PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 45:e07501, 2025
DOI: 10.1590/1678-5150-PVB-7501

Original Article
Livestock Diseases

Detection of ESBL-producing strains at the veterinary necropsy environment[1]

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ABSTRACT.- Pinto L.B., Holmström T.C.M., David L.A., Mendes M.B., Makita M.T., Rocha-de-Souza C.M., Brito M.F., Melo D.A. & Souza M.M.S. 2025. **Detection of ESBL-producing strains at the veterinary necropsy environment.** *Pesquisa Veterinária Brasileira 45:e07501, 2025.* Laboratório de Bacteriologia Veterinária, Departamento de Microbiologia e Imunologia Veterinária, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Rodovia BR-465 Km 7, Seropédica, RJ 23890-000, Brazil. E-mail: theresseholmstrom@yahoo.com.br

The emergence and spread of resistance to antimicrobials is one of the three main threats to public health in the 21st century. It must be analyzed using an integrated One Health approach, as it is a health risk shared by people, animals, and the environment. Among these, the necropsy space represents a point of cohesion, being an extremely relevant place for research and understanding the circulation of the bacterial microbiota and its resistance genes. The present study evaluated the occurrence of superbugs in samples from animals necropsied at the Federal Rural University of Rio de Janeiro, considering the priority criteria established by the World Health Organization (WHO). Of the 198 samples collected from 45 animals, 20 pet animals, 20 production animals and three wild animals, 325 strains were isolated, of which 51.38% (167/325) were Enterobacterales, 31.69% (103/325) *Staphylococcus* spp., 12.62% (41/325) *Enterococcus* spp., 2.46% (8/325) *Streptococcus* spp. and 1.85% (6/325) n*on-fermenting Gram-negative bacilli* (NFGNB). In 29.13% (30/103) of *Staphylococcus* spp., the *bla*Z gene was detected, and in enterobacteria, the presence of bla_{SHV} was detected in 10.18% (17/167), bla_{TEM} in 6.59% (11/167) and $bla_{CTX\text{-}M\text{-}1}$ in 4.19% (7/167). These results reveal the occurrence of species characterized as critical superbugs by the WHO in the necropsy environment and reinforce the need to monitor these strains in the veterinary environment not only for the adoption of appropriate control and treatment measures for animals but also for the implementation of protocols safe for the disposal of their carcasses.

INDEX TERMS: ESBL production, necropsy environment, antimicrobial resistance, superbugs.

RESUMO.- [**Detecção de bactérias produtoras de ESBL em ambientes de necropsia veterinária.**] O surgimento e a disseminação da resistência aos antimicrobianos é uma das três principais ameaças à saúde pública no século XXI, e deve ser analisado em uma abordagem integrada de Saúde Única, por se tratar de um risco à saúde compartilhado por pessoas, animais e meio ambiente. Dentre estes, o espaço de necropsia representa um ponto de coesão, sendo um local de extrema relevância para pesquisa e compreensão da circulação da microbiota bacteriana e seus genes de resistência. O presente estudo avaliou a ocorrência de superbactérias em amostras de animais necropsiados na Universidade Federal Rural do Rio de Janeiro, considerando os critérios de prioridade estabelecidos pela Organização Mundial de Saúde (OMS). Das 198 amostras coletadas de 45 animais, sendo 20 animais de companhia, 20 de produção e três selvagens, foram isoladas 325 cepas, das quais 51,38% (167/325) foram Enterobacterales, 31,69% (103/325)

[1] Received on July 20, 2024.
Accepted for publication on September 30, 2024.

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Staphylococcus spp., 12,62% (41/325) *Enterococcus* spp., 2,46% (8/325) *Streptococcus* spp. e 1,85% (6/325) bacilos Gram-negativos não fermentadores (BGNNF). Em 29,13% (30/103) dos *Staphylococcus* spp. houve detecção do gene *blaZ* e nas enterobactérias detectou a presença de bla_{SHV} em 10,18% (17/167), bla_{TEM} em 6,59% (11/167) e $bla_{CTX\text{-}M\text{-}1}$ em 4,19% (7/167). Esses resultados revelam a ocorrência de espécies caracterizadas como superbactérias críticas pela OMS em ambiente de necropsia e reforçam a necessidade de monitoramento dessas cepas no ambiente veterinário não apenas para a adoção de medidas de controle e tratamento adequados dos animais, mas também para a implementação de protocolos seguros para o descarte de suas carcaças.

TERMOS DE INDEXAÇÃO: Produção de ESBL, ambiente de necropsia, resistência antimicrobiana, superbactérias.

