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DOTTORATO DI RICERCA IN SANITA' PUBBLICA, IGIENE VETERINARIA E DELLE PRODUZIONI ANIMALI

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Multidrug Resistance and Production of Extended Spectrum β-lactamases and Plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* isolates from diseased cats in Sicily

Tesi di Dottorato

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Dedication

For my family,

who helped me in all things great and small

Contents

Abstract

Introduction: In human and veterinary medicine, *Enterobacteriaceae* are common causes of enteric and extra-intestinal opportunistic infections and their resistance to multiple antimicrobials is a major global threat. Multidrug-resistant (MDR) *Enterobacteriaceae* are increasingly reported in companion animals, thus raising great concerns for animal and public health (Bogaerts et al., 2015). The β-lactam resistance in *Enterobacteriaceae* is associated mainly with production of enzymes hydrolyzing these antibiotics, among which the extended-spectrum β-lactamases (ESBLs), Plasmid-mediated AmpC β-lactamases (*p*AmpC) and carbapenemases are the most important resistance mechanisms (Rubin and Pitout, 2014).

Objectives: This study aimed to investigate the antimicrobial resistance of *Enterobacteriaceae* isolates from a Sicilian population of cats affected by diseases commonly encountered in practice, with emphasis on multidrug resistance, and to detect the occurrence of ESBLs and Plasmid-mediated AmpC β-lactamases *p*AmpC producers.

Materials and Methods: Clinical samples were collected from *n*=101 cats affected by several clinical conditions (58.4% diarrhoea, 30.7% rhinitis, 3.9% otitis, 2.9% conjunctivitis, 1% abscess, 2% stomatitis, 1% cystitis). Bacterial susceptibility testing to *n*= 8 antimicrobial classes and interpretation were performed according to EUCAST clinical breakpoints (EUCAST, 2015). ESBLs and *p*AmpC genes were identified by PCR and DNA sequencing. Phylogenetic groups of *Escherichia coli* (*E. coli*) harbouring resistance genes were determined according to Doumith et al. *(*2012).

Results: A total of *n*=125 *Enterobacteriaceae* were isolated from *n*=90 cats. *E. coli (*52%) was the most frequently isolated, followed by *Enterobacter* spp. (16%), *Proteus* spp. (10%) and *Citrobacter* spp. (10%). The higher prevalences of resistance among isolates were against amoxicillin-clavulanic acid (49%) and third-generation cephalosporines (40%). Although lower, resistance to aztreonam (32%), ciprofloxacin (23%), amikacin (31%), chloramphenicol (24%) and sulphamethoxazole-thrimetoprim (37%) were also significant, whereas all isolates were susceptible to meropenem.

Forty-five percent (*n*=56) of isolates were multidrug-resistant, showing *n*= 29 different MDR profiles, and were isolated from the 47% (*n*=42) of cats.

PCR and DNA sequencing confirmed a total of *n* = 26 MDR isolates as ESBLs/*p*AmpC β-lactamases producers, representing the 21% of total isolates and recovered from the 20% (*n*=18) of cats, affected by diarrhoea, rhinitis, abscess, otitis, stomatitis and cystitis.

Twenty-three isolates were confirmed as ESBLs-producers, harbouring several *bla* genes, namely: *bla*CTX-M-group1 (*n*=12), -group2 (*n*=1) and –group9 (*n*=1); *bla*SHV (*n*=1), *bla*TEM (*n*=8) and *bla*OXA-1 (*n*=6).

Ten isolates were *p*AmpC *bla*CMY-producers, with *n*=7 isolates also harbouring *bla*TEM (*n*=4), *bla*CTX-M (*n*=2) and *bla*OXA-1 (*n*=1).

ESBL/*p*AmpC-producing *E. coli* (*n*=12) belonged to phylogenetic groups B2 and D and were collected from *n*=6 diarrheic cats, *n*=1 cat with rhinitis, *n*=1 with cystitis and *n*=1 with otitis. Two MDR non β-lactamase producing *E. coli* belonged to phylogenetic groups B2 and D as well and were isolated from *n*=1 cat with rhinitis and *n*=1 cat with diarrhea. Six *E. coli* belonged to phylogenetic groups A and B1and were isolated from *n*=3 cats with rhinitis, *n*=1 cat with

diarrhea, *n*=1 with abscess and *n*=1 with stomatitis. One MDR non β-lactamase producing *E. coli* belonged to phylogenetic group B1 was isolated from *n*=1 cat with diarrhea.

Discussion and Conclusions: This study showed the prevalence of MDR and β-lactamases producing *Enterobacteriaceae* isolated in a variety of common clinical conditions in a feline population in Southern Italy, with a high degree of diversity between antimicrobial resistance profiles.

To the best of knowledge, occurrence of MDR ESBLs/*p*AmpC producing *E. coli* in cats affected by rhinitis and detection of gene *bla*CTX-M-79 in a member of *Enterobacteriaceae* isolated from companion animals are described for the first time in literature.

The emergence of ESBL/*p*AmpC-producing MDR *Enterobacteriaceae* poses major limitations in companion animals' therapeutic options. Furthermore, it raises great concerns regarding the bi-directional transmission of MDR bacteria between pets and humans, and awareness should be raised among companion animal practitioners. Resort to appropriate bacteriological isolation, identification and susceptibility testing is essential to address antimicrobial treatment of commonly encountered bacterial

infections. This could avoid the resort to ineffective compounds, thus reducing selective pressure excerted by antimicrobials on resistant strains, helping the control and monitoring of antimicrobial resistance in companion animals' medicine.

CHAPTER 1

Introduction

1.1 *Enterobacteriaceae*

The family *Enterobacteriaceae* belongs to the class γ-proteobacteria and includes a very large group of biochemically and genetically related microorganisms, which are provided with heterogeneity in terms of ecology, host range and pathogenic potential.

Taxonomically, it comprises 56 genera and over 170 named species (J.P. Euzéby: List of Prokaryotic Names with Standing in Nomenclature, http://www.bacterio.cict.fr (accessed November 11, 2016).

Enterobacteriaceae are spread worldwide and inhabit a wide spectrum of environmental niches, some of them being recovered in water, soil and sewage (Johnson et al., 2008; Schmiedel et al., 2014; Picao et al., 2013).

Most are part of the normal commensal gut flora of humans and animals. They can be isolated from several clinical conditions in companion animals, such as urinary, respiratory, skin and soft tissue, gastrointestinal, joint and opportunistic infections (Bogaerts et al., 2015, Greiner et al., 2007; Costa et al., 2008; Suchodolski , 2011).

Members of this family are Gram-negative, medium sized (0.3–1.0 × 1.0–6.0 μm), non spore-forming, straight rods.

Essential biochemical characteristics of most organisms include fermentation of glucose, reduction of nitrate to nitrite, catalase positivity and oxidase negativity. This latter characteristic, due to the absence of the cytochrome-oxidase activity, allows for a quick differentiation of *Enterobacteriaceae* from other Gram-negative bacilli (Murray et al., 2008).

Enterobacteriaceae can be motile or non-motile (e.g. *Klebsiella*, *Shigella* and *Yersinia* species), depending on the presence or absence of peritrichous flagella, long filaments distributed on the entire surface of the organism and fixed to a proteinic disc, which is integrated in the inner cell membrane.

Flagella are constituted by helically looped subunits of flagellin and they number usually 5-10 per cell. They possess antigenic properties, representing the H antigen of motile species.

Several traits of the cellular structure and cellular products of *Enterobacteriaceae* are important from a medical point of view.

An inner and an outer membrane, a thin peptidoglycan layer and a periplasm constitute their cell wall.

The outer membrane is an asymmetric bilayer with phospholipids on its inner surface, and lipid A, the hydrophobic anchor of lipopolysaccharide (LPS), on the outer one.

LPS is a potent endotoxin, inducer of host's innate immune response. Its main endotoxic principle is lipid A, which is released after death and lysis of bacteria, eliciting severe toxic reactions due to its effects on the innate immune and coagulatory systems (Park et al., 2009).

Once released, lipid A binds to serum LPS-binding protein, which converts oligomeric micelles of LPS into a monomer for delivery to the cluster of differentiation 14 (CD14).

CD14 concentrates lipid A for binding to the Toll-like receptor 4 (TLR4) – myeloid differentiation factor 2 (MD2) complex, found on the surfaces of macrophages, dendritic cells and endothelial cells.

Binding of lipid A to CD14 triggers a signal transduction cascade that results in expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6).

This enables the expression of tissue factor by endothelial cells and B7 proteins induced costimulatory molecules by macrophages and dendritic cells.

These proinflammatory and procoagulatory responses are responsible, in part, for the clinical signs associated with endotoxemia: fever, leukopenia followed by leukocytosis and hyperglycemia, with a subsequent fall in blood sugar and lethal shock after a latent period.

The outermost region of LPS consists of the hydrophilic O antigen polysaccharide region. It is on the cell surface and appears to be a major target for both immune system and bacteriophages.

Enterobacteriaceae possess other virulence factors that are part of the cell structure.

Many members express adhesins, which consist of proteins embedded in the outer cell membrane, composed of subunits and assembled into organelles, such as fimbriae (pili) and afimbrial (nonfimbrial) adhesins.

Fimbriae are hair-like appendages diffusely arranged on the surface of bacterial cells. They usually number 100–1000 per cell.

Fimbriae bind to receptors on the surface of host cells, and different types of fimbriae vary in their binding specificities.

A single bacterial isolate can express multiple fimbrial types.

Most *Enterobacteriaceae* have type 1 fimbriae, which enable bacterial adhesion to epithelial cells and represent the F antigen.

Enterobacteriaceae often express a capsule that consists of an acidic polysaccharide.

Two types of capsular polysaccharides may be produced: the M antigen, consisting of colanic acid, is produced by most strains and is thought to provide protection against desiccation; the K antigen may provide antiphagocytic, serum resistance and mucosal adherence properties (Euzeby, 2013).

Enterobacteriaceae have simple nutritional requirements and most grow well at 22–35°C, under aerobic or anaerobic conditions.

This ability reflects both a respiratory and fermentative metabolism, although fermentation is the more common method of utilization of carbohydrates, often with production of acid and gas.

Blood agar and MacConkey agar are the solid culture media routinely used to isolate *Enterobacteriaceae* in diagnostic laboratories.

MacConkey agar is a selective medium, which contains lactose as fermentable sugar, bile salts and crystal violet, in order to inhibit Gram-positive bacteria, and neutral red as pH indicator.

After aerobic incubation of the organism at 37°C for 24-48 hours, in case of lactose fermentation, acid metabolic products are generated and the medium and colonies appear pink (lactose-positive). If the organism is unable to use the lactose, then it attacks the peptone in the medium, with release of alkaline products.

Members that ferment lactose are traditionally indicated with the term “coliform”, such as *E. coli* and *Klebsiella*, *Citrobacter* and *Enterobacter* species, to distinguish them from the non-lactose fermenters, such as *Shigella*, *Yersinia*, *Proteus* and *Salmonella* species.

Other useful selective media to isolate *Enterobacteriaceae* include Brilliant Green Agar, Hektoen Enteric Agar and Xylose Lysine Deoxycholate Agar.

Enrichment media like selenite F broth are commonly used to increase the possibility of detecting *Salmonella* and *Shigella* species, whose numbers in fecal specimens may be too low to be detected on the primary plating media.

Genera and species of the family *Enterobacteriaceae* have traditionally been differentiated based on biochemical tests, used to

identify isolates after a preliminary examination of their morphology, motility, and growth responses.

Commonly used tests are those for the type of fermentation, lactose and citrate utilization, indole production from tryptophan, urea hydrolysis, and hydrogen sulfide production.

The usefulness of biochemical tests in identifying enteric bacteria is synthetized in commercial identification systems, such as the Enterotube and API 20-E systems, which are based on these tests.

Other methods include immunological tests: the great variability of O, H and K antigens provides the major basis for the internationally recognized serotyping schemes of *Enterobacteriaceae*. Hence, it is possible to distinguish several serotypes within a species (Baron et al., 1996).

Molecular methods are used to identify bacteria at taxonomic levels from the family down to the strain; furthermore, molecular tests based on virulence and pathogenicity genes can be used to distinguish pathogenic and non-pathogenic isolates (Keer and Birch, 2003).

Enterobacteriaceae can be divided into three main groups based on their pathogenicity for animals:

• Major pathogens such as *Salmonella* species, *E. coli* and *Yersinia* species.

• Opportunistic pathogens and commensals that occasionally cause infections, like species within the genera *Escherichia*, *Klebsiella*, *Enterobacter*, *Proteus*, *Serratia*, *Edwardsiella*, *Citrobacter*, *Morganella* and *Shigella*.

• Organisms of uncertain significance, including *Buttiauxella agrestis*, *Leclercia adecarboxylata*, *Kluyvera* and *Providencia* species.

1.1.1 *Escherichia coli*

The genus *Escherichia* includes straight Gram-negative rods that are approximately 0.5 μm in diameter and 1.0–3.0 μm in length.

It comprises six species: *albertii*, *coli*, *fergusonii*, *hermannii*, *marmotae* and *vulneris*. The species *blattae* has been recently moved into the *Shimwellia* genus (Priest and Baker, 2010).

E. coli is undoubtedly the best-studied bacterium and the experimental organism of choice for many microbiological research laboratories.

The species comprises commensal variants, which belong to the normal gut flora of humans and warm-blooded animals. Additionally, several pathogenic variants have been identified as responsible for different types of intestinal or extraintestinal opportunistic infections in both humans and animals.

In order to produce disease, *E. coli* must possess genes encoding virulence factors. Nonpathogenic strains may also acquire genes through transduction, conjugation or transformation, thus gaining a pathogenic potential.

This form of gene acquisition, often realized through bacteriophages or plasmids, is particularly important for the occurrence of new pathogenic types.

E. coli strains can be classified based on serology, using the antigenic differences in the structure of the LPS somatic antigen (O antigen), flagellar antigens (H antigen) and capsular antigens (K antigen). The existence of 170 O antigens, 56 H antigens and 80 K antigens has been reported (Ruffo G., 1998) and over 700 antigenic serotypes of *E. coli* are documented.

Moreover, numerous fimbrial adhesins (F antigens) have been described, providing strains with the ability to adhere to and colonize the epithelial cells of intestinal mucosa.

Although serotyping is still widely used for the epidemiological investigation of *E. coli* disease, a number of molecular methods for *E. coli* strain characterization are now available and increasingly employed.

Among all, PCR based methods are used to assign strains to major phylogroups A, B1, B2, D and E (Boyd and Hartl 1998, Clermont et al. 2000).

According to this classification, extra intestinal pathogenic strains belong to phylogroups B2 and D, whereas intestinal pathogenic strains and commensals belong to groups A and B1.

E. coli that cause gastrointestinal disease are classified into pathogenic categories, also called pathovars.

Each pathovar is defined by a characteristic set of virulence factors that act in concert to determine the clinical, pathologic, and epidemiologic features of the disease they cause. Pathovars can be broadly divided into diarrheagenic (DEC) and extra intestinal pathogenic (ExPEC).

DEC pathovars isolated in companion animals include enterotoxigenic *E. coli* (ETEC), enterohemorragic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), necrotoxigenic *E. coli* (NTEC) and adherent invasive *E. coli* (AIEC).

In contrast to ETEC strains, which are obligate pathogens, ExPEC and EPEC strains form part of the normal flora in animals and are considered opportunistic pathogens (Gyles and Fairbrother, 2010).

ETEC strains are responsible of the enterotoxigenic diarrhea, which occurs in pigs, calves and lambs and has been reported in dogs, cats and horses (Beutin, 1999; Olson et al., 1984). In human medicine,

they cause infantile diarrhea (in developing countries) and a syndrome known as "traveler's diarrhea" (Navarro Garcia et al., 2001).

ETEC strains produce fimbrial adhesins, which promote attachment to surface glycoproteins of jejunal epithelial cells and ileum, which appears from the first to the sixth week of life, explaining the highest incidence of disease in young animals.

Some strains also produce curli fimbriae, which mediate adherence to extracellular matrix proteins, whose exposition is determined by concurrent viral or parasitic infections. This determines an increase in the window of age of susceptibility to enterotoxigenic disease.

