Griffin AK, et al. Treatment of respiratory infections in horses with ceftiofur sodium. Equine Vet J. 1992 Jul;24(4):300-4.

Fontes LC, Neves PR, Oliveira S, et al. Isolation *of Pseudomonas aeruginosa* coproducing metallo-β-lactamase SPM-1 and 16SrRNA methylase RmtD1 in an urban river. Antimicrob Agents Chemother. 2011 Jun;55(6):3063-4.

Food Drug Administration (FDA). [Internet homepage]. United States. Cephalosporin Order of Prohibition Questions and Answers. Last Updated: oct, 2014. Last access: April, 04, 2016. Available from: http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/ucm421538.htm

Francisco GR, Nora ST, Bueno MF, et al. Identification of aminoglycoside-resistant *Pseudomonas aeruginosa* producing RmtG 16S rRNA methyltransferase in a cystic fibrosis patient. Antimicrob Agents Chemother. 2015 May;59(5):2967-8.

Frye JG, Fedorka-Cray PJ. Prevalence, distribution and characterisation of ceftiofur resistance in *Salmonella* enteric isolated from animals in the USA from 1999 to 2003. Intern J Antimicrob Agents. 2007; 30: 134-42.

Gannon VP, Rashed M, King RK, et al. Detection and haracterization of theeae gene of Shiga-like toxin-producing *Escherichia coli* using polymerase chainreaction. J Clin Microbiol. 1993 May;31(5):1268-74.

García-Fernández A, Carattoli A. Plasmid double locus sequence typing for IncHI2 plasmids, a subtyping scheme for the characterization of IncHI2 plasmids carrying extended-spectrum beta-lactamase and quinolone resistance genes. J Antimicrob Chemother. 2010 Jun;65(6):1155-61.

García Fernández A, Cloeckaert A, Bertini A, et al. Comparative analysis of IncHI2 plasmids carrying *bla*$_{CTX-M-2}$ or *bla*$_{CTX-M-9}$ from *Escherichia coli* and *Salmonella enterica* strains isolated from poultry and humans. Antimicrob Agents Chemother. 2007 Nov;51(11):4177-80.

Tsodikova Garneau-S, Labby KJ. Mechanisms of Resistance to Aminoglycoside Antibiotics: Overview and Perspectives. Medchemcomm. 2016 Jan 1;7(1):11-27.

Geser N, Stephan R, Hächler H. Occurrence and characteristics of extended spectrum b-lactamase (ESBL) producing Enterobacteriaceae in food producing animals, minced meat and raw milk. BMC Vet Research. 2012;8:21:2-9

Gibson JS, Morton JM, Cobbold RN, et al. Risk factors for multidrug-resistant *Escherichia coli* rectal colonization of dogs on admission to a veterinary hospital. Epidemiol Infect. 2011 Feb;139(2):197-205).

Gibson JS, Cobbold RN, Trott DJ. Characterization of multidrug-resistant *Escherichia coli* isolated from extraintestinal clinical infections in animals. J Med Microbiol. 2010: 59: 592–598.

Giguere S, Prescott JF, Baggot JD. Antimicrobial therapy in veterinary medicine. Ames, Iowa State University Press: 2010.

Girlich D, Poirel L, Carattoli A, et al. Extended-Spectrum β-Lactamase CTX-M-1 in *Escherichia coli* Isolates from Healthy Poultry in France. Appl Environ Microbiol, 2007, 73(14), 4681–4685.

González-Sanz R, Herrera-León S, de la Fuente M, et al. Emergence of extened-spectrum β-lactamases and AmpC-type β-lactamases in human *Salmonella* isolated in Spain from 2001 to 2005. J Antimicrob Chemother. 2009;64:1181–1186.

Gonullu N, Aktas Z, Kayacan CB, et al. Dissemination of CTX-M-15 beta-lactamase genes carried on Inc FI and FII plasmids among clinical isolates of *Escherichia coli* in a university hospital in Istanbul, Turkey. J Clin Microbiol. 2008 Mar;46(3):1110-2.

Grami R, Mansour W, Dahmen S, et al. The *bla*$_{CTX-M-1}$ IncI1/ST3 plasmid is dominant in chickens and pets in Tunisia. J Antimicrob Chemother. 2013 Dec;68(12):2950-2.

Greene C. Infectious Diseases in dogs and cats. 4ed, 2012 Elsevier. USA

Guardabassi L, Schwarz S, Lloyd DH. Pet animals as reservoirs of antimicrobial-resistant bacteria. J Antimicrob Chemother. 2004; Aug 54(2):321-332.

Haenni M, Poirel L, Kieffer N, et al. Co-occurrence of extended spectrum β lactamase and MCR-1 encoding genes on plasmids. Lancet Infect Dis. 2016 Mar;16(3):281-2.

Haenni M, Saras E, Métayer V, et al. High prevalence of *bla*$_{CTX-M-1}$/IncI1/ST3 and *bla*$_{CMY-2}$/IncI1/ST2 plasmids in healthy urban dogs in France. Antimicrob Agents Chemother. 2014 Sep;58(9):5358-62.

Haenni M, Saras E, Ponsin C, et al. High prevalence of international ESBL CTX-M-15-producing *Enterobacter cloacae* ST114 clone in animals. J Antimicrob Chemother. 2016 Feb 4. [Epub ahead of print]

Hall TL, Tell LA, Wetzlich SE, et al. Pharmacokinetics of ceftiofur sodium and ceftiofur crystalline free acid in neonatal foals. J Vet Pharmacol Ther. 2011 Aug;34(4):403-9.

Hansen KH, Damborg P, Andreasen M, et al. Carriage and fecal counts of cefotaxime M-producing *Escherichia coli* in pigs: a longitudinal study. Appl Environ Microbiol. 2013 Feb;79(3):794-8

Harada K, Nakai Y, Kataoka Y. Mechanisms of resistance to cephalosporin and emergence of O25b-ST131 clone harboring CTX-M-27 β lactamase in extraintestinal pathogenic *Escherichia coli* from dogs and cats in Japan. Microbiol Immunol. 2012 Apr 9. 56: 480–485.

Harada K, Morimoto E, Kataoka Y, et al. Clonal spread of antimicrobial-resistant *Escherichia coli* isolates among pups in two kennels. Acta Vet Scand. 2011; Feb;17;53:11:1-7.

Harris AD, McGregor JC, Johnson JA, et al. Risk factors for colonization with extended-spectrum beta-lactamase-producing bacteria and intensive care unit admission. Emerg Infect Dis. 2007, Aug;13(8):1144-9.

Harris PN, Ferguson JK. Antibiotic therapy for inducible AmpC β-lactamase-producing Gram-negative bacilli: what are the alternatives to carbapenems, quinolones and aminoglycosides? Int J Antimicrob Agents. 2012 Oct;40(4):297-305.

Hartman AB, Venkatesan M, Oaks EV, et al. Sequence and molecular characterization of a multicopy invasion plasmid antigen gene, ipaH, of *Shigella flexneri*. J Bacteriol. 1990 Apr;172(4):1905-15.

Hashimoto A, Nagamatsu M, Ohmagari N, et al. Isolation of OXA-48 carbapenemase-producing *Klebsiella pneumoniae* ST101 from an overseas traveler returning to Japan. Jpn J Infect Dis. 2014;67(2):120-1.

Hemalatha V, Padma M, Sekar U, Vinodh TM, Arunkumar AS. Detection of Amp C beta lactamases production in *Escherichia coli ; Klebsiella* by an inhibitor based method. Indian J Med Res 2007;126:220-3.

Heuer OE, Jensen VF, Hammerum AM. Antimicrobial drug consumption in companion animals. Emerg. Infect. Dis. 2005, 11, 344-345.

Hiroi M, Yamazaki F, Harada T, et al. Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in food-producing animals. J Vet Med Sci. 2012; Feb;74(2):189-195.

Holman DB, Chénier MR. Antimicrobial use in swine production and its effect on the swine gut microbiota and antimicrobial resistance. Can J Microbiol. 2015 Nov;61(11):785-98.

Hordijk J, Schoormans A, Kwakernaak M, et al. High prevalence of fecal carriage of extended spectrum β-lactamase/AmpC-producing Enterobacteriaceae in cats and dogs. Front Microbiol. 2013 Aug 16;4:242.

Hornish RE, Kotarski SF. Cephalosporins in veterinary medicine - ceftiofur use in food animals. Curr Top Med Chemi. 2002;2(7):717-731.

Horton RA, Randall LP, Snary EL, et al. Fecal Carriage and Shedding Density of CTX-M Extended-Spectrum β-Lactamase-Producing *Escherichia coli* in Cattle, Chickens, and Pigs: Implications for Environmental Contamination and Food Production. Appl Environ Microbiol. 2011 77(11), 3715–3719.

Hu FP, Guo Y, Zhu DM, et al. Resistance trends among clinical isolates in China reported from CHINET surveillance of bacterial resistance, 2005-2014. Clin Microbiol Infect. 2016 Mar;22 Suppl 1:S9-S14.