INTRODUCTION

Antimicrobial resistance (AMR) represents a serious public health problem. It hinders the treatment of infections caused by multi-resistant bacteria, threatening established protocols for managing infectious diseases (Nesme et al. 2014). The World Health Organization (WHO) has considered the emergence and spread of AMR as one of the three main threats to public health in the 21st century (Alós 2015, Hernando-Amado et al. 2019). O'Neill (2016) estimated a significant increase in mortality from untreatable bacterial infectious processes, reaching 10 million people per year worldwide by 2050.

As a threat shared by humans and animals, in addition to representing a potential risk to the environment, Bordier et al. (2020) assert that monitoring and controlling AMR should transition from isolated, sectoral, and linear actions to systemic and transdisciplinary approaches. The Centers for Disease Control and Prevention (CDC 2018) considers AMR one of the key issues to address in an integrated One Health approach.

The contribution of animal-related environments to the emergence of multi-resistant bacteria is related to the selection pressure exerted by the increased use of antimicrobials at therapeutic levels in companion animals, which can serve as natural reservoirs of resistant microorganisms (Guardabassi et al. 2004). Additionally, the indiscriminate use of these drugs at subtherapeutic levels as growth promoters in animal production also plays a role in this phenomenon (Robinson et al. 2016).

Due to their diverse nature in terms of animal species and cause of death, pathology services concentrate a wide diversity of bacterial specimens and consequent resistance genes, thus allowing the assessment of specific resistance profile circulation. The inclusion of companion animals, production animals and wild animals provides a comprehensive representation of different species and potential health issues.

Extended-spectrum beta-lactamases (ESBL) are enzymes capable of hydrolyzing the beta-lactam ring of antimicrobials that possess the oxyimino group in their molecular structure (Andrade et al. 2018). There are over 180 types of identified ESBL, mainly detected in bacteria such as *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* (Gomes & Casalini 2018). Gram-negative enteric bacteria from the Enterobacterales order have become resistant to the class of β-lactam agents by acquiring genes that encode and produce ESBL-type enzymes. These enzymes are widespread worldwide, with over 1.5 billion people colonized by ESBL-producing Enterobacterales (Woerther et al. 2013). Recently, many ESBL-produced strains have been described in veterinary medicine (Medeiros et al. 2021, Campana et al. 2024), which makes research and identification of animal origin samples relevant.

The highest frequency ESBL belongs to the TEM, SHV, and CTX-M families, encoded by both chromosomal and mainly plasmid genes (Andrade & Darini 2017). The SHV family has a chromosomal origin, while the TEM family has a plasmid origin (Andrade et al. 2018). Initially, the bla_{SHV} and bla_{TEM} genes encoded the synthesis of cephalosporinases; however, due to genetic mutations, these enzymes expanded their spectrum of action, becoming ESBLs (Andrade & Darini 2017). The CTX-M family is one of the most predominant and belongs to the group of class A molecular serine beta-lactamases, differing in at least six subgroups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25, and KLUC (Andrade et al. 2018). This heterogeneous group intrinsically features the hydrolysis of broad-spectrum cephalosporins and originates from chromosomal $bla_{CTX\text{-}M}$ genes from *Kluyvera* spp. (Andrade & Darini 2017).

Beta-lactamases are classified in two ways: by molecular structure and substrate preference. The scheme proposed by Ambler divides them into four molecular classes: class A (including ESBLs, penicillinases, and carbenicillinases), B (metallo-beta-lactamases – MBLs), C (chromosomal cephalosporinases AmpC), and D (oxacillinases). On the other hand, the classification by Bush, Jacob, and Medeiros separates them into four functional groups and subgroups, which is the most accepted and widely used (Gomes & Casalini 2018).

A significant portion of ESBLs belongs to Ambler's molecular class A and functional subgroup 2be of Bush, Jacob, and Medeiros (Andrade & Darini 2017).

In general, these enzymes are encoded by genes found on mobile plasmids, facilitating the horizontal transfer of genetic material between species and increasing dissemination potential (Miranda et al. 2019). Additionally, they often carry additional genes that confer resistance to other classes of antimicrobials (Andrade et al. 2018). In this context, the present study investigated carbapenem-resistant and extended-spectrum beta-lactamases producers Enterobacterales (ESBL), bacterial agents classified as "critically important" by the World Health Organization (WHO 2017).

MATERIALS AND METHODS

Ethical approval. This study was approved by the Ethics Committee on Animal Use of the "Universidade Federal Rural do Rio de Janeiro" (UFRRJ), under number CEUA 6239180418.

