Faculdade de Medicina de São José do Rio Preto
Programa de Pós-Graduação em Ciências da Saúde

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Ocorrência de enterobactérias produtoras de β-lactamases de Espectro Estendido (ESBL) em animais da região de São José do Rio Preto, SP.

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SUMÁRIO

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"Quando o homem compreende a sua realidade, pode levantar hipóteses sobre o desafio dessa realidade e procurar soluções. Assim, pode transformá-la e o seu trabalho pode criar um mundo próprio, seu Eu e as suas circunstâncias."

Paulo Freire

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Lista de Abreviaturas e Símbolos

%	Porcentagem
<	Menor que
≤	Menor ou igual que
ºC	Graus Celsius
®	Marca registrada
B	Beta
µg	Micrograma
µL	Microlitro
A.F.	Frequência absoluta
ACT	Topo AmpC
ANVISA	Agência Nacional de Vigilância Sanitária
Bla	β-lactamase
BrCAST	Comitê Brasileiro de Teste de Sensibilidade aos Antimicrobianos
CECON	Centro de Controle e Produtos para Diagnóstico
UFC/mL	Unidade formadora de colônia por mililitro
CIAPAV	Centro de Análises e Patologia Veterinária
CMY	Cefamicinase
CTX	Cefotaximase
CTX-M	Cefotaximase-Munique

DDST	Teste de sinergismo de disco duplo
DNA	Ácido desoxirribonucleico
EDTA	Ácido etilenodiamino tetra-acético
ESBL	β-lactamase de espetro estendido
EUCAST	Comitê Europeu de Testes de Susceptibilidade Antimicrobiana
FOX	Cefoxitina
I	Sensível aumentando a exposição
IBGE	Instituto Brasileiro de Geografia e Estatística
IMP	Imipenemase
IncF	Grupo de incompatibilidade F
KPC	*Klebsiella pneumoniae* carbapenemase
MBL	Metalo-β-lactamase
MIR-1	Miriam Hospital em Providence β-lactamase
Mm	Milímetro
N	Número
N.I.	Não informado
OMS	Organização mundial da saúde
OXA	Oxacilinase
PBPs	Proteínas ligadoras de penicilina

PIB	Produto interno bruto
Piper + Tazo	Piperacilina Sódica-Tozabactam Sódico
R	Resistente
R.F.	Frequência relativa
SHV	Família β-lactamase variável sulfidrila
S	Sensível
spp.	Espécies
TEM	β-lactamase Temoneira
VIM	Verona imipenemase
WHO	World Health Organization

Resumo

Introdução: O mecanismo mais importante de resistência aos antibióticos β-lactâmcios entre as enterobactérias Gram-negativas são as β-lactamases. Dentre elas, as β-lactamases de espetro estendido (ESBL), que são enzimas que hidrolizam o anel β-lactâmico, entretanto, diferenciam-se pela extensão da eficácia e por inativar grande parte das cefalosporinas de terceira e quarta geração. O uso indiscriminado de antibióticos favorece a ocorrência e a disseminação dessas enterobactérias nos animais , o que é motivo de grande preocupação, pois além de aumentar a morbimortalidade, implica, sobretudo, em opções terapêuticas cada vez mais desafiadoras, restritas e onerosas. **Objetivos:** Investigar a presença de enterobactérias produtoras de ESBL nas diferentes espécies animais atendidas em clínicas veterinárias da região de São José do Rio Preto, São Paulo, Brasil, e nas amostras biológicas encaminhadas a um laboratório de análises clínicas no período de janeiro de 2019 a junho 2022, bem como avaliar o perfil de resistência bacteriana das cepas isoladas aos diferentes antibióticos. **Material e Métodos.** Foi realizado um estudo retrospectivo, transversal e descritivo, por meio de uma abordagem predominantemente quantitativa. Foram avaliados 105 laudos de cultura e antibiograma de amostras biológicas positivas para a presença de bactérias produtoras de ESBL, as quais foram obtidas de animais atendidos em clínicas veterinárias do noroeste paulista. **Resultados:** Foram identificadas amostras com cepas de *Escherichia coli* (35,2%), *Klebsiella* spp. (56,2%) e *Proteus mirabilis* (8,6%) produtoras de ESBL. Apenas *E. coli* foi isolada em amostras de todas as espécies animais avaliadas (cão, gato, equino, asinino, ave, bovino e suíno). Foi possível identificar essas enterobactérias em diferentes amostras biológicas, sendo que 63,8% esteve presente na urina. As cepas isoladas apresentaram resistência a diferentes antibióticos testados, bem como alta sensibilidade aos

carbapenêmicos. **Conclusões:** Todas as espécies animais avaliadas nestes estudo são suscetíveis a essas enterobactérias produtoras de ESBL, entretanto a ocorrência foi maior em cães e gatos. A bactéria produtora de ESBL predominante foi *Klebsiella* spp. e a de menor ocorrência foi *P. mirabilis*. As enterobactérias foram identificadas em sua maioria em amostras de urina. As cepas isoladas apresentaram notável resistência aos antibióticos β-lactâmicos e as fluoroquinolonas, e apresentaram 100% de sensibilidade ao imipenem.

Palavras-chave: resistência bacteriana a β-lactâmicos, *Escherichia coli*, *Klebsiella* spp., *Proteus mirabilis.*

Abstract

Introduction: The most important mechanism of resistance to β-lactam antibiotics among Gram-negative enterobacteria are β-lactamases. Among them, extended-spectrum β-lactamases (ESBL), which are enzymes that hydrolyze the β-lactam ring, however, differ by the extent of their effectiveness and by inactivating a large part of third and fourth generation cephalosporins. The indiscriminate use of antibiotics favors the occurrence and dissemination of these enterobacteria in animals, which is a cause for great concern, as in addition to increasing morbidity and mortality, it implies, above all, increasingly challenging, restricted, and costly therapeutic options. **Objectives:** To investigate the presence of ESBL-producing enterobacteria in different animal species treated in veterinary clinics in the region of São José do Rio Preto, São Paulo, Brazil, and in biological samples sent to a clinical analysis laboratory from January 2019 to June 2022, as well as evaluating the bacterial resistance profile of the isolated strains to different antibiotics. **Material and methods:** A retrospective, cross-sectional and descriptive study was carried out using a predominantly quantitative approach. 105 culture and antibiogram reports of biological samples positive for the presence of ESBL-producing bacteria were evaluated, which were obtained from animals treated in veterinary clinics in the northwest of São Paulo. **Results:** Samples were identified with strains of Escherichia coli (35.2%), Klebsiella spp. (56.2%) and Proteus mirabilis (8.6%) producing ESBL. Only E. coli was isolated in samples from all animal species evaluated (dog, cat, horse, donkey, bird, bovine and swine). It was possible to identify these enterobacteria in different biological samples, with 63.8% being present in urine. The isolated strains showed resistance to different antibiotics evaluated, as well as high sensitivity to carbapenems. **Conclusions:** All animal species evaluated in these studies

are susceptible to these ESBL-producing enterobacteria, however the occurrence was higher in dogs and cats. The predominant ESBL-producing bacteria was Klebsiella spp. and the one with the lowest occurrence was P. mirabilis. Enterobacteria were mostly identified in urine samples. The isolated strains showed remarkable resistance to β-lactam antibiotics and fluoroquinolones, and were 100% sensitive to imipenem.

