FULL ARTICLE

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Detection of beta-lactamase-producing Enterobacteriaceae in a veterinary hospital environment

Detecção de Enterobactérias produtoras de beta-lactamases em ambiente hospitalar veterinário

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ABSTRACT

Due to the strong selective pressure resulting from the misuse of antibiotics, the natural process of bacterial resistance has been accelerated, leading to the increasingly constant appearance of multiresistant isolates. The high number of multi-resistant bacteria is a one health problem. Enterobacteriaceae are usually commensal bacteria of the gastrointestinal tract. However, they can cause infections, and the most important resistance characteristic among them is the production of β-lactamases. This study aimed to identify ESBL-producing Enterobacteriaceae of types of *TEM*, *SHV*, and the *CTX-M* groups. To isolate the enterobacteria, swabs were collected by swiping objects that had contact with the patients and professionals, and the water of the hospital environment. Ten collections were carried out, yielding 306 samples, from which 118 enterobacteria were identified: *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Proteus mirabilis*, *Serratia* spp., and *Citrobacter* spp. Isolates. The genes *TEM* and *CTX-M*, for the production of β-lactamases, were detected in 12.7% of the 118 enterobacterial isolates. It is very important to know the bacterial population circulating in the veterinary hospital environment and its resistance to antimicrobials so that professionals can take appropriate measures to minimize the risks of transmission, especially from cages and consultation tables. In addition, the correct control of the microbiological quality of the supply water, as well as environmental cleaning procedures, are essential to prevent the transmission of these microorganisms.

Keywords: TEM. CTX-M. Antimicrobial resistance. Nosocomial infection.

RESUMO

Devido à grande pressão seletiva decorrente do uso indevido de antibióticos, tem se acelerado o processo natural de resistência das bactérias, levando ao aparecimento cada vez mais constante de isolados multirresistentes. O elevado número de bactérias multirresistentes identificadas é um problema da saúde única. As enterobactérias são bactérias geralmente comensais do trato gastrointestinal, entretanto podem causar infecções, e a característica de resistência mais importante entre elas é a produção de β-lactamases. Buscando caracterizar melhor os microrganismos circulantes e potencialmente causadores de infecções em ambiente hospitalar veterinário, este estudo objetivou identificar as enterobactérias produtoras de ESBL do tipo *TEM*, *SHV* e os cinco grupos de *CTX-M* presentes em isolados circulantes em hospital veterinário. Foi realizada coleta de suabes de arrasto de objetos que entram em contato com os pacientes e com os profissionais que ali trabalham, bem como de água, para a identificação das enterobactérias. Foram realizadas 10 coletas, obtendo-se 306 amostras, dessas, 118 enterobactérias foram identificadas: *Escherichia coli*, Enterobacter, Klebsiella, *Proteus mirabilis*, Serratia e Citrobacter. Dentre as enterobactérias identificadas, alguns isolados possuíam genes para a produção de β-lactamases, do tipo *TEM* e *CTX-M*. É de grande importância conhecer a população bacteriana circulante no ambiente hospitalar veterinário, e a sua resistência aos antimicrobianos, para que os profissionais possam tomar medidas apropriadas para minimizar os riscos de transmissão, principalmente a partir de gaiolas e mesas de atendimento. Além disso, o correto controle da qualidade microbiológica da água de abastecimento, bem como dos procedimentos de higienização do ambiente, são fundamentais para evitar a transmissão destes microrganismos.

Palavras-chave: TEM. CTX-M. Resistência antimicrobiana. Infecção hospitalar.