In order to cause disease, they must also synthesize enterotoxins, protein exotoxins encoded by genes usually carried on transmissible plasmids (e.g. heat labile enterotoxin (LT), heat-stable (ST) enterotoxins, and EAST1).

Most of the canine ETEC strains express ST enterotoxins. The bacteria adhere to the proximal small intestinal mucosa and produce plasmid-encoded enterotoxins, which bind to the extracellular domain of guanylyl cyclase C, which leads to accumulation of

intracellular cyclic GMP and ultimately secretion of chloride and decreased absorption of NaCl, with resultant osmotic diarrhea.

Two different ST enterotoxins have been identified, STa and STb. ST-producing ETEC have been detected in young dogs with diarrhea (Drolet et al., 1994; Hammermueller et al., 1995; Beutin, 1999)

After ingestion by the host, ETEC strains adhere to epithelial cells without damaging them, then multiply and secrete enterotoxins.

Following the action of enterotoxins, fluid and electrolytes accumulate in the lumen of the intestine, resulting in watery and nonbloody diarrhea, dehydration, and electrolyte imbalances. Peristalsis determines the infecting strains to move distally, away from the target cell, and the disease process stops. Nevertheless, unless fluid and electrolyte imbalances are corrected, the disease has high mortality.

EPEC strains adhere to mucosal cells of the small intestine and colon, with a typical intestinal lesion, characterized by intimate adherence of bacteria to the epithelium, microvilli destruction and reorganization of the cytoskeletal actin, which leads to the formation of actin-rich pedestals (DeVinney et al., 1999).

They carry the eaeA gene on their chromosome, which encodes a 94-kDa protein, intimin, which is mainly responsible for the intimate attachment, by forcing the host cells to form the attachments with the bacteria using their own actin.

EPEC are one of the most important causes of infantile diarrhea in humans in the world and infections occur naturally in pigs, calves, dogs and cats as well. In animals, they colonize and cause lesions in both the distal small intestine and the large intestines. They do not produce enterotoxins and are responsible for watery diarrhea.

They have been isolated in healthy cats and in one diarrheic cat in Brazil (Morato et al., 2009), and several serotypes have been identified, two of which were recognised as human pathogens.

EHEC strains act with the same attaching mechanisms used by EPEC and cause the same type of lesions.

The higher severity of clinical signs is due to the production of two forms of toxin called Shiga-like toxins (SLT 1 and SLT 2), also known as verocytotoxins VT-I and VT-II, which have chemical and biological similarity with the toxin elaborated by *Shigella dysenteriae* type 1 (Buchanan and Doyle, 1997).

Genes encoding Shiga-like toxins are harboured in phages within bacteria, which mediate lysis of bacteria and release of toxins if a damage of DNA occurs.

One of the most known EHEC serotypes is *E. coli* O157:H7, often called verotoxigenic (VTEC), which is responsible for foodborne and waterborne infections in humans and is recognized as the primary cause of hemorrhagic colitis or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome.

E. coli O157:H7 is naturally harboured by cattles, which are resistant to infection due to the lack of Stx-receptors, and it has been isolated in dogs (Kataoka et al., 2010; Hogg et al., 2009).

A study conducted in the USA by Smith et al. (1998) identified an overall prevalence of 12.3% of *E. coli* O157:H7 in a feline population, composed by both healthy and ill cats and some of the isolated serotypes were similar to those found in people and cattle, suggesting that cats might be reservoirs for human infection.

NTEC strains produce cytotoxic necrotizing factors (CNFs), protein toxins CNF1 and CNF2, which have been associated with diarrhea, bacteremia, and urinary tract infections in humans.

E. coli producing CNF1 have been isolated from stools of normal dogs and cats, as well as from dogs with enteritis (Starcic et al., 2002; Mainil et al., 2003).

AIEC strains have been implicated in approximately 37% of human Crohn's patients (Barnich and Darfeuille-Michaud, 2007).

They adhere to carcinoembryonic antigen-related cell adhesion molecule 6 in the ileum, which is overexpressed in patients with Crohn's disease.

AIEC then translocate into the lamina propria, where they live and replicate within macrophages, stimulating production of large amounts of TNF- α. Granuloma formation is thought to be the consequence of aggregation and fusion of infected macrophages, with subsequent recruitment of lymphocytes.

An association has been made between histiocytic ulcerative (granulomatous) colitis in Boxer dogs and intramucosal colonization by *E. coli* that phylogenetically resembles Crohn's disease – associated *E. coli* LF-82 (Simpson et al., 2006).

Others diarrheagenic pathovars implicated in humans' disease and not documented in small animals are enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli*

(DAEC), neonatal meningitis *E. coli* (NMEC) and cell-detaching *E. coli* (CDEC).

ExPEC strains possess virulence characteristics that allow them to invade, colonize and induce disease in body sites other than the gastrointestinal tract.

In most animal species, ExPEC infections commonly occur in the urinary tract, umbilicus, blood, lung, and wounds.

Extraintestinal diseases may result from infection with strains causing invasive conditions, as it is the case of septicaemia (SEPEC), and uropathogenic *E. coli* (UPEC), and with commensals.

Moreover, Köhler & Dobrindt (2011) recently suggested two new animal pathogenic groups: mammary pathogenic *E. coli* (MPEC), causing infections of the mammary gland, and endometrial pathogenic *E. coli* (EnPEC), affecting the uterus.

1.1.2 *Salmonella*

The genus *Salmonella* includes bacteria with a size of 0.7-1.5 x 2.0-5.0 microns, generally motile because of the presence of peritrichous flagella, with exceptions like *S. enterica* ssp. *enterica* ser. Gallinarum and *S. enterica* ssp. *enterica* ser. Pullorum.

They harbour the intestine of warm and cold-blooded animals, as well as the environment, where they can survive more than nine months, particularly in water and moist soil.

Infection occurs through the gastrointestinal way and the most common source is the contact with contaminated food, water or fomites. The airborne transmission, which determines the respiratory infection, can occasionally occur, since the microorganism is able to survive on dry air particles in absence of organic material. In fact, the biological cycle of *Salmonella* also comprises "environmental guests" that act as a link between ecological niches formed by wild animals and domestic ones.

Environmental reservoirs are natural sources such as wastewater, shallow lakes, seas, sewage and other sources such artificial surfaces or instruments.

The genus currently comprises three species: *S. bongori*, *S. enterica*, and *S subterranea*.

S. enterica contains six subspecies: *enterica* (ssp. I), *salamae* (ssp. II), *arizonae* (ssp. IIIa), *diarizonae* (ssp. IIIb), *houtenae* (ssp. IV), and *indica* (ssp. V). Subspecies V has been reclassified as *S. bongori*. The type species is *S. enterica* ssp. *enterica* and the type strain is *S. enterica* ssp. *enterica* serotype Typhimurium strain LT2 (Lilleengen strain type 2).

There are currently 2,463 serotypes of *Salmonella* (also known as serovars or varieties), based on O and H antigens (Popoff et al., 2000) and nearly 60% of these falls within subspecies I, whose strains are commonly isolated from humans and warm-blooded animals.

S. bongori and *S. enterica* subspecies II, IIIa, IIIb, IV, and VI generally infect cold-blooded vertebrates and live in the environment.

S. subterranea is a recent addition to the genus and was isolated from low pH subsurface sediment contaminated with nitrate and hexavalent to tetravalent uranium.

From an epidemiological point of view, *Salmonellae* are classified in host-specific and non-host-specific strains, the first ones being

responsible of more severe clinical forms compared to non-host-specific ones.

Among non-host-specific ones, *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium cause infection in humans and animals, with a variety of clinical forms, although they are usually self-limiting diseases.

A further subdivision within host-specific *Salmonellae* includes host-restricted and host-adapted strains.

These latter are confined to a limited number of guests, as it is the case of *S. enterica* ser. Dublin in cattles and *S. enterica* ser. Choleraesuis in pigs, which can also infect humans.

Host-restricted *Salmonellae* are associated with severe systemic forms in a single host species, as it is the case of *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi in humans, *S. enterica* ser. Gallinarum in poultry and *S. enterica* ser. Abortusovis in sheeps.

Infection of dogs and cats with *Salmonella* has been associated with the feeding of raw meat diets (Finley et al., 2000; Lenz et al., 2009), although commercial dry and raw dog food and pig ear pet treats have also been contaminated with the organism (Behravesh et al., 2010; Selmi et al., 2011).

The clinical signs of salmonellosis vary depending on the number of infecting organisms, host's immune status and the complicating factors or concomitant diseases.

The syndrome may comprise of gastroenteritis, bacteremia and endotoxemia, organ localization and persistence of asymptomatic carrier state.

In particular, metastatic infection can occur because of clinical or subclinical bacteremia. The microorganisms can be located in a particular organ for a certain period before producing overt clinical signs, which are related to the bacterial location.

Salmonellosis is a significant disease of ruminants, mainly cattle. The disease affects commonly young and adult animals in feedlots and dairies. The disease may present as septicemia or be limited to an enteritis or enterocolitis. Pneumonia can be hematogenously acquired. Abortion may follow septicemia.

Salmonellosis is uncommon in dogs and cats. When outbreaks in companion animals occur, they are usually associated with a common source, such as contaminated pet food or “treats”.

Dogs and cats infected with *Salmonella* spp. may show no signs or they may develop enterocolitis, focal suppurative infection, or severe

systemic illness. The majority of dogs are chronically and subclinically infected.

Occasionally salmonellae localize in a particular organ. Rodriguez et al. (1993) reported the case of a cat that developed pneumonia caused by *S. choleraesuis* without enteric events or positive results of stool cultures. Moreover, it has been described in a short-haired cat with severe pneumonia without gastrointestinal or cutaneous manifestations, so it should be considered as a possible cause of lung disease in cats, especially if immunocompromised (Callegari et al., 2014).

1.1.3 *Yersinia*

The genus *Yersinia* includes 11 species, of which three key members infect dogs, cats and humans: *Y. enterocolitica*, *Y. pestis* and *Y. pseudotubercolosis*.

Y. enterocolitica is a Gram-negative, mobile coccobacillus, measuring 0.5-1.0 × 1.0-3.0 μm that causes enterocolitis in humans. An unusual feature of this bacterium is that it replicates in culture at refrigeration temperature.

The prevalence of isolation from animals increases in colder months. Since *Y. enterocolitica* was isolated from dogs' feces and clinically healthy cats, it is thought to be a commensal organism (Fenwick et al., 1994; Salamah, 1994).

Y. pseudotuberculosis is the cause of enteritis in many animals, especially during winter and spring months (Black et al., 1996). Many animals, including birds, rodents, cats and pigs, have been indicated as reservoirs (Fukushima et al., 1989; Salamah, 1994). Humans are more severely affected and develop mesenteric lymphadenitis and septicemia (Fukushima et al., 1989).

Y. pestis is the type species of the genus and it is the cause of plague, a septicemic disease of major importance in humans, rodents, and occasionally domestic animals, mainly cats.

Humans and pets are alternative hosts for *Y. pestis*, which is maintained in nature through a chronic bacteremia in wild rodents and transmitted by fleas. Cases in pets are more frequent from February to August, when rodents and their fleas are most active and humans and their companion animals are more likely to be outdoors. Transmission is less commonly due to the contact with mucous membranes or broken skin or inhalation of droplets from animals with pneumonic plague.

In both humans and cats, three clinical forms of the disease have been described: bubonic plague, septicemic plague and pneumonic plague. The most common is bubonic plague, which in cats is usually acquired through ingestion of infected rodents and is associated with fever (40.6 to 41.2 ° C), dehydration and adenopathy of submandibular, retropharyngeal and cervical lymph nodes, which become swollen and abscessed.

Cats with spontaneously draining abscesses have more chances of surviving.

In case of progress of the bubonic form (without draining of abscesses), infection can spread via blood or through the lymph to become a septicemic form.

This can determine involvement of any organ, although more frequently involved are the spleen and lungs in humans and cats.

In cats, fever, shock, disseminated intravascular coagulation and severe leukocytosis are characteristic findings of the septicemic form, which is deadly and normally occurs 1-2 days after bacteremia.

1.1.4 *Klebsiella*

The genus *Klebsiella* comprises of straight rods, measuring 0.3–1.0x0.6–6.0 mm, arranged singly, in pairs or short chains.

They are often surrounded by a capsule and are Gram negative, nonmotile (except *K. mobilis*) and facultatively anaerobic.

The type species is *Klebsiella pneumoniae*, which, together with *K. oxytoca*, is the most common pathogens in veterinary medicine and a commensal of the intestinal tract of animals.

In humans, as well as in veterinary medicine, this species has been frequently associated with hospital-acquired infections and with many forms of opportunistic infections. Contaminated obstetric equipment, surgical equipment, cleaning devices, and clinic surfaces may contribute to the occurrence of the infection.

Virulence factors associated with *Klebsiella* spp. are similar to other *Enterobacteriaceae*.

The capsule is essential for resistance to host defense mechanisms (phagocytosis, opsonization, and cytolysis).

Endotoxins, adhesins, enterotoxins, siderophores, and cell wall components have an also significant role.

Members of the genera have been involved in a wide range of canine diseases such as pneumonia (Haenni et al., 2012), otitis externa (Brothers et al., 2002), prostatitis (White and Williams, 1995), meningoencephalomyelitis (Radaelli and Platt, 2012), cholangiophepatitis (Forrester et al., 1992; Farrar et al., 1996) and pyoderma.

Klebsiella spp. are the second most common cause of canine cystitis (Ling et al., 2001; Johnson et al., 2003). They are also reported in canine mastitis (Schäfer-Somi et al., 2003).

Neonatal puppies are particularly predisposed to infections: sources of infection include the environment, vaginal discharge, maternal faeces, oropharynx and skin (Münnich, 2008).

Systemic infections in dogs are common with these bacteria and may present with multiorgan dysfunction. Treatment often require aggressive antimicrobial therapy and supportive treatment for pneumonia (Cavana et al., 2009).

In cats, *Klebsiella* spp. have been involved in cat flu (Adler et al., 2007) and cystitis (Ghantous and Crawford, 2006), as well as in hospital-associated infections (Bowlt et al., 2013).

1.1.5 *Citrobacter*

Citrobacter spp. are usually considered to be of low pathogenicity. They are commonly present in water, soil and food, whilst they occasionally colonise the gastrointestinal tract of animals and humans.

However, in immunocompromised human hosts, a range of infections such as urinary tract infections, pneumonia, skin and soft-tissue infections, sepsis and meningitis are likely to occur (Lipsky et al., 1980).

The genus includes 11 different species, of which *C. freundii* and *C. diversus* are the most significant and responsible for healthcare-associated opportunistic infections.

The high incidence of mortality, as a result of infections by these microrganisms, has been associated with multidrug-resistant strains (Pepperell et al., 2002).

Citrobacter spp have been associated with cystitis in the dog and cat (Euclid et al., 2011).

They are considered an opportunistic or secondary pathogens of the skin, gastrointestinal and respiratory tracts (Farmer and Kelly, 1991).

In dogs, they are part of the normal biotome of the oropharynx (Kasempimolporn et al., 2003) and gastrointestinal flora.

Species which are pathogenic for dogs include *C. freundii, C. diversus* and *C. koserii*.

In dogs, *Citrobacter* spp have been commonly involved in recurrent cystitis, with reports of emphysematous cystitis occurring rarely (Chang et al., 2007).

Localized infections associated with indwelling intravenous catheters (Lobetti et al., 2002) and secondary infections with respiratory diseases have been reported (Johnson and Fales, 2001).

However, septicemia is not uncommon, with a number of puppies and immunocompromised adult dogs reportedly suffering acute hemorrhagic diarrhea, followed by septicemia, peritonitis (Galarneau et al., 2003), myocarditis (Cassidy et al., 2002) and fibrinous pericarditis (Stafford Johnson et al., 2003).

1.1.6 *Enterobacter*

Enterobacter species are found in the natural environment including water, sewage, vegetables, and soil. In human medicine, *Enterobacter* spp. are frequently encountered as nosocomial pathogens, probably due to a greater resistance to disinfectants and antimicrobial agents than that of other members of the *Enterobacteriaceae*.