Huber H, Zweifel C, Wittenbrink MM, et al. ESBL-producing uropathogenic *Escherichia coli* isolated from dogs and cats in Switzerland. Vet Microbiol. 2013; Mar 23;162(2-4):992-6.

Hughes LA, Pinchbeck G, Callaby R, Dawson S, Clegg P, Williams N. Antimicrobial prescribing practice in UK equine veterinary practice. Equine Vet J. 2013 Mar;45(2):141-7.

Knothe H, Shah P, Krcmery V, et al. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection. 1983, 11, 315-7.

Iredell J, Brown J, Tagg K. Antibiotic resistance in Enterobacteriaceae: mechanisms and clinical implications. BMJ. 2016 Feb 8;352:h6420.

Jacoby GA. Mechanisms of resistance to quinolones. Clin Infect Dis. 2005 Jul 15;41 Suppl 2:S120-6.

Jacoby Ga.; Sutton, L. Properties of plasmids responsible for production of extended-spectrum β-lactamases. Antimicrob Agents Chemother. 1991;35:164–169.

Jacoby Ga, Gacharna N, Black Ta, et al. Temporal appearance of plasmid-mediated quinolone resistance genes. Antimicrob Agents Chemother. 2009 Apr;53(4):1665-6.

Jiang, HX, Tang, D, Liu, YH, et al. Prevalence and characteristics of beta-lactamase and plasmid-mediated quinolone resistance genes in *Escherichia coli* isolated from farmed fish in China. J Antimicrob Chemother. 2012; 67(10): 2350–2353.

Johnson TJ, Wannemuehler YM, Johnson SJ, et al. Plasmid replicon typing of commensal and pathogenic *Escherichia coli* isolates. Appl Environ Microbiol 2007; 73:1976-83.

Johns I, Verheyen K, Good L, et al. Antimicrobial resistance in faecal Escherichia coli isolates from horses treated with antimicrobials: a longitudinal study in hospitalised and non-hospitalised horses. Vet Microbiol. 2012, Oct 12;159(3-4):381-9.

Jordan D, Chin JJ, Fahy VA, et al. Antimicrobial use in the Australian pig industry: results of a national survey. Aust Vet J. 2009 Jun;87(6):222-9.

Jørgensen CJ, Cavaco LM, Hasman H, Emborg HD, Guardabassi L. Occurrence of CTX-M-1-producing Escherichia coli in pigs treated with ceftiofur. J Antimicrob Chemother. 2007; 59:1040-1042.

Kaartinen L, Panu S, Pyörälä S. Pharmacokinetics of enrofloxacin in horses after single intravenous and intramuscular administration. Equine Vet J. 1997 Sep;29(5):378-81.

Kadlec K, Schwarz S. Analysis and distribution of class 1 and class 2 integrons and associated gene cassettes among *Escherichia coli* isolates from swine, horses, cats and dogs collected in the BfT-GermVet monitoring study. J Antimicrob Chemother 2008, 62(3):469-473.

Kiratisin P, Henprasert A. Resistance phenotype-genotype correlation and molecular epidemiology of *Citrobacter, Enterobacter, Proteus, Providencia, Salmonella and Serratia* that carry extended-spectrum β-lactamases with or without plasmid-mediated AmpC β-lactamase genes in Thailand. Trans R Soc Trop Med Hyg. 2011 Jan;105(1):46-51.

Knothe H, Shah P, Krcmery V, et al. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection. 1983Nov-Dec;11(6):315-7.

Kowalski RP, Dhaliwal DK, Karenchak LM. Gatifloxacin and moxifloxacin: an in vitro susceptibility comparison to levofloxacin, ciprofloxacin, and ofloxacin using bacterial keratitis isolates. Am J Ophthalmol. 2003;136:500–505.

Lahlaoui H, Ben Haj Khalifa A, Ben Moussa M. Epidemiology of Enterobacteriaceae producing CTX-M type extended spectrum β-lactamase (ESBL). Med Mal Infect. 2014 Sep;44(9):400-4.

Larsen MV, Cosentino S, Rasmussen S, et al. Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol. 2012 Apr;50(4):1355-61.

Lartigue MF, Poirel L, Nordmann P. Diversity of genetic environment of bla_{CTX-M} genes. FEMS Microbiol Lett. 2004 May 15;234(2):201-7.

Le Bouguenec C, Archambaud M, Labigne A. Rapid and specific detection of the pap, afa, and sfa adhesin-encoding operons in uropathogenic *Escherichia coli* strains by polymerase chain reaction. J Clin Microbiol. 1992 May;30(5):1189-93.

Ledbetter EC, Hendricks LM, Riis RC, et al. In vitro fluoroquinolone susceptibility of *Pseudomonas aeruginosa* isolates from dogs with ulcerative keratitis. Am J Vet Res June. 2007; Vol. 68, No. 6, Pages 638-642.

Lee W, Chung HS, Lee H, et al. CTX-M-55-type extended-spectrum β-lactamase-producing *Shigella sonnei* isolated from a Korean patient who had travelled to China. Ann Lab Med. 2013; Mar;33(2):141-144

Lee J, Peck K, Ko K. J. advantages of two major clones of carbapenem-resistant Pseudomonas aeruginosa isolates (CC235 and CC641) from Korea: antimicrobial

resistance, virulence and biofilm-forming activity. J. Med. Microbiol., July 2013 62: 1015-1024 Med. Microbiol. 62(7):1015-1024Selective

Leigue-dos-Santos L, Moura RA, Ramires PA, et al. Current status of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae in animals. In: Méndez-Vilas A, editor. Microbial pathogens and strategies for combating them: science, technology and education. Badajoz: Formatex Research Center, Spain; 2013. p. 1600–7.

Leigue L, Warth JF, Melo LC, et al. MDR ST2179-CTX-M-15 *Escherichia coli* co-producing RmtD and AAC(6')-Ib-cr in a horse with extraintestinal infection, Brazil. J Antimicrob Chemother. 2015 Apr;70(4):1263-5.

Li XZ, Mehrotra M, Ghimire S, et al. Beta-Lactam resistance and beta-lactamases in bacteria of animal origin. Vet Microbiol. 2007 Apr 15;121(3-4):197-214.

Limbert M, Isert D, Klesel N, et al. Antibacterial activities in vitro and in vivo and pharmacokinetics of Cefquinome (HR 111V), a new broad-spectrum cephalosporin. Antimicrob Agents Chemother. 1991; 35: 14-19.

Liu YY, Wang Y, Walsh TR, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis. 2016 Feb;16(2):161-8.

Liu BT, Liao XP, Yue L. Prevalence of β-lactamase and 16S rRNA methylase genes among clinical *Escherichia coli* isolates carrying plasmid-mediated quinolone resistance genes from animals. Microb Drug Resist 2013; 19: 237-245.

Livermore DM, Canton R, Gniadkowski M, et al. CTX-M: changing the face of ESBLs in Europe. J Antimicrob Chemother. 2007;59:165–174.

Locatelli C, Scaccabarozzi L, Pisoni G, et al. CTX-M-1 ESBL-producing *Klebsiella pneumoniae subsp. pneumoniae* isolated from cases of bovine mastitis. J Clin Microbiol. 2010;48(10):3822-3823.

Lu SY, Zhang YL, Geng SN, et al. High Diversity of Extended-Spectrum Beta-Lactamase-Producing Bacteria in an Urban River Sediment Habitat. Appl Environ Microbiol, 2010, 76(17), 5972–5976.

Ma J, Liu JH, Lv L, et al. Characterization of extended-spectrum β-lactamase genes found among *Escherichia coli* isolates from duck and environmental samples obtained on a duck farm. Appl Environ Microbiol. 2012; 78(10):3668-73.

Ma J, Zeng Z, Chen Z, et al. High prevalence of plasmid-mediated quinolone resistance determinants *qnr, aac(6')-Ib-cr*, and *qepA* among ceftiofur-resistant Enterobacteriaceae isolates from companion and food-producing animals. Antimicrob Agents Chemother. 2009; 53: 519-24.

Maatallah M, Cheriaa J, Backhrouf A, et al. Population Structure of Pseudomonas aeruginosa from Five Mediterranean Countries: Evidence for Frequent Recombination and Epidemic Occurrence of CC235. PLoS ONE 2011, 6(10): e25617.

Maddox TW, Clegg PD, Diggle PJ, et al. Cross-sectional study of antimicrobial-resistant bacteria in horses. Part 1: Prevalence of antimicrobial-resistant Escherichia coli and methicillin-resistant *Staphylococcus aureus*. Equine Vet J. 2012; 44: 289-96.

Maddox TW, Pinchbeck GL, Clegg PD, et al. Cross-sectional study of antimicrobial-resistant bacteria in horses. Part 2: Risk factors for faecal carriage of antimicrobial-resistant *Escherichia coli* in horses. Equine Vet J. 2012 May;44(3):297-303.