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Introduction

The number of multidrug-resistant bacteria is increasing globally, making untreatable infections a real possibility (Blair et al., 2015). Such microorganisms are being identified not only in hospital settings but also in the community (Munita & Arias, 2016). Furthermore, antibiotic resistance is a natural phenomenon that has been accelerated due to the misuse of these drugs (Loureiro et al., 2016). The Enterobacteriaceae family is composed of Gram-negative bacteria, widely distributed in nature, found in soil, water, vegetables, and in the intestinal tract of animals. Those bacteria can be isolated from many specimens received in the laboratory and can cause virtually any type of infection, especially in immunosuppressed patients (Koneman et al., 2012). Among the members of this family, resistance to broad-spectrum cephalosporins, together with the production of carbapenemases, represent the most important resistance trait. Resistance to broad-spectrum cephalosporins (ceftazidime, cefotaxime, ceftriaxone) is mainly due to the production of ESBL (Extended Spectrum Beta-Lactamases), disseminated worldwide (Poirel et al., 2016). They are enzymes capable of hydrolyzing the β-lactam ring of third and fourth-generation cephalosporins and penicillin (Rocha, 2015). Resistance to cephalosporins is a public health concern since these drugs had great importance for human and animal health. ESBLs are often mediated by plasmids, so these genes can be transferred via horizontal transfer (Johard et al., 2015).

β-lactamases receive two classifications, the Ambler classification, which classifies them according to their peptide chain structure (classes A, B, C, and D) (Ambler, 1980), and the functional classification of Bush and Jacoby, which takes into account the preference of enzymes for the substrate and inactivation by β-lactamase inhibitors. In this second classification, β-lactamases are clustered into groups 1, 2, and 3, which also have subgroups (Bush & Jacoby, 2010). ESBLs are included in Amble Class A and Bush 2b group (Poirel et al., 2012), and the three main variants are *TEM*, *SHV*, and *CTX-M* (Rubin & Pitout, 2014), the latter being currently predominant. The five most important classes of the *CTX-M* variant, according to the protein structure of the enzyme, are *CTX-M-1*, *CTX-M-2*, *CTX-M-8*, *CTX-M-9*, and *CTX-M-25* (Bonnet, 2004; Livermore, 2012).

β-lactamase-producing enterobacteria have already been reported in companion and production animals, in clinical samples, and healthy animals (Johard et al., 2015). These bacteria can be transmitted from animals to people in the veterinary hospital environment. Due to the importance of this group of bacteria to animal and public health, the present study aimed to identify potentially ESBL-producing enterobacteria of types of *TEM*, *SHV*, and *CTX-M* present in a veterinary teaching hospital.

Material and Methods

Sample collection

Ten collections were carried out, at intervals of no less than 10 days, at the Veterinary Hospital of the Federal University of Jataí (UFJ) between January and July 2018. The collections included swabs on consultation tables, surgical tables, door handles, inpatient cages, stethoscopes, thermometers, muzzles, ultrasound device transducer, and ultrasound table, in addition to 50 mL of water from the clinic sinks. The swabs were placed in tubes containing nutrient broth, which were taken to the Laboratory of Veterinary Microbiology at the Federal University of Jataí, and incubated at 37°C for 24 h. The water was filtered through a Millipore membrane, and, after filtration, the membranes were placed on MacConkey agar plates and incubated together with the tubes.

Bacterial identification

The tubes with swabs that showed positive culture had an aliquot seeded on MacConkey agar plates, which were incubated at 37°C for 24 h. Afterward, five isolated colonies were used for species identification. The tests used for biochemical identification were: TSI (Triple Sugar Iron), MIO (motility, indole, and ornithine), MRVP (for the Methyl Red and Vorges-Proskauer test), citrate, urea, phenylalanine, and lysine. The readings of biochemical tests were performed within a period of 24 h to 48 h, respecting the recommendations by the manufacturer and Koneman et al. (2012). The isolates were stored in microtubes containing

750 µL of culture and 750 µL of 40% glycerol and stored in a freezer at -30°C.

Antibiogram test

Antibiogram of the isolates was performed using the disk diffusion method on a plate with Mueller Hinton agar. Antibiotics from several generations of β-lactams were tested: amoxicillin 30 mcg + clavulanic acid, piperacillin + tazobactam 110 mcg, ampicillin 10 mcg (penicillin), cephalotin 10 mcg (1st generation cephalosporin), cefoxitin 30 mcg (2nd generation cephalosporin), cefotaxime 30 mcg (3rd generation cephalosporin), 30 mcg cefepime (4th generation cephalosporin), and ertapenem 10 mcg (carbapenem) (Clinical and Laboratory Standards Institute, 2018).