E. cloacae predominates, followed by *E. agglomerans*, *E. sakazakii*, and others.

They are a common cause of nosocomial infections of surgical wounds and burns, whereas other infections include cellulitis, fasciitis, abscesses, emphysema, myositis and urinary tract infections, from asymptomatic bacteriuria to pyelonephritis and urosepsis.

In veterinary medicine, *Enterobacter* spp. have been associated to neonatal mortality (Münnich and Küchenmeister, 2014), urinary tract infections (Marsh-Ng et al., 2007; Bubenik et al., 2007), urinary catheterization (Bubenik and Hosgood, 2008) and as commensals in dogs affected by tracheal collapse (Johnson and Fales, 2001).

They have also been associated with pancreaticobiliary duct infections (Quian et al., 1993) and post-operative empyemas (De Stefani et al., 2008). Zoonotic infections in humans have been attributed to these bacteria, which are normal residents of the canine oropharynx (Saphir and Carter, 1976).

1.1.7 *Proteus*

The genus comprises of straight rods, measuring 0.4–0.8x1.0–3.0 μm. They are Gram negative and motile by peritrichous flagella.

Most strains, in solid culture media, swarm with periodic cycles of migration producing concentric zones, or spread in a uniform film.

The type species is *Proteus vulgaris*.

They are commensal bacteria normally found on dogs' skin and gastrointestinal tract.

Clinically, *Proteus* spp. are regularly involved in bacterial infections in neonatal puppies (Münnich, 2008), but are sometimes associated also with cystitis (Ball et al., 2008), paronychia and otitis externa (Zamankhan Malayeri et al., 2010).

1.1.8 *Buttiauxella*

Members of the genus *Buttiauxella* are straight rods, measuring 0.5–0.7 x 2–3 μm. They are Gram negative, motile with peritrichous flagella and facultatively anaerobic.

Buttiauxella spp. are widely distributed in nature, may be isolated from food and are occasionally isolated from human sources. Although the natural habitat of *Buttiauxella* spp. was originally thought to be water, the majority of strains have been isolated from the intestines of snails and slugs (Muller et al., 1996).

The type species is *B. agrestis*, which has been recently reported as a cause of infection in human medicine (Antonello et al., 2014).

1.1.9 *Hafnia*

Members of this genus have the common characteristics of *Enterobacteriaceae*.

The type species is *Hafnia alvei*, which occurs in humans and animals, including birds, and in natural environments such as soil, sewage, and water.

Kume (1962) described a case of equine abortion in which *H. alvei* was isolated from a fetus and lochia in pure culture.

Riggio et al. (2013) detected found it associated to ovine “broken mouth” periodontitis.

In human medicine, *H. alvei* has been reported to cause septicemia (Englund, 1969; Mobley, 1971), respiratory tract infections (Klapholz et al., 1994; Fazal et al., 1997), meningitis (Mojtabaee and Siadati, 1978), abscesses (Agustin and Cunha, 1995), urinary tract infections (Whitby and Muir, 1961), wound infections (Berger et al., 1977), periodontal disease with tissue destruction (Vieira Colombo et al., 2016).

The intestinal tract of animals, in particular mammals, appears to be a very common ecologic habitat for this bacterium (Janda and Abbot, 2006). Moreover, *H. alvei* has been isolated from reptiles (snakes

and skinks), fish, invertebrates, insects and avian species (Goldstein et al. 1981; Goatcher et al. 1987; Cassel-Beraud and Richard, 1988; Okada and Gordon 2003).

1.1.10 *Kluyvera*

The genus *Kluyvera* includes *Kluyvera ascorbata*, *K. cryocrescens*, and *K. georgiana*.

Fainstein et al. (1982) isolated strains of *Kluyvera* spp. from human patients with and without diarrhea, and suggested that *Kluyvera* strains might have had a role in some of the diarrhea cases.

The presence of *Kluyvera* in food and water is a possible source of intestinal isolates.

The respiratory tract has been the most common source for *Kluyvera* spp., but there is no strong evidence that it is clinically significant at this site (however, one isolate of *K. ascorbata* was from a lung at autopsy). The respiratory tract (particularly sputum) is notoriously difficult to evaluate for clinical significance. The urinary tract has been the next most common source, but it has also been difficult to document clinical significance (Tristram and Forbes, 1988).

1.1.11 *Leclercia*

The type species is *Leclercia adecarboxylata*, previously named *Escherichia adecarboxylata* by Leclerc (1962), which proposed that it should have been recognized as a separate species in the genus *Escherichia*.

Tamura et al. (1986) used DNA–DNA hybridization to show that *E. adecarboxylata* was only 26% related to the type strain of *Escherichia coli*. Hence, they proposed a new genus *Leclercia* with one species *Leclercia adecarboxylata*.

Isolated from human clinical specimens, environmental samples, food and water, its clinical significance is not fully documented but its potential role as a pathogen is suggested by isolates from blood and similar specimens that are normally sterile.

However, it may be colonizing rather than infecting nonsterile body sites.

The isolates from food, drinking water, feces, and an intravenous fluid bottle suggest ways that humans are exposed to it. There is no evidence that it can cause diarrhea or intestinal infections.

It should be considered a rarely isolated species of *Enterobacteriaceae*, and a possible opportunistic pathogen (extraintestinal infections only) for humans.

1.1.12 *Providencia*

The type species is *P. alcalifaciens* and the genus includes *P. rustigianii, P. heimbachae, P. stuartii* and *P. rettgeri*. Some strains are opportunistic pathogens in humans and can cause urinary tract infections, particularly in patients with long-term indwelling urinary catheters or extensive severe burns.

In companion animals, *P. alcalifaciens* has been reported as a cause of diarrhea in dogs and cats (Kròl et al., 2007; Tribe and Rood, 2002) and *P. stuarti* in a dog with severe skin ulceration and cellulitis (Papadogiannakis et al., 2007).

1.1.13 *Serratia*

The type species is *Serratia marcescens*, which has been reported as possibly associated with a subgroup of granulomatous/pyogranulomatous skin lesions in dogs (Cornegliani et al., 2015).

It can be considered as an opportunistic pathogen: Lobetti et al. (2002) reported that IV catheters might be colonized with bacteria including *S. marcescens* in 22% of young dogs suspected to have Parvovirus infection.

Perez et al. (2011) reported the case of a 2 years old Dalmatian, referred for evaluation of acute lethargy, fever, neurologic signs, and a heart murmur, whose echocardiography and blood cultures revealed a nonhospital-acquired *Serratia marcescens* bacteremia and aortic valve endocarditis.

1.2 Antimicrobial resistance

Antimicrobial resistance is the ability of bacteria to be, or become, resistant to antimicrobials, therefore managing to survive and multiply in presence of the drug (Cantón et al., 2011).

The ability of bacteria to develop resistance was described soon after the first antimicrobials were introduced during the 1930s and 1940s. Bacteria are ubiquitous in the environment, including on the skin and mucous membranes as well as in the gastrointestinal tract of animals. Their ecologic success is largely attributable to their ability to survive hostile conditions and adapt to changes in the environment. Therefore, development of antimicrobial resistance does not represent a recent phenomenon but an unavoidable result of microbial cell evolution.

Development of antimicrobial resistance by pathogens and commensals represents a major threat to both animal and public health, due to its alarming development rates and its quick spread across the globe among different species of bacteria, as highlighted by the World Health Organization (WHO, 2014).

The decreased efficacy of commonly used antibacterial agents and the need to use more expensive drugs leads to disposal of limited therapeutic options and increase in treatment costs.

Moreover, the arsenal of antibacterial drugs available to treat infections caused by resistant bacteria may be so restricted that the ability to cure an infection without producing toxicity is compromised.

In fact, current concerns related to antimicrobial resistance arise principally from the rapid rate of development of resistance relative to the slow rate at which new antibiotics are introduced and the conviction that development of resistance is accelerated by overuse of antimicrobials.

Furthermore, because of the acquisition of resistance determinants against different antimicrobials, multi-drug resistance in common bacterial pathogens is being reported worldwide, extremely compromising the future usefulness of antimicrobials in treating bacterial infections.

The ease with which resistant genes are transferred between bacteria accelerates the emergence of antimicrobial resistance in a particular

animal species and increases the risk of spread of resistance to other species, including human beings.

Obviously, if the development and spread of resistance are to be retarded, it is necessary that public health workers, including veterinarians, understand the mechanisms that bacteria use to resist antibacterial agents.

Antimicrobial resistance can be intrinsic or acquired.

Intrinsic resistance is the resistance of all members of a bacterial species without any genetic extra-modification and it is due to either lack of the target for the action of the drug or to the inability of the drug to enter the bacterial cell (Normark and Normark, 2002; Greenwood et al., 2006).

Knowledge of the intrinsic resistance of pathogens is important in practice, in order to avoid resort to inappropriate and ineffective therapies for infections caused by an intrinsically resistant microrganism.

Some examples of intrinsic resistance and their respective mechanisms (Forbes et al., 1998; Giguere et al., 2006) are:

- resistance of anaerobic bacteria to aminoglycosides, due to lack of oxidative metabolism which drives the uptake of aminoglycosides

- resistance of aerobic bacteria to metronidazole, due to their inability to anaerobically reduce the drug to its active form

- resistance of Gram-positive bacteria to aztreonam, due to the lack of penicillin binding proteins (PBPs) that bind and are inhibited by this beta-lactam antimicrobial

- resistance of Gram-negative bacteria to vancomycin, due to lack of uptake, resulting from inability of vancomycin to penetrate their outer membrane.

Acquired resistance occurs when a microorganism gains the ability to resist the activity of an antimicrobial agent to which it was previously susceptible.

Acquired resistance can result from:

- mutations in chromosomal genes (Martinez et al., 1998)

- acquisition of new genes by horizontal gene transfer (Jacoby and Sutton, 1991)

- a combination of these two mechanisms (e.g. mutations in previously acquired genes) (Jacoby and Medeiros, 1991).

Mutational resistance occurs by point mutations, deletions, inversions or insertions in the bacterial genome.

Transferable resistance occurs when a resistance gene is transfered from a resistant to a susceptible bacterial cell by several mobile genetic elements, such as plasmids, bacteriophages, transposons and integrons (Normark and Normark, 2002; Greenwood et al., 2006).

Selection and expression of resistance can also result from exposure to antimicrobial agents.

Generally, antibiotic exposure does not cause a susceptible strain to mutate to a resistant one.

Nevertheless, exposure to antimicrobial agents promotes emergence of resistance by facilitating the survival of resistant strains or inducing the expression of existing antimicrobial resistance genes. Classically, resistance in a bacterial population can be identified by the existence of at least two distinct subpopulations separated on the basis of Minimal Inhibitory Concentration values.

Survival of the relatively resistant subpopulation is promoted by exposure to concentrations of antibiotics that inhibit only the susceptible subpopulation.

As a result of this differential effect, resistant strains increase in number until they represent a larger proportion of the population as

a whole, thus increasing the likelihood that they cause infectious diseases.

Within environments that are subject to frequent and consistent antibacterial use patterns, such as intensive care units, the emergence of predominant populations of resistant strains is accelerated, particularly when little care is taken to prevent transfer of resistant strains between patients.

Antibiotic exposure not only promotes the survival of drug-resistant pathogenic bacteria, but increases the population of drug-resistant nonpathogenic bystanders, many of which are commensals in the upper respiratory and gastrointestinal tracts, thus increasing the reservoir of resistance in the bacterial population as a whole and increasing the opportunity for resistance to be transferred to pathogenic bacteria by processes like conjugation and transposition.

Aside from the effect of antimicrobial exposure on survival of resistant mutants, antimicrobial agents may also induce the expression of existing resistance genes (Palzkill, 2001).

For example, beta-lactamases are present in virtually all Gram-negative bacilli. However, in some bacterial strains, such as *E. coli* and *Klebsiella* spp., the β-lactamase is produced at a low level and

cannot be induced to greater production by the presence of β-lactams. In other species, β-lactamase production occurs at low levels, but is inducible when exposed to certain β-lactams, commonly resulting in resistance to these agents. These inducible β-lactamases are frequently found in *Enterobacter* spp., *Citrobacter freundii*, *Providencia* spp., *Morganella* spp. and *Serratia* spp., often termed the 'ESCPM' group, which may express high levels of chromosomally determined AmpC β-lactamases following exposure to β-lactams, either by induction or selection for derepressed mutants. This may lead to clinical failure even if an isolate initially tests susceptible in vitro (Harris and Ferguson, 2012).

1.3 β-lactam antimicrobials

In veterinary medicine, antimicrobial use is directed towards farm animals, companion animals, wildlife and animals raised in aquaculture.

Nine classes of antimicrobials are exclusively used in animals (Pagel, 2012), but several classes are commonly prescribed in both veterinary and human medicine, namely: penicillins, cephalosporins, tetracyclines, chloramphenicols, aminoglycosides, macrolides, nitrofuranes, nitroimidazoles, sulphonamides, trimethoprim, polymyxins and quinolones (Prescott, 2000).

Due to their diversity, broad spectrum of activity and low toxicity, β-lactams are the most prescribed antimicrobials worldwide (Livermore and Woodford, 2006) and in companion animals medicine they represent the most widely used antimicrobials for treating bacterial infections also caused by *Enterobacteriaceae* (Escher et al., 2010; Mateus et al., 2011).

All β-lactam antimicrobials share the presence, in their molecular structure, of the β-lactam ring, a four-atom ring that serves as a substrate for the transpeptidase target enzymes of bacteria and is therefore vital for the antimicrobial activity.

The cross-linked peptidoglycan layer in the bacterial cell wall is vital for the protection of the cell shape and rigidity.

The cross-linking of peptidoglycan units is catalysed by a group of bacterial enzymes, the cell wall transpeptidases (Fisher et al, 2005; Wilk et al, 2005), which are traditionally named penicillin binding proteins (PBPs) (Spratt, 1994), because of their affinity for and binding of the β-lactam penicillin, which has a stereochemical similarity to the D-alanine residues of peptidoglycan units.

Through the creation of a covalent complex between PBPs and β-lactams, PBPs are inactivated and the peptidoglycan cross-linking is inhibited.

Consequently, this produces irregularities in the cell wall synthesis, such as elongation, lesions and loss of selective permeability, leading to loss of integrity and finally cell lysis (Tipper and Strominger, 1965).

1.3.1 Mechanisms of resistance to β-lactam antimicrobials

There are four main ways bacteria can avoid the effect of β-lactam antimicrobials.

The first way involves the production of β-lactamases, bacterial enzymes that hydrolyze the β-lactam ring and cause the antimicrobial to be inactive before it reaches the transpeptidases/PBPs target (Babic et al., 2006).

The second way, typical of Gram-positive bacteria, is the existence of modified transpeptidases/PBPs, which are not susceptible or are less susceptible to inhibition by β-lactams (Chambers, 1997).

The third way, which is characteristic of Gram-negative bacteria, is the lack of expression of outer membrane proteins (OMPs), transmembrane protein structures that provide access to relatively water-soluble antibacterial agents.

Loss of OMPs causes impermeability of the cell wall or cell membranes, impeding the entry of β-lactams into the periplasmic space of Gram-negative bacteria and therefore the access to PBPs on the inner membrane.

The fourth mechanism is the overexpression of efflux pumps, which actively transport drugs from the inner phospholipid layer of the

inner cytoplasmic membrane, a site that is sequestered from the aqueous cytoplasm and is therefore accessible primarily to relatively lipid-soluble drugs. Overexpression of efflux pumps results in the rapid expulsion of an antimicrobial from the cell.

In contrast to mutational changes in the structure of antibacterial target sites, which confer resistance to similar drugs that meet stringent stereospecific characteristics, changes in porin expression and the action of efflux pumps generally are less specific for individual antimicrobial agents but discriminate only on the basis of general physicochemical characteristics, such as lipid solubility.

For example, multidrug-resistant efflux pumps exist that have wide substrate activities across a variety of different chemical groups of antibacterial agents.

The hydrolytic inactivation of β-lactam antimicrobials by β-lactamases is a major determinant of resistance in Gram-negative pathogens, particularly among *Enterobacteriaceae* (Babic et al., 2006) and mechanisms for the efflux of these agents and lack of expression of OMPs contribute often in conjunction to the first one.