Keywords: bacterial resistance to β-lactams, *Escherichia coli*, *Klebsiella* spp., *Proteus mirabilis*.

1.INTRODUÇÃO

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1.2 Resistência bacteriana

A resistência bacteriana aos antibióticos está aumentando para níveis sem precedentes em todo o mundo e a adoção de ações contra esse crescimento é urgente, pois caminha-se para uma era pós-antibióticos, na qual infecções comuns poderão resultar em óbito (1). Portanto, é considerada uma ameaça global à saúde e ao desenvolvimento e é notavelmente alarmante a rápida disseminação de bactérias multi e pan-resistentes. A Organização Mundial de Saúde (OMS), em 2019, identificou 32 antibióticos em desenvolvimento clínico que atendem à lista de patógenos prioritários, porém apenas seis foram considerados inovadores. Isto é motivo de grande preocupação, pois muitas bactérias multirresistentes e pan-resistentes, de forma frequente, causam infecções que não são debeladas com o uso dos antibióticos existentes (2)

1.3 Mecanismos de Resistência

A resistência a antibióticos pode ser intrínseca ou adquirida (3). A resistência intrínseca a um antibiótico caracteriza a resistência que é um atributo inerente a uma determinada espécie de bactéria, ou seja, o microrganismo pode não ter o alvo suscetível ao medicamento ou possuir barreiras naturais que o impede de atingir o alvo. Entretanto, a resistência adquirida reflete uma mudança na composição genética da bactéria, assim o medicamento que antes era eficaz deixa de ser efetivo, resultando em resistência (4).

As bactérias podem desenvolver algumas estratégias para evitar as ações dos antibióticos, como a limitação da concentração intracelular do agente antimicrobiano pela diminuição do influxo ou aumento do efluxo, a alteração do alvo para que o medicamento

não atue ou a eliminação total do alvo pela elaboração de novas vias metabólicas, e a neutralização do antibiótico por enzimas que inativam o medicamento (5–9).

1.3.1 β-lactamases

β-lactamases são enzimas produzidas por bactérias que hidrolizam o anel β-lactâmico, fundamental para atividade antimicrobiana dos antibióticos β-lactâmicos, sendo considerado o mecanismo de resistência mais comum que contribui para uma resistência generalizada entre as bactérias Gram-negativas (10).

Ambler (11) dividiu as β-lactamases de acordo com suas estruturas primárias em quatro classes (A, B, C e D). Estas classes foram inseridas em dois grandes grupos bioquimicamente distintos, classificadas de acordo com os mecanismos catalíticos em serina-β-lactamases (classes A, C e D) e metalo-β-lactamases (MBLs) (classe B).

Entretanto, as β-lactamases podem também ser classificadas em três grupos, segundo suas características funcionais (10).

As Cefalosporinases, que pertencem à classe molecular C e são codificadas nos cromossomos de várias enterobactérias. As enzimas incluídas nesses grupos são das famílias AmpC, CMY, ACT, FOX e MIR-1 e muitas são mediadas por plasmídeos (12).

As serina β-lactamases, que é o maior grupo e pertencem à classe molecular A e D. Possuem amplo espectro de ação, atuando nas penicilinas, cefalosporinas e carbapenêmicos. As enzimas presentes neste grupo são das famílias TEM, SHV, CTX, OXA e KPC e é neste grupo que estão presentes as β-lactamases de espectro estendido (ESBLs). Os genes destas enzimas podem estar situados em cromossomos, entretanto, também podem estar presentes em plasmídeos, sendo transmitidos horizontalmente para outros gêneros de bactérias (10,13).

As metalo-β-lactamases (classe molecular B) compõem o terceiro grupo e diferem estruturalmente das demais, pois necessitam de um íon zinco no sítio ativo (10). Como algumas serina β-lactamase, possuem a capacidade de hidrolisar os carbapenêmicos, porém possuem baixa capacidade de hidrolisar os monobactamicos e não são inibidas pelo ácido clavulânico ou tazobactam, mas sim por quelantes de íons metálicos, como o ácido etilenodiamino tetra-acético (EDTA) e o ácido dipicolínico (14,15). Foram identificadas primeiramente como enzimas cromossômicas, mas algumas são codificadas por plasmídeos, como as enzimas das famílias IMP e VIM que estão entre as principais famílias de metalo-β-lactamases (16).

1.3.2 β-lactamases de espetro estendido (ESBL)

As ESBL inativam os antibióticos β-lactâmicos antes de adentrarem no microrganismo pois atuam no anel β-lactâmico presente na estrutura molecular do fármaco. Entretanto, diferenciam-se pela extensão da eficácia e por conseguirem inativar grande parte das cefalosporinas de terceira e quarta geração. São produzidas, em sua maioria, pela codificação de genes plasmidiais e são geralmente bloqueadas pelos inibidores de β-lactamases, como o ácido clavulânico, sulbactam e tazobactam (17). O ácido clavulânico, por exemplo, é um inibidor de β-lactamase muitas vezes utilizado como recusrso para a detecção das ESBLs em laboratórios clínicos (18)

O primeiro relato de uma cepa produtora de ESBL em animais de companhia foi em 1998, na Espanha. O animal era um cão com infecção no trato urinário e a bactéria, uma *E. coli* produtora de β-lactamase do tipo SHV-12 (19).

1.4 Antibióticos β-lactâmicos

Os antibióticos β-lactâmicos são caracterizados por possuírem um anel β-lactâmico em sua estrutura química (20). As diferentes cadeias lineares associadas às características do esqueleto básico formado por dois anéis (núcleo), alteram as propriedades do composto e determinam os grupos β-lactâmicos. São divididos em cinco grandes grupos, as cefalosporinas, os carbapenêmicos, as penicilinas, os monobactâmicos e os inibidores das β-lactamases (21).