Madec JY, Poirel L, Saras E, et al. Non-ST131 *Escherichia coli* from cattle harbouring human-like $bla_{CTX\text{-}M\text{-}15}$-carrying plasmids. J Antimicrob Chemother. 2012 Mar;67(3):578-81.

Magdesia, KG. Update on antimicrobial selection and use. In Current Therapy in Equine Medicine. Sprayberry, KA; Robinson, NE. USA: Elsevier, 7ed, 2015.

Magiorakos AP, Srinivasan A, Carey RB, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012 Mar;18(3):268-81.

Mahrouki S, Belhadj O, Chihi H, et al. Chromosomal $bla_{CTX\text{-}M\text{-}15}$ associated with IS*Ecp1* in *Proteus mirabilis* and *Morganella morganii* isolated at the Military Hospital of Tunis, Tunisia. J Med Microbiol. 2012 Sep;61(Pt 9):1286-9.

Majiduddin FK, Materon IC, Palzkill TG. Molecular analysis of beta-lactamasestructure and function. Int J Med Microbiol. 2002 Jul;292(2):127-37.

MAPA: Ministério da Agricultura e Abastecimento. Coordenação De Fiscalização de Produtos Veterinários Relatório de Produtos com Licença Vigente no Brasil, 2014 [Internet Homepage]. Last access: March, 25, 2016. Available in: http://www.agricultura.gov.br/arq_editor/file/Registros_Autorizacao/listas%20de%20produtos/Produtos%20Vigentes-%20Abril-2014.pdf

Marchandin H, Carriere C, Sirot D, et al. TEM-24 produced by four different species of Enterobacteriaceae, including Providencia rettgeri, in a single patient. Antimicrob Agents Chemother. 1999; 43:2069-2073.

Marín M, Gudiol F. Beta-Lactam antibiotics. Enferm Infecc Microbiol Clin.2003 Jan;21(1):42-55.

Markovska RD, Stoeva TJ, Bojkova KD, et al. Epidemiology and molecular characterization of extended-spectrum beta-lactamase-producing *Enterobacter* spp*., Pantoea agglomerans,* and *Serratia marcescens* isolates from a Bulgarian hospital. Microb Drug Resist. 2014 Apr;20(2):131-7.

Mathers AJ, Peirano G, Pitout JD. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. Clin Microbiol Rev. 2015 Jul;28(3):565-91.

Matsumoto Y, Ikeda F, Kamimura T, et al. Novel plasmid-mediated β-lactamase from *Escherichia coli* that inactivates oxyimino-cephalosporins. Antimicrob Agents Chemother. 1988; 32:1243-1246.

Meacham KJ, Zhang L, Foxman B, et al. Evaluation of Genotyping Large Numbers of *Escherichia coli* Isolates by Enterobacterial Repetitive Intergenic Consensus-PCR. J Clin Microbiol. 2003;41(11):5224-5226.

Melegh S, Schneider G, Horváth M, et al. Identification and characterization of CTX-M-15 producing *Klebsiella pneumoniae* clone ST101 in a Hungarian university teaching hospital. Acta Microbiol Immunol Hung. 2015 Sep;62(3):233-45.

Melo LC. Commensal Microbiota of Companion Animals as Reservoirs of Extended-Spectrum Beta-Lactamase (ESBL) and Plasmid-Mediated Quinolone Resistance (PMQR) Genes. 2014 83p. Masters dissertation (Microbiology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2014.

Meunier D, Jouy E, Lazizzera C, et al. CTX-M-1- and CTX-M-15-type beta-lactamases in clinical *Escherichia coli* isolates recovered from food-producing animals in France. Int JAntimicrob Agents. 2006; 28(5):402-7.

Meyer E, Gastmeier P, Kola A, et al. Pet animals and foreign travel are risk factors for colonisation with extended-spectrum β-lactamase-producing Escherichia coli. Infection. 2012. Dec;40(6):685-7.

Meyer S, Giguère S, Rodriguez R, et al. Pharmacokinetics of intravenous ceftiofur sodium and concentration in body fluids of foals. J Vet Pharmacol Ther. 2009 Aug;32(4):309-16.

Mccabe WR, Jackson GG. Gram–negative bacteremia. I Etiology and ecology. Arch Int Med. 1962; 110: 847–55

Molstad S, Cars O, Struwe J. Strama--a Swedish working model for containment of antibiotic resistance. Euro Surveill. 2008 Nov 13;13(46).

Moore Cp, Collins, B.K.; Fales, W.H. Antibacterial susceptibility patterns for microbial isolates associated with infectious keratitis in horses: 63 cases (1986-1994). J Am Vet Med Assoc. 1995 Oct 1;207(7):928-33.

Moreno A, Bello H, Guggiana D, et al. Extended-spectrum beta-lactamases belonging to CTX-M group produced by *Escherichia coli* strains isolated from companion animals treated with enrofloxacin. Vet Microbiol. 2008, May 25;129(1-2):203-8.

Moore CP, Naisse MP. Clinical Microbiology. In : Gellat, K.N. VetOphthal, 3°Ed, 1999. Molstad S, Cars O, Struwe J. Strama--a Swedish working model for containment of antibiotic resistance. Euro Surveill. 2008 Nov 13;13(46).

Morosini MI, García-Castillo M, Coque TM, et al. Antibiotic Coresistance in Extended-Spectrum-β-Lactamase-Producing Enterobacteriaceae and In Vitro Activity of Tigecycline. Antimicrob Agents Chemother. 2006, 50(8), 2695–2699.

Moura I, Torres C, Silva N et al. Genomic Description of Antibiotic Resistance in Escherichia coli and Enterococci Isolates from Healthy Lusitano Horses. Journal of Equine Vet Sci, XXX (2013) 1-7.

Naeem A, Badshah SL, Muska M, et al. The Current Case of Quinolones: Synthetic Approaches and Antibacterial Activity. Molecules. 2016 Mar 28;21(4).

Naseer U, Haldorsen B, Simonsen GS, et al. Sporadic occurrence of CMY-2-producing multidrug-resistant *Escherichia coli* of ST-complexes 38 and 448, and ST131 in Norway. Clin Microbiol Infect. 2010 Feb;16(2):171-8.

National Office of Animal Healthy (NOAH) Anonymus. [Internet Homepge]. Last access: Aug, 30, 2016. Available in: http://www.noah.co.uk/medicine-topics/antibiotics-for-animals/resistance/

Navon-Venezia S, Chmelnitsky I, Leavitt A, et al. Dissemination of the CTX-M-25 family beta-lactamases among *Klebsiella pneumoniae, Escherichia coli and Enterobacter cloacae* and identification of the novel enzyme CTX-M-41 in *Proteus mirabilis* in Israel. J Antimicrob Chemother. 2008 Aug;62(2):289-95.

Nebbia P, Tramuta C, Odore R, et al. Genetic and phenotypic characterisation of *Escherichia coli* producing cefotaximase-type extended-spectrum β-lactamases: first evidence of the ST131 clone in cats with urinary infections in Italy. J Feline Med Surg. 2014 Dec;16(12):966-71.

Nedjai S, Barguigua A, Djahmi N, et al. Prevalence and characterization of extended spectrum β-lactamases in *Klebsiella-Enterobacter-Serratia* group bacteria, in Algeria. Med Mal Infect. 2012 Jan;42(1):20-9.

Nemmetz LP, Lennox AM. Zosyn. a replacement for Pipracil in the avian patient. In: Exotic Animal Formulary. 4ed. USA: Elsevier, 2004.

Neu HC. Beta-Lactam antibiotics: structural relationships affecting in vitro activity and pharmacologic properties. Rev Infect Dis. 1986 Jul-Aug;8 Suppl 3:S237-59.

Nilsen E, Haldorsen BC, Sundsfjord A, et al. Norwegian Study Group on Invasive *Enterobacter*. Large IncHI2-plasmids encode extended-spectrum β-lactamases (ESBLs) in *Enterobacter* spp. bloodstream isolates, and support ESBL-transfer to *Escherichia coli.* Clin Microbiol Infect. 2013 Nov;19(11):E516-8

Nunnery J, Angulo FJ, Tollefson L: Public health and policy. Prev Vet Med. 2006, 73(2-3):191-195.

O'Hara JA, McGann P, Snesrud EC, et al. Novel 16S rRNA methyltransferase RmtH produced by *Klebsiella pneumoniae* associated with war-related trauma. Antimicrob Agents Chemother. 2013 May;57(5):2413-6.

O'Keefe A, Hutton TA, Schifferli DM, et al. First detection of CTX-M and SHV extended-spectrum beta-lactamases in *Escherichia coli* urinary tract isolates from dogs and cats in the United States. Antimicrob Agents Chemother. 2010, Aug;54(8):3489-92.

O'Neil H. An overview of aminoglycoside usage in horse. Companion Animal. 2012, (17) :9 – 4-7.