1.3.2 Classification and history of β-lactam antimicrobials

β-lactam antimicrobials have a long history in the treatment of infectious diseases, although their use has been and continues to be threatened by the development of resistance in target organisms.

The β-lactam antimicrobial class includes amino-, carboxy-, idanyl, and ureido-penicillins, first- to fourth-generation cephalosporins, monobactams and carbapenems (Babic et al., 2006).

Penicillins were the first β-lactams to be discovered and introduced to clinical use. They are active against most Gram-positive bacteria such as staphylococci and streptococci, against spirochetes (*Treponema pallidum* and *Leptospira* spp), gonococci and meningococci.

Natural penicillins are inactive against Gram-negative bacteria, whereas semi-synthetic penicillins, such as ampicillin and amoxicillin, have a broader spectrum of activity, even against some Gram-negative bacteria.

Penicillin G, or benzylpenicillin, is the product of fermentation of *Penicillium* spp. and progenitor of all penicillins. Its structural core is the 6-aminopenicillanic acid, which comprises a thiazolidine ring and a β-lactam ring.

Penicillin G was discovered by Sir Alexander Fleming in 1927, but it was not until the early 1940s, through the work of Drs. Florey, Chain and Heatley from Oxford University, that it was purified and shown to cure specific bacterial infections.

Within a few years after its introduction, *Staphylococcus aureus* strains showed resistance to penicillin (Rammelkamp and Maxon, 1942), due to the production of penicillinase, a β-lactamase enzyme. This drove the search for new forms of β-lactams that were not inhibited by penicillinase and had a wider spectrum of activity against both Gram-positive and Gram-negative bacteria.

The inclusion of different side chains gave rise to the many existing semi-synthetic penicillins.

Semi-synthetic penicillins, such as ampicillin and carbenicillin, were introduced by the early 1960s, showing much more efficacy against Gram-negative bacteria than natural penicillins.

Moreover, many other chemical derivatives have been developed from penicillin to combat resistance that has arisen in bacteria.

These derivatives, commonly referred to as the extended-spectrum β-lactams, include cephalosporins, carbapenems and monobactams.

Cephalosporins were developed with the objective of combining the broad-spectrum activity of ampicillin and achieving stability to staphylococcal penicillinase.

They are all semi-synthetic derivatives of a compound called cephalosporin C that is produced by the mould *Cephalosporium acremonium* (*Acremonium chrysogenum*).

A natural progenitor of cephalosporins, cephalosporin C was immediately interesting for its broad spectrum of activity, being active against both Gram-positive and Gram-negative bacteria. Furthermore, compared to penicillin G, it had the advantage of being resistant to β-lactamases.

Although it did not find application in the therapeutic field, it turned out to be of industrial interest for the production of the 7-aminocephalosporanic acid, a base compound to obtain the clinically used semi-synthetic cephalosporins, whose antibacterial activity is much more potent than that of the original compound.

The production of cephalosporines evolved between 1960 and 1980 and they are classified into first, second, third and fourth generation cephalosporines, based upon the spectrum of antibacterial activity

and their stability against β-lactamase-producing Gram-negative bacteria.

First-generation cephalosporins, including cephaloridine, cephalothin, cefazolin and cephadrine, are more effective against Gram-positive bacteria such as streptococci and staphylococci and present a moderate activity against Gram-negatives like *E. coli* and *P. vulgaris*.

Second-generation cephalosporins, such as cefuroxime, cefoxitin and cefotetan, are less active against staphylococci and streptococci but more active against Gram-negative bacilli.

The third generation of cephalosporins includes cefotaxime, ceftriaxone, ceftazidime, and cefoperazone, which are more resistant to β-lactamases and are provided with an increased activity against strains of *Haemophilus* spp. and *Neisseria* spp. producers of β-lactamase as well as against *Citrobacter* spp., *Serratia marcescens* and *Providencia* spp. Some of these compounds, in particular ceftazidime and cefoperazone, are also active against *Pseudomonas aeruginosa*.

Fourth generation cephalosporines, such as cefepime and cefpirome, are active against staphylococci and Gram-negative bacteria including *P. aeruginosa*.

In contrast to the earlier cephalosporins, cefepime penetrates the bacterial cell more rapidly and escapes the effects of many chromosomal and plasmid-mediated β-lactamase enzymes due to their low affinity for this cephalosporin.

Monobactams are monocyclic β-lactams characterized by a unique β-lactam ring, not fused with another ring, unlike many other β-lactam antimicrobials, which have at least two rings.

Naturally occurring monobactams exhibit poor antimicrobial acitivity. However, modification of the monocyclic monobactam results in a potent antibacterial agent.

The only commercially available compound is aztreonam, which is active mainly against aerobic and facultatively anaerobic Gram-negative bacteria such as *Neisseria* spp. and *Pseudomonas* spp.

The advantage of the narrow-spectrum is the absence of damage to the patient's normal protective flora.

Carbapenems represent the most recently developed sub-class of β-lactam agents and exhibit the broadest spectrum of antibacterial

activity. These agents have been isolated from the fermentation products of a variety of *Streptomycetes*.

The most commonly administered compounds are meropenem, imipenem, doripenem and ertapenem.

They are active against many Gram-positive and Gram-negative, aerobic and anaerobic bacteria.

1.4 Beta-lactamases

Production of β-lactamases represents the predominant method of resistance to β-lactam antimicrobials among *Enterobacteriaceae*.

They are distributed in both Gram-negative and Gram-positive bacteria (Bush, 1997; Ambler, 1980).

In Gram-positive bacteria, β-lactamases are secreted extracellularly, whereas in Gram-negative bacteria they remain in the periplasmic space (Samaha-Kfoury and Araj, 2003).

The first report of a β-lactamase dates 1940, when an enzyme produced by a strain of *E. coli* was shown to compromise the ability of penicillin to kill bacterial cells (Abraham and Chain, 1940).

This represented the first report of β-lactamase activity before widespread use of penicillin, suggesting the existence of β-lactam-inactivating enzymes in the natural environment.

To date, over 1300 β-lactamases have been reported (Bush, 2013).

The genes encoding β-lactamases (*bla* genes) are located on either the bacterial chromosome or in mobile genetic elements, such as plasmids, transposons and integrons (Wright, 2005; Babic et al., 2006; Drawz, 2010).

The two most commonly used classification schemes for the β-lactamase enzymes are the Ambler scheme and the Bush-Jacoby-Medeiros (BJM) scheme.

The Ambler scheme divides β-lactamases into four groups based on amino acid sequences (Ambler, 1980).

Ambler class A, C and D β-lactamases are named “serine β-lactamases” as they possess in their active site a serine residue to bind to the β-lactam ring. Amber Class B β-lactamases are called “metallo-β-lactamases” (MBLs) as they possess zinc ions in their active site (Livermore, 1995; Bush, 1997).

In the BJM scheme, β-lactamases are classified based on their substrate and inhibitor profiles. It includes groups 1, 2 and 3, and several subgroups (e.g. 2a, 2c, 3a, etc) (Bush and Jacoby, 2010).

The major clinically important β-lactamases in Gram-negative bacteria are grouped in Table 1.

Table 1. Major clinically important β-lactamases in Gram-negative bacteria (adapted from Bush, 2010)

			β-Lactams to which resistance is conferred	
BJM[a]	**Ambler[b]**	**Common name**	**Primary[c]**	**Secondary[d]**
1	C	Cephalosporinase	Penicillins, cephalosporins	Carbapenems, monobactams
2b	A	Penicillinase	Penicillins, early cephalosporins	β-lactamase inhibitor combinations
2be	A	Extended-spectrum β-lactamase	Penicillins, cephalosporins, monobactams, β-lactamase inhibitor combinations	None
2d	D	Cloxacillinase	Penicillins, including oxacillin and cloxacillin	None
2df	D	Carbapenemase	Carbapenems and other β-lactams	None
2f	A	Carbapenemase	All current β-lactams	None
3	B	Metallo-β-lactamase	All β-lactams except monobactams	None

[a]BJM classification scheme. [b]Ambler classification scheme. [c]β-lactams that are resistant solely as a function of β-lactamase production. [d]β-lactams that are resistant as a function of β-lactamase production, usually at high levels, in combination with efflux or porin modifications.

There are two primary ways to overcome β-lactamases action: either through inhibitors (or inactivators) or by finding a new β-lactam antimicrobial that has a greater affinity for the target PBP and is resistant to β-lactamases.

There are currently three inhibitors used to this aim in combination with β-lactamase antimicrobials: clavulanic acid, sulbactam and tazobactam (Babic et al., 2006).

All three of these compounds resemble penicillin structurally, they exhibit high affinity for PBP's and are poorly hydrolyzed by β-lactamases (Helfand et al., 2003).

As previously described, in order to address the challenge posed by the production of β-lactamases confering resistance to β-lactam antimicrobials, newer extended spectrum β-lactams with a greater resistance to β-lactamase activity were introduced in the 1980's, including cephalosporins and carbapenems (Philippon et al., 2002). Some of the β-lactamases target the newer broad-spectrum β-lactams, such as the cephalosporins, carbapenems and aztreonam.

These enzymes comprise:

- extended spectrum β-lactamases (ESBLs), mostly belonging to Ambler classes A (Bradford, 2001)

- AmpC cephalosporinases, belonging to Ambler class C (Philippon et al., 2002)

- carbapenemases, belonging to Ambler classes A, B and D (Poirel and Nordmann, 2002).

1.4.1 ESBLs

Classically, ESBLs are defined as enzymes that have hydrolytic activity against the extended spectrum cephalosporins (ceftazidime or cefotaxime), the penicillins and aztreonam, but not the cephamycins (cefoxitin) or carbapenems, and are inhibited by β-lactamase inhibitors including clavulanic acid (Bush et al., 1995).

Most ESBLs contains a serine in their active site and belongs to the Ambler class A. In the BJM scheme, the ESBLs are inserted in the 2be functional group (Bush et al., 1995).

The most clinically important groups of ESBLs are CTX-M enzymes, followed by SHV- and TEM-derived ESBLs.

The majority of ESBLs are acquired enzymes, encoded by genes on plasmids, and they are expressed at various levels.

The level of expression, properties of a specific enzyme and the co-presence of other resistance mechanisms (other β-lactamases, overexpression of efflux pumps, lack of porins) result in a large variety of resistance phenotypes observed among ESBL-positive isolates.

1.4.1.1 TEM

The first plasmid-mediated β-lactamase was detected in Greece in the 1960s and was designated TEM after the name of the patient (Temoneira) who carried the pathogen, an *E. coli*.

The original TEM-type β-lactamases (TEM-1, TEM-2 and TEM-13) confer resistance to all penicillins, first generation cephalosporins (e.g. cephalothin) and are susceptible to β-lactam inhibitors such as clavulanic acid.

Amino acid substitutions in the TEM enzyme sequence cause alterations of the substrate profile and result in hydrolysis of the extended-spectrum cephalosporins.

The first TEM-type ESBL was reported in the late 1980's in Germany (Kliebe et al., 1985) and was originally designated CTX-1, due to the enzyme's ability to hydrolyse the third generation cephalosporin cefotaxime, but, shortly afterwards, the enzyme was renamed TEM-3. The extended-spectrum TEM enzymes also confer resistance to aztreonam and are susceptible to β-lactamase inhibitors such as clavulanic acid.

TEM-type ESBLs are most often found in *E. coli* and *K. pneumoniae*, but also in other species of Gram-negative bacteria (Bradford, 2001).

1.4.1.2 SHV

The designation ‘SHV’ refers to the term ‘Sulfhydryl Variable’, initially used to describe a biochemical property of the enzyme now known as ‘SHV’.

SHV-1, the parent enzyme, is present on the chromosome of *K. pneumoniae* and determines the 20% of ampicillin resistance in this species.

In 1983, Knothe found a single nucleotide mutation in a SHV that represented the first plasmid-encoded β-lactamase that could hydrolyze the extended-spectrum third-generation cephalosporins in an isolate of *K. ozaenae*, and this type was named SHV-2 (Knothe et al., 1983).

Outbreaks of *Klebsiella* spp. infections with mutated SHV enzyme derivates were reported from French hospitals at the end of the 1980s (Sirot et al., 1987; Philippon et al., 1989).

Mobilization of the *bla*SHV gene from the chromosome of *Klebsiella* species has been associated with the rapid dissemination of the enzyme to other members of the *Enterobacteriaceae* such as *E. coli*, *Enterobacter* spp. and other non-*Enterobacteriaceae*, such as *Pseudomonas aeruginosa* and *Acinteobacter* spp. (Paterson and

Bonomo, 2005; Bradford, 2001; Velasco et al., 2007; Morosini et al., 2006).

To date, more than 114 varieties of SHV are currently recognized (Jacoby and Bush, 2008).

1.4.1.3 CTX-M

The most common group of ESBLs is termed CTX-M (Jacoby and Munoz-Price, 2005).

Unlike TEM and SHV ESBLs, which have arisen from point mutations of parent enzymes, the CTX-M class is thought to have developed because of incorporation of pre-existing chromosomal ESBL genes from *Kluyvera* species onto a mobile plasmid (Bonnet, 2004).

They can be divided into five groups based on their amino acid identities: the CTXM- 1 group, the CTX-M-2 group, the CTX-M-8 group, the CTX-M-9 group, and the CTX-M-25 group (Pitout et al., 2007).

The designation ‘CTX-M’ refers to ‘cefotaximase’, as they demonstrate greater activity against cefotaxime than against ceftazidime (Babic et al., 2006, Jacoby and Munoz-Price, 2005).

CTX-M ESBLs are most often found in *E. coli*, with CTX-M variants being identified also in other members of the *Enterobacteriaceae* such as *Klebsiella*, *Serratia*, *Enterobacter* and *Salmonella* species (Bradford, 2001). At present, over 100 CTX-M-type enzymes have been identified (Bonnet, 2004).

1.4.1.4 OXA

The OXA-type enzymes are another growing family of ESBLs.

They differ from TEM and SHV enzymes, belonging to molecular Ambler Class D and functional BJM group 2d (Bush et al., 1995).

The OXA-type β-lactamases confer resistance to ampicillin, first generation cephalosporins, oxacillin and cloxacillin, but they are poorly inhibited by clavulanic acid (Bush et al., 1995), with the exception of OXA-18 (Philippon et al., 1997).

While most of ESBLs have been found in *E. coli*, *K. pneumoniae* and other *Enterobacteriaceae*, the OXA-type ESBLs were mainly found in *Pseudomonas aeruginosa*.

Many of the OXA-type ESBLs are derived from OXA-10 (OXA-11, -14, -16 and -17) (Danel et al., 1999; Danel et al., 1995; Hall et al., 1993; Mugnier et al., 1998).

OXA-14 differs from OXA-10 for a single amino acid residue, OXA-11 and OXA-16 differ for two, and OXA-13 and OXA-19 differ for nine.

Unlike most of the OXA-type ESBLs, which confer resistance to ceftazidime, the β-lactamase OXA-17 confers resistance to

cefotaxime and ceftriaxone, but offers only marginal protection against ceftazidime (Danel et al., 1999).

Recently a number of non-ESBL OXA has been described, including OXA-20 (Naas et al., 1998), OXA-22 (Nordmann et al., 2000), OXA-24 (Bou et al., 2000), OXA-25, -26, and -27 (Afazal- Shah et al., 2001), OXA-30 (Siu et al., 2000).

OXA-1 gene has been found in plasmids and integrons in a large variety of Gram-negative bacteria, frequently associated with genes encoding other ESBLs.

OXA-1 β-lactamase, like most OXAs, significantly hydrolyzes amino and ureidopenicillins (piperacillin) and weakly hydrolyzes narrow-spectrum cephalosporins. In addition, it hydrolyzes broad-spectrum cephalosporins, conferring reduced susceptibility to cefepime and cefpirome.

Recent studies have reported very frequent association of *bla*OXA-1 with the worldwide-spread CTX-M-15 ESBL determinant found among human *E.coli* isolates from diverse geographical origins (Poirel et al., 2011). This association of *bla*OXA-1 with *bla*CTX-M genes makes isolates resistant to β-lactam-β-lactamase inhibitor combinations.