As penicilinas possuem um anel tiazolidina ligada ao anel β-lactâmico. Nas cefalosporinas, o anel β-lactâmico se funde a um anel de dihidrotiazina. Já nos carbapenêmicos, há uma estrutura de anel duplo. Os monobactamicos não possuem um anel adicional (20,21). Os inibidores de β-lactamases são estruturalmente relacionados à penicilina, contendo a ligação amida do grupo β-lactâmico e uma cadeia lateral modificada, e com isso podem se ligar de forma irreversível às β-lactamases, tornando-as inativas (22).

Os antibióticos do grupo β-lactâmico são muito utilizados na medicina veterinária devido a sua alta eficácia terapêutica e baixa toxicidade (23). Seu mecanismo está relacionado com o bloqueio da síntese da parede celular, especificamente na reação de transpeptidação na formação da cadeia de peptidoglicano. Estes antimicrobianos se ligam às transpeptidases conhecidas como proteínas ligadoras de penicilina (PBPs) e impedem a reação de transpeptidação necessária à formação do peptidoglicano para a construção da parede celular, resultando em orifícios na fase da fissão binária e consequentemente a morte do microrganismo (24). Alguns antibióticos como os carbapenêmicos, que são pertencentes ao grupo dos β-lactâmicos, são utilizados como medicamentos de último

recurso em seres humanos para combater bactérias multirresistentes, inclusive as produtoras de ESBL (25)

1.5 Enterobactérias

O mecanismo mais importante de resistência aos antibióticos β-lactâmicos entre as enterobactérias Gram-negativas são as β-lactamases (26).

Membros da família Enterobacteriacea produtoras de ESBL, têm sido considerados a principal causa de infecções nosocomiais desde a década de 1980 (27). Entretanto, infecções adquiridas na comunidade por essas bactérias vem sendo relatados nos últimos anos (13,27,28).

Segundo Logan e Weinstein (29), infecções causadas por bactérias que expressam ESBL e carbapenemases vem aumentando significativamente nas ultimas décadas. Em 2017 a OMS classificou entrerobactérias produtoras de ESBL, incluindo *E. coli, Klebsiella* spp. e *Proteus* spp., como agentes microbianos de máxima prioridade (30).

O uso indiscriminado de antibióticos favorece a ocorrência e a disseminação de enterobactérias produtoras de ESBL em animais de companhia, o que é motivo de grande preocupação, pois além de aumentar a morbimortalidade, implica, sobretudo, em opções terapêuticas cada vez mais desafiadoras, restritas e onerosas (31), além da rápida disseminação dessas enzimas e o surgimento constante de novas variantes (32). Apesar da frequente utilização de antibióticos em animais domésticos, o impacto causado pela resistência aos antimicrobianos nesses animais ainda não tem recebido a atenção necessária (33). Entretanto, a Organização Mundial de Saúde Animal vem recolhendo informações sobra a utilização de antimicrobianos em animais desde 2015 (34)

1.6 Objetivos

1. Investigar a presença de enterobactérias produtoras de ESBL consideradas pela OMS como agentes microbianos de máxima prioridade, em espécies animais atendidas em clínicas veterinárias da região de São José do Rio Preto, São Paulo, Brasil;

2. Investigar a ocorrência de enterobactérias produtoras de ESBL nas diferentes amostras biológicas obtidas desses animais e encaminhadas a um laboratório de análises clínicas de São José do Rio Preto, São Paulo, Brasil, no período de janeiro de 2019 a junho 2022;

3. Avaliar o perfil de resistência bacteriana das cepas isoladas aos diferentes antibióticos.

2.ARTIGO CIENTÍFICO

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Os resultados desta tese estão apresentados na forma de artigo submetido à publicação.

ARTIGO 1

Título: Occurrence of Extended Spectrum β-lactamases (ESBL)-Producing Bacteria in Animals Treated at Veterinary Clinics in the Northwest of the State of São Paulo, Brazil.

Autores: Sanches L, Delmaschio-Oliveira IB, Varallo GR, Andrade LA, Brandão CC

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ARTIGO CIENTÍFICO

Periódico: Veterinary Microbiology, Qualis A1

Occurrence of Extended Spectrum β-lactamases (ESBL)-Producing Bacteria in Animals Treated at Veterinary Clinics in the Northwest of the State of São Paulo, Brazil.

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Abstract

Despite the frequent use of antibiotics in domestic animals, the impact caused by antimicrobial resistance has not received the necessary attention. The indiscriminate use of antibiotics favors the occurrence and dissemination of these enterobacteria, which is a cause for great concern, as it increases morbidity and mortality and implies more challenging therapeutic options. The objective of this study was to investigate the presence of ESBL-producing enterobacteria in animal species treated in veterinary clinics and in different biological samples, as well as to evaluate the profile of bacterial resistance to different antibiotics. A retrospective, cross-sectional and descriptive study was carried out using a predominantly quantitative approach. 105 culture and antibiogram reports of biological samples positive for the presence of ESBL-producing bacteria, obtained from animals treated in veterinary clinics in the northwest of São Paulo, were evaluated. Samples with strains of Escherichia coli (35.2%), Klebsiella spp. (56.2%) and Proteus mirabilis (8.6%) producing ESBL. Only E. coli was isolated in samples from all animal species evaluated. These enterobacteria were identified in different biological samples. The isolated strains showed resistance to different antibiotics tested, as well as high sensitivity to carbapenems. It was concluded that all animal species evaluated in these studies are susceptible to these enterobacteria, however the occurrence was greater in dogs and cats. The predominant ESBL-producing bacteria was Klebsiella spp. Enterobacteria were mostly identified in urine samples. The isolated strains showed remarkable resistance to β-lactams and fluoroquinolones, and showed 100% sensitivity to imipenem.

Key words: *E. coli*; *Klebsiella* spp.; *Proteus mirabilis*; Bacterial resistance; β-lactams; domestic animals

Introduction

Antibiotic resistance is a global threat to health and development and the rapid spread of multi- and pan-resistant bacteria is alarming. In 2019, the World Health Organization (WHO) identified 32 antibiotics in clinical development that meet the list of priority pathogens, but only six were considered innovative, which is a cause for great concern since many of these pathogens frequently cause infections that are not resolved with the use of existing antibiotics (WHO, 2021).