Sampling. Samples were collected from 45 animals necropsied at the UFRRJ, including 40 from the "Setor de Patologia Animal" (Pathological Anatomy Sector – SAP) and five broilers from the Poultry Sector. Out of the 40 animals necropsied at the SAP, 20 were companion animals, comprising 16 dogs, three cats, and one rabbit (*Oryctolagus cuniculus*); 17 were production animals, including 10 bullfrogs (*Lithobates catesbeianus*), four cattle, two birds, and one horse; and three were wild animals, consisting of one puma (*Puma concolor*), one black-eared skunk (*Didelphis aurita*), and one free-living frog. The five animals necropsied in the Poultry Sector were chickens of the *Gallus gallus* species. The animals had different clinical suspicions, not only related to bacterial infections (Table 1). The animals were chosen according to the demand of the sector, and in the period from August/2020 to September/2021, all were selected.

Samples were collected from sites previously determined for each species and carried out in two ways: with sterile swabs packed in a culture medium for transport and through an organ fragment packed in sterile 0.9% saline. According to the cause of death, other sites were included. The animals from which the samples were collected were males and females of different species, breeds and ages ranging from six months to 15 years. A total of 198 samples were collected from the 45 animals taken for this study.

Bacterial isolation. The samples collected were sent to the "Laboratório de Bacteriologia Veterinária" (Laboratory of Veterinary Bacteriology – LABACVET), UFRRJ, where they underwent inoculation in selective culture media. Specifically, MacConkey agar (Biokar®) and *Salmonella Shigella* agar (Kasvi®) were utilized, with the latter being employed only after a 12-hour sample enrichment in Tetrathionate broth (Acumedia®) at 37 °C. Subsequently, the inoculated samples were incubated at 37 °C for 24 hours, as referenced by Markey et al. (2013) and Procop et al. (2018).

Phenotypic identification. Gram-negative rods were selected and submitted to different tests, including the 3% Potassium Hydroxide test and biochemical analyses using Triple Sugar Iron agar (Himedia®), MR-VP broth (VETEC®), Simmon Citrate agar (MicroMed®) and Indole Sulfide Motility agar (MicroMed®). In addition, some complementary tests, such as the evaluation of the urea hydrolysis using Urea Broth (Himedia®) were performed. Isolates of the order Enterobacterales were characterized and phenotypically identified according to Markey et al. (2013) and Procop et al. (2018).

Table 1. List of animal groups, animal species and samples collected at each site of necropsy

Animal group (N)*	Species (N)*	Collection site (N)*
Wild animals (3)	*Puma concolor* (1)	Ear canal (2)
		Nasal canal (2)
		Lung (1)
		Rectum (1)
	Didelphis aurita (1)	Ear canal (2)
		Nasal canal (2)
		Lung (1)
		Rectum (1)
	Anuro – frog (1)	Liver (1)
		Lung (1)
		Kidney (1)
Companion animals (20)	Canine (16)	Ear canal (32)
		Nasal canal (32)
		Lung (16)
		Rectum (16)
		Muzzle lesion (1)
	Feline (3)	Ear canal (6)
		Nasal canal (6)
		Lung (3)
		Rectum (3)
	Leporine (1)	Nasal canal (2)
		Lung (1)
		Rectum (1)
Production animals (22)	*Lithobates catesbeianus* (10)	Liver(10)
		Lung(8)
		Kidney (10)
	Bovine (4)	Nasal canal (8)
		Sinuses (4)
		Lung (2)
		Abscess (1)
	Gallus gallus (6)	Cloaca (6)
		Trachea (6)
		Liver (1)
	Anser cygnoides (1)	Cloaca (1)
		Trachea (1)
	Equine (1)	Nasal Canal (2)
		Sinuses (1)
		Trachea (1)
		Cecum (1)
		Small intestine (1)

* N = number of samples.

Characterization of Enterobacterales species by proteomic analysis (MALDI-TOF MS). Isolates were inoculated on Brain Heart Infusion (BHI) agar at 37 °C for 24 h. Each culture was transferred to a microplate (96 MSP, Bruker® - Billerica, USA). Bacterial sediment was covered by a lysis solution (70% formic acid; Sigma-Aldrich®). Additionally, a 1-μL aliquot of matrix solution (alpha-ciano-4-hidroxicinamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, Sigma-Aldrich®) was added to each sediment. The spectra of each sample were generated in a mass spectrometer (MALDI-TOF LT Microflex, Bruker®) equipped with a 337 nm nitrogen laser in a linear path controlled by the FlexControl 3.3 (Bruker®) program. The spectra were collected in a mass range between 2,000-20,000 m/s and analyzed by the MALDI Biotyper 2.0 (Bruker®) program, using the standard configuration for bacteria identification, by which the spectrum of the sample is compared with the references in the database. The results vary on a 0-3 scale, where the highest value means a more precise match and reliable identification.