1.4.2 AmpC β-lactamases

Ambler class C (Bush Group 1) β-lactamases are the second largest class of β-lactamases, also called AmpC beta-lactamases (Bulychev and Mobashery, 1999).

Like Ambler class A and D, class C enzymes also possess a serine residue within their active site (Jacoby and Munoz-Price, 2005). They can be chromosomally or plasmid-encoded.

They were initially coded on the chromosomes of enteric and non-enteric Gram-negative bacteria (Tenover et al., 2003).

Expression of the chromosomal AmpC gene is normally low. However, gene amplification within the chromosome, or mutations in the promoter region, may lead to an over-expression of the AmpC β-lactamase (Siu et al., 2003; Caroff et al., 1999; Fernandez-Cuenca et al., 2005; Tracz et al., 2005), and this over-expression is inducible by the presence of β-lactams (Hanson and Sanders, 1999).

The phenotype of bacteria over-expressing AmpC β-lactamases is similar to ESBLs, in that they hydrolyze first, second and third generation cephalosporins (Philippon et al., 2002).

However, unlike ESBLs, these isolates are poorly inhibited by β-lactamase inhibitor and they hydrolyze cefoxitin (Nelson and Elisha, 1999).

AmpC β-lactamase genes have recently been found on plasmids that transfer non-inducible cephalosporin resistance.

These mobile AmpC genes originate from natural producers, such as the *Enterobacter* group (MIR, ACT), the *Citrobacter freundii* group (CMY-2-like, LAT, CFE), the *Morganella morganii* group (DHA), the *Hafnia alvei* group (ACC), the *Aeromonas* group (CMY-1-like, FOX, MOX) and the *Acinetobacter baumannii* group (ABA).

The most prevalent and widely disseminated are the CMY-2-like enzymes.

Plasmids harboring genes encoding AmpC β-lactamases frequently carry resistance genes for other classes of antimicrobials, such as aminogylcosides, chloramphenicol, sulfonamides, tetracyclines and trimethoprim (Philippon et al., 2002).

Clinical isolates frequently produce an AmpC β-lactamase in addition to another β-lactamase, harboured either on the same plasmid or on a different plasmid (Philippon et al., 2002).

Therefore, the phenotypic detection of plasmid-encoded AmpC β-lactamases is difficult, and frequently these β-lactamases can be misidentified as ESBLs (Hanson, 2003).

1.4.3 Carbapenemases

The spread of β-lactamase producing *Enterobacteriaceae*, able to hydrolyze almost all cephalosporins except carbapenems (Pitout and Laupland, 2008), highlighted how essential is to maintain clinical efficacy of carbapenems, which have become the "last resort" antimicrobial drugs.

These agents are essential keys to prevent and treat nosocomial infections that are often associated with techniques developed in the field of modern medicine like transplants, admissions to intensive care units and highly technical surgeries (Nordmann et al., 2011).

Nonetheless, isolation of *Enterobacteriaceae* resistant to carbapenems is nowadays reported worldwide (Queenan and Bush, 2007).

Carbapenemases producing *Enterobacteriaceae* do not produce specific clinical infections.

The role of these bacteria is linked more to the difficulty of treating infections rather than to the expression of specific traits of virulence.

The first carbapemenase-producing species (NmcA-producing *Enterobacter cloacae*) was identified in 1993 (Naas and Nordmann, 1994).

Since then, several carbapenemases have been identified and they belong to Ambler Classes A, B, and D.

In addition, a rare chromosome-encoded cephalosporinase belonging to class C may express a certain activity against carbapenems, although its clinical role remains unknown (Queenan and Bush, 2007; Giske et al., 2009).

1.4.3.1 Class A Carbapenemases

To date, numerous class A carbapenemases have been described, some encoded by chromosomes (NmcA, Sme, IMI-1, SFC-1) and others encoded by plasmids (*Klebsiella pneumoniae* carbapenemase [KPC], IMI, KPC-2, GES and derivatives).

All hydrolyze effectively carbapenems and are partially inhibited by clavulanic acid (Queenan and Bush, 2007).

The first producer of KPC, a KPC-2 producing *K. pneumoniae*, was identified in 1996 in the eastern United States (Yigit et al., 2001).

Numerous producers of KPC were later reported, especially nosocomial strains of *K. pneumoniae* but also strains of *E. coli* and other species of *Enterobacteriaceae* (Nordmann et al., 2009).

1.4.3.2 Class B Carbapenemases (Metallo β-lactamase)

Class B metallo-β-lactamases (MBLs) are represented by the metallo-β-lactamase Verona (VIM), encoded by integrons, the IMP types and more recently, the New Delhi metallo-β-lactamase-1 (NDM-1) (Queenan and Bush, 2007; Walsh et al., 2005).

The first acquired MBL, called IMP-1, was reported in *Serratia marcescens* in Japan in 1991 (Ito et al., 1995). In recent years MBLs have been described worldwide (Queenan and Bush, 2007; Walsh et al., 2005).

These enzymes hydrolyze all β-lactams except aztreonam (Walsh et al., 2005) and their activity is inhibited by EDTA, but not by clavulanic acid (Walsh et al., 2005).

Several producers of MBLs are nosocomial multidrug-resistant strains of *K. pneumoniae* (Walsh et al., 2005), which are associated to a high mortality rate (18-67% of human patients) (Daikos et al., 2009).

Discovered in 2008 in Sweden in an Indian patient with a history of previous hospitalization in New Delhi (Yong et al., 2009), NDM-1-producing *Enterobacteriaceae* are the new focus of world attention today (Daikos et al., 2009; Nordmann et al., 2011).

Compared to other carbapenemases, NDM-1 has several features that are deeply worrying to public health in the world (Nordmann et al., 2011).

Occurrence of *bla*NDM-1 gene is not in a single species but in many unrelated species, as it spreads in the environment.

It is frequently acquired by *K. pneumoniae*, a typical nosocomial pathogen, and by *E . coli*, which is by far the most widespread human pathogen.

Moreover, dangerous human reservoirs have been identified (e.g. in some areas of Pakistan, ≤ 20% of the population hosts NDM-1 producers) (Nordmann et al., 2011).

Of particular interest was the identification of NDM-1 strains of *E. coli* type ST-131, responsible for community infections (Poirel et al., 2010), because this organism can increase the risk of releasing drug-resistant strains into the environment.

The level of resistance to carbapenems can vary.

Plasmids carrying *bla*NDM-1 gene are different and can harbour a large number of resistance genes associated with other carbapenemases genes (OXA48 types, other types of VIM), plasmid-mediated cephalosporinases genes, ESBLs genes, genes of resistance

to aminoglycoside (16S RNA methylase), resistance genes to macrolides (esterase), rifampicin resistance genes (rifampin modifying enzymes) and sulfamethoxazole resistance determinants. Therefore, they represent a source of multidrug resistance (Nordmann et al., 2011; Kumarasamy et al., 2010).

1.4.3.3. Class D Carbapemenases (OXA-48 type)

The first reported OXA-48 producer was a strain of *K. pneumoniae* isolated in Turkey in 2003 (Poirel et al., 2004).

Since then, OXA-48 producers have been widely reported in Turkey as a source of nosocomial outbreaks (Carrer et al., 2010; Poirel et al., 2011).

Their distribution throughout the world now includes countries in Europe, in the southern and eastern part of the Mediterranean Sea, and Africa (Poirel et al., 2004; 2011).

OXA-48 is unique because it weakly hydrolyzes carbapenems, third generation cephalosporins, such as ceftazidime, and aztreonam (Poirel et al., 2004; Castanheira et al., 2011) and its activity is not inhibited by EDTA or clavulanic acid.

Although reported in various species of enteric bacteria, producers of OXA-48 are mostly *K. pneumoniae* and *E. coli*.

The mortality rate attributed to infection by producers of OXA-48 is unknown (Nordmann et al., 2011).

1.5 β-lactamases producing *Enterobacteriaceae* in companion animals

Emergence of antimicrobial-resistant bacteria in food animals and the risk posed to human consumers when these resistant bacteria contaminate food products have been a subject of considerable concern in the veterinary and human health communities.

In comparison to people and food animals, relatively few studies have addressed the emergence of resistance in small companion animals and the relationships between the use of antibacterial agents and development of resistance.

According to the statistics of FEDIAF – The European Pet Food Industry (2014), the estimated number of European households owning at least one pet animal is 75 millions.

The most represented species among household pet animals in Europe is the feline species, with an estimated total population of 99.195.251 cats, followed by 81.013.940 dogs, 54.718.038 ornamental birds, 29.302.020 small mammals, 12.651.000 ornamental fishes and 7.288.500 reptiles (FEDIAF, Facts and Figures, 2014).

These data reflect changes in the relationships between humans and companon animals, which possess nowadays a well-established status of family members.

Companion animals live in close contact with humans and they might contribute substantially to the exposure of humans to ESBL-producing *Enterobacteriaceae* (Guardabassi et al., 2004).

This is associated with unique risks that are broader than risks associated with farm animals. In addition, both humans and pet animals are exposed to antimicrobial agents for the treatment and prophylaxis of diseases (DeVincent and Reid-Smith, 2006).

Veterinarians frequently use first-line antibacterials, such as amoxicillin-clavulanate, cephalosporins, and fluoroquinolones, in pet animals and the resultant resistance in pathogenic bacteria as well as commensals presents a significant risk for zoonotic transfer between pets and people (Guardabassi et al., 2008)

Such transfer poses a risk to human health, because pets can then serve as reservoirs of these microorganisms and as a source of infection for other susceptible people and animals living in the same household.

Similar ESBLs gene types, i.e., CTX-M-14, CTX-M-15, SHV-12, and CMY-2, were found in strains originating from humans and companion animals (Ewers et al., 2012). Additionally, transmission of CTX-M-15-carrying ST131 and ST648 *Escherichia coli* strains between dogs and humans has been suggested (Ewers et al., 2010; Ewers et al., 2014).

This underlines the importance of investigating the role of companion animals in the epidemiology of ESBL-producing *Enterobacteriaceae*.

β-lactamases producing *Enterobacteriaceae* isolates from companion animals have been reported worldwide, both in healthy (Costa et al., 2004; Guardabassi et al., 2004; Albrechtova et al., 2012; Gandolfi-Decristophoris et al., 2013; Belas et al., 2014) and diseased animals (Carattoli et al., 2005; Dierikx et al., 2011; Hordijik et al., 2013; Huber et al., 2013; O'Keefe et al., 2010; Schink et al., 2011).

Studies evaluating the colonization rates of healthy companion animals with β-lactamases producers have been small, including at most just over 200 dogs and 50 cats, as reported in a study by Albrechtova et al. (2012).

In this study, *n*=2 MDR ESBL-positive *E. coli* were isolated from two healthy cats, harbouring both *bla*CTX-M-15 and *bla*OXA-1, in Northern Kenya, representing the 4% of total *n* =50 isolates from rectal swabs of *n*=50 cats. In the same study, a total of 47 (22%, *n* = 216) ESBL-positive *E. coli* isolates were obtained from dogs.

In a study conducted in Tunisia, *n*= 8 *bla*CTX-M-1 producing *E. coli* were isolated from fecal samples of 39 healthy cats (Sallem et al., 2013).

In a canadian study, the prevalence and patterns of antimicrobial susceptibility of fecal *Escherichia coli* and extended-spectrum β-lactamase producing *E. coli* were determined for healthy cats (*n* = 39) from veterinary hospitals in southern Ontario.

The prevalence of antimicrobial resistance in *E. coli* was obtained against streptomycin (2%), ampicillin (4%), cephalothin (< 1%), and tetracycline (2%), with the 15% of cats harbouring isolates resistant to at least 2 antimicrobials. However, none harboured an ESBL (Murphy et al., 2009).

In Europe, in a study conducted in Switzerland among healthy animals in community and living in nursing homes, only 2% (n=4) of *n*=202 cats were carrying ESBL producing *Enterobacteriaceae*

(Gandolfi-Decristophoris et al., 2013), represented by *E. coli*, *K. pneumoniae*, and *Enterobacter* spp.

In Portugal, a rate of 12.1% of ESBLs producing *E.coli* has been reported on a population of *n*=36 healthy cats (Costa et al., 2008).

Surveys of diagnostic isolates have identified β-lactamases producing *Enterobacteriaceae* also in diseased cats.

In the Netherlands, Dierikx et al. (2012) reported a total of *n*=11 *Enterobacteriaceae* isolates from diseased cats, expressing an ESBL/AmpC resistance phenotype.

Among members showing an ESBL resistance profile, *n*=2 *bla*CTX-M-1 harbouring *E. coli* from urine, *n*=1 *bla*CTX-M-2 and *bla*TEM-1 harbouring *E. coli* from urine and *n*=1 *bla*TEM-52 harbouring *S. enterica* from faeces, were isolated.

Among isolates with an AmpC profile, *n*=1 *bla*CMY-2 harbouring *P. mirabilis* from urine, *n*=1 *bla*CMY-2 and *bla*SHV-1 harbouring *E. coli*, *n*=1 *E. coli* from bile with chromosomal *ampC* mutation and *n*=2 *bla*TEM-1harbouring *E. cloacae* from wounds, were isolated. *N*=1 *bla*CTX-M-9 harbouring *E. cloacae* isolated from a wound presented a combined ESBL/AmpC profile, while *n*=1 *bla*TEM-1

and *bla*OXA-1 harbouring *E. coli* from a wound showed inconclusive results in the combination disc test.

Authors from Switzerland identified *n*=1 *bla*CTX-M-15 harbouring *E. coli* and *n*=2 *bla*CTX-M-15 and *bla*TEM harbouring *E. coli* among *n*=40 feline urinary isolates (Huber et al., 2013), belonging to phylogroup A and D, respectively.

Also in Europe, in a small Dutch study of healthy (*n*=20) and diarrheic (*n*=20) cats, the frequency of colonization with ESBLs and AmpC producing *Enterobacteriaceae* was 0% and 12.5%, respectively, and *bla*CTX-M-1 producers predominated (Hordijk et al., 2013).

The first detection of CTX-M ESBLs in uropathogenic *E. coli* in the United States has been reported by O'Keefe et al. (2010): a total of *n*=12 isolates producing *bla*CMY and *bla*CTX-M-15 were found, accounting for *n*=10 and *n*=4 feline urinary isolates, respectively, with *n*=2 isolates harbouring both *bla*CMY and *bla*CTX-M-15.

Only one study has been conducted that included the sole feline species (Nebbia et al., 2014), the most represented species among household pet animals, nowadays accounting for the 26% of total household pet animals in Europe.

Indeed, most of the studies included also other domestic animals like dogs, horses and farm animals (Carattoli et al., 2005; Costa et al., 2004; Dierikx et al., 2012; Huber et al., 2013; Murphy et al., 2009; O'Keefe et al., 2010; Sallem et al., 2013; Schink et al., 2011).

Moreover, in these studies, isolates were obtained from organs at necropsy (Carattoli et al., 2005), faeces from healthy cats (Costa et al., 2004; Murphy et al., 2009; Sallem et al., 2013), urinary tract infections (Huber et al., 2013; O'Keefe et al., 2010) and in one study (Schink et al., 2011), results of isolates from dogs and cats were presented together.

In Italy, only few studies (Carattoli et al., 2015; Donati et al.,2014; Nebbia et al., 2014) have been conducted about detection of beta-lactamases in companion animals including cats.

Carattoli et al. (2005) reported three multidrug-resistant *bla*CTX-M-1 and *bla*TEM producing *E. coli* isolated at necropsy from organs of diseased cats.

More recently, Nebbia et al. (2014) described *E. coli* isolates from cats with urinary tract infection, characterized by multidrug-resistance, production of ESBLs and *p*AmpC genes (namely, *bla*CTX-M-1, -14, -15, *bla*CMY-2 and *bla*TEM-1) and possession of

virulence factors linked to the pathogenicity of extraintestinal pathogenic *E. coli*. Moreover, the first uropathogenic *bla*CTX-M-producing *E. coli* ST131 in cats in Italy was described in the same study.

