Universidade Nova de Lisboa
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Carbapenem resistance in Veterinary healthcare and in sick companion animals

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- **Joana Moreira da Silva.** *Hospital Infection and Control - What´s new?*. Oral communication at 8th International Congress of Veterinary Nursing. 19-20 January 2024, Elvas, Portugal
- **Joana Moreira da Silva**; Juliana Menezes; Laura Fernandes; Cátia Marques; Andreia J. Amaral; Constança Pomba. *Emerging challenges: environmental and staff colonization by carbapenem-resistant Gram-negative bacteria and MRSA in veterinary practices*. Flash poster communication at 34th ECCMID. 27-30 April 2024, Barcelona, Portugal
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To my family

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"You can, you should, and if you’re brave enough to start, you will."

Stephen King

Resumo

Os carbapenemos são antibióticos de última linha em medicina humana e o seu uso é proibido em medicina veterinária, para que a sua utilização terapêutica continue a ser possível. Contudo, os níveis de resistência estão a aumentar mundialmente e têm surgido descrições de resistência a esta classe em animais de companhia.

Na primeira parte desta tese, foi feita uma avaliação da prevalência de Enterobacterales produtores de carbapenemases em Portugal, provenientes de estirpes clínicas de animais companhia, assim como uma caracterização dos seus elementos genéticos móveis. Dos 977 isolados clínicos de Enterobacterales originários de um laboratório de diagnóstico veterinário, 261 isolados resistentes foram estudados e a sequenciação do genoma completo foi feita para estirpes produtoras de carbapenemases. A frequência observada foi de 0,51% (n=5/977) e incluiu uma *Klebsiella pneumoniae* ST273 produtora de OXA-181, duas *K. pneumoniae* ST147 produtoras de KPC-3, uma *K. pneumoniae* ST392 produtora de KPC-3 e uma *Escherichia coli* ST127 produtora de OXA-48. O gene *bla*$_{\text{KPC-3}}$ encontrava-se no transposão Tn*4401d* em plasmídeos do tipo IncFIA e IncN, enquanto o gene *bla*$_{\text{OXA-181}}$ encontrava-se num plasmídeo do tipo IncX3. Todos os plasmídeos e transposões eram homólogos aos descritos em medicina humana. Estas descobertas sugerem a ocorrência de uma disseminação horizontal destas bactérias de humanos para animais de companhia, realçando a importância de realizar o rastreio de resistência aos carbapenemos em laboratórios de diagnóstico veterinário e a implementação de medidas de prevenção e controlo de infeção (PCI) para que se evite a disseminação destas bactérias na comunidade.

Com o aumento do número de animais de companhia por família, acompanhado pela evolução dos níveis de cuidados prestados em centros de atendimento médico-veterinários (CAMV), é expectável que o número de infeções nosocomiais provocadas por bactérias multirresistentes venha a aumentar, à semelhança do que ocorre em hospitais humanos. Contudo, contrariamente ao que existe em medicina humana, onde protocolos de PCI monitorizam o surgimento de bactérias multirresistentes no ambiente, em medicina veterinária a implementação destes protocolos está aquém do expectável.

Assim, a avaliação do nível de contaminação ambiental e colonização das equipas foi realizada em diferentes CAMV em Portugal. Catorze CAMV foram avaliados, com amostragem de superfícies críticas e colheita de amostras nasais, retais e de mãos da equipa. Para todas as amostras, foram rastreadas bactérias Gram-negativas produtoras de beta-lactamases de espetro alargado e/ou carbapenemases. As concentrações mínimas inibitórias de imipenemo e meropenemo foram calculadas para bactérias produtoras de carbapenemases. A sequenciação do genoma completo foi feita para bactérias resistentes aos carbapenemos.

A avaliação ambiental demonstrou que 6,5% das superfícies testadas estava contaminada com bactérias Gram-negativas multirresistentes, nomeadamente: i) um CAMV tinha *Acinetobacter* spp. produtor de OXA-23 (n=5); ii) outro CAMV tinha *Pseudomonas juntendi* produtora de IMP-8; iii) isolados de *Stenotrophomonas maltophilia* (n=12) e *Pseudomonas aeruginosa* (n=3) foram encontrados em várias superfícies de múltiplos CAMV. Estirpes de *P. aeruginosa* resistentes aos carbapenemos, que apresentavam mutações na porina OprD, foram isoladas de duas amostras retais e uma amostra de mãos. Estirpes de *S. maltophilia* foram encontradas no total de quatro amostras (duas retais, duas de mãos). Uma amostra nasal testou positivo para *K. pneumoniae* ST11 resistente aos carbapenemos por truncatura da OmpK36. Estes resultados apontam para o papel que os CAMV poderão ter na disseminação de bactérias multirresistentes. Adicionalmente, torna-se claro que é fundamental implementar protocolos de PCI, auditados com regularidade, em conjunto com *workshops* educacionais para estudantes e profissionais de medicina veterinária.