Phenotypic test for ESBL

All isolates were submitted to the confirmatory phenotype test for ESBL production by the double-disk diffusion method, also called disk approximation. Isolates were tested with antibiotic disks containing 3rd generation cephalosporins and a β-lactamase inhibitor. The disks used were ceftriaxone, aztreonam, ceftazidime, cefotaxime, and amoxicillin + clavulanic acid. The β-lactamase inhibitor, amoxicillin + clavulanic acid, was placed in the center of the Mueller Hinton agar plate, and the other disks around it, respecting a distance of 20 mm from the central disk. The production of ESBL was positive when there was a deformation of the β-lactamase inhibition halo or the appearance of an irregular zone, called the phantom zone.

PCR

DNA extraction from the isolates was performed by thermal lysis, where 2 mL of pure culture of each isolate were centrifuged at 5,000 rpm for 4 min, discarding the supernatant, and washed with 1 mL of autoclaved distilled water. The washing process was carried out three times. After washing, the cells were subjected to a temperature of 100°C for 10 min, centrifuged at 5,000 rpm for 30 sec, and 500 µL of the supernatant was collected and stored in a freezer at -30°C. Conventional PCR was also performed for the positive isolates in the ESBL phenotypic test, investigating the *SHV*, *TEM*, and five *CTX-M* groups. The primer pairs used for the *SHV* gene were 5' AGG ATT GAC TGC CTT TTT G and 5'ATT TGC TGA TTT CGC TCG (392 bp), and for the *TEM* gene they were 5' TTG GGT GCA CGA GTG GGT TA and 5' TAA TTG CCG GGA AGC TA (465 bp) (Bajpai et al., 2017). For the five classes of *CTX-M*, the primers were: class 1, 5' AAA AAT CAC TGC GCC AGT TC and 5' AGC TTA TTC ATC GCC ACG TT (415 bp); class 2, 5' CGA CGC TAC CCC TGC TAT T and 5' CCA GCG TCA GAT TTT TCA GG (552 bp); class 9, 5' CAA AGA GAG TGC AAC GGA TG and 5' ATT GGA AAG CGT TCA CC (205 bp); class 08 TCG CGT TAA GCG GAT GC and 5' AAC CCA CGA TGT GGG TAG C (666 bp), and class 25, 5' GCA CGA TGA CAT TCG GG and 5' AAC CCA CGA TGT GGG TAG C (327 bp) (Woodford et al., 2006). Strains belonging to the bacterial collection of the Laboratory of Veterinary Microbiology of the Federal University of Jataí were used as a positive control.

PCR reactions were assembled with a final volume of 25 µL, 12.5 µL of which were Promega Mix (Promega Corporation, USA), 0.5 µL of each primer, 5 µL of ultrapure water, and 2.5 µL of DNA. The conditions for amplification were according to the methodology by Bajpai et al. (2017) for *TEM* and *SHV* and by Woodford et al. (2006) for *CTX-M*.

Results

Of the 306 samples collected, 118 were identified as Enterobacteriaceae, which represents a percentage of 19.6%. Those enterobacteria came from the following samples: thermometer, table, cage, water, doorknob, gutter, and noseband. The species identified were *Escherichia coli* (56/47.5%), and *Enterobacter* spp. (25/21.2%), *Klebsiella* spp. (19/16.1%), *Proteus mirabilis* (13/11.0%), *Serratia* spp. (3/2.5%), and *Citrobacter* spp. (2/1.7%). The distribution of species and the collection sites are described in Table 1. The antibiotic sensitivity test detected a high frequency of resistance to cephalothin (53.4%) and ampicillin (44.9%). Concerning ampicillin + clavulanic acid (20.9%), cefoxitin (19.4%), cefepime (16.1%), and cefotaxime (14.4%), lower resistance frequencies were observed. None of the enterobacteria showed resistance to ertapenem or the association of piperacillin with tazobactam. It can also be seen that 34 isolates were multiresistant, i.e., resistant to three or more classes of antibiotics.