β-lactam antibiotics are widely used in veterinary medicine due to their high therapeutic efficacy and low toxicity (Bush and Bradford, 2016). These substances act disrupting bacterial synthesis inhibiting cell wall transpeptidation by binding to penicillin-binding proteins (PBPs) and preventing the transport of peptidoglycans required for cell wall synthesis, resulting in gaps in the DNA replication phase and consequently the death of the microorganism (Ealand et al., 2018). Some antibiotics such as carbapenems, which belong to the β-lactam group, are used as last resort drugs to combat multidrug-resistance, including of Extended Spectrum β-lactamases (ESBL)-producing bacteria (Liu et al., 2017).

In this context, antibiotic resistance can be intrinsic or acquired. Intrinsic resistance to an antibiotic characterizes the resistance that is an attribute contained in a certain species of bacteria, that is, the microorganism may not have a target susceptible to the medicine or have natural barriers that prevent it from reaching the target. However, acquired resistance reflects a change in the genetic makeup of the bacteria, so the medicine that was previously effective is no longer effective, resulting in resistance (Kaye et al., 2004). Bacteria develop some strategies to avoid the actions of antibiotics, such as limiting the intracellular concentration of the antimicrobial agent by decreasing the influx or

increasing the efflux, changing the target so that the medicine does not act or the total elimination of the target by the development of new metabolic pathways, and neutralization of the antibiotic by enzymes that inactivate the medicine (Pfeifer et al., 2010).

β-lactamases are enzymes produced by bacteria that hydrolyze the β-lactam ring, which is essential for the antimicrobial activity of β-lactam antibiotics, this is considered the most common resistance mechanism contributing to widespread resistance of Gram-negative bacteria (Bush and Jacoby, 2010). ESBL are notable by the extent of their effectiveness and by being able to inactivate most third- and fourth-generation cephalosporins. These enzymes are mostly produced by encoding plasmid genes and are generally disrupted by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Ur Rahman et al., 2018). Clavulanic acid is a β-lactamase inhibitor often used to detect ESBL in clinical laboratories (BrCAST-EUCAST, 2017).

Despite the common use of antibiotics in domestic animals, the impact caused by antimicrobial resistance in these animals has not yet received the necessary attention (Shimizu et al., 2017). However, the World Organization for Animal Health has been collecting information on the use of antimicrobials in animals since 2015 (WOAH, 2023). Indiscriminate use favors the occurrence and dissemination of ESBL-producing enterobacteria in domestic animals, which is a cause for great concern as, more important than the increased morbidity and mortality caused, Is the fact that therapeutic options are becoming increasingly challenging, restricted, and costly (Ewers et al., 2014).

Therefore, the objective of this study was to investigate the presence of ESBL-producing enterobacteria in different animal species treated in veterinary clinics in the region of São José do Rio Preto, São Paulo, Brazil, and in biological samples sent to a clinical analysis

laboratory from January 2019 to June 2022, as well as evaluating the bacterial resistance profile of the isolated strains to different antibiotics.

Materials and methods

A retrospective, cross-sectional and descriptive study, using a predominantly quantitative approach, was carried out at the Veterinary Analysis and Pathology Center (CIAPAV), located in the city of São José do Rio Preto, São Paulo.

In this study, 4.793 culture and antibiogram reports of biological samples were evaluated, and only positive reports (n = 105) for the presence ESBL-producing bacteria obtained from animals treated at veterinary clinics in northwestern region of São Paulo State and sent to the aforementioned laboratory were considered. The inclusion criteria adopted in the methodology of this study were: culture and antibiogram reports released from August 2019 to July 2022 of biological samples from different animal species positive for ESBL-producing bacteria. The exclusion criteria were cultures and antibiogram reports that did not isolate ESBL-producing bacteria.

The instrument used for data collection was the physical report provided by the laboratory with the data being compiled in an Excel spreadsheet for statistical analysis.

The dependent variables of this study were the animal species, type or tecidual location where the biological material was obtained, bacterial genus or species isolated and the bacterial sensitivity profile identified by antibiogram. Change to independent variables in this study were the type or tecidual location where the biological material was obtained and anima species.

During the period covered by this study, different biological samples were sent to the laboratory, including: urine, feces, bronchoalveolar lavage, pleural fluid (pleural

effusion), abdominal effusion, subcutaneous cavity fluid (subcutaneous effusion), nasal secretion, oral secretion, ocular secretion, in addition to samples of the contents of the auditory canal, skin lesions, surgical lesions, bones, kidneys and abscesses.

Phenotypic characterization of enterobacteria

Bacterial cultivation was carried out according to the type of sample, in bacteriological incubator at 35º C for 24 to 48 hours.

For samples of colony growths on Blood Agar and Chocolate Agar, smears were prepared on glass slides and Gram staining was applied for the morphological characterization of Gram-negative bacteria, with subsequent biochemical tests being carried out for presumptive identification of enterobacteria. Biochemical tests were performed using Modified Rugai medium (CECON®) and incubation in a bacteriological incubator for 24 hours; Bactray systems (Laborclin®) were used for inconclusive cases to perform additional biochemical tests. The stages of enterobacteria identification followed National Health Surveillance Agency standards (ANVISA, 2013).

An antibiotic sensitivity test was performed after identifying the enterobacteria.

Antimicrobial Sensitivity Test

First, three to five colonies isolated from the culture were collected from the Blood Agar medium with a sterile 10-µL calibrated loop and suspended in 3 mL of 0.9% saline solution in a sterile tube in order to obtain a suspension with turbidity equivalent to the 0.5 McFarland standard (1-2 x 10^8 CFU/mL), according to the Brazilian Committee for Antimicrobial Susceptibility Testing rules, which are based on the European Committee for Antimicrobial Susceptibility Testing (BrCAST-EUCAST, 2021).

The susceptibility profile of enterobacteria to antimicrobials was determined by disk diffusion according to the method of BrCAST-EUCAST (2021). Using a sterile swab, bacterial suspensions were seeded in Petri dishes containing Müeller-Hinton agar with antibiotic discs being applied after five minutes. The choice of antibiotics followed the laboratory's internal policy and considered aspects such as the type of sample, the type of bacteria characterized and the site of action of the antibiotic. The antibiotics discs of interest tested were: amikacin (30µg), amoxicillin-clavulanic acid (30µg), amoxicillin (10µg), ampicillin (10µg), ampicillin-sulbactam (20µg), aztreonam (30µg), cefadroxil (30µg), cephalexin (30µg), cefepime (30µg), cefotaxime (30µg), cefoxitin (30µg), ceftazidime (30µg), ceftiofur (30µg), ceftriaxone (30µg), cefovecin (30µg), ciprofloxacin (5µg), chloramphenicol (30µg), doxycycline (30µg), enrofloxacin (5µg), florfenicol (30µg), fosfomycin (200µg), gentamicin (10µg), imipenem (10µg), levofloxacin (5µg), marbofloxacin (5µg), meropenem (10µg), metronidazole (4µg), neomycin (30µg), nitrofurantoin (300µg), norfloxacin (10µg), ofloxacin (5µg), piperacillin sodium/tazobactam sodium (100µg), polymyxin B (300µg), tetracycline (30µg), tobramycin (10µg) and sulfazotrim (25µg). The Petri dishes were incubated for 16-18 hours at 35°C. Breakpoints recommended by EUCAST were used to evaluate the inhibition halos with the results being read using a caliper and classified as sensitive (S), sensitive increased by exposure (I) or resistant (R) (EUCAST, 2022).