Em conclusão, bactérias resistentes aos carbapenemos estão presentes em animais de companhia doentes e no ambiente dos CAMVs, evidenciando o papel que estes setores têm na disseminação destas bactérias no contexto da Uma Só Saúde.

Palavras-chave: Carbapenemos; Animais de Companhia; Medicina Veterinária; Bactérias Gram-Negativas; PCI

Abstract

Carbapenems are last-line antibiotics in human medicine and are of prohibited use in veterinary medicine as a way to preserve their therapeutic use for humans. Nonetheless, resistance levels are increasing and reports of resistance in companion animals are appearing.

In the first part of this dissertation, an evaluation on the prevalence of carbapenemase-producing Enterobacterales (CPE) clinical strains in companion animals in Portugal and characterization of their mobile genetic elements was performed.

Out of the 977 Enterobacterales clinical strains collected from a Portuguese veterinary diagnostic laboratory, 261 resistant strains were studied and whole-genome sequencing was conducted for carbapenemase-producing strains. The frequency of CPE in companion animals was 0.51% (n=5/977) and included one OXA-181-producing *Klebsiella pneumoniae* ST273, two KPC-3-producing *K. pneumoniae* ST147, one KPC-3-producing *K. pneumoniae* ST392, and one OXA-48-producing *Escherichia coli* ST127. The $bla_{\text{KPC-3}}$ gene was located on transposon Tn*4401d* on IncFIA and IncN type-plasmids, while the $bla_{\text{OXA-181}}$ gene was on an IncX3 type-plasmid, with all plasmids and transposons harbouring carbapenemase genes homologous to those in human healthcare. Such finding suggested an horizontal dissemination from humans to companion animals, stressing the importance of implementing carbapenemase-screening methods in veterinary diagnostic laboratories and implementation of infection, prevention and control (IPC) measures to avoid the spreading of resistant bacteria onto the community, prompting the second part of this dissertation.

With the increasing number of companion animals per family and the evolution of care offered by veterinary practices, it is expected that the number of nosocomial infections to increase, similarly to human medicine. Yet, contrary to what exists in human medicine, where IPC protocols exist to monitor the appearance of multidrug-resistant (MDR) bacteria, in veterinary medicine there is still a gap in the implementation of IPC.

Thus, environmental contamination and staff carriage of multidrug-resistant organisms (MDROs) in veterinary practices across Portugal was evaluated. A total of fourteen SAVPs were enrolled, and environmental samples were collected from critical areas. Additionally, veterinary team members provided nasal, hand, and rectal swabs. All samples were screened for ESBL- and carbapenemase-producing Gram-negative bacteria, while minimal inhibitory concentrations (MIC) for imipenem and meropenem were determined for the latter. Whole-genome sequencing was performed for carbapenem-resistant strains.

Environmental evaluation showed that 6.5% (n=32/490) of surface swabs were contaminated with multidrug-resistant Gram-negative bacteria, specifically: i) OXA-23-producing *Acinetobacter* spp. (n=5) in one SAVP; ii) IMP-8-producing *Pseudomonas juntendi* (n=2) strains in one SAVP; iii) *Stenotrophomonas maltophilia* (n=12) and *Pseudomonas aeruginosa* (n=3) strains were found on multiple surfaces of different SAVPS. Carbapenem-resistant *P. aeruginosa* strains with *oprD* mutations were found on two rectal and one hand samples, while *S. maltophilia* strains were recovered from four samples (two rectal, two hands). One nasal swab was positive for carbapenem-resistant *K. pneumoniae* ST11 by ompK36 truncation.

These findings indicate that SAVPs may significantly contribute to the dissemination of MDROs. It is clear that strict IPC strategies must be implemented and regularly revised, together with educational workshops for veterinary healthcare students and professionals.

In conclusion, carbapenem-resistant bacteria were found in clinical strains from sick companion animals and in the environment of veterinary healthcare, showing the role of small animal quadrant in the dissemination of this type of resistance under the One Health context.