Phenotypic characterization of the resistance profile to beta-lactams. The phenotypic detection of resistance to beta-lactams was performed using disk diffusion tests as recommended by CLSI (2018) and CLSI (2020). The inoculum was prepared after incubating the isolates in BHI agar culture medium (Absorb®) for 18 to 24 hours at 35 °C. After this time, colonies were suspended in sterile 0.9% saline with turbidity equivalent to McFarland's 0.5 scale. Subsequently, the sowing of the isolates was carried out on Müeller-Hinton agar (Kasvi®), followed by the deposition of selected antimicrobial discs used for prediction and analysis of resistance by ESBL expression. After incubation at 35 °C for 18 hours, the diameters formed around the discs were measured and interpreted according to the CLSI (2018) and CLSI (2020) documents.

The screening test for ESBL production was performed with the following antimicrobials: amoxicillin + clavulanic acid (30 μg), ampicillin (10 μg), aztreonam (30 μg), cefotaxime (30 μg), ceftazidime (30 μg) and cefepime (30 μg). Isolates that did not show sensitivity to one or more cephalosporins and/or monobactam were submitted to the confirmatory test for ESBL. In this test the following antimicrobials were used: cefotaxime (30 μg), ceftazidime (30 μg), cefotaxime + clavulanic acid (30/10 μg) and ceftazidime + clavulanic acid (30/10 μg) and the result was considered positive for the production of ESBL when observed an increase of, at least, 5 mm in the diameter of the inhibition halo for any of the antimicrobials combined with clavulanate compared to the diameter of the halo of inhibition of the same agent when tested alone.

DNA extraction. Isolates were cultured in 1.5 mL of *brain heart infusion broth* (BHIB) at 35 °C for 24 h. Microtubes were centrifuged for 2 min at 8,000 g, and the supernatant was discarded; this procedure was repeated three times. Cells were resuspended in 200 μL of ultrapure water and vortexed, being incubated at 100 °C for 10 min. Microtubes were cooled at room temperature and centrifuged for 2 min at 8,000 g. Approximately 180 μL of the supernatant was transferred to a new microtube (600 μL) and stored at -20 °C (Buyukcangaz et al. 2013).

Detection of ESBL genes. All isolated strains were further analyzed by polymerase chain reaction (PCR) protocols used to search for the most important ESBL genes described in literature encoding genes belonging to bla_{TEM} (Minarini et al. 2007), bla_{SHV} (Shahid 2010) and $bla_{CTX\text{-}M\text{-}1}$ (Geser et al. 2012), as described in Table 2.

Statistical analysis. A prevalence analysis was conducted, calculated as the number of affected individuals at a given time divided by the total number of individuals (Nascimento et al. 2023). Data were tabulated using Excel software, version 2019.

RESULTS

Phenogenotypic identification of strains

The identification of Gram-negative rods through biochemical tests revealed the isolation of 167 strains belonging to the Enterobacterales order, of which *Escherichia coli* was the predominant species, totaling 39.52% (66/167). *Klebsiella pneumoniae* was the second most frequent species, identified in 13.17% (22/167) of the strains, followed by *Proteus mirabilis*, which represented 11.38% (19/167), and *Enterobacter cloacae*, with 7.19% (12/167) of the total isolated specimens. Nine other species of enterobacteria were phenotypically identified with an isolation frequency equal to or less than 3.59% (6/167). It was not possible to characterize 14.97% (25/167) of the isolated strains through biochemistry. Further analyses were carried out using the MALDI-TOF MS technique to identify the 25 strains that were not phenotypically characterized and to compare the results obtained in this test with those of the classical method. The agreement between phenotypic and proteomic techniques in identifying strains of the Enterobacterales order was 92.96%.

Six strains of non-glucose-fermenting Gram-negative rods were isolated, with one of them being identified through phenotypic analyses and confirmed by the MALDI-TOF technique as *Pseudomonas aeruginosa*. It was not possible to characterize the other five strains through biochemical methods. Their identification at the genus or species level was achieved through results from the proteomic technique, identifying them as *Pseudomonas* sp., *Acinetobacter* sp., *Acinetobacter baumannii*, and *Acinetobacter lactucae*.