Of the 118 enterobacteria, 17 isolates (14.4%) were positive for the production of β-lactamases. Among them, six isolates of *Enterobacter* spp., six of *Escherichia coli,* and five of *Klebsiella* spp. Of the 17 ESBL isolates, 10 (58.8%) came from the hospital area; four (23.5%), from the isolation area; two (11.7%), from the ultrasound room, and one, from the doctor's office. Table 2 shows the species identified as ESBL and their respective collection sites. Among the 17 isolates positive in the ESBL phenotypic test, none was positive for the *SHV* gene. Fifteen ESBL isolates (88.23%) were positive for the *TEM* gene: *Escherichia coli* (4), *Enterobacter* spp. (6), and *Klebsiella* spp. (5). And 15 ESBL isolates were also positive for the *CTX-M* gene, as

Table 1 – Enterobacteria circulating in the veterinary hospital environment and their respective collection sites and the corresponding percentage of species. Veterinary Hospital of the Federal University of Jataí (UFJ), Goias State, Brazil, between January and July 2018

	Wa*	Ut	Mu	Ca	Dk	Tb	The	Total (%)
Citrobacter spp.	02	—	—	—	—	—	—	02 (1.7)
Enterobacter spp.	04		03	04	03	11		25 (21.1)
Escherichia coli	—	02	—	10	03	13	28	56 (47.5)
Klebsiella spp.	07	01	—	—	04	07	—	19 (16.1)
Proteus mirabilis	—	—	—	—	—	—	13	13 (11.0)
Serratia spp.							03	03 (2.6)
Total (%)	13 (11)	03 (2.6)	03 (2.6)	14 (11.8)	10 (8.5)	31 (26.2)	44 (37.3)	118

*Wa: water, Ut: ultrasound table, Mu: muzzle, Ca: cage, Dk: doorknob, Tb: table, The: thermometer.

Table 2 – Species identified as ESBL, their respective collection sites, resistance phenotype, and genotypic profile. Veterinary Hospital of the Federal University of Jataí (UFJ). Goias State, Brazil, between January and July 2018

Isolate	Species	*TEM gene*	*CTX-M gene*	Resistance Phenotype*	Location (object)
1	*Escherichia coli*	+	+ (Group 1)	CPL-CTA-CEP-AMP	Isolation area (cage)
2	*Escherichia coli*	+	+ (Group 1)	CPL-CTA-CEP-AMP	Isolation area (cage)
3	*Escherichia coli*	+	+ (Group 1)	CPL-CTA-CEP-AMP	Isolation area (cage)
4	*Escherichia coli*	+	+ (Group 1)	CPL-CTA-CEP-AMP	Isolation area (table)
5	*Escherichia coli*	-	+ (Group 9)	CPL-CTA-CEP-AMP	Ultrasound room (table)
6	*Escherichia coli*	-	+ (Group 9)	CPL-CTA-AMC-CEP-AMP	Ultrasound room (table)
7	*Enterobacter* sp.	+	+ (Group 1)	CPL-CTA-CEP-AMP	Hospitalization area (cage)
8	*Enterobacter* sp.	+	+ (Group 1)	CPL-CTA-CEP-AMP	Hospitalization area (cage)
9	*Enterobacter* sp.	+	+ (Group 1)	CPL-CTA-CEP-AMP	Hospitalization area (cage)
10	*Enterobacter* sp.	+	+ (Group 1)	CXI-CPL-CTA-AMC-CEP-AMP	Hospitalization area (table)
11	*Enterobacter* sp.	+	-	CXI-CPL-CTA-AMC-CEP-AMP	Hospitalization area (table)
12	*Enterobacter* sp.	+	+ (Group 1)	CXI-CPL-CTA-AMC-CEP-AMP	Hospitalization area (table)
13	*Klebsiella* sp.	+	-	CPL-CTA-CEP-AMP	Doctor's office (table)
14	*Klebsiella* sp.	+	+ (Group 1)	CXI-CPL-CTA-AMC-CEP-AMP	Hospitalization area (water)
15	*Klebsiella* sp.	+	+ (Group 1)	CXI-CPL-CTA-AMC-CEP-AMP	Hospitalization area (water)
16	*Klebsiella* sp.	+	+ (Group 9)	CPL-CTA-AMC-CEP-AMP	Hospitalization area (water)
17	*Klebsiella* sp.	+	+ (Group 1)	CXI-CPL-CTA-AMC-CEP-AMP	Hospitalization area (water)