Oliphant CM, Green GM. Quinolones: a comprehensive review. Am Fam Physician. 2002 Feb 1;65(3):455-64.

Oliveiva GD, Park CH, Robicsek A, et al. Prevalence in the United States of *aac(6')-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob Agents Chemother. 2006 Nov;50(11):3953-5.

Olowe OA, Adewumi O, Odewale G, et al. Phenotypic and Molecular Characterisation of Extended-Spectrum Beta-Lactamase Producing *Escherichia coli* Obtained from Animal Fecal Samples in Ado Ekiti, Nigeria. J Environ Public Health. 2015; 2015:497980.

Orsini JA, Moate PJ, Boston RC, et al. Pharmacokinetics of imipenem cilastatin following intravenous administration in healthy adult horses. J Vet Pharmacol Ther. 2005 Aug;28(4):355-61.

Papich M.G. Current Concepts in Antimicrobial Therapy for Horses. In: Current Concepts In Selection and Use of Antimicrobials. 2001 / Vol. 47 / IVIS Proceedings of the Annual Convention of the AAEP 2001. [Internet Homepage]. Last access: April, 02, 2016. Available in: http://www.ivis.org/proceedings/AAEP/2001/91010100094.pdf

Papich M.G. Saunders Handbook of Veterinary Drugs: Small and Large Animals. 1st Ed. St. Louis: Elsevier [S.I.], 2002.

Park YJ, Kim SY, Yu JK, et al. Spread of *Serratia marcescens* coharboring *aac(6')-Ib-cr,* bla_{CTX-M}, *armA*, and bla_{OXA-1} carried by conjugative IncL/M type plasmid in Korean hospitals. Microb Drug Resist. 2009 Jun;15(2):97-102.

Park YJ, Yu JK, Kim SI, et al. Accumulation of plasmid-mediated fluoroquinolone resistance genes, *qepA* and *qnrS1*, in *Enterobacter aerogenes* co-producing *RmtB* and class A beta-lactamase LAP-1. Ann Clin Lab Sci. 2009 Winter;39(1):55-9.

Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: A clinical update. Clin Microbiol Rev. 2005;18:657–86.

Peña C, Pujol M, Ardanuy C, et al. Epidemiology and successful control of a large outbreak due to *Klebsiella pneumoniae* producing extended-spectrum beta-lactamases. Antimicrob Agents Chemother. 1998; Jan;42(1):53-58

Pérez- Pérez FJ, Hanson ND. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol. 2002 Jun;40(6):2153-62.

Peyrou M, Bousquet-Melou A, Laroute V, et al. Enrofloxacin and marbofloxacin in horses: comparison of pharmacokinetic parameters, use of urinary and metabolite data to estimate first-pass effect and absorbed fraction. J Vet Pharmacol Ther. 2006 Oct;29(5):337-44.

Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type beta-lactamases. Antimicrob Agents Chemother. 2002 Jan;46(1):1-11.

Pinto N, Schumacher J, Taintor Boothe LD. Pharmacokinetics of amikacin in plasma and selected body fluids of healthy horses after a single intravenous dose. Equine Vet J. December 2010, 43(1):112-6 •

Platell JL, Johnson JR, Cobbold RN, et al. Multidrug-resistant extraintestinal pathogenic *Escherichia coli* of sequence type ST131 in animals and foods. Vet Microbiol. 2011; 153(1-2):99–108.

Poeta P, Radhouani H, Gonçalves A, et al. Genetic characterization of antibiotic resistance in enteropathogenic *Escherichia coli* carrying extended-spectrum beta-lactamases recovered from diarrhoeic rabbits. Zoonoses Public Health. 2010 May;57(3):162-70.

Poirel L, Kieffer N, Liassine N, et al. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. Lancet Infect Dis. 2016 Mar;16(3):281.

Poirel L, Nordmann P, Ducroz S, et al. Extended-spectrum β-lactamase CTX-M-15-producing *Klebsiella pneumoniae* of sequence type ST274 in companion animals. Antimicrob Agents Chemother. 2013 May;57(5):2372-5.

Pomba C, Fonseca JD, Baptista BC, et al. Detection of the Pandemic O25-ST131 Human Virulent *Escherichia coli* CTX-M-15-Producing Clone Harboring the *qnrB2* and *aac(6')-Ib-cr* Genes in a Dog. Antimicrob Agents Chemother. 2009 Jan;53(1):327-8.

Poppe C, Martin LC, Gyles CL, et al. Acquisition of resistance to extendedspectrum cephalosporins by *Salmonella enterica* subsp. Enterica serovar Newport and *Escherichia coli* in Turkey poult intestinal tract. Appl. Environ. Microbiol.2005, 71:1184-1192.

Pournaras S, Köck R, Mossialos D, et al. Detection of a phylogenetically distinct IMP-type metallo-β-lactamase, IMP-35, in a CC235 *Pseudomonas aeruginosa* from the Dutch-German border region (Euregio). J Antimicrob Chemother. 2013 Jun;68(6):1271-6.

Prescott JF: Antimicrobial drug resistance and its epidemiology. In Antimicrobial Therapy in Veterinary Medicine, edn 3. Edited by Prescott JF, Baggot JD, Walker RD. Ames: Iowa State University, Press; 2000:27-49.

Quinn PJ, Carter ME, Markey B, et al. Antimicrobial agents, p.95-117. In: Ibid.(Eds), Clinical Veterinary Microbiology. 1994. Wolfe, London.

Radice M, Power J, Di Conza G, et al. Early dissemination of CTX-M-derived enzymes in South America. Antimicrob Agents Chemother. 2005, 46:602-604.

Rankin SC, Whichard JM, Joyce K, et al. Detection of a bla_{SHV} Extended-Spectrum β-Lactamase in *Salmonella enterica* Serovar Newport MDR-AmpC. J Clin Microbiol. 2005; (43): 11: 5792–5793

Rawat D, Nair D. Extended-spectrum β-lactamases in Gram Negative Bacteria. J Glob Infect Dis. 2010 Sep;2(3):263-74.

Rice LB. Mechanisms of resistance and clinical relevance of resistance to β-lactams, glycopeptides, and fluoroquinolones. Mayo Clin Proc. 2012 Feb;87(2):198-208.

Robinson NE, Sprayberry KA. Current therapy in equine medicine. USA. Saunders Elesevier. 5ed, 2009.

Rocha FR, Pinto VP, Barbosa FC. The Spread of CTX-M-Type Extended-Spectrum β-Lactamases in Brazil: A Systematic Review. Microb Drug Resist. 2015 Dec 15. [Epub ahead of print] PubMed PMID: 26669767.

Rodriguez I, Barownick W, Helmuth R, et al. Extended-spectrum beta-lactamases and AmpC beta-lactamases in ceftiofur resistant *Salmonella enterica* isolates from food and livestock obtained in Germany during 2003-07. J Antimicrob Chemother. 2009; 64:301-309.

Saenz Y, Brinas L, Dominguez E, et al. Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. Antimicrob Agents Chemother. 2004, 48(10):3996-4001.

Saladin M, Cao VT, Lambert T, et al. Diversity of CTX-M b-lactamases and their promoter regions from Enterobacteriaceae isolated in three Parisian hospitals. FEMS Microbiol. Lett. 2002, 209, 161–168.

Sallem RB, Gharsa H, Slama KB, et al. First Detection of CTX-M-1, CMY-2, and QnrB19 Resistance Mechanisms in Fecal *Escherichia coli* Isolates from Healthy Pets in Tunisia. Vector Borne Zoonotic Dis. 2013, 13 (2): 98-102.

Sanchez Teran AF, Rubio-Martinez LM, Villarino NF, et al. Effects of repeated intra-articular administration of amikacin on serum amyloid A, total protein and nucleated cell count in synovial fluid from healthy horses. Equine Vet J Suppl. 2012 Dec;(43):12-6.

Schmid A, Hormansdorfer S, Messelhausser U, et al. Prevalence of extended-spectrum beta-Lactamase-producing *Escherichia coli* on bavarian dairy and beef cattle farms. Appl Environ Microbiol. 2013;May;79(9):3027-3032.

Seki LM, Pereira PS, de Souza Mda P, et al. Molecular epidemiology of KPC-2-producing *Klebsiella pneumoniae* isolates in Brazil: the predominance of sequence type 437. Diagn Microbiol Infect Dis. 2011 Jun;70(2):274-7.

Shah PM. Bactericidal activity of continuously changing cefotaxime concentrations in human blood. J Antimicrob Chemother. 1984 Sep;14 Suppl B:103-8

Shaheen BW, Nayak R, Foley SL, et al. Molecular characterization of resistance to extended-spectrum cephalosporins in clinical *Escherichia coli* isolates from companion animals in the United States. Antimicrob Agents Chemother. 2011. Dec;55(12):5666-75.

Shaikh S, Fatima J, Shakil S, et al. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi J Biol Sci. 2015 Jan;22(1):90-101.