Confirmatory Test for ESBL

This step was carried out following the guidelines of BrCAST-EUCAST (2017). The detection of ESBL-producing enterobacteria was based on the resistance profile of bacterial isolates to broad-spectrum cephalosporins on the antibiogram. The ESBL

suggestive halos defined were for ceftriaxone (30 µg) < 23 mm, cefotaxime (5 µg) < 21 mm and ceftazidime (10 µg) < 22 mm. From this profile, a phenotypic test, the double disk synergy test (DDST), was performed to confirm ESBL production.

Disks containing cefotaxime, ceftazidime and ceftriaxone were applied to the Petri dish 20 mm from an amoxicillin disk (center to center) containing a β-lactamase inhibitor (clavulanic acid). Samples are considered positive when zones of inhibition around any cephalosporin disc are increased towards the disc containing clavulanic acid. This deformation zone, known as the phantom zone, characterizes the inhibitory action of β-lactamase.

Statistical analysis

Collected data were tabulated in Excel spreadsheets.

Descriptive statistical analysis was performed based on calculations of measures of central tendency, dispersion and frequency counts. The chi-square test was used for inferential statistical analysis.

For all analyses, a statistically significant value was considered with p-value ≤ 0.05. SPSS (IBM®, version 23, 2014) and GraphPad Instat (3.10, 2009) software was used.

Results

In total, 4793 culture and antibiogram reports from different species of animals were evaluated. Of these, the presence of ESBL-producing enterobacteria was identified in 105 cases. The samples were obtained from one donkey (1%), one pig (1%), three birds (*Nymphicus hollandicus*) (2.9%), two cattle (1.9%), five horses (4.8%), 19 cats (18.1%) and 74 dogs (70.5%), as shown in Table 1.

Of the 105 positive samples, 37 (35.2%) strains of *E. coli*, 59 (56.2%) of *Klebsiella* spp. and 9 (8.6%) strains of *Proteus mirabilis* were identified using the methodology employed in the aforementioned laboratory (Table 2). The prevalence of ESBL-producing enterobacteria was 2.19% (105/4,793).

It was observed that only *E. coli* was common to all the different animal species evaluated; 67.7% from dogs (n = 25), 16.2% from cats (n = 6), 5.4% from horses (n = 2), 2.7% from a donkey (n = 1), from birds (n = 1), from cattle (n = 1) and pig (n = 1). *P. mirabilis* was only present in biological samples obtained from dogs (n = 8; 88.9%) and cats (n = 1; 11.1%), while the species *Klebsiella* spp. was isolated in samples from dogs (n = 41; 69.5%), cats (n = 13; 22%), birds (n = 2; 3.4%), horses (n = 2; 3.4%) and cattle (n = 1; 1.7%) (Figure 1). There was no statistically significant relationship between the animal species affected and the occurrence of different enterobacteria.

Bacteria were isolated from different biological samples. There was high occurrence of *E. coli* (23.8%), *Klebsiella* spp. (33.3%) and *P. mirabilis* (6.7%) in urine samples (Table 3). Notably, the occurrence of these enterobacteria was higher in urine samples (63.8%) compared to other samples (Table 3). A total of 6.7% of the bacterial agents were isolated from samples not identified by the person responsible for sending them to the laboratory. Low frequencies were observed in stool (4.7%), skin lesion (4.7%), nasal secretion (2.9%), auditory canal (3.8%), bronchoalveolar lavage (1.9 %) and surgical wound samples (1.9%). In these sampling sites, only *E. coli* and *Klebsiella* spp. were isolated. Occurrence was only 1% in other samples including *E. coli* in abdominal effusion, abscess and uterine secretion samples, *Klebsiella* spp. in oral secretion, milk, subcutaneous fluid, bone, ocular secretion and renal secretion samples, and *P. mirabilis* in pleural effusion specimens. No significant correlation was identified between the evaluated samples and

the bacterium isolated, that is, the occurrence of specific bacterial species is not related to the type of sample.

Resistance to β-lactams was notable for the *E. coli* isolates. There was 100% resistance to amoxicillin (n = 13), ampicillin (n = 17), aztreonam (n = 2), cefadroxil (n = 37), cephalexin (n = 36), cefepime (n = 3), cefotaxime (n = 3), ceftiofur (n = 5), ceftriaxone (n = 33), cefovecin (n = 25), ofloxacin (n = 4) and metronidazole (n = 2). Resistance to doxycycline and tetracycline was above 90% and to fluoroquinolones it was above 80%; resistance to sulfazotrim was 91.9% (n = 37). It is also noteworthy that resistance to other antibiotics was considered high, including ampicillin associated with sulbactam (75%; n = 4), cefoxitin (50%; n = 2), ceftazidime (73.3%; n = 15), chloramphenicol (60%; n = 5), gentamicin (66.7%; n = 36), polymyxin B (66.7%; n = 3) and tobramycin (66.7%; n = 3). Resistance was below 50% for the other antibiotics tested (Table 4).

As previously mentioned, the choice of antibiotics used for the antibiogram followed the laboratory's internal policy and there was no standardization regarding the antibiotics tested, that is, the same antibiotics were not necessarily tested for all isolates.

Resistance to some classes of antibiotics was extremely high for *Klebsiella* spp.; resistance to cefadroxil (n = 57), cephalexin (n = 57), cefotaxime (n = 9), cefovecin (n = 47), metronidazole (n = 2), tetracycline (n = 15) and tobramycin (n = 4) was 100%. It is important to emphasize that this species presents intrinsic resistance to amoxicillin, ampicillin and the combination of ampicillin and sulbactam. Furthermore, resistance to fluoroquinolones (with the exception of ofloxacin), aztreonam (n = 8), ceftiofur (n = 10), ceftriaxone (n = 54), doxycycline (n = 58), fosfomycin (n = 8), gentamicin (n = 58) and sulfazotrim (n = 57) was above 80%. There was also considerable resistance to amoxicillin associated with clavulanic acid (69.5%; n = 59), ceftazidime (72.2%; n = 18),

cefepime (50%; n = 8), neomycin (53.8 %; n = 13), nitrofurantoin (73%; n = 37), marbofloxacin (81.4%; n = 59) and ofloxacin (57.1%; n = 7). Other antibiotics were tested, but resistance was below 50% (Table 4).