Phenogenotypic characterization of beta-lactam resistance

The susceptibility analysis to beta-lactams demonstrated that 59.88% (100/167) of the strains showed resistance to ampicillin, and 16.17% (27/167) were resistant to amoxicillin/clavulanic acid, a beta-lactamase inhibitor compound. Regarding cephalosporins, cefotaxime presented the highest percentage of resistance at 16.17% (30/167), followed by ceftazidime and cefepime, both with 10.18% resistance (17/167). Aztreonam,

Table 2. List of primers used to detect *bla* genes in this study

Gene (amplicon size)	Sequence (5'-3')	Amplification condition	Reference
$bla_{CTX\text{-}M\text{-}1}$ (862 bp)	AAAAATCACTGCGCCAGTTC CCGTCGGTGACGATTTTAGCC	94 °C for 5 min; 40 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; and 72 °C for 5 min	Geser et al. (2012)
bla_{SHV} (930 bp)	TTTATCGGCCCTCACTCAAGG GCTGCGGGCCGGATAACG	94 °C for 3 min; 32 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min; and 72 °C for 10 min	Shahid (2010)
bla_{TEM} (831 bp)	ATGAGTATTCAACATTTCCGTG TTACCAATGCTTAATCAGTGAG	94 °C for 5 min; 40 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; and 72 °C for 5 min	Minarini et al. (2007)

the only representative of the monobactams class, showed 10.78% (18/167) resistance.

In the Enterobacterales order, the expression of ESBLs was detected in 11.98% (20/167) of the strains, and in 70% (14/20) of them, the confirmatory test result was positive. As for non-glucose-fermenting Gram-negative rods (NGF-GNRs), phenotypic resistance was observed in 16.67% (1/6) in the screening test, with an *Acinetobacter* spp. isolated from a dog's lung swab. This resistance was confirmed through positivity in the confirmatory test for ESBL expression.

Additionally, the analysis of bacterial DNA quality through PCR of the *16S* rRNA gene and subsequent PCR for the detection of bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, and bla_{TEM} genes were performed. These genes represent the most abundant coders of beta-lactamases in the order Enterobacterales. They are known to confer resistance to beta-lactam antibiotics, including penicillins and cephalosporins, and their global dissemination has been a significant concern regarding the rise of antimicrobial resistance (Pérez-Etayo et al. 2018, Ejaz et al. 2021).

Phenotypic resistance indicating ESBL expression was detected in 11.98% (20/167) of the strains (Fig.1), with 80% (16/20) of them showing a positive result in the confirmatory test (Fig.1). The presence of the bla_{SHV} gene was detected in 10.18% of the strains, followed by the bla_{TEM} gene in 6.59% and $bla_{CTX\text{-}M\text{-}1}$ in 4.19%. It is noteworthy that more than one of these genes was present in 32% of the strains with positive genotypic detection. Additionally, one strain showed phenotypic resistance but did not exhibit any of these genes upon genotypic detection. The strains that showed resistance in at least one of the phenotypic assays, either screening or confirmatory, or genotypic assays, through the detection of bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{TEM} genes have their results described in Table 3.

Out of 45 necropsied animals, ESBL genes were detected in 13 animals across four species: five broiler chickens, four dogs, three bullfrogs, and one cat. Therefore, the prevalence of resistance gene detection was 27.66%.

DISCUSSION

The strains that showed sensitivity to amoxicillin+clavulanate and were not susceptible to at least one representative antimicrobial from third-generation cephalosporins (ceftazidime and cefotaxime), fourth-generation (cefepime), or monobactam (aztreonam) were considered positive in the phenotypic screening test for extended-spectrum beta-lactamase (ESBL) production. Since ESBLs degrade third-generation cephalosporins and are characterized by being sensitive to beta-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Sawa et al. 2020), an increase in the zone of inhibition of bacterial growth is observed in the disks containing clavulanate when compared to the disks impregnated only with cephalosporins (CLSI 2020).

Regarding the prevalence of enzymatic variants, ESBLs of the SHV and TEM types were initially recognized in the 1980s and are widely disseminated in different species of Gram-negative bacteria, mainly among Enterobacterales. These variants have been gradually replaced by CTX-M type enzymes, the most dominant type of enzyme (Paterson &