Shiraki Y, Shibata N, Doi Y, et al. *Escherichia coli* Producing CTX-M-2 β-Lactamase in Cattle, Japan. Emerg Infec Dis. 2004;10(1):69-75.

Silva KC, Fontes LC, Moreno AM, et al. Emergence of extended-spectrum-β-lactamase CTX-M-2-producing *Salmonella enterica* serovars Schwarzengrund and Agona in poultry farms. Antimicrob Agents Chemother. 2013 Jul;57(7):3458-9.

Silva KC, Lincopan N. Epidemiologia das betalactamases de espectro estendido no Brasil: impacto clínico e implicações para o agronegócio. J. Bras. Patol. Med. Lab. 2012, 48(2): 91-99.

Silva KC, Moreno M, Cabrera C, et al. First Characterization of CTX-M-15-Producing *Escherichia coli* Strains Belonging to Sequence Type (ST) 410, ST224, and ST1284 from Commercial Swine in South America. Antimicrob Agents Chemother. 2016 Mar 25;60(4):2505-8.

Sidjabat HE, Hanson ND, Smith-Moland E, et al. Identification of plasmid-mediated extended-spectrum and AmpC beta-lactamases in *Enterobacter* spp. isolated from dogs. J Med Microbiol. 2007; Mar;56 (3):426-34.

Sidjabat HE, Seah KY, Coleman L, et al. Expansive spread of IncI1 plasmids carrying bla_{CMY-2} amongst *Escherichia coli*. Int J Antimicrob Agents. 2014 Sep;44(3):203-8.

Six R, Cherni J, Chesebrough R, et al. Efficacy and safety of cefovecin in treating bacterial folliculitis, abscesses, or infected wounds in dogs. J Am Vet Med Assoc. 2008 Aug 1;233(3):433-9.

Smet A, Van Nieuwerburgh F, Vandekerckhove TT, et al. Complete nucleotide sequence of CTX-M-15-plasmids from clinical *Escherichia coli* isolates: insertional events of transposons and insertion sequences. PLoS One. 2010 Jun 18;5(6):e11202.

Song W, Bae IK, Lee YN, et al. Detection of extended-spectrum beta-lactamases by using boronic acid as an AmpC beta-lactamase inhibitor in clinical isolates of *Klebsiella* spp. and *Escherichia coli*. J Clin Microbiol. 2007 Apr;45(4):1180-4.

Song W, Jeong SH, Kim JS, et al. Use of boronic acid disk methods to detect the combined expression of plasmid-mediated AmpC beta-lactamases and extended-spectrum beta-lactamases in clinical isolates of *Klebsiella* spp., *Salmonella* spp., and *Proteus mirabilis*. Diagn Microbiol Infect Dis. 2007 Mar;57(3):315-8.

Song W, Kim J, Bae IK, et al. Chromosome-encoded AmpC and CTX-M extended-spectrum β-lactamases in clinical isolates of *Proteus mirabilis* from Korea. Antimicrob Agents Chemother. 2011 Apr;55(4):1414-9.

Sotgiu G, D'Ambrosio L, Centis R, et al. Carbapenems to Treat Multidrug and Extensively Drug-Resistant Tuberculosis: A Systematic Review. Int J Mol Sci. 2016 Mar 12;17(3).

Srinivasan V, Nam HM, Sawant AA, et al. Distribution of tetracycline and streptomycin resistance genes and class 1 integrons in Enterobacteriaceae isolated from dairy and nondairy farm soils. Microb Ecol. 2008, 55(2):184-193.

Sripreechasak P, Tamura T, Shibata C, et al. *Streptomyces andamanensis* sp. nov., isolated from soil of island. Int J Syst Evol Microbiol. 2016 Feb 23.

Steen SI, Webb PJ, Extended-spectrum beta-lactamase producing bacteria isolated from companion animals. Vet Rec. 2007, Nov 17:161:703.

Stegmann MR, Sherington J, Blanchflower S. Pharmacokinetics and pharmacodynamics of cefovecin in dogs. J Vet Pharmacol Ther. 2006 Dec;29(6):501-11.

Strahilevitz J, Jacoby GA, Hooper DC, et al. Plasmid-mediated quinolone resitance: a ultifaceted threat. Clin Microbiolo Rev. 2009; 22: 664-89.

Sugawara M, Komori J, Kawakami M, et al. Molecular and phenotypic characteristics of CMY-2 beta-lactamase-producing *Salmonella enterica* serovar Typhimurium isolated from cattle in Japan. J Vet Med Sci. 2011; 73:345–349.

Sun Y, Zeng Z, Chen S, et al. High prevalence of bla_{CTX-M} extended-spectrum β-lactamase genes in *Escherichia coli* isolates from pets and emergence of CTX-M-64 in China. Clin Microbiol Infect. 2010 Sep;16(9):1475-81.

Svarm-Swedres. 2014. [Internet homepage]. Use of antimicrobials and occurrence of antimicrobial resistance in Sweden. Solna/ Uppsala Last access: jan, 30, 2016. Available from:
http://www.sva.se/globalassets/redesign2011/pdf/om_sva/publikationer/swedres_svarm2013.pdf

Tada T, Miyoshi-Akiyama T, Kato Y, et al. Emergence of 16S rRNA methylase-producing *Acinetobacter baumannii and Pseudomonas aeruginosa* isolates in hospitals in Vietnam. BMC Infect Dis. 2013 May 30;13:251.

Tamang MD, Nam HM, Jang GC, et al. Molecular Characterization of Extended-Spectrum β-Lactamase and Plasmid mediated AmpC β-Lactamase-producing *Escherichia coli* Isolated from Stray Dogs from Korea. Antimicrob Agents Chemother 2012, May;56(5):2705-12.

Teshager T, Dominguez L, Moreno MA, et al. Isolation of a SHV-12 ß-lactamase-producing *Escherichia coli* strain from a dog with recurrent urinary tract infections. Antimicrob Agents Chemother. 2000; 44: 3483-3484.

Thal D. Horse Side Vet Guide Site [Internet homegape]. United States. 2016. Last acess: April, 20, 2016. Available in: http://horsesidevetguide.com/drv/Treatment/90/antibiotics-systemic-antibacterials-amp-antimicrobials/

Tian GB, Wang HN, Zou LK, et al. Detection of CTX-M-15, CTX-M-22, and SHV-2 extended-spectrum beta-lactamases (ESBLs) in *Escherichia coli* fecal-sample isolates from pig farms in China. Foodborne Pathog Dis. 2009; 6: 297-304

Timofte D, Dandrieux J, Wattret A, et al. Detection of extended-spectrum-beta-lactamase-positive *Escherichia coli* in bile isolates from two dogs with bacterial cholangiohepatitis. J Clin Microbiol. 2011; Sep 49(9):3411-3414.

Tissera S, Lee SM. Isolation of Extended Spectrum β-lactamase (ESBL) Producing Bacteria from Urban Surface Waters in Malaysia. Malays J Med Sci. 2013;20(3):14-22.

Todar, K. Bacterial Resistance to Antibiotics. In Todar's Online Textbook of Bacteriology. [Internet Homepage]. Last access: April, 25, 2016. Available from: http://textbookofbacteriology.net/resantimicrobial.html.

Tofteland S, Dahl KH, Aasnæs B, et al. A nationwide study of mechanisms conferring reduced susceptibility to extended-spectrum cephalosporins in clinical *Escherichia coli* and *Klebsiella* spp. isolates. Scand J Infect Dis. 2012; Dec;44(12):927-933.

Toleman MA, Walsh TR. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. FEMS Microbiol Rev. 2011 Sep;35(5):912-35.

Tollentino FM, Polotto M, Nogueira ML, et al. High prevalence of *bla*$_{\text{CTX-M}}$ extended spectrum beta-lactamase genes in *Klebsiella pneumoniae* isolates from a tertiary care hospital: first report of *bla*$_{\text{SHV-12}}$, *bla*$_{\text{SHV-31,}}$ *bla*$_{\text{SHV-38}}$, and *bla*$_{\text{CTX-M-15}}$ in Brazil. Microb Drug Resist. 2011 Mar;17(1):7-16.

Tomasz A. From penicillin-binding proteins to the lysis and death of bacteria: a 1979 view. Rev Infect Dis. 1979 May-Jun;1(3):434-67.

Trabulsi LR,Alterthum F. Microbiologia. 4. ed. – São Paulo: Editora Atheneu, 2004.

Trott D. Beta-lactam resistance in gram-negative pathogens isolated from animals. Curr Pharm Des. 2013;19(2):239-249.

Valenzuela de Silva EM, Mantilla Anaya JR, Reguero Reza MT, et al. Detection of CTX-M-1, CTX-M-15, and CTX-M-2 in Clinical Isolates ofEnterobacteriaceae in Bogotá, Colombia. J Clin Microbiol, 2006, 44(5), 1919–1920.