P. mirabilis was isolated in only nine samples and as previously mentioned, there was no standardization regarding the antibiotics used for antibiograms. This genus has intrinsic resistance to certain antibiotics (ANVISA, 2020), three of them were tested (nitrofurantoin, polymyxin B and tetracyclines). Sulfazotrim was tested in nine isolates and the resistance was 100%. Resistance to ampicillin (n = 6), cefadroxil (n = 7) and cephalexin (n = 8) was also 100%. Cefepime and levofloxacin were tested only once in different isolates with both being resistant to these antibiotics. Neomycin and tobramycin were also tested only once, but in the same isolate which was resistant to both. It is also notable that resistance to amoxicillin-clavulanic acid (55.6%,; n = 9), aztreonam (75%; n = 4), cefotaxime (80%; n = 5), cefovecin (88.9%; n = 9), ceftiofur (50%; n = 2), ciprofoxacin (87.5%; n = 8), enrofloxacin (88.9%; n = 8), gentamicin (62.5%; n = 8), marbofloxacin (85.7%; n = 7) and norfloxacin (75%; n = 8) was considerable. Resistance to the other antibiotics tested was below 50% (Table 4).

The evaluated bacteria showed high sensitivity to carbapenems. *P. mirabilis* isolates were 100% sensitive to imipenem and meropenem. *E. coli* and *Klebsiella* spp. were also 100% sensitive to imipenem, whereas resistance to meropenem was 8.1% (n = 15) and 1.8% (n = 55), respectively. Resistance to amikacin (*Klebsiella* spp. - 5.5%, *E. coli* - 14.3%, and *P. mirabilis* = 37.5%) was also insignificant. It is important to emphasize that *E. coli* resistance was relatively low to the association of piperacillin sodium/tazobactam sodium (7.7%; n = 13), nitrofurantoin (16%; n = 25) and florfenicol (25%; n = 4). *Klebsiella* spp.

also showed low resistance to florfenicol (14.3%; n = 7) and polymyxin B (33.3%; n = 12), and *P. mirabillis* had low resistance to ceftazidime (16.7%; n = 6).

Discussion

The present study investigated the occurrence of ESBL-producing bacteria in animals treated at veterinary clinics in northwestern São Paulo. The occurrence of ESBL-producing enterobacteria was higher in dogs (70.5%) and cats (18.1%). Salgado-Caxito et al. (2021a) performed a scoping review and meta-analysis to compare the occurrence prevalence of ESBL-producing *E. coli* in dogs and cats across continents and found that 67.2% (86/128) of the studies were performed only in companion animals with the others (32.8%) evaluating samples from production and wild animals.

An interesting point is that some studies (Melo et al., 2018; Sfaciotte et al., 2021) show that in clinical and hospital environments the occurrence of ESBL-producing enterobacteria in dogs is higher than in cats, which corroborates the results obtained in the current analysis. This can be justified, in part, by the greater number of domesticated dogs. According to figures collected in 2019 by the Brazilian Institute of Geography and Statistics (IBGE), approximately 33.8 million households in the country have one or more dogs; in the municipality of São Paulo, this figure is about 1.5 million. However, only 14.2 million households in Brazil approximately have one or more cats, 661 thousand of which are in the municipality of São Paulo.

The prevalence of ESBL-producing enterobacteria in this study was 2.19%. Studies carried out on dogs in other countries, including Beijing (Chen et al., 2019) and Portugal (Carvalho et al., 2021), show higher rates of isolation; 6.73% and 13.02%, respectively.

However, in the scope review carried out by Salgado-Caxito et al. (2021), the overall prevalence of ESBL-producing *E. coli* was 6.87% in dogs and 5.04% in cats.

The current study also found low rates of ESBL-producing enterobacteria in samples obtained from production animals. The excessive use of antibiotics in animal production, either prophylactically or therapeutically, has been described as one of the main causes of resistance acquired by enterobacteria (Pellegrino et al., 2006). However, the sporadic isolation of these agents and their low occurrence may be due to underdiagnosis, either due to the absence of clinical suspicion or to the lack of sending samples to specialized laboratories.

The presence of ESBL-producing enterobacteria in wild and companion birds has already been described in Brazil and worldwide (Borges et al., 2017; Yılmaz and Dolar, 2017). In this study, these bacteria were identified in three cockatiels (*Nymphicus hollandicus*). It is important to emphasize that the potential for ESBL transmission from birds to humans still needs further investigation, however, due to the proximity of these animals, especially when kept in captivity (whether they are companion animals, on farms or in zoos), there is a risk to public health (Yılmaz and Dolar, 2017).

In this study, only *E. coli* was common to all the different animal species evaluated. According to Alonso et al. (2017), most studies focus on *E. coli*, as it is generally considered an appropriate indicator of antibiotic resistance, due to its medical importance and its presence in a wide range of hosts. However, there was a higher occurrence of *Klebsiella* spp. (56.2%) in the samples of this study. The results obtained by Shnaiderman-Torban et al. (2021), corroborate our findings, as they also found a higher occurrence of *Klebsiella* spp. (35.89%). This genus is reported to cause infections in animals and humans worldwide, and these infections are linked to resistance to important

antimicrobial agents (Marques et al., 2019), mainly through the production of ESBL (Hendrik et al., 2015). ESBL in animals has also been described by other researchers (Shnaiderman-Torban et al., 2021; El-Tarabili et al., 2022).

Most of the bacteria in the present study were isolated from urine samples. According to Melo et al. (2018), urinary tract infections by ESBL-producing enterobacteria are more frequent than other infections. The occurrence in the other specimens was low, however, ESBL-producing enterobacteria are often associated with infections in different organs after translocating from the gastrointestinal tract to other body sites (Kanamori et al., 2011). An important point to mention is that 6.7% of ESBL-producing enterobacteria were isolated from samples not identified by the person responsible for sending them to the laboratory. In addition to the extreme importance of providing basic patient information to adequately record and process samples, these data can be used in studies aimed at recognizing the epidemiological characteristics of different diseases.