Another study (Donati et al., 2014) described isolates of *Klebsiella pneumoniae* and *K. oxytoca* from cats at necropsy, which showed extended-spectrum cephalosporin resistance and harboured ESBLs or *p*AmpC genes and plasmid-mediated quinolone resistance genes.

In summary, antimicrobial resistance of small animal pathogens varies considerably depending on geographic location and the specific microorganism of interest.

Although resistance of microorganisms affecting small animals may be less widespread than in human beings and food animals, probably because of differences in antibacterial exposure, there are nevertheless sufficient data to conclude that the prevalence of resistance in dogs and cats is high enough to pose therapeutic challenges and to justify development and implementation of strategies to retard further development of resistance.

Chapter 2

Study

2.1 Aims

1. To investigate the antimicrobial resistance of *Enterobacteriaceae* isolates from a population of diseased cats in Sicily, with emphasis on multidrug resistance

2. To detect and characterize the ESBLs and Plasmid-Mediated AmpC-β-lactamases resistance mechanisms

2.2 Materials and Methods

2.2.1 Study design, animals and sampling

From November 2014 to February 2015, clinical samples were collected from *n*=101 cats admitted to the Veterinary Teaching Hospital of the Department of Veterinary Sciences of the University of Messina (Italy) and to private veterinary practices of the cities Palermo and Messina (Italy).

Samples were collected using sterile Amies medium swabs (rectal, nasal, etc.) and only one sample per animal was collected, for a total of *n*=100 swabs. One sample was represented by urine collected in a container.

The animals' owners were questioned verbally on data concerning race, age, sex, Feline Immunodeficiency Virus (FIV) and Feline Leukaemia Virus (FeLV) infection status, previous hospitalization, currently and previously administered antimicrobial treatment.

2.2.2 Bacterial isolation and identification

Samples were transported to the Laboratory of Bacteriology, Department of Veterinary Sciences, Messina (Italy), where analyses were carried out immediately.

Swab material was inoculated in buffered peptone water, incubated 18-24h at 37°C and subcultured onto MacConkey agar 18-24 hours at 37°C.

The species of each isolate was determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS).

2.2.3 Antimicrobial susceptibility testing

Susceptibility testing was performed using the disk diffusion method.

The density of bacterial inoculum (a saline suspension) used in the test was equivalent to a 0.5 McFarland standard.

Bacteria were spread to a Mueller-Hinton agar plate and antimicrobial agent disks were placed on the surface of the medium. Plates were incubated for 18 hours at 37°C in aerobic conditions, and the diameters of complete growth inhibition zones were measured.

The following antimicrobial disks were used: 30 µg amikacin (AK), 30 µg amoxicillin-clavulanic acid (AUG), 30 µg aztreonam (ATM), 5 µg cefotaxime (CTX), 10 µg ceftazidime (CAZ), 30 µg ceftriaxone (CRO), 5 µg ciprofloxacin (CIP), 30 µg chloramphenicol (C), 10 µg meropenem (MRP), 25 µg thrimethoprim-sulfamethoxazole (SXT).

Isolates were classified as susceptible or resistant according to the clinical breakpoints of the European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters (EUCAST, 2015).

Isolates displaying resistance to at least one antimicrobial in at least three categories were considered as multidrug-resistant (Schwarz et al., 2010).

The analysis was performed for eight antimicrobial categories: penicillin with β-lactamase inhibitor (amoxicillin/clavulanic acid), extended-spectrum cephalosporines (cefotaxime, ceftazidime, ceftriazone), carbapenems (meropenem), monobactams (aztreonam), aminoglycosides (amikacin), fluoroquinolones (ciprofloxacin), folate pathway inhibitors (sulfamethoxazole/trimethoprim) and phenicols (chloramphenicol).

2.2.4 Detection of β-lactamases production

Isolates resistant to 3rd generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone) were considered as ESBLs and AmpC producers.

ESBLs production was confirmed by phenotypic synergy test (Combination Disk Test), in which disks containing a cephalosporin alone (cefotaxime, ceftazidime, cefepime) and in combination with clavulanic acid are applied, according to EUCAST guidelines for the detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance (www.eucast.org).

2.2.5 Bacterial genotyping for resistance genes

This study focused on plasmid-mediated ESBL genes.

Biomolecular analyses were conducted at the Laboratory of Antibiotic and Biocide Resistance, Faculty of Veterinary Medicine, Lisbon (Portugal).

Isolates resistant to 3rd generation cephalosporins and positive to phenotypic confirmation method for ESBLs detection were subjected to PCRs targeting the presence of ESBLs encoding genes *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{OXA-1}}$ (Edelstein et al., 2003; Pomba et al., 2006). The *bla*CTX-M-group1, -group9, -group2, -group8 and -group25 genes were identified by PCR with specific primers and positive amplicons were submitted to nucleotide sequencing (Woodford et al., 2005).

A multiplex-PCR for plasmid-borne genes encoding AmpC β-lactamases (*bla*$_{\text{CIT}}$, *bla*$_{\text{FOX}}$, b*la*$_{\text{DHA}}$, *bla*$_{\text{MIR}}$, *bla*$_{\text{ACT}}$, *bla*$_{\text{MOX}}$) was performed, using specific primers as previously described (Pérez and Hanson, 2002). Isolates positive for the *p*AmpC group CIT were submitted to nucleotide sequencing after a specific PCR targeting the entire *bla*$_{\text{CMY}}$ (Belas et al., 2014).

2.2.6 Detection of Phylogenetic groups and Detection of clonal group O25b:H4-B3-ST131

E. coli harbouring resistance genes were categorised into phylogroups (A, B1, B2 or D) by multiplex PCR as decribed by Doumith et al. (2012).

Isolates belonging to group B2 were screened for detection of the human pandemic clone O25b:H4-B3-ST131 by PCR with specific primers for O25b rfb, allele 3 of pabB gene to identify as described by Clermont et al. (2009).

2.2.7 Statistical analyses

Statistical analyses were performed using EPITools (http://epitools.ausvet.com.au/content.php?page=PrevalenceS).

Results are presented as prevalence with a Wilson 95% confidence interval, expressed in percentage.

2.3 Results

2.3.1 Study population

The study population included cats showing clinical signs of several diseases, as shown in Table 2.

Table 2. Diseases affecting sampled animals

Disease	N° Cats
Diarrhoea	59
Rhinitis	31
Otitis	4
Conjunctivitis	3
Stomatitis	2
Cystitis	1
Abscess	1
Total	101

Diarrheic cats (*n*= 59) represented the most numerous group of the study population (58.4%), followed by *n*= 31 cats (30.7%) with rhinitis, *n*= 4 cats with otitis (3.9%), *n*= 3 cats with conjunctivitis (2.9%), *n*= 2 cats with stomatitis (2%), *n*= 1 cat with abscess (1%) and *n*= 1 cat with cystitis (1%).

Cats with diarrhea and rhinitis were chronically affected with recurring symptoms.

Cats were all European Shorthair, with a median age of 3.7 years (8 months – 12 years).

Fifty-one percent (*n*=51) were males, 49% (*n*=50) were females. Sixty-three percent lived in shelters (*n*=64), 37% (*n*=37) were household cats.

Eight cats (7.9%) were positive for FIV infection (*n*=1 with abscess, *n*=3 with rhinitis, *n*=4 with diarrhea).

Eighty-five percent of cats (*n*=86) had never received an antimicrobial treatment.

Fifteen percent (*n*=14) had been treated during the last year with compounds belonging to several antimicrobial classes, namely: amynoglycosides (streptomycin), β-lactams (cefadroxil, cefovecin,

ceftiofur, penicillin G), phenicols (florfenicol), fluoroquinolones (enrofloxacin), macrolides (spiramycin), nitroimidazoles (metronidazole), polymyxins (polymyxin B) and tetracyclines (doxycycline).

At the moment of sample collection, 17% (*n*=17) of cats were under antimicrobial treatment with various compounds, namely: amoxicillin-clavulanic acid, cefovecin, ceftiofur, doxycycline, enrofloxacin, spiramycin+metronidazole. No prior antimicrobial susceptibility test was ordered by treating veterinarians before starting the antimicrobial treatment.

Cats under antimicrobial treatment included *n*=10 cats with rhinitis (treated with amoxicillin-clavulanic acid, enrofloxacin, ceftiofur and doxycycline), *n*=5 cats with diarrhea (treated with spiramycin+metronidazole, cefovecin and enrofloxacin), *n*=1 cat with otitis and *n*=1 cat with stomatitis (both treated with cefovecin).

Cats with diarrhoea had a median age of 3.5 years (8 months – 8 years). Twenty-eight were females (47.4%), *n*= 31 (52.6%) were males; 47.5% (*n*=28) were household cats, three of which were FIV positive; 52.1% (*n*=31) lived in shelters, one of them being FIV positive. At the time of sample collection, *n*=5 diarrheic cats (8.5%)

were under antimicrobial treatment (cefovecin, enrofloxacin, spyramycin and metronidazole) and other five cats had received an antimicrobial treatment in the last year (amoxicillin-clavulanic acid, cefovecin, enrofloxacin).

Cats affected by rhinitis (*n*=31) had a median age of 3.5 years (1-8 years); 54.8% (*n*=17) were females, 45.2% (*n*=14) were males. Eighty percent (*n*=25) were living in shelters, three of which were FIV positive cats, 20% (*n*=6) were household cats. Thirty-eight point seven percent (*n*=12) were under antimicrobial treatment (amoxicillin-clavulanic acid, ceftiofur, doxycycline, enrofloxacin) and 22.6% (*n*=7) had an antimicrobial treatment in the last year (amoxicillin-clavulanic acid, benzilpenicilline-diidrostreptomicine, cefadroxil, ceftiofur, cefovecin, doxicicline, florfenicol, spyramycin-metronidazole).

2.3.2. *Enterobacteriaceae* isolates

A total of 101 samples were screened, and 91 samples were culture positive on MacConkey Agar, resulting in 125 isolates.

A single bacterial species was isolated in *n*=81 samples (89%); two or more bacterial species were isolated in *n*=10 samples (11%).

All isolated bacterial species are displayed in Table 3.

Fifty-two percent *(n*=65) were *E. coli*, 16 % (*n*=20) *Enterobacter* spp., 10.4 % (*n*=13) *Proteus* spp., 9.6 % (*n*=12) *Citrobacter* spp. and 4.8 % (*n*=6) *Providencia* spp.

Two strains per each were isolated for *Buttiauxiella agrestis*, *Kluyvera* spp. and *Serratia liquefaciens*.

A single strain per each was isolated for *Hafnia alvei*, *Klebsiella oxytoca* and *Leclercia adecarboxylata*.

Table 3. *Enterobacteriaceae* isolates and disease condition

Species	**Rhinitis**	**Diarrhoea**	**Other conditions***
E. coli (*n*=65)	12	40	13
Enterobacter species (*n*=20)	6	14	-
Proteus species (*n*=13)	1	12	-
Citrobacter species (*n*=12)	5	7	-
Providencia species (*n*=6)	1	5	-
Buttiauxella agrestis (*n*=2)	1	1	-
Kluyvera species (*n*=2)	-	2	-
Serratia liquefaciens (*n*=2)	2	-	-
Hafnia alvei (*n*=1)	-	1	-
Klebsiella oxytoca (*n*=1)	1	-	-
Leclercia adecarboxylata (*n*=1)		1	-

*abscess, conjunctivitis, cystitis, otitis, stomatitis

Eighty-three *Enterobacteriaceae*, which represented the 66.4% of total isolates, were isolated from samples of fifty-eight cats with diarrhea.

Species isolated from cats with diarrhea were represented by *E. coli* (*n*=40), *Enterobacter* spp. (*n*=14), *Proteus* spp. (*n*=12), *Citrobacter* spp. (*n*=7), *Providencia* spp. (*n*=5), *Kluyvera* spp. (*n*=2), *Buttiauxella agrestis* (*n*=1), *Hafnia alvei* (*n*=1) and *Leclercia adecarboxylata* (*n*=1).

Twenty-nine strains were isolated from samples of twenty-four cats with rhinitis, accounting for the 24% of total isolates.

They were represented by *E. coli* (*n*=12), *Enterobacter* spp. (*n*=6), *Citrobacter* spp. (*n*=5), *Proteus mirabilis* (*n*=1), *Serratia liquefaciens* (*n*=2), *Buttiauxella agrestis* (*n*=1), *Klebsiella oxytoca* (*n*=1) and *Providencia rettgeri* (*n*=1).

The rest of the strains (9.6%), represented by thirteen *E. coli*, were isolated from a cat with abscess (*n*=1), two cats with conjunctivitis (*n*=3), one cat with cystitis (*n*= 3), four cats with otitis (*n*=4) and two cats with stomatitis (*n*=2).

2.3.2 Antimicrobial susceptibility

The highest rates of resistance among all *Enterobacteriaceae* isolates (*n*=125) were observed to amoxicillin-clavulanic acid (48.8%), ceftazidime (40%), ceftriaxone (40.8%) and cefotaxime (38.4%).

Although lower, resistances to amikacin (31.2%), aztreonam (32%), chloramphenicol (24%), ciprofloxacin (23.2%) and sulphamethoxazole-trimethoprim (33.6%) were also significant.

All isolates were clinically susceptible to meropenem.

Overall prevalence of resistance is shown in Table 4.

Table 4. Overall prevalence % (95% CI) of resistance among *n*=125 *Enterobacteriaceae* isolates

Molecule	95% CI Prevalence
AUG	48.8 (40.2 – 57.5) *n*=61
CTX	38.4 (30.3 – 47.2) *n*=48
CAZ	40 (31.8 – 48.8) *n*=50
CRO	40.8 (32.6 – 49.6) *n*=51
ATM	32 (24.5 – 40.6) *n*=40
CIP	23.2 (16.7 – 31.3) *n*=29
AK	31.2 (23.7 – 39.8) *n*=39
C	24 (17.4 – 32.2) *n*=30
SXT	33.6 (25.9 – 42.3) *n*=42
MRP	0
MDR	44.8 (36.4 – 53.5) *n*=56

Prevalence of resistant *E. coli*, *Citrobacter* spp., *Enterobacter* spp. and *Proteus* spp., isolated in cats with diarrhea and rhinitis, which represented the most numerous groups, are shown in Table 5 and Table 6.

Among *n*=67 isolates resistant to 3rd generation cephalosporines, *n*=49 (73.1%) were confirmed as ESBLs producers phenotypically by Combination Disk Test, represented by *n*=21 *E. coli*, *n*=9 *Proteus* spp., *n*=8 *Enterobacter* spp., *n*=5 *Citrobacter* spp., *n*=2 *Kluyvera* spp., *n*=2 *Serratia* spp., *n*=1 *Providencia rettgeri* and *n*=1 *Hafnia alvei*.

Multidrug-resistance was displayed by 44.8% (*n*=56) of total isolates, accounting for *n*=27 *E. coli*, *n*=11 *Proteus* spp., *n*=3 *Citrobacter* spp., *n*=7 *Enterobacter* spp., *n*=2 *Providencia* spp., *n*=2 *Buttiauxella agrestis*, *n*=2 *Kluyvera* spp., *n*=1 *Serratia liquefaciens* and *n*=1 *Klebsiella oxytoca*. Overall, they displayed twenty-nine different resistance profiles (Table 7).

Among total *E. coli* (*n*=65), *Proteus* spp (*n*=13) *Enterobacter* spp. (*n*=20) and *Citrobacter* spp. (*n*=12) isolated in the study, 41.5% (*n*=27), 85% (*n*=11), 35% (*n*=7) and 25% (*n*=3) were MDR, respectively.