Van Bambeke F, Michot JM, Van Eldere J, et al. Quinolones in 2005: an update. Clin Microbiol Infect. 2005 Apr;11(4):256-80.

Van Der Bogard AE, Stobberingh EE. Antibiotic Usage in Animals: Impact on Bacterial Resistance and Public Health. Drugs. 1999;58:589–607.

Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 1991, 19:6823–6831.

Viana FAB. Guia terapêutico veterinário. 2ed., Editora CEM, Lagoa Santa, 2007.

Vo AT, van Duijkeren E, Fluit AC, et al. Characteristics of extended-spectrumcephalosporin-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates from horses. Vet Microbiol. 2007 Oct 6;124(3-4):248-55.

Vragović N, Bazulić D, Njari B. Risk assessment of streptomycin and tetracycline residues in meat and milk on Croatian market. Food Chem Toxicol. 2011 Feb;49(2):352-5.

Wachino J, Arakawa Y. Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: an update. Drug Resist Updat. 2012 Jun;15(3):133-48.

Walker PR, Dowling PM. Fluoroquinolonas. In: Terapia Antimimicrobiana em Medicina Veterinária. Giguere, Prescott, Baggot, Walker, Dowling. 4ed. Roca Edition, Sao Paulo, Brasil, 2010.

Walther B, Janssen T, Gehlen H, et al. Infection control and hygiene management in equine hospitals. Berl Munch Tierarztl Wochenschr. 2014 Nov-Dec;127(11-12):486-97.

Warren RE, Ensor VM, O'Neill P, et al. Imported chicken meat as a potential source of quinolone-resistant *Escherichia coli* producing extended-spectrum beta-lactamases in the UK. J Antimicrob Chemother. 2008; 61(3):504-8

Wassef M, Behiry I, Younan M, et al. Genotypic Identification of AmpC β-Lactamases Production in Gram-Negative Bacilli Isolates. Jundishapur J Microbiol. 2014;7(1):e 8556.

Watson E, Jeckel S, Snow L, et al. Epidemiology of extended spectrum beta-lactamase *E. coli* (CTX-M-15) on a commercial dairy farm. Vet Microbiol. 2012; 154, 339–346.

Webster P. The perils of poultry. Canadian Medical Association. 2009, Jul 7: (181) 1-2.

Wedley AL, Maddox TW, Westgarth C, et al. Prevalence of antimicrobial-resistant *Escherichia coli* in dogs in a cross-sectional, community-based study. Vet Rec. 2011 Apr 2;168(13):354.

Weese S. Antimicrobial resistance in companion animals. Anim Health Res Rev. 2008, 9(2):169-176

Weill FX, Lailler R, Praud K, et al. Emergence of extended-spectrum-beta-lactamase (CTX-M-9)-producing multiresistant strains of *Salmonella enterica* serotype Virchow in poultry and humans in France. J Clin Microbiol. 2004 Dec;42(12):5767-73.

Winokur PL, Brueggemann A, DeSalvo DL, et al. Animal and human multidrug-resistant, cephalosporin-resistant salmonella isolates expressing a plasmid-mediated CMY-2 AmpC beta-lactamase. Antimicrob Agents Chemother. 2000 Oct;44(10):2777-83.

Wilson, D. Rational Selection of Antimicrobials for Use in Horses. In: Current Concepts in Selection and use of Antimicrobials. AAEP PROCEEDINGS/ Vol.47/2001. [Internet homepage]. Last Access: April, 02, 2016. Available from: http://www.ivis.org/proceedings/aaep/2001/91010100075.pdf?origin=publication_detail

Wirth T, Falush D, Lan R, et al. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol.Microbiol. 2006. 60(5), 1136-1151

Wittum TE, Mollenkopf DF, Daniels JB, et al. CTX-M-type extended-spectrum beta-lactamases present in *Escherichia coli* from the feces of cattle in Ohio, United States. Foodborne Pathog Dis. 2010;Dec;7(12):1575-1579.

World health organization (WHO). Anonymous. Antimicrobial resistance. [Internet homepage]. Last access: Oct, 14, 2016. Available from: http://www.who.int/mediacentre/factsheets/fs194/en/

Wu S, Chouliara E, Hasman H, et al. Detection of a single isolate of CTX-M-1-producing *Escherichia coli* from healthy pigs in Denmark. J Antimicrob Chemother. 2008;61:747-749.

Yamane K, Wachino J, Suzuki S, et al. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrob Agents Chemother. 2007 Sep;51(9):3354-60.

Yum JH, Kim S, Lee H, et al. Emergence and wide dissemination of CTX-M-type ESBLs, and CMY-2- and DHA-1-type AmpC beta-lactamases in Korean respiratory isolates of *Klebsiella pneumoniae*. J Korean Med Sci. 2005 Dec;20(6):961-5.

Yuan L, Liu JH, Hu GZ, et al. Molecular characterization of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates from chickens in Henan Province, China. J Med Microbiol. 2009; 58:1449-1453.

Zheng H, Zeng Z, Chen S, et al. Prevalence and characterisation of CTX-M beta-lactamases amongst *Escherichia coli* isolates from healthy food animals in China. Intern J Antimicrob Agents. 2012; Apr;39(4):305-310.

Putney. Cefpodoxime printed directions for the use of medicine. [Internet Homepage]. USA. 2012. Last access: April, 14, 2016. Available from: http://putneyvet.com/sites/default/files/product_docs/cefpodoxime_proxetil_brochure_for_veterinarians.pdf

Zoetis (a) - Amikacin printed directions for the use of medicine. [Internet Homepage]. USA. 2013. Last access: April, 14, 2016. Available from: https://www.zoetisus.com/products/horses/amiglyde-v.aspx

Zoetis (b) - Convenia printed directions for the use of medicine. [Internet Homepage]. USA. 2013. Last access: April, 14, 2016. Available from: https://www.zoetisus.com/products/dogs/convenia/documents/convenia_pi.pdf

Zoetis (c) - Excede printed directions for the use of medicine. [Internet Homepage]. USA. Last access: April, 14, 2016. 2009 Available from: https://www.zoetisus.com/_locale-assets/mcm-portal-assets/msds_pi/pi/excede-horses.pdf

Zurfluh K, Jakobi G, Stephan R, et al. Replicon typing of plasmids carrying *bla*$_{CTX-M-1}$ in Enterobacteriaceae of animal, environmental and human origin. Front Microbiol. 2014 Oct 30;5:555.

Appendix I Primers sequence used in this study:

Target site	DNA Sequence - Primer sequence (5′ to 3′)	Size (pb)	$T_{anneling}$
	ESBL/pAmpc		
bla$_{TEM}$	F:5'GAGTATTCAACATTTCCGTGTC 3' R:5'TAATCAGTGAGGCACCTATCT 3'	861	51
bla$_{SHV}$	F:5' ATGCGTTATATTCGCCTGTG 3' R:5' GTTAGCGTTGCCAGTGCTCG 3'	573	53
bla$_{CTX-M}$	F:5' CGCTTTGCGATGTGCAG 3' R:5'ACCCGCATATGCTTGTG 3'	544	56
bla$_{CTX-M-2}$	F:5' GCGACCTGGTTAACTACAATC 3' R:5'CGGTAGTATTGCCCTTAAGCC 3'	351	55
bla$_{CTX-M-8}$	F: 5' CTGGAGAAAAGCAGCGGGGG3' R:5' ACCCAcGATGTGGGTAGCCC3'	320	55
bla$_{CTX-M-1}$	F: 5' CACACGTGGAATTTAGGGACT3' R: 5' TTAGGAARTGTGCCGCTGYA 3'	668	60
bla$_{CMY}$	F:5' CGATATCGTTGGTGGTRCCAT 3' R:5'CAGTAGCGAGACTGCGCA3'	631	58
bla$_{MIR/ACT}$	F:5'TCGGTAAAGCCGATGTTGCCG3' R:5'CTTCCACTGCGGCTGCCAGTT3'	302	56
bla$_{DHA}$	F:5'TCTGCCGCTGATAATGTCC3' R:5'GCCGCCGGATCATTCAGCGC3'	262	52
bla$_{DHA-2}$	F:5'TCTGCCGCTGATAATGTCG3' R:5'TCTGCCGGGTCATTCAACAT3'	298	52
bla$_{FOX}$	F:5'AACATGGGGTATCAGGGAGATG3' R:5'CAAAGCGCGTAACCGGATTGG3"	190	52
Mutlipex reaction CTX-M group (used for French strains) (Dalene et al., 2010).			
MultiCTXMp1_for MultiCTXMp1-2_rev	5′-TTAGGAARTGTGCCGCTGYA-3′ 5′-CGATATCGTTGGTGGTRCCAT-3′	688	
MultiCTXMp2_for	5′-CGTTAACGGCACGATGAC-3′	404	60
MultiCTXMp9_for MultiCTXMp9_rev	5′-TCAAGCCTGCCGATCTGGT-3′ 5′-TGATTCTCGCCGCTGAAG-3′	561	
Quinolones 5'- 3' (Ref. Jacoby et al., 2009/Park et al., 2009)			
QnrA mult	F ATTTCTCACGCCAGGATTTG R TGCCAGGCACAGATCTTGAC	570	55
QnrB mult-F	F CGACCTKAGCGGCACTGAAT R GAGCAACGAYGCCTGGTAGYTG	510	60
QnrC	F GGGTTGTACATTTATTGAATCG R CACCTACCCATTTATTTTCA	305	48
QnrD mult	F CGAGATCAATTTACGGGGAATA R AACAAGCTGAAGCGCCTG	580	53
QnrS mult	F ACTGCAAGTTCATTGAACAG R GATCTAAACCGTCGAGTTCG	430	53
Aac(6')-1b	F TTGCGATGCTCTATGAGTCGCTA R CTCGAATGCCTGGCGTGTTT	480	55
QepA	F AACTGCTTGAGCCCGTAGAT R GTCTACGCCATGGACCTCAC	595	57
oqxA	F CTTGCACTTAGTTAAGCGCC R GAGGTTTTGATAGTGGAGGTAGG	865	55
oqxB	F GCGGTGCTGTCGATTTTA R TACCGGAACCCATCTCGAT	785	55