In this study, the profiles of resistance to antibiotics exhibited by the bacterial strains of interest were also investigated. *E. coli* isolates showed high resistance rates to β-lactams, including 3rd and 4th generation cephalosporins and fluoroquinolones. This same pattern has been observed in other studies on ESBL-producing *E. coli* in dogs and cats in Brazil (Sfaciotte et al., 2021) and in other countries (Chen et al., 2019; Johansson et al., 2022), as well as in other animal species (Sartori et al., 2017; Yılmaz and Dolar, 2017). It is currently recognized that *E. coli* resistance to fluoroquinolones in the treatment of urinary tract infections is widespread, including in some countries treatment with this antibiotic is ineffective in more than half of the human patients (WHO, 2021).

High resistance rates to other non-β-lactam antibiotics, such as aminoglycosides, tetracyclines, polymyxin B, sulfazotrim and metronidazole (despite having been tested in

only two isolates) were also observed. In another study also carried out in Brazil, Sfaciotte et al. (2021) reported that, similar to this study, ESBL-producing *E. coli* isolates identified in dogs and cats were resistant to aminoglycosides and tetracyclines.

The current study also showed high resistance rates of *Klebsiella* spp. to β-lactams and fluoroquinolones. Indeed, resistance to fluoroquinolones does occur as ESBL strains that exhibit co-resistance to these two classes of antibiotics have often been isolated (Johansson et al., 2022). According to Rozwandowicz et al. (2018), it is likely that this high occurrence of fluoroquinolone-resistance genes is due to the conjugative dissemination of the blaCTX-M-15 gene by IncF plasmids. This study also found a high occurrence of resistance of these bacteria to other non-β-lactam antibiotics, such as aminoglycosides, tetracyclines, metronidazole, fosfomycin and nitrofurantoin.

The rate of resistance to cephalosporins, fluoroquinolones, aminoglycosides, penicillins, monobactams and sulfazotrim found for *P. mirabilis* isolates was also high. Similar results were obtained by El-Tarabili et al. (2022), who reported in their study that species of ESBL-producing *Proteus* spp. isolated from dogs showed resistance to penicillins, cephalosporins and monobactams.

The isolates identified in this study showed high sensitivity to carbapenems, which are generally only used in the clinical treatment of severe infections (Hawkey, 2015). However, 8.1% of the *E. coli* and 1.8% of the *Klebsiella* spp. isolates tested showed resistance to meropenem. This may reflect the increased use of carbapenems due to high levels of resistance to the most frequently used antibiotic classes and, consequently, the emergence and dissemination of carbapenemase-producing enterobacteria may occur. Sfaciotte et al. (2021) suggest that these bacteria had decreased permeability or presence of enzymes responsible for hydrolyzing the antibiotics of this group. According to

Escandón-Vargas et al. (2017), reports of enterobacteria resistance to carbapenems have increased alarmingly in American countries.

Finally, the high resistance rates to the different antibiotics found in this study highlight a worrying and serious issue: the treatment options available for infections caused by ESBL-producing bacteria are more challenging, restricted and costly. This reinforces the imperative need for the rational and judicious use of antibiotics in veterinary medicine.

This study is the first epidemiological investigation on this issue in the region of São José do Rio Preto, São Paulo, Brazil. Further studies on this theme are needed, as complications related to microorganisms resistant to multiple antibiotics are a single and global health emergency.

Conclusion

All animal species evaluated in these studies are susceptible to these ESBL-producing enterobacteria, however the occurrence was higher in dogs (70.5%) and cats (18.1%). Under the conditions of this study, the predominant ESBL-producing bacteria was Klebsiella spp. and the one with the lowest occurrence was P. mirabilis. E. coli was the only enterobacteria present in all animal species evaluated. ESBL-producing enterobacteria were mostly identified in urine samples (63.8%). Furthermore, the isolated strains showed remarkable resistance to β-lactam antibiotics, as well as fluoroquinolones, however, they showed 100% sensitivity to imipenem.

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Table 1: Relative and absolute frequencies of the presence of ESBL-producing enterobacteria isolated in different species.

Species	Absolute frequency	Relative frequency (%)
Bird	3	2.9
Cattle	2	1.9
Dog	74	70.5
Donkey	1	1.0
Horse	5	4.8
Cat	19	18.1
Swine	1	1.0
Total	105	100

Table 2: Relative and absolute frequencies of isolated ESBL-producing enterobacteria species.

Enterobacteria	Absolute frequency	Relative frequency (%)
E. coli	37	35.2
Klebsiella spp.	59	56.2
Proteus mirabilis	9	8.6
Total	105	100

Table 3: Relative frequency of enterobacteria species isolated in the different samples.

	Sample content																		
Bacteria identified	Auditory canal	Oral secretion	Abdominal effusion	Surgical wound	Feces	Bronchoalveolar lavage	Milk	Skin lesion	Abscess	Subcutaneous effusion	Pleural effusion	Bone	Nasal secretion	Ocular secretion	Kidney secretion	Uterine secretion	Urine	N.I.	Total
E. coli	1	0	1	0	1.9	1	0	1.9	1	0	0	0	1	0	0	1	23.8	1.9	35.2
Klebsiella spp.	2.8	1	0	1.9	2.8	1	1	2.8	0	1	0	1	1.9	1	1	0	33.3	3.8	56.2
P. mirabilis	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	6.7	1	8.6
Total	3.8	1	1	1,9	4.7	1.9	1	4.7	1	1	1	1	2.9	1	1	1	63.8	6.7	100