Table 5. Prevalence % (95% CI) of resistant *E. coli* and *Enterobacter* spp. isolated from cats with diarrhea and rhinitis

Molecule	*E. coli* (Diarrhea) *n*=40	*E. coli* (Rhinitis) *n*=12	*Enterobacter* spp. (Diarrhea) *n*=14	*Enterobacter* spp. (Rhinitis) *n*=6
AUG	52.5 (37.5 – 67.1) *n*=21	66.7 (39.1 – 86.2) *n*=8	I.R.	I.R.
CTX	25 (14.2 – 40.2) *n*=10	41.7 (19.3 – 68) *n*=5	42.9 (21.4 – 67.4) *n*=6	16.7 (3 – 56.4) *n*=1
CAZ	22.5 (12.3 – 37.5) *n*=9	16.7 (4.7 – 44.8) *n*=2	42.9 (21.4 – 67.4) *n*=6	16.7 (3 – 56.4) *n*=1
CRO	32.5 (20.1 – 48) *n*=13	41.7 (19.3 – 68) *n*=5	28.6 (11.7 – 54.6) *n*=4	33.3 (9.7 – 70) *n*=2
ATM	25 (12.3 – 37.5) *n*=10	25 (8.9 – 53.2) *n*=3	42.9 (21.4 – 67.4) *n*=6	16.7 (3 – 56.4) *n*=1
CIP	7.5 (2.6 – 19.9) *n*=3	16.7 (4.7 – 44.8) *n*=2	42.9 (21.4 – 67.4) *n*=6	0
AK	35 (22.1 – 50.5) *n*=14	33.3 (13.8 – 60.9) *n*=4	14.3 (4 – 39.9) *n*=2	33.3 (9.7 – 70) *n*=2
C	12.5 (5.5 – 26.1) *n*=5	0	21.4 (7.6 – 47.6) *n*=3	33.3 (9.7 – 70) *n*=2
SXT	25 (14.2 – 40.2) *n*=10	58.3 (32 – 80.7) *n*=7	35.7 (16.3 – 61.2) *n*=5	0
MRP	0	0	0	0
MDR	30 (18.1 – 45.4) *n*=12	41.7 (19.3 – 68) *n*=6	35.7 (16.3 – 61.2) *n*=5	33.3 (9.7 – 70) *n*=2

Table 6. Prevalence % (95% Wilson CI) of resistant *Citrobacter* spp. isolated from cats with diarrhea and rhinitis and of resistant			
Molecule	*Citrobacter* spp. (Diarrhea) *n*=7	*Citrobacter* spp. (Rhinitis) *n*=5	*Proteus* spp. (Diarrhea) *n*=12
AUG	I.R.	I.R.	100 (75.8 – 100) *n*=12
CTX	71.4 (35.9 – 91.8) *n*=5	20 (3.6 – 62.4) *n*=1	75 (46.8 – 91.1) *n*=9
CAZ	71.4 (35.9 – 91.8) *n*=5	40 (11.8 – 76.9) *n*=2	75 (46.8 – 91.1) *n*=9
CRO	57.1 (25 – 84.2) *n*=4	40 (11.8 – 76.9) *n*=2	50 (25.4 – 74.6) *n*=6
ATM	28.6 (8.2 – 64.1) *n*=2	40 (11.8 – 76.9) *n*=2	33.3 (13.8 – 60.9) *n*=4
CIP	14.3 (2.6 – 51.3) *n*=1	20 (3.6 – 62.4) *n*=1	41.7 (19.3 – 68) *n*=5
AK	57.1 (25 – 84.2) *n*=4	20 (3.6 – 62.4) *n*=1	16.7 (4.7 – 44.8) *n*=2
C	14.3 (2.6 – 51.3) *n*=1	20 (3.6 – 62.4) *n*=1	75 (46.8 – 91.1) *n*=9
SXT	14.3 (2.6 – 51.3) *n*=1	40 (11.8 – 76.9) *n*=2	75 (46.8 – 91.1) *n*=9
MRP	0	0	0
MDR	28.6 (8.2 – 64.1) *n*=2	20 (3.6 – 62.4) *n*=1	91.7 (64.6 – 98.5) *n*=11

Twenty-nine different resistance profiles were displayed (Table 7).

Resistance to third generation cephalosporins was associated to resistance to sulphamethoxazole-thrimetoprim, ciprofloxacin, amikacin or chloramphenicol in the 56.5% (*n*=35), 38.7% (*n*=24), 35.5% (*n*=22) and 42% (*n*=26) of MDR strains.

Table 7. Resistance patterns detected in *n*=56 multidrug-resistant *Enterobacteriaceae* isolated from *n*=42 cats– (A.C. = antimicrobial classes; CEPH= third generation cephalosporines)

A.C.	Phenotypes	Isolates	Disease	N°Animals
3	AUG, AK, SXT	*E. coli* (n=2); *Proteus* spp. (n=1)	Diarrhea, Rhinitis; Diarrhea	N=2
	AUG, CEPH, SXT	*E. coli* (n=2)	Rhinitis, Diarrhea	N=2
	AUG, CEPH, CIP	*E. coli* (n=1); *Proteus* spp. (n=1)	Rhinitis; Diarrhea	N=2
	AUG, CEPH, C	*Kluyvera* spp. (n=1); *Serratia* spp. (n=1)	Diarrhea; Rhinitis	N=2
	AUG, CEPH, ATM	*E. coli* (n=2)	Conjunctivitis, Diarrhea	N=2
	CEPH, CIP, SXT	*Buttiauxella* spp. (n=1)	Diarrhea	N=1
	CEPH, C, SXT	*Citrobacter* spp. (n=1)	Diarrhea	N=1
	CEPH, AK, C	*Enterobacter* spp. (n=1)	Rhinitis	N=1
	CEPH, ATM, AK	*Enterobacter* spp. (n=1)	Rhinitis	N=1
	ATM, CIP, C	*Klebsiella oxytoca* (n=1)	Rhinitis	N=1
4	AUG, CEPH, ATM, SXT	*E. coli* (n=3)	Rhinitis (n=1), Diarrhea (n=2)	N=2
	AUG, CEPH, CIP, C	*Proteus* spp. (n=1) ; *Kluyvera* spp. (n=1)	Diarrhea; Diarrhea	N=2
	AUG, CEPH, C, SXT	*Proteus* spp. (n=2)	Diarrhea	N=2
	AUG, CEPH, CIP, AK	*E. coli* (n=1)	Conjunctivitis	N=1
	CEPH, ATM, CIP, AK	*Citrobacter* spp. (n=1)	Diarrhea	N=1
	CEPH, ATM, AK, SXT	*E. coli* (n=1)	Otitis	N=1
	CEPH, ATM, C, SXT	*Enterobacter* spp. (n=1)	Diarrhea	N=1
	CEPH, ATM, CIP, SXT	*Enterobacter* spp. (n=1)	Diarrhea	N=1
5	AUG, CEPH, ATM, AK, SXT	*E. coli* (n=4)	Diarrhea (n=2), Rhinitis (n=1), Stomatitis (n=1)	N=4
	AUG, CEPH, ATM, CIP, SXT	*E. coli* (n=3)	Abscess (n=1), Diarrhea (n=1), Rhinitis (n=1)	N=3
	AUG, CEPH, ATM, AK, C	*E. coli* (n=3)	Cystitis (n=1), Diarrhea (n=2)	N=3
	AUG, CEPH, ATM, C, SXT	*Proteus* spp. (n=3)	Diarrhea	N=2
	AUG, CEPH, ATM, CIP, AK	*Buttiauxella* spp. (n=1); *Providencia* spp. (n=1)	Diarrhea; Rhinitis	N=2
	AUG, CEPH, CIP, C, SXT	*Proteus* spp. (n=2)	Diarrhea	N=2
	CEPH, ATM, CIP, C, SXT	*Citrobacter* spp. (n=1); *Enterobacter* spp. (n=2)	Rhinitis; Diarrhea	N=2
	CEPH, ATM, CIP, AK, SXT	*Enterobacter* spp. (n=1); *Providencia* spp. (n=1)	Diarrhea; Rhinitis	N=2
6	AUG, CEPH, ATM, AK, C, SXT	*E. coli* (n=1)	Otitis	N=1
	AUG, CEPH, ATM, CIP, C, SXT	*E. coli* (n=1)	Cystitits	N=1
7	AUG, CEPH, ATM, CIP, AK, C, SXT	*E. coli* (n=3); *Proteus* spp. (n=1)	Cystitis (n=1), Diarrhea (n=2); Diarrhea	N=4

Multidrug-resistant *Enterobacteriaceae* were harboured by the 47% (*n*=42) of cats (*n*=90).

These cats were affected by diarrhoea (*n*=24), rhinitis (*n*=12), otitis (*n*=2), abscess (*n*=1, conjunctivitis (*n*=1) cystitis (*n*=1) and stomatitis (*n*=1).

Among the *n*=24 diarrheic cats haurbouring MDR strains, *n*=12 were from shelters, with only one cat being under antimicrobial treatment; *n*=12 were household; one cat tested positive for FIV and *n*=4 cats were receiving an antimicrobial treatment (Table 8).

They represented the 42%% of diarrheic cats included in the study from which *Enterobacteriaceae* members were isolated (*n*=57).

Table 8. Clinical data of *n*=24 diarrheic cats harbouring MDR isolates (M=male; F=female; H=household; S=shelter; Antimicrobial therapy = therapy at the moment of sample collection)

ID	MDR isolates	Age (y)	Sex	Habitat	FIV/FeLV	Antimicrobial therapy
2588	*E. coli*	4	M	H	neg	-
2589	*Proteus mirabilis* (n=2)	5	M	H	neg	Enrofloxacin, Spiramycin, Metronidazol
2590	*E. coli*	2	F	H	neg	-
2597	*E.coli*	1	F	H	neg	-
2607	*Citrobacter* spp.	5	F	S	neg	-
2613	*E.coli*	2	F	S	neg	-
2616	*E.coli* (n=2)	3	F	S	neg	-
2619	*Proteus* spp. (n=2)	2	F	H	FIV+	-
2622	*Proteus* spp.	3	M	H	neg	Cefovecin
2639	*E.coli*	6	M	H	neg	Spiramycin+Metronidazol
2644	*E.coli*	5	F	S	neg	Cefovecin
2650	*Kluyvera* spp. (n=2)+ *E.coli*	7	M	S	neg	-
2653	*Proteus* spp.	7	M	H	neg	Cefovecin
2657	*Proteus* spp. (n=2)	5	M	S	neg	-
2660	*Citrobacter* spp	2	M	S	neg	-
2664	*Proteus* spp. (n=2)	3	F	H	neg	-
2665	*Proteus* spp.	4	F	S	neg	-
2667	*E.coli*	3	M	S	neg	-
2670	*E.coli*	4	F	S	neg	-
2674	*Enterobacter* spp. (n=2)	4	M	S	neg	-
2675	*Buttiauxiella* spp.	3	M	S	neg	-
2677	*E.coli*	2	F	H	neg	-
2686	*Providencia* spp.	4	M	H	neg	-
2855	*Enterobacter* spp. (n=3)	3	F	H	neg	-

Among cats affected by rhinitis and harbouring MDR isolates (*n*=12), *n*=11 were from shelters, *n*=1 was household. Among shelter cats, *n*=2 had tested positive for FIV and *n*=8 were under antimicrobial treatment (Table 9).

They represented the 50% of total *n*=24 cats with rhinitis included in the study from which *Enterobacteriaceae* members were isolated.

Table 9. Clinical data of *n*=12 cats affected by rhinitis and harbouring MDR strains (y=years; M=male; F=female; H=household; S=shelter, Antimicrobial therapy = therapy at the moment of sample collection)

ID	MDR isolates	Age (y)	Sex	Habitat	FIV/FeLV	Antimicrobial therapy
2626	*Buttiauxiella* spp.	3	F	S	Neg	Ceftiofur
2627	*Citrobacter* spp.	6	M	S	Neg	Amoxicillin/Clavulanate
2608	*E. coli*	5	F	S	Neg	Amoxicillin/Clavulanate
2635	*E. coli*; *Enterobacter* spp.	2	F	S	Neg	Amoxicillin/Clavulanate
2642	*E.coli*	6	F	S	Neg	-
2618	*E.coli*	4	F	S	Neg	-
2637	*E.coli*	5	F	S	Neg	-
2652	*E.coli*	4	M	S	Neg	Enrofloxacin
2640	*Enterobacter* spp.	4	M	S	FIV+	Amoxicillin/Clavulanate
2592	*Klebsiella oxytoca*	1	M	H	Neg	-
2620	*Providencia* spp.	3	M	S	Neg	Enrofloxacin
2631	*Serratia* spp.	6	M	S	FIV+	Ceftiofur

The rest of cats harbouring MDR isolates had otitis (*n*=2), stomatitis (*n*=1), cystitis (*n*=1), conjunctivitis (*n*=1) and abscess (*n*=1) (Table 10).

Four were shelter cats (*n*=1 with abscess, *n*=1 with conjunctivitis, *n*=1 with otitis, *n*=1 with stomatitis), of which one had tested positive for FIV and *n*=2 were under antimicrobial treatment. Two were household cats (*n*=1 cat with otitis and *n*=1 cat with cystitis).

Table 10. Clinical data of cats with abscess, conjunctivitis, cystitis, otitis and stomatitis, harbouring MDR isolates (y=years; M=male; F=female; H=household; S=shelter, Antimicrobial therapy = therapy at the moment of sample collection)

ID	Disease	MDR isolates	Age (y)	Sex	Habitat	FIV/FeLV	Antimicrobial therapy
2606	Conjunctivitis	*E. coli*	5	F	S	neg	-
2610	Abscess	*E. coli*	8	M	S	FIV+	-
2600	Otitis	*E. coli*	12	F	H	neg	-
2612	Otitis	*E. coli*	2	F	S	neg	Cefovecin
2621	Stomatitis	*E. coli*	3	M	S	neg	Cefovecin
2977	Cystitis	*E. coli* (n=3)	4	M	H	neg	-

2.3.4 ESBL/AmpC determinants and phylogeny of *E. coli*

A total of *n*=26 isolates were confirmed as ESBLs/*p*AmpC β-lactamases producers by PCR and DNA sequencing.

They were represented by *n*=18 *E. coli*, *n*=5 *Proteus* spp. and *n*=3 *Enterobacter* spp. (Table 11).

Twenty-three isolates were confirmed as ESBLs-producers, harbouring different types of *bla* genes.

Three types of CTX-M ESBLs were detected, belonging to CTX-M-1 (*n*=12), CTX-M-2 (*n*=1) and CTX-M-9 (*n*=1) groups.

The rest of ESBLs producers harboured *bla*SHV (*n*=1), *bla*TEM (*n*=8) and *bla*OXA-1 (*n*=6).

Ten isolates were *p*AmpC *bla*CMY-producers, with *n*=7 isolates also harbouring *bla*TEM (*n*=4), *bla*CTX-M (*n*=2) and *bla*OXA-1 (*n*=1).

All β-lactamases producing isolates were MDR, showing resistance to at least three antimicrobial classes.

Twelve ESBL/*p*AmpC-producing *E. coli* belonged to phylogenetic groups B2 and D, and were collected from *n*=6 diarrheic cats (*n*=3 living in shelter, *n*=3 household), *n*= 1 shelter cat with rhinitis, *n*= 1 household cat with cystitis and *n*= 1 household cat with otitis.

The human O25b:H4-B3-ST131 pandemic clone was not detected among *E. coli* strains belonging to phylogenetic group B2.

Six ESBL/pAmpC-producing *E. coli* belonged to phylogenetic groups A and B1, collected from *n*=1 FIV positive cat with abscess, *n*=3 cats with rhinitis, *n*=1 cat with stomatitis and *n*=1 cat with diarrhoea.

ESBLs/pAmpC β-lactamases-producing isolates (*n*=26) were recovered from *n*=18 cats (Table 12) of total cats from which *Enterobacteriaceae* members were isolated (*n*=90), with a prevalence of 20% (95% Confidence Interval 13 – 29.4).

The prevalence of ESBLs/*p*AmpC β-lactamases producers in diarrheic cats and cats with rhinitis was 17.5% (95 % Confidence Interval 9.8 – 29.4) and 16.7% (95% Confidence Interval 6.7 – 35.9), respectively.