*CXI: Cefoxitin, CPL: Cephalotin, CTA: Cefotaxime, AMC: Clavulanic Acid + Amoxicillin, PIT: Piperacillin + Tazobactam, CEP: Cefepime, AMP: Ampicillin, ERT: Ertapenem.

follows: 12 isolates carried genes from group 1 and three isolates carried genes from group 9. Thirteen isolates had genes for the TEM and *CTX-M* variants concomitantly. The *CTX-M* group, predominant in the study, was group 1, corresponding to 80% of the *CTX-M* found.

Discussion

Antimicrobial resistance is observed in several species of microorganisms, threatening human and animal health, and being a unique one health challenge. In this sense, microorganisms can be naturally resistant to antibiotics or acquire this resistance through genetic mutation or horizontal gene transfer (Blair et al., 2015). The direct consequences of infection by resistant microorganisms can be serious, including longer illness and hospitalization periods, increased mortality, loss of antibiotic prophylaxis efficiency for patients undergoing operations, and, consequently, increased medical procedures and costs (Mendelson & Matsoso, 2015). β-lactam antibiotics, mainly cephalosporins, are the widest drugs used for animal treatment, so the increase in this resistance poses a problem for veterinary medicine (Wieler et al., 2015).

The veterinary medical environment, therefore, can be a site for the spread of resistant isolates. In this work, the most contaminated objects were the thermometers, from which 37.3% of the identified enterobacteria came, followed by the service desks, with 28.8%. Equipment and fomites can serve as reservoirs for infectious agents (Milton et al., 2014), as well as inadequately decontaminated thermometers can serve as a vehicle for pathogens, potentially causing hospital infections (Kanamori et al., 2017; van den Berg et al., 2000). The transmission of microorganisms through contaminated surfaces can occur directly or indirectly; in the latter case, through the hands of health professionals, through water and food (Kanamori et al., 2017; Lopéz-Cerero, 2014). Enterobacteria, identified in

this veterinary hospital environment, are the same ones of human medical importance already reported as an etiologic agent of hospital infections (Agência Nacional de Vigilância Sanitária, 2017). They are also of veterinary importance, as they cause urinary tract infections (Carvalho et al., 2014; Moyaert et al., 2017; Wong et al., 2015) and bloodstream infections (Stull & Weese, 2015). Therefore, it is essential to properly carry out the decontamination of these surfaces and fomites before and after the treatments, seeking to avoid the transmission of these microorganisms to patients.

In this study, 43 (36.44%) isolates did not show resistance to any of the antibiotics tested, whereas 34 (28.8%) were resistant to three or more categories of the β-lactams tested, being classified as multiresistant (Magiorakos et al., 2012). Cephalotin and ampicillin, respectively, were the least effective antibiotics. The association of piperacillin with tazobactam was more efficient than amoxicillin with clavulanic acid. Both are an association of a β-lactam with a β-lactamase inhibitor, but tazobactam has greater inhibitory activity on β-lactamases than clavulanic acid (Bush & Jacoby, 2010).

Enterobacteriaceae can carry antibiotic resistance genes, which can lead to a failure or delay in treatment and, in some cases, trigger death. Among the identified isolates, 17 were positive for the production of β-lactamases. Of those, five isolates were *Klebsiella* sp., which corresponds to 26.3% of the total *Klebsiella* sp. identified in the study. The second highest frequency of ESBL-producing isolates was found among *Enterobacter* sp. (24%). As in the study by Guzman-Blanco et al. (2014), the genus *Klebsiella*. was found to produce the most ESBL.