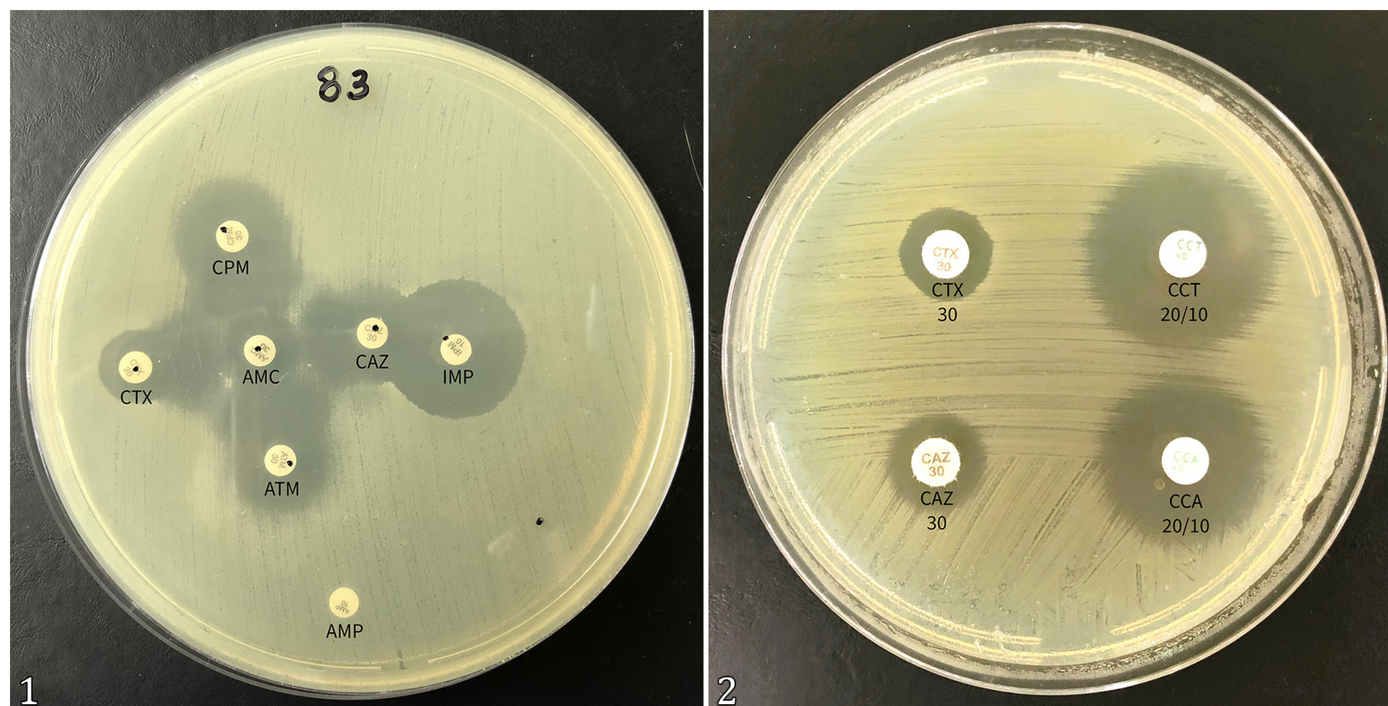

Fig.1-2. The screening and confirmatory test results for assessing resistance to beta-lactams mediated by extended-spectrum beta-lactamases (ESBL). (**1**) Positive result in the screening test: zones of resistance to the antimicrobials CAZ, CPM, CTX and ATM. (**2**) Positive result in the confirmatory test for assessing resistance to beta-lactams mediated by ESBL due to the *Enterobacter cloacae* increased zone of sensitivity to the antimicrobials in the presence of clavulanic acid, both in CAZ and CTX. AMC = amoxicillin + clavulanic acid, AMP = ampicillin, ATM = aztreonam, CAZ = ceftazidime, CCA = ceftazidime + clavulanic acid, CCT = cefotaxime + clavulanic acid, CPM = cefepime, CTX = cefotaxime, IMP = imipenem. CLSI (2018) and CLSI (2020). Source: personal file.

Bonomo 2005, Minarini et al. 2007, Ewers et al. 2010). The close frequency of detection of the three genes studied reveals that these different types of enzymes remain in circulation, both in companion animals and in production animals, in a fairly homogeneous manner.

Although many researchers report the occurrence of resistance to beta-lactam antibiotics due to the genetic expression of ESBLs in wild animals (Guenther et al. 2010, Wasyl et al. 2018, Islam et al. 2021), this study did not yield phenotypic results of resistance due to ESBL production, nor did it detect the targeted genes. This finding may be related to the limited availability of samples from wild animals, which led to a smaller number of isolated Enterobacterales strains (6.59%, 11/167) compared to those obtained from production animals (47.31%, 79/167) and companion animals (46.11%, 77/167). Additionally, the research was conducted with convenience sampling, and the COVID-19 pandemic presented challenges that restricted access to necropsies, as the demand for this examination significantly decreased during the pandemic, especially at defined sample collection times.