Target site	DNA Sequence - Primer sequence (5′ to 3′)	Size (pb)	$T_{anneling}$
16S Ribossomal RNA Methyltransferase (Doi et., 2006)			
armA	F 5' – ATT CTG CCT ATC CTA ATT GG – 3' R 5' – ACC TAT ACT TTA TCG TCG TC – 3'	635	55
rmtA	F 5' – CTA GCG TCC ATC CTT TCC TC – 3' R 5' - TTG CTT CCA TGC CCT TGC C – 3'	173	55
rmtB	F 5' – GCT TTCTGC GGG CGA TGT AA – 3' R 5' – ATG CAA TGC CGC GCT CGT ATT – 3'	711	55
rmtC	F 5' – CGA AGA AGT AAC AGC CAA AG – 3' R 5´- ATC CCA ACA TCT CTC CCA CT – 3'	920	55
rmtD	F 5' – CGG CAC GCG ATT GGG AAG C – 3' R 5' – CGC AAA CGA TGC GAC GAT – 3'		
Plasmid Molecular Typing - PBRT (PCR-based replicon typing) (Caratolli et al., 2005)			
HI1	F: GGAGCGATGGATTACTTCAGTAC R: TGCCGTTTCACCTCGTGAGTA	471	60
HI2	F: TTTCTCCTGAGTCACCTGTTAACAC R: GGCTCACTACCGTTGTCATCCT	644	60
I1	F: CGAAAGCCGGACGGCAGAA R: TCGTCGTTCCGCCAAGTTCGT	139	60
X	F: AACCTTAGAGGCTATTTAAGTTGCTGAT R: TGAGAGTCAATTTTTATCTCATGTTTTAGC	376	60
L/M	F: GGATGAAAACTATCAGCATCTGAAG R: CTGCAGGGGCGATTCTTTAGG	785	60
N	F: GTCTAACGAGCTTACCGAAG R: GTTTCAACTCTGCCAAGTTC	559	60
FIA	F: CCATGCTGGTTCTAGAGAAGGTG R: GTATATCCTTACTGGCTTCCGCAG	462	60
FIB	F: GGAGTTCTGACACACGATTTTCTG R: CTCCCGTCGCTTCAGGGCATT	702	60
FIC	F: GTGAACTGGCAGATGAGGAAGG R: TTCTCCTCGTCGCCAAACTAGAT	262	60
FII	F:CTGTCGTAAGCTGATGGC R: CTCTGCCACAAACTTCAGC	270	60
F_{rep}	F: TGATCGTTTAAGGAATTTTG R: GAAGATCAGTCACACCATCC	270	52
W	F: CCTAAGAACAACAAAGCCCCCG R: GGTGCGCGGCATAGAACCGT	242	60
Y	F: AATTCAAACAACACTGTGCAGCCTG R: GCGAGAATGGACGATTACAAAACTTT	765	60
P	F: CTATGGCCCTGCAAACGCGCCAGAAA R: TCACGCGCCAGGGCGCAGCC	534	60
A/C	F: GAGAACCAAAGACAAAGACCTGGA R: ACGACAAACCTGAATTGCCTCCTT	465	60
T	F: TTGGCCTGTTTGTGCCTAAACCAT R: CGTTGATTCACCTTAGCTTTGGAC	750	60
K/B K	F: GCGGTCCGGAAAGCCAGAAAAC R: TCTTTCACGAGCCCGCCAAA	160	60
K/B B/O	F: GCGGTCCGGAAAGCCAGAAAAC R: TCTGCGTTCCGCCAAGTTCGA	159	60

Target site	DNA Sequence - Primer sequence (5′ to 3′)	Size (pb)	$T_{anneling}$
	Mobile genetic elements (Dropa, 2012)		
IS*Ecp1*	F: GCAGGTCTTTTTCTGCTCC R: TTTCCGCAGCACCGTTTGC	717	60
IS*26*	F: CAGCGTGACATCATTCTGTG R: TCTGCTTACCAGGCGCATTT	662	60
IS*Ecp1* P2D	Used for French CTX-M-1 *E.coli* strains F: AAAATGATTGAAAGGTGGT R:CAGCGCTTTTGCCGTCTAAG	1,100	50
	Phylogroup by Clermont et al., 2013.		
chuA	5′-ATGGTACCGGACGAACCAAC-3′ 5′-TGCCGCCAGTACCAAAGACA-3′	288	59
yjaA	5′-CAAACGTGAAGTGTCAGGAG-3′ 5′-AATGCGTTCCTCAACCTGTG-3′	211	59
TspE4C2	5′-CACTATTCGTAAGGTCATCC-3′ 5′-AGTTTATCGCTGCGGGTCGC-3′	152	59
AceK.f *ArpA1.r*	5′-AACGCTATTCGCCAGCTTGC-3′ 5′-TCTCCCCATACCGTACGCTA-3′	400	57
ArpAgpE	5′-GATTCCATCTTGTCAAAATATGCC-3′ 5′-GAAAAGAAAAAGAATTCCCAAGAG-3′	301	59
trpAgpC.	5′-TCTGCGCCGGTCACGCCC-3′ 5′-TCTGCGCCGGTCACGCCC-3′	219	59
	Phylogroup by Clermont et al., 2000.		
ChuA	F: 5´- ACGAACCAACGGTCAGGAT-3´ R: 5´-TGCCGCCAGTACCAAAGACA-3	279	57
YjaA	F: 5´-TGAAGTGTCAGGAGACGCTG-3’ R: 5´-ATGGAGAATGCGTTCCTCAAC-3´	211	55
TspE4C2	F: 5´-GAGTAATGTCGGGGCATTCA-3´ R: 5´-CGCGCCAACAAAGTATTACG-3´	152	55
	MLST - *E.coli* - (Wirth et al., 2006)		
adk	F 5'-ATTCTGCTTGGCGCTCCGGG-3' R 5'-CCGTCAACTTTCGCGTATTT-3'	583	54° C
fumC	R1 5'-TCCCGGCAGATAAGCTGTGG-3' F 5'-TCACAGGTCGCCAGCGCTTC-3'	806	54° C
gyr	F 5'-TCGGCGACACGGATGACGGC-3' R 5'-GTCCATGTAGGCGTTCAGGG-3'	911	60° C
icd	F 5'-ATGGAAAGTAAAGTAGTTGTTCCGGCACA-3' R 5'-GGACGCAGCAGGATCTGTT-3'	878	54° C
mdh	F: ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG R: TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	932	60° C
purA	F: TCGGTAACGGTGTTGTGCTG R: CATACGGTAAGCCACGCAGA	816	54° C
recA	F: ACCTTTGTAGCTGTACCACG R: TCGTCGAAATCTACGGACCGGA	780	58° C

PCR: 2 min at 95°, 30 cycles of 1 min at 95°, 1 min at annealing temp, 2 min at 72° followed by 5 min at 72°. The PCR reaction contains 50 ng of chromosomal DNA, 20 pmol of each primer, 200 umol (10 ul of a 2 mM solution) of the dNPTs, 10 ul of 10x PCR buffer, 5 units of Taq polymerase and water to 100 ul. (Wirth et al., 2006)

Target site	DNA Sequence - Primer sequence (5′ to 3′)	Size (pb)	$T_{anneling}$
MLST - *Klebsiella pneumonia* (Brisse et al., 2009; Diancourt et al., 2005)			
rpoB	F: GGCGAAATGGCWGAGAACCA R: GAGTCTTCGAAGTTGTAACC	1075	50°C
gapA	F: TGAAATATGACTCCACTCACGG R: CTTCAGAAGCGGCTTTGATGGCTT	662	60°C
mdh	F: CCCAACTCGCTTCAGGTTCAG R: CCGTTTTTCCCCAGCAGCAG	756	50°C
pgi	F: GAGAAAAACCTGCCTGTACTGCTGGC R: CGCGCCACGCTTTATAGCGGTTAAT	566	50°C
phoE	F: ACCTACCGCAACACCGACTTCTTCGG R: TGATCAGAACTGGTAGGTGAT	602	50°C
infB	F: CTCGCTGCTGGACTATATTCG R: CGCTTTCAGCTCAAGAACTTC	462	50°C
tonB	F: CTTTATACCTCGGTACATCAGGTT R: ATTCGCCGGCTGRGCRGAGAG	539	45°C

PCR: 2 min at 94°, 35 cycles of: 20 sec at 94°, 30 sec at annealing temp, 30 sec at 72° followed by 5 min at 72°. For gene tonB, it was used MgCl2 at 50 mM, whereas for all other genes the primers work with MgCl2 at 25 mM.