Most β-lactamase-producing isolates were identified from the hospitalization area (58.8%), followed by the isolation area (23.5%). The hospital environment requires adaptations to bacterial cells (Walther et al., 2017), as it is a large ecological niche for resistant bacteria due to the widespread use of antibiotics in this location (Oliveira et al., 2017). The hospitalization area is the place with the greatest flow and permanence of animals, while the isolation area is the place with the most debilitated animals. Both locations are where microorganisms are more likely to circulate and these are subject to horizontal gene transfer mechanisms.

Extended-spectrum β-lactamases hydrolyze penicillins and cephalosporins of 1st, 2nd, 3rd, and generally 4th generation, sparing carbapenems (Bush & Jacoby, 2010). In this work, the isolates identified as ESBL exhibited this phenotype, except for the 2nd generation cephalosporins, as 11 isolates were sensitive to it. Cefoxitin, the 2nd generation cephalosporin tested, belongs to the group of cefamycins, which are generally stable to a variety of β-lactamases (Nordmann & Poirel, 2017; Rubin & Pitout, 2014; Shaikh et al., 2015; Woerther et al., 2018).

Oliveira et al. (2016) found ESBL isolates with resistance patterns similar to those identified in this work, whereas Bogaerts et al. (2015) found 20% of isolates resistant to 4th generation cephalosporins, showing that resistance can be distributed differently among the bacterial populations studied.

CTX-M comprises the largest group of β-lactamases and is currently the most reported worldwide (Bevan et al., 2017; Dropa et al., 2016). Not much is known about the spread of isolates producing these enzymes in Brazil (Rocha et al., 2016). In recent decades, the profile of ESBL-producing isolates has changed, with *TEM* and *SHV* profiles being less reported than the *CTX-M* profile, which is also the most isolated type of ESBL in companion animals, while the class *CTX-M 1* is the most prevalent (Wieler et al., 2015). However, in a study carried out in a veterinary hospital in Brazil, a different scenario was found, where 100% of its ESBL isolates had the *TEM* variant, while 78.02% had some group of the *CTX-M* variant (Sfaciotte et al., 2021). In this work, most isolates carried both genes (Table 2). This demonstrates the importance of having more studies characterizing the profiles of ESBL isolates that circulate in veterinary hospitals, as the epidemiological reality of the various regions may be different. Studies carried out with companion animals in Europe (Bogaerts et al., 2015) and Switzerland (Zogg et al., 2018) also found the *CTX-M* variant as predominant among ESBL isolates, and the *CTX-M-1* type was the most frequent. In contrast, in this study, an equal prevalence of MET and *CTX-M* was found, but among the *CTX-M* identified, class 1 was the most prevalent, in agreement with the works mentioned above. That demonstrates that the circulation of isolates carrying *CTX-M-1* is also high in the veterinary hospital environment in Brazil.

The presence of multiresistant isolates carrying genes linked to the production of β-lactamases in the service desks (isolates 10 and 12) is also noteworthy. This highlights the importance of proper cleaning of these places, especially between medical consultations, as there is a risk that the hospital environment will contaminate the patients treated there. In addition, detection of multiresistant ESBL isolates and carriers of the *TEM* and *CTX-M-1* and *-9* genes in the water of the hospital room (isolates 14, 15, and 17), alerts to the possibility of contamination of the hospital environment being transmitted this way. This indicates that the microbiological quality control of the water supply of a veterinary hospital is a fundamental item in any hospital

biosafety control program and should never be neglected. This work also highlights the need for research seeking to better identify the prevalence of multiresistant bacteria in veterinary hospital environments.

Conclusion

Bacterial resistance is currently a major problem in one health. Multiresistant ESBL-producing enterobacteria (*Escherichia coli*, *Enterobacter* sp., and *Klebsiella* sp.), carrying the genes *TEM* and *CTX-M-1* and *-9*, were identified in the veterinary hospital environment. The establishment of good hygiene and disinfection practices in the veterinary hospital environment (adequate decontamination of surfaces and fomites), as well as the control of the microbiological quality of the water supply, is essential to minimize the possibility of transmission of those microorganisms among the animal and human populations.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics Statement

The authors declare no direct animal use in the experiment.

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