Concerning the single strain of *Acinetobacter* spp. that showed resistance in phenotypic tests, the detection of the *blaSHV* gene confirmed the resistance to beta-lactam antibiotics mediated by ESBL production in this n*on-fermenting Gram-negative bacilli* (NFGNB). Over the last decade, *Acinetobacter* spp. have emerged as one of the most clinically important agents due to their potential as nosocomial pathogens, their high intrinsic resistance to various routine antimicrobials in both veterinary and human medicine, and the increasing cases of multidrug resistance, making them highly infectious and difficult to control (Vijayakumar et al. 2019). Determining resistance to beta-lactam antibiotics mediated by ESBL production in *Acinetobacter* spp. is crucial for guiding the therapeutic protocol to be followed, as in this case, carbapenems represent the last-resort option within the class of beta-lactam antibiotics. The choice of last-generation cephalosporins or aztreonam may be responsible for selecting resistant strains capable of spreading their genes to other species and even other bacterial genera.

Although a strain of *Enterobacter cloacae* (strain 83 – Table 3) showed a phenotype of resistance due to ESBL production in both the screening and confirmatory tests, none of the researched genes were detected. In this case, the vast molecular diversity found in the enzymes of the SHV, TEM, and CTX-M types may explain the absence of phenogenotypic correlation. In a study that evaluated the diversity and virulence of ESBL-producing *Escherichia coli* strains in companion animals, Bortolami et al. (2019) reported that the $bla_{CTX\text{-}M}$ genotype was the most commonly found, with $bla_{CTX\text{-}M\text{-}15}$ being the most prevalent (46.9%, 30/64), followed by $bla_{CTX\text{-}M\text{-}14}$ (21.9%, 14/64) and

Tabela 3. Analysis of phenogenotypic resistance to beta-lactams by extended-spectrum beta-lactamases (ESBL) production in strains of the order Enterobacterales

Strain	Species	Source Animal (c) – site	Phenotypic tests		PCR		
			Screening	Confirmatory	bla_{SHV}	$bla_{CTX\text{-}M\text{-}1}$	bla_{TEM}
14	*Klebsiella pneumoniae*	Canine (1) – *swab* rectum	(+)	(+)*	(+)	neg.	neg.
22	*Escherichia coli*	Canine (2) – *swab* rectum	(+)	(+)*	(+)	(+)	neg.
23	*E. coli*	Canine (2) – *swab* rectum	(+)	(+)*	(+)	(+)	(+)
40	*Citrobacter freundii*	*Lithobates catesbeianus* (4) – liver fragment	(+)	neg.	(+)	neg.	neg.
44	*C. freundii*	*L. catesbeianus* (4) – lung fragment	(+)	neg.	(+)	neg.	neg.
45	*Edwardsiella tarda*	*L. catesbeianus* (4) – lung fragment	(+)	neg.	(+)	neg.	neg.
64	*K. pneumoniae*	*L. catesbeianus* (11) – lung fragment	(+)	neg.	(+)	neg.	neg.
82	*Leclercia adecarboxylata*	*L. catesbeianus* (9) – kidney fragment	(+)	neg.	(+)	neg.	neg.
83	*Enterobacter cloacae*	*L. catesbeianus* – lung fragment	(+)	(+)*	neg.	neg.	neg.
138	*E. coli*	Canine (17) – *swab* rectum	(+)	(+)**	neg.	neg.	(+)
140	*K. pneumoniae*	Canine (17) – *swab* lung	(+)	(+)*	(+)	(+)	(+)
227	*E. cloacae*	Feline (32) – *swab* lung	(+)	(+)*	neg.	neg.	(+)
228	*E. cloacae*	Feline (32) – *swab* lung	(+)	(+)*	neg.	(+)	(+)
270	*K. pneumoniae*	*Gallus gallus* (27) – *swab* trachea	neg.	NR	(+)	neg.	neg.
272	*E. coli*	*G. gallus* (27) – *swab* trachea	neg.	NR	(+)	neg.	(+)
273	*K. pneumoniae*	*G. gallus* (28) – *swab* trachea	(+)	(+)*	(+)	(+)	neg.
275	*E. coli*	*G. gallus* (29) – *swab* trachea	neg.	NR	(+)	(+)	neg.
277	*E. coli*	*G. gallus* (30) – *swab* trachea	(+)	(+)***	(+)	neg.	(+)
280	*E. coli*	*G. gallus* (26) – *swab* cloaca	(+)	(+)*	(+)	neg.	neg.
281	*E. coli*	*G. gallus* (27) – *swab* cloaca	neg.	NR	(+)	neg.	(+)
284	*E. coli*	*G. gallus* (28) – *swab* cloaca	(+)	neg.	neg.	neg.	(+)
285	*E. coli*	*G. gallus* (29) – *swab* cloaca	neg.	NR	neg.	neg.	(+)
289	*E. coli*	*G. gallus* (30) – liver fragment	(+)	(+)*	(+)	neg.	neg.
331	*K. pneumoniae*	Canine (36) – *swab* rectum	(+)	(+)***	neg.	neg.	(+)
334	*E. cloacae*	Canine (36) – *swab* lung	(+)	(+)*	neg.	(+)	neg.
TOTAL	26 strains	-	20	14	17	7	11