Dideoxyoligonucleotide primers used for *P. aeruginosa* MLST (Curran et al., 2004)

Locus and function	Primer sequence (5′ to 3′)		Amplicon size (bp)
	Forward	Reverse	
acsA			
Amplification	ACCTGGTGTACGCCTCGCTGAC	GACATAGATGCCCTGCCCCTTGAT	842
Sequencing	GCCACACCTACATCGTCTAT	GTGGACAACCTCGGCAACCT	390
aroE			
Amplification	TGGGGCTATGACTGGAAACC	TAACCCGGTTTTGTGATTCCTACA	825
Sequencing	ATGTCACCGTGCCGTTCAAG	TGAAGGCAGTCGGTTCCTTG	495
guaA			
Amplification	CGGCCTCGACGTGTGGATGA	GAACGCCTGGCTGGTCTTGTGGTA	940
Sequencing	AGGTCGGTTCCTCCAAGGTC	TCAAGTCGCACCACAACGTC	372
mutL			
Amplification	CCAGATCGCCGCCGGTGAGGTG	CAGGGTGCCATAGAGGAAGTC	940
Sequencing	AGAAGACCGAGTTCGACCAT	ATGACTTCCTCTATGGCACC	441
nuoD			
Amplification	ACCGCCACCCGTACTG	TCTCGCCCATCTTGACCA	1,042
Sequencing	ACGGCGAGAACGAGGACTAC	TTCACCTTCACCGACCGCCA	366

Reaction conditions for all the primers were as follows: initial denaturation at 96°C for 1 min; 30 cycles of denaturation at 96°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 1 min; followed by a final extension step of 72°C for 10 min. Each 50 µl amplification reaction mixture comprised 2.0 µl chromosomal DNA (5-20 ng/µl), 2.0 µl forward primer (10 pmol/µl), 2.0 µl reverse primer (10 pmol/µl), 5.0 µl 10x PCR buffer (Qiagen, contains 15 mM MgCl2), 1.0 µl dNTP solution (Qiagen, 10 mM each dNTP), 0.25 µl Taq polymerase (Qiagen, 5units/µl) and 37.75 µl PCR-grade water.

Appendix II - Abstracts and proceedings in national and international meetings

1) Second Biennial Symposium for the International Society for Companion Animal Infectious Diseases 2012, San Francisco (USA), 2012. LEIGUE DOS SANTOS, Lucianne. ; CASTRO, A. P.; BARBATO, L.; MELO L.; MOURA, R.; LINCOPAN, N.. Emergence of extended-spectrum beta-lactamase (ESBL) producing Escherichia coli in domestic animals, Brazil.

2) Second Biennial Symposium for the International Society for Companion Animal Infectious Diseases 2012, San Francisco (USA), 2012. LEIGUE DOS SANTOS, Lucianne. ; LIMA, Leandro; DE BARROS FILHO, IVAN R.; LANGE, R. R.; MONTIANI, Fabiano. Antimicrobial susceptibility and minimal inhibitory concentration of Pseudomonas aeruginosa isolated from eyes of different species of animals in Brazil.

3) Second Biennial Symposium for the International Society for Companion Animal Infectious Diseases 2012, San Francisco (USA), 2012. v. 2.MELO, L.; LEIGUE DOS SANTOS, L.; BARBATO, L.; LINCOPAN, N. High prevalence of CTX-M-producing Gram-negative bacteria in companion animals with urinary tract infections, Brazil.

4) ICAAC 2013: N° C2-1613; Denver, CO, USA: LEIGUE DOS SANTOS, L.; WARTH, J.F. ; MELO, L.; BARBATO, L.; PESTANA DE CASTRO, A.F.; LINCOPAN, N. Isolation of Escherichia coli sequence type 2179 (ST2179) co-producing CTX-M-15, RmtD1 and AAC(6'-Ib-CR in a horse, Brazil.

5) ICAAC 2013: N° C2-1613; Denver, CO, USA: MELO, L C..; BARBATO, L.; LEIGUE DOS SANTOS, L.; MOURA, R.; AGUILAR, P.; LINCOPAN, N. Commensal Microbiota of Companion Animals as Reservoirs of blaCTX-M Extended-Spectrum Beta-lactamase Gene.

6) 27º Congresso Brasileiro de Microbiologia, 2013, Natal, Brasil: MOURA, Q. ; VIANELLO, A. ; LEIGUE DOS SANTOS, L. ; NEVES, P. ; CASTRO, A. P. ; LINCOPAN, N. . Alta prevalência de bactérias produtoras de CTX-M em uma comunidade remota da região Amazônica.

7) Conference on Environment, Industrial and Applied Microbiology, 2013, Madrid. Book of Abstracts, 2013. v. V. p. 444. LEIGUE DOS SANTOS, L. ; MOURA, R. ; Warth, J.f. ; MOURA, Q. ; VILLABONA, C. J. ; ZOPPA, A. ; SPAGNOLO, J. ; CASTRO, A. P. ; LINCOPAN, N. . Emergence of Multidrug-Resistant Gram-negative Bacteria Co-producing Exteded-Spectrum B-lactamase and Methylases in Horses. In: V International - Oral presentation

Appendix III – Scientific Production

- Book Chapter

Leigue dos Santos L, Moura RA, Ramires PA, Pestana de Castro AF, Lincopan N. Current Status of extended-spectrum-beta-lactamase-producing Enterobacteriaceae in animals. In: Méndez-Vilas. Microbial pathogens and strategies for combating them: science technology and education. Badajoz: Formatex Research Center, Spain; 2013. p.1600-7. Available in: http://www.formatex.info/microbiology4/vol3/1600-1607.pdf)

- Papers published:

Leigue L, Warth JF, Melo LC, Silva KC, Moura RA, Barbato L, Silva LC, Santos AC, Silva RM, Lincopan N. MDR ST2179-CTX-M-15 *Escherichia coli* co-producing RmtD and AAC(6')-Ib-cr in a horse with extraintestinal infection, Brazil. J Antimicrob Chemother. 2015 Apr;70(4):1263-5.

Aizawa J, Neuwirt N, Barbato L, Neves PR, Leigue L, Padilha J, Pestana de Castro AF, Gregory L, Lincopan N. Identification of fluoroquinolone-resistant extended-spectrum β-lactamase (CTX-M-8)-producing *Escherichia coli* ST224, ST2179 and ST2308 in buffalo (*Bubalus bubalis*). J Antimicrob Chemother. 2014 Oct;69(10):2866-9.

- Manuscript in progression

Leigue L, Dropa M, Cunha MPV, Knobl T, Matté MH, Lincopan N. Multidrug-resistant *Escherichia coli* ST2113-CTX-M-2 belonging to the virulence phylogroup D from a domestic fighting cock (*Gallus gallus domesticus*) with colisepticemia.

Leigue L, Melo LC, Moura Q, Nascimento T, Sartori L, Silva KC, Dropa M, Lincopan N. Diversity of CTX-M genotypes in *Escherichia coli* isolated from food, animals, human and environmental sources, Brazil (2008-2015).

Leigue L, Cerdero L, Fernandes M, Dropa M, Lincopan N. Complete Sequencing characterization of conjugative plasmid harboring CTX-M-15 from a Multidrug-Resistant *Escherichia coli* strain isolated from horse, Brazil.

Appendix IV – Collaborating Institutions

- Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil;
- Department of Public Heath, University of São Paulo, São Paulo, SP, Brazil;
- Department of Veterinary Medicine, University of São Paulo, São Paulo, SP, Brazil;
- Department of Microbiology, Immunology and Parasitology, Universidade Federal de São Paulo, São Paulo, Brazil;
- Department of Veterinary Medicine, Universidade Federal do Paraná, Curitiba, Paraná, Brazil;
- ANSES - French Agency for Food, Environmental and Occupational Health; Safety, Unit AVB (*Antibiorésistance et Virulence Bactérienne*), Lyon, France;
- Colégio Bandeirantes, English Department.