N.I.: Not informed

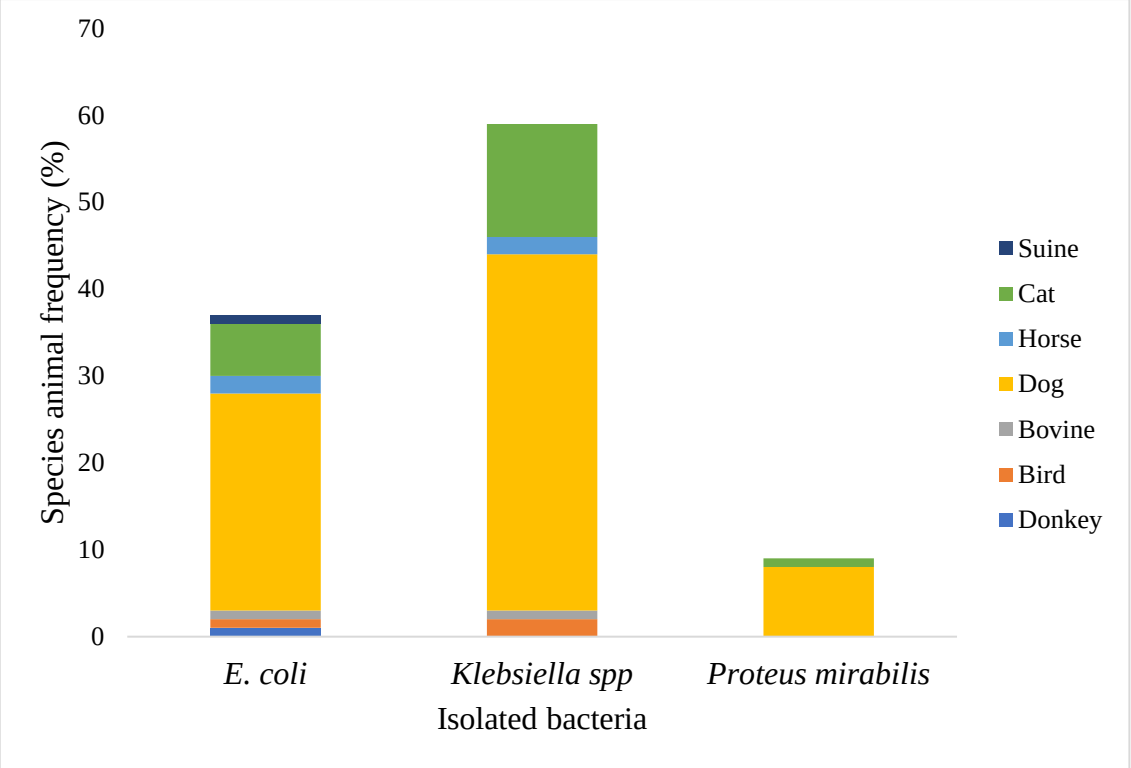
Table 4: Relative and absolute frequencies of different species of ESBL-producing enterobacteria resistant to different antibiotics.

Antibiotics	*E. coli*		*Klebsiella* spp.		*P. mirabilis*	
	A.F.	R.F.	A.F.	R.F.	A.F.	R.F.
Amikacin	35	14.3	55	5.5	8	37.5
Amoxicillin-Clavulanic Acid	37	37.8	59	69.5	9	55.6
Amoxicillin	13	100.0	41	100.0	0	0.0
Ampicillin	17	100.0	45	100.0	6	100.0
Ampicillin-Sulbactam	4	75.0	3	100.0	0	0.0
Aztreonam	2	100.0	8	87.5	4	75.0
Cefadroxil	37	100.0	57	100.0	7	100.0
Cephalexin	36	100.0	57	100.0	8	100.0
Cefepime	3	100.0	8	50.0	1	100.0
Cefotaxime	3	100.0	9	100.0	5	80.0
Cefoxitin	2	50.0	7	42.9	0	0.0
Ceftazidime	15	73.3	18	72.2	6	16.7
Ceftiofur	5	100.0	10	80.0	2	50.0
Ceftriaxone	33	100.0	54	96.3	7	42.9
Cefovecin	25	100.0	47	100.0	9	88.9
Ciprofoxacin	37	83.8	59	91.5	8	87.5
Chloramphenicol	5	60.0	11	45.5	0	0.0
Doxycycline	36	94.4	58	96.6	5	100.0
Enrofloxacin	35	91.4	59	96.6	9	88.9
Florfenicol	4	25.0	7	14.3	0	0.0

Fosfomycin	9	0.0	8	87.5	1	0.0
Gentamicin	36	66.7	58	81.0	8	62.5
Imipenem	15	0.0	30	0.0	4	0.0
Levofloxacin	17	82.4	24	91.7	1	100.0
Marbofloxacin	36	86.1	59	81.4	7	85.7
Meropenem	37	8.1	55	1.8	9	0.0
Metronidazole	2	100.0	2	100.0	0	0.0
Neomycin	7	42.9	13	53.8	1	100.0
Nitrofurantoin	25	16.0	37	73.0	7	100.0
Norfloxacin	30	86.7	50	84.0	8	75.0
Piper + Tazo	13	7.7	17	0.0	1	0.0
Polymyxin B	3	66.7	12	33.3	1	100.0
sulfazotrim	37	91.9	57	96.5	9	100.0
Tetracycline	10	90.0	15	100.0	7	100.0
Tobramycin	3	66.7	4	100.0	1	100.0
Ofloxacin	4	100.0	7	57.1	0	0.0

A.F.: Absolute frequency; R.F.: Relative frequency; Piper + Tazo: Piperacillin sodium/ Tazobactam Sodium

Figure 1: Frequency of ESBL-producing enterobacterial strains isolated from different animal species.



3. CONCLUSÕES

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ARTIGO CIENTÍFICO

1. Todas as espécies animais avaliadas nestes estudo são suscetíveis a essas enterobactérias produtoras de ESBL;
2. A ocorrência foi maior em cães (70,5%) e gatos (18,1%);
3. Nas condições deste estudo, a bactéria produtora de ESBL predominante foi *Klebsiella* spp. e a de menor ocorrência foi *P. mirabilis*;
4. A *E. coli* foi a única enterobactéria presente em todas as espécies animais avaliadas;
5. As enterobactérias produtoras de ESBL foram identificadas em sua maioria em amostras de urina (63,8%);
6. As cepas isoladas apresentaram notável resistência aos antibióticos β-lactâmicos, bem como as fluoroquinolonas;
7. As enterobactérias avaliadas neste estudo apresentaram 100% de sensibilidade ao imipenem e apenas 8,1% dos isolados de *E. coli* e 1,8% de *Klebsiella* spp. apresentaram resistência ao meropenem.

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5. ANEXOS

Anexo I. Submissão do artigo científico “Prevalence of Extended Spectrum β-lactamases (ESBL)-Producing Bacteria in Animals Treated at Veterinary Clinics in the Northwest of the State of São Paulo, Brazil”

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Corresponding Author: Veterinarian Isabela Belei Delmaschio de Oliveira
Co-Authors: Leonardo Sanches; Giovanna Rossi Varallo; Luana Augusto Andrade; Cinara Cássia Brandão
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FUMVET Fundação Medicina Veterinária

DECLARAÇÃO

Declaramos para os devidos fins que o artigo intitulado "**In sickness and in health: the intestinal microbiome of dogs**", de autoria de Giovanna Rossi Varallo, Gabriela Marchiori Bueno, Cinara de Cassia Brandão, Leonardo Sanches, Isabela Belei Delmaschio de Oliveira, sob o número de protocolo **203612**, foi aceito para publicação na revista *Brazilian Journal of Veterinary Research and Animal Science* no Volume 60 (2023).

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Atenciosamente,

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