(c) = animal code, PCR = polymerase chain reaction, (+) positive, neg. = negative; * Confirmation with ceftazidime and cefotaxime, ** confirmation with ceftazidime only, *** confirmation with ceftotaxime only; NR = unrealized.

bla$_{CTX-M-55}$ (12.5%, 8/64), among other less frequent ones such as *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-24}$, and *bla*$_{CTX-M-28}$, demonstrating the molecular variety found in this beta-lactamase. The SHV and TEM enzyme types also have many molecular variants, which, combined with the lack of genotypic detection in a strain with a well-characterized phenotype of resistance due to ESBL production, suggest that the chosen primers for the study did not amplify the gene responsible for encoding the synthesis of ESBL present in this bacterium.

The discrepancies in the phenotypic and genotypic analyses of the isolated strains are noticeable in the case of the five strains isolated from *Lithobates catesbeianus* organ fragments, which were resistant in the phenotypic screening analysis but tested negative in the confirmatory test, yet with detection of the *bla*$_{SHV}$ gene (strains 40, 44, 45, 64 and 82 – Table 3). This may indicate the presence of this gene even in strains that did not show resistance in the confirmatory test. As for the discrepancies observed in the phenogenotypic analyses, the five strains isolated from broiler chicken samples showed sensitivity to antimicrobials in the screening test but tested positive in the genotypic search, including three of them carrying more than one of the researched genes (strains 270, 272, 275, 281 and 285 – Table 3), suggesting the possibility of complex genetic variations in these bacterial isolates. These discrepancies highlight the importance of an integrated approach that considers phenotypic and genotypic aspects in evaluating antimicrobial resistance, aiming to understand better the characteristics and resistance mechanisms of these bacterial strains. The data shows that these findings are increasingly being identified in animal samples, which should be worked on together with the veterinary clinic, and these bacteria should be notified, but this is still not the reality of the veterinary clinical routine.

CONCLUSIONS

The necropsy environment for animals is a location with a wide diversity of potentially pathogenic microorganisms, where the circulation of bacteria crucial to One Health has been observed. These include *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Citrobacter freundii*, *Edwardsiella tarda*, and *Leclercia adecarboxylata* exhibiting resistance to beta-lactams due to extended-spectrum beta-lactamase (ESBL) production encoded by genes *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{CTX-M-1}$. These findings serve as a warning and underscore the need for data dissemination, as in some cases, bacteria may not exhibit phenotypic resistance. However, since genotypic results are considered the gold standard, they should be routinely employed in veterinary clinical practice to assist in more effective therapy for animals.

Another crucial point is the proper disposal of deceased animals to reduce biological risks and inactivate genetic elements responsible for antimicrobial resistance (AMR) dissemination, thereby disrupting horizontal transmission cycles of resistance genes. Incineration represents a safe and efficient method for disposing of necropsy residues, ensuring complete elimination of both microorganisms and the resistance genes they harbor.

Acknowledgments.- "Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro" (FAPERJ): Support for Thematic Projects in the State of Rio de Janeiro E-26/210.085/2020, Scientist of Our State E-26/202.604/2019, Project Antimicrobial Resistance Network E-26/211.554/2019, Regional Scientific and Technological Development Project E-26/210.111/2022 and Technological Development (CNPq) supported this work.

Conflict of interest statement.- The authors declare no conflict of interest.

Credit author statement.- All authors contributed to the study conception and design and also to the final manuscript. Miliane M.S. de Souza, Dayanne A. de Melo and Letícia B. Pinto developed the methodology. Material preparation, data collection and analysis were performed by Letícia B. Pinto, Thérèsse C. N. Holmström, Luria A. David and Mario T. Makita. Letícia B. Pinto, Thérèsse C. N. Holmström and Marcela B. Mendes performed the writing, reviewing and editing the manuscript. Miliane M. S. de Souza were responsible for the acquisition of the financial support for the project leading to this publication.

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