Table 11. Resistance determinants and antimicrobial resistance profile of ESBLs/pAmpC β-lactamases producing *Enterobacteriaceae* strains and phylogrouping of resistant *E. coli*. (PG=phylogenetic group)

Species	Sample ID	Resistance profile	PG	Genes	Disease
E. coli	1	AUG-CAZ-CRO-ATM-CIP-SXT	A	SHV-12	Abscess
E. coli	9	AUG-CTX-CAZ-CRO-ATM-AK-SXT	A	CTX-M-15,OXA-1	Stomatitis
E. coli	13	AUG-CTX-CAZ-CRO-ATM-SXT	B2	CTX-M-3/22/162	Rhinitis
E. coli	15	AUX-CTX-CAZ-CRO-ATM-CIP-SXT	B1	TEM, OXA-1, CMY-2	Rhinitis
E. coli	18	AUG-CTX-CRO-ATM-AK-SXT	B1	CTX-M-group2	Rhinitis
E. coli	24	AUG-CTX-CAZ-CRO-ATM-CIP-C-SXT	D	CTX-M-79, OXA-1	Otitis
E. coli	25	AUG-CTX-CRO-SXT	B1	CTX-M-9, CMY-2	Rhinitis
E. coli	30	AUG-CTX-CAZ-CRO-ATM-CIP-AK-C-SXT	D	CTX-M-79, CMY-2	Diarrhea
E. coli	32	AUG-CTX-CRO-ATM-CIP-AK-C-SXT	D	TEM	Diarrhea
E. coli	40	AUG-CTX-CAZ-CRO-ATM-AK-C	D	CMY-2	Diarrhea
E. coli	41	AUG-CTX-CAZ-CRO-ATM-SXT	B2	CTX-M-15	Diarrhea
E. coli	42	AUG-CTX-CAZ-CRO-ATM-SXT	B2	CTX-M-15	Diarrhea

E. coli	47	AUG-CTX-CAZ-CRO-ATM-CIP-SXT	B2	CTX-M-15	Diarrhea
E. coli	48	AUG-CTX-CAZ-CRO-ATM-AK-SXT	B2	TEM, CTX-M-15	Diarrhea
E. coli	61	AUG-CTX-CAZ-CRO-ATM	B1	CTX-M-15	Diarrhea
E. coli	65	AUG-CTX-CAZ-ATM-AK-C	B2	CMY-2	Cystitis
E. coli	71	AUG-CTX-CAZ-CRO-ATM-AK-C-SXT	B2	OXA-1, CMY-2	Cystitis
E. coli	72	AUG-CTX-CAZ-ATM-AK-C-SXT	B2	CMY-2	Cystitis
Enterobacter cloacae	27	CTX-CAZ-ATM-CIP-AK-C-SXT	-	CTX-M-15, OXA-1	Diarrhea
Enterobacter cloacae	28	CTX-CAZ-CRO-ATM-C-SXT	-	CTX-M-15, OXA-1	Diarrhea
Enterobacter spp.	29	CTX-CAZ-CRO-ATM-CIP-C-SXT	-	CTX-M-15	Diarrhea
Proteus mirabilis	43	AUG-CTX-CAZ-CRO-CIP-C-SXT	-	TEM,CMY-2	Diarrhea
Proteus mirabilis	44	AUG-CTX-CRO-CIP	-	TEM	Diarrhea
Proteus mirabilis	46	AUG-CTX-CAZ-CRO-ATM-CIP-AK-C-SXT	-	TEM	Diarrhea
Proteus mirabilis	66	AUG-CTX-CAZ-CRO-ATM-C-SXT	-	TEM,CMY-2	Diarrhea
Proteus mirabilis	67	AUG-CTX-CAZ-ATM-C-SXT	-	TEM,CMY-2	Diarrhea

Table 12. Clinical informations of *n*=18 cats harbouring ESBLs/*p*AmpC β lactamases producing *Enterobacteriaceae* (y=years; M=male; F=female; H=household; S=shelter, Antimicrobial therapy = therapy at the moment of sample collection)

ID	Disease	Isolates	*bla* Gene	Age (y)	Sex	Habitat	FIV/FeLV	Antimicrobial therapy
2588	Diarrhea	*E. coli*	CTX-M-79, CMY-2	4	M	H	Neg	-
2590	Diarrhea	*E. coli*	TEM	2	F	H	Neg	-
2600	Otitis	*E. coli*	CTX-M-79, OXA-1	12	F	H	Neg	-
2608	Rhinitis	*E. coli*	CTX-M-9, CMY-2	5	F	S	Neg	Amoxicillin/Clavulanate
2610	Abscess	*E. coli*	SHV-12	8	M	S	FIV+	-
2613	Diarrhea	*E.coli*	CMY-2	2	F	S	Neg	-
2616	Diarrhea	*E.coli* (n=2)	CTX-M-15	3	F	S	Neg	-
2618	Rhinitis	*E.coli*	TEM, OXA-1, CMY-2	4	F	S	Neg	-
2619	Diarrhea	*Proteus* spp. (n=2)	TEM, CMY-2; TEM	2	F	H	FIV+	-
2621	Stomatitis	*E.coli*	CTX-M-15, OXA-1	3	M	S	Neg	Cefovecin
2622	Diarrhea	*Proteus* spp.	TEM	3	M	H	Neg	Cefovecin
2637	Rhinitis	*E.coli*	CTX-M-2-group	5	F	S	Neg	-
2639	Diarrhea	*E.coli*	CTX-M-15	6	M	H	Neg	Spiramycin+Metronidazol
2642	Rhinitis	*E.coli*	CTX-M-3/22/162	6	F	S	Neg	-
2657	Diarrhea	*Proteus* spp. (n=2)	TEM, CMY-2	5	M	S	Neg	-
2670	Diarrhea	*E.coli*	CTX-M-15	4	F	S	Neg	-
2855	Diarrhea	*Enterobacter* spp. (n=3)	CTX-M-15, OXA-1 (n=2) ; CTX-M-15 (n=1)	3	F	H	Neg	-
2977	Cystitis	*E.coli* (n=3)	CMY-2 (n=2) ; OXA-1, CMY-2 (n=1)	4	M	H	Neg	-

Discussion

This study highlights the large number of cats carrying MDR and ESBLs/*p*AmpC producing *Enterobacteriaceae* in a variety of clinical conditions commonly encountered in practice, reaching the 45% and the 20% of isolates studied, respectively.

Moreover, MDR isolates and ESBLs/*p*AmpC producers were harboured by the 47% and 19% of cats included in the study population, representing also an alarming data.

Resistance prevalence to several antimicrobial classes was significantly high, especially to those of antimicrobials that in human medicine are categorized as critically important antimicrobials (amoxicillin-clavulanic acid, third generation cephalosporins and fluoroquinolones) and are commonly used as first line molecules in small animal practice.

Overall, a very high prevalence of resistance to amoxicillin-clavulanic acid (48.8%), third generation cephalosporins (40%) and fluoroquinolones (23%) was found among total isolates, and this prevalence was still high when considering single species.

Fifty-trhee percent and 66.7% of *E. coli* isolated from diarrheic cats and cats with rhinitis, respectively, and 100% of *Proteus* spp. isolated

in diarrheic cats in the study displayed resistance to amoxicillin-clavulanic acid.

This compound represents by far the most prescribed antimicrobial to treat companion animals worldwide (Guardabassi et al., 2004; Murphy et al., 2012) and results of this study confirmed the high frequency of resistance to amoxicillin-clavulanic acid reported among clinical *E. coli* isolates of different origin worldwide (Belmar-Liberato et al., 2011).

Authors have previously associated resistance to amoxicillin-clavulanic acid with the activity of class C β-lactamases or inhibitor-resistant TEM β-lactamases (Philippon et al., 2002; Thomson, 2010). This association was observed in this study through detection of *bla*CMY/*bla*TEM producing isolates displaying resistance to amoxicillin-clavulanic acid.

ESBLs/*p*AmpC production, which is the main mechanisms of resistance to third generation cephalosporins, was detected in the 46% of isolates resistant to third-generation cephalosporins.

Most likely, other resistance mechanisms, such as lack of expression of outer membrane proteins or overexpression of efflux pumps, may be implicated.

Resistance to fluoroquinolones was presented by the 46% of strains in which an ESBL/*p*AmpC gene was detected.

Shaheen et al. (2013) have recently showed an association between ESBLs production and resistance to fluoroquinolones, identifiying new mechanisms of resistance, represented by novel mutations in topoisomerase and *qepA* genes, and plasmid-mediated fluoroquinolone resistance determinants in ESBL-producing *E. coli* of canine and feline origin.

Although in our study we did not charachterize the mechanisms of resistance to fluoroquinolones, the percentage of ESBLs/*p*AmpC producers showing resistance to fluoroquinolones was high and this result should stimulate a more focused monitoring of fluoroquinolone resistance.

A high prevalence of resistance to aminoglycosides (31.2%) was also detected in the study. Use of these drugs in the treatment of infections in companion animals has been tempered by toxicity considerations (ototoxicity, nephrotoxicity) and by the need to administer them parentally. Neverthless, our results indicate that *Enterobacteriaceae* strains harboured in cats could be a substantial reservoir of the aminoglycoside resistance genes.

MDR was displayed by a high percentage of isolates (45%) and all ESBLs/*p*AmpC producers detected in the study were MDR.

Results obtained in this work could constitute additional evidence about the association between ESBLs/*p*AmpC production and MDR, which allows a co-selection process, because these genes are on plasmids that frequently carry aminoglycoside, tetracycline, sulfonamide or fluorquinolone resistant genes (Canton and Coque, 2006).

Thirty-eight percent of cats with rhinitis (n=12/31) carried MDR strains.

Most were shelter cats, while only one cat was a household cat and had never received an antimicrobial treatment before sample collection.

All shelter cats were treated with several compounds at the time of sample collection or had been treated in the last year, without bacterial isolation and susceptibility testing to address the treatment, and four of these cats were harbouring ESBLs/*p*AmpC genes.

These results underline the role of an unrational use of antimicrobials for the selection of MDR ESBLs/*p*AmpC harbouring isolates.

Forty-one per cent of *n*=59 cats with diarrhea carried MDR strains: 50% were household cats, 50% were from shelter, though no difference in the frequency of isolation of MDR bacteria between shelter and household diarrheic cats was shown.

Except for one cat, shelter cats had never received a previous antimicrobial treatment before sample collection and four of them harboured ESBLs/*p*AmpC genes.

Only four cats on thirteen household cats were receiving an antimicrobial treatment and ESBLs/*p*AmpC genes were found on both treated and untreated cats.

These data may suggest that diarrheic cats living either in shelter/community or in a household may harbour MDR and ESBLs/*p*AmpC producing strains, also without a selective pressure by antimicrobial treatment.

The CTX-M enzymes were the most common ESBLs and different types of these enzymes were detected, indicating a high diversity of CTX-M-encoding genes in *Enterobacteriaceae* from diseased cats, whereas the CMY-2 was the only *p*AmpC β-lactamase found in this study. The high prevalence of *p*AmpC CMY-2 is of concern since these β-lactamases confer resistance to a wide range of extended-

spectrum cephalosporins used for the treatment of serious infections in humans and animals, and because they are not affected by β-lactamase inhibitors and can easily be transferred between different bacterial species and between animals and humans (Guardabassi et al., 2004; Carattoli et al., 2005; Shaheen et al., 2011).

Among ESBLs genes, the *bla*CTX-M-79 gene has been detected.

CTX-M-79 harbouring isolates were from n=1 cat with diarrhea and n=1 cat with otitis**,** which were both household cats and had not received any antimicrobial treatment.

To the best of knowledge, detection of *bla*CTX-M-79 harbouring *E. coli* is described for the first time from a companion animal in literature.

Previos reports described the gene from *Enterobacteriaceae* isolated from house crows, hospital waste, human patients and farmed fish in Asia (Hasan et al., 2015; Guo et al., 2013; Jiang et al., 2012) and from faeces of cattle in United States (Wittum et al., 2010).

Feline upper respiratory tract disease represented the second most common disease of cats included in the study, and it is frequently encountered in clinical practice.

In the study, the detection of MDR ESBLs/*p*AmpC producing *E. coli* in cats with upper respiratory tract disease is described for the first time in literature, accounting for n=4 shelter cats that had never received an antimicrobial. Moreover, one of these isolates harboured a *bla*CTX-M-15 gene and belonged to phylogenetic group B2, while others belong to phylogenetic group B1.

These results show that chronic rhinitis in cats may be complicated by MDR extraintestinal pathogenic *E. coli* and that ESBLs/*p*AmpC genes harbouring *E. coli* may be involved, even without a previously excerted antimicrobial selective pressure.

In the study, besides common pathogens, such as *E. coli*, *Enterobacter* spp., *Citrobacter* spp. and *Proteus* spp., members that are less commonly reported in literature, such as *Buttiauxiella agrestis*, *Kluyvera* spp., *Hafnia alvei*, *Leclercia adecarboxylata*, *Providencia* spp. and *Serratia* spp., are described. Furthermore, among them, *Buttiauxiella* spp. (from a cat with diarrhea and a cat with upper respiratory tract disease), *Serratia liquefaciens* (from a cat with upper respiratory tract disease), *Kluyvera* spp. and *Providencia* spp. (from two diarrheic cats) isolates were MDR.

Although the above-mentioned microrganisms rarely determine clinical infections in companion animals, as reported in literature, the detection of MDR members in this study warn of the possibility that these bystanders could become a source of resistance determinants or could determine opportunistic infections, which can be difficult to treat due to their MDR.

Carriage of unusually non-pathogenic members of *Enterobacteriace* among companion animals intestinal flora in concomitance with major antimicrobial resistant pathogens may favorite acquisition of resistance genes from antimicrobial resistant strains through mobile genetic elements. Hence, these rarely reported bacteria may become reservoirs of resistance genes and express the acquired resistance when causing opportunistic infections.

All isolates in the study were susceptible to carbapenems.

The resistance to carbapenems has so far remained a rare phenomenon among Gram-negative bacteria isolated from companion animals, but recently several studies showed that they could harbour carbapenemase-producing bacteria, which represents an additional hazard to public health and veterinary medicine.

The first producer of beta-lactamase-type NDM-1 bacterium of veterinary interest was an *E. coli* isolated from a cat (Shaheen et al., 2012). Furthermore, the first *E. coli* and *K. pneumoniae* producers of OXA-48-like carbapenemase were also recently identified (Stolle et al., 2013). In 2014, the first strain of *Acinetobacter baumanii* producing an OXA-23 carbapenemase was reported from a urinary tract infection in a cat that lived outdoor and had a 1.5-year history of skin and soft tissues infections, for which multiple courses of amoxicillin-clavulanic acid had been prescribed (Pomba et al., 2014). Reports of carbapenemase-producing bacteria are today anecdotal in companion animals, but they could raise in the future, based on the advancing spread of this type of resistance in humans isolates.

The prevalence of carriage of ESBL- or *p*AmpC-producing *Enterobacteriaceae* identified in this study was higher than that documented in previous reports in Italy, which may indicate that ESBL- and *p*AmpC-producing *Enterobacteriaceae* might be widespread in the Palermo and Messina regions.

The spread of these enzymes can be attributed to the transfer of plasmids and other mobile genetic elements between species.

The findings suggest that diseased cats may suffer from infections caused by cephalosporin-resistant *Enterobacteriaceae* and act as reservoirs for resistant bacteria.

Conclusions

Our results confirm that the phenomenon of antimicrobial resistance has spread so rapidly that it represents today one of the most dire threats to public health (E.P., 2013).

The problem posed by the emerging resistance, underlined in this study by the discovery of a new β-lactamase gene not previously described from companion animals, the prospect of losing these drugs and the possibility of an already attested interspecies spread emphasize the urgent need to define the epidemiology of β-lactamases producing bacteria in companion animals.

The emergence of ESBL/*p*AmpC-producing MDR *Enterobacteriaceae* poses major limitations in companion animals' therapeutic options.

Furthermore, it raises great concerns regarding the bi-directional transmission of MDR bacteria between pets and humans.

Awareness should be raised among companion animals' general practitioners about the threat they may encounter in their daily clinical activities.

Moreover, these results suggest that veterinary practitioners should ask for a bacteriological examination and a susceptibility testing

before setting up antimicrobial therapy, in order to establish precisely treatment of commonly encountered bacterial infections.

This could reduce costs by avoiding the resort to ineffetctive compounds and play a role in the control and monitoring of antimicrobial resistance in companion animals' medicine.

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