Keywords: Carbapenems; Companion animals, Veterinary healthcare, Gram-Negative Bacteria, IPC

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Abbreviation List

- ABC - ATP binding cassette
- AMR – Antimicrobial resistance
- AST – Antimicrobial susceptibility testing
- BTC - Blue Carba test
- CFU – Colony forming unit
- CG – Clonal group
- CHDL - Carbapenem-hydrolysing class D β-lactamases
- CIM – Carbapenem inactivation method
- CP – Carbapenemase-producing
- CPE – Carbapenemase-producing Enterobacterales
- CRBSI - Catheter-related bloodstream infection
- CRE – Carbapenem-resistant Enterobacterales
- CRAB – Carbapenem-resistant *Acinetobacter baumannii*
- EARS-Vet - European Antimicrobial Resistance Surveillance network in veterinary medicine
- ECDC – European Centre for Disease Prevention and Control
- EMA – European Medicines Agency
- ESCMID - European society of clinical microbiology and infectious disease
- ESBL - Extended-spectrum β-lactamases
- EU – European Union
- HAI – Healthcare-associated infection
- HGT – Horizontal gene transfer
- IDSA - Infectious diseases society of America
- IPC – Infection, prevention and control
- IS – Insertion sequence
- LAMP - Loop-mediated isothermal amplification
- MALDI-TOF MS - Matrix-assisted laser desorption ionization-time of flight mass spectrometry
- MATE - Multidrug and toxin extrusion
- MBL – Metallo-β-lactamases

- MDR – Multidrug-resistant
- MDROs – Multidrug-resistant organisms
- MFS - Major facilitator superfamily
- MIC - Minimal inhibitory concentration
- mNGS - Metagenomic next-generation sequencing
- MRSA – Methicillin-resistant *Staphylococcus aureus*
- PBP – Penicillin-binding protein
- PPE – Personal protective equipment
- RND - Resistance nodulation-division
- SMR - Small multidrug resistance
- SNP – Single-nucleotide polymorphisms
- SSTI - Skin soft tissue infection
- ST – Sequence type
- URTI - Upper respiratory tract infections
- UTI – Urinary tract infection
- WGS – Whole-genome sequencing
- WHO – World Health organization

Chapter 1 General introduction

1.1 Healthcare-associated infections and ESKAPE pathogens

In the last years, it is estimated that 1.27 million deaths, globally, were attributable to antimicrobial resistance (AMR) (1). The microorganisms responsible for the majority of deaths are *Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa.*

Some of these organisms are also the most commonly isolated from healthcare-associated infections (HAI). These are defined as infections acquired by patients during hospitalization, occurring within the first 48 hours post-admission or within 30 days after discharge (2). Nowadays, HAIs pose difficulties for society and healthcare organizations as they increase patient morbidity and mortality, leading to extended treatment periods, consequently increasing economic costs (3).

According to the latest report on HAIs from the European Centre for Disease Prevention and Control (ECDC), 4.3 million human patients in Europe acquired at least one HAI in 2022-2023, resulting in a prevalence of 8% (4). Within the reported organisms, *E. coli* was the most prevalent (12.7%), followed by *Klebsiella* spp. (11.7%). Furthermore, *P. aeruginosa* and *A. baumannii* were also isolated, albeit in lower percentages (7.9% and 3.2%, respectively).

K. pneumoniae, *Acinetobacter* spp., and *P. aeruginosa* are part of the ESKAPE pathogens group, a significant cluster of microorganisms responsible for HAIs. The Infectious Diseases Society of America (IDSA) coined the acronym ESKAPE to highlight the clinical relevance of these bacteria due to their ability to evade antibiotic action, setting new standards for pathogenesis, transmission, and antibiotic resistance (5,6). ESKAPE stands for *Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species. Other bacteria, such as *E. coli* and *Stenotrophomonas maltophilia*, should also be considered part of this group due to their recent links to HAIs (1,4). Although *S. maltophilia* has a significant lower prevalence, its intrinsic resistance to multiple classes of antibiotics, via efflux pumps, makes treatment challenging, so ECDC recommends its monitorization despite its low prevalence (4).

Also recognizing the critical threat posed by these pathogens, in 2017, the World Health Organization (WHO) published a priority list of pathogenic agents needing urgent research and development of antibiotics (7). This list includes carbapenem-resistant *P. aeruginosa*, carbapenem-resistant *A. baumannii* (CRAB), and carbapenem-resistant and extended-spectrum β-lactamase-producing Enterobacterales (ESBL) as top priorities due to their significant threat to healthcare systems. In 2024, an update to this list was published and while no changes were observed to Enterobacterales and carbapenem-resistant *A. baumannii* categorization, carbapenem-resistant *P. aeruginosa* was demoted to high priority due to an apparent decrease in resistance in at least a region, as well as lower transmission of resistant strains when compared to other carbapenem-resistant species (8). Another major change was the discrimination of Enterobacterales bacterial species and its resistance, with carbapenem-resistant *K. pneumoniae* being first priority, followed by 3rdgeneration cephalosporin resistant *E. coli* and CRAB.

Resistant Enterobacterales, mainly *E. coli* and *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* have also been described as causative agents of infection in companion animals (9,10). Considering these pathogenic bacteria's ability to evade antibiotic treatment, with some antibiotics overlapping in human and veterinary medicine (11) and with veterinary medicine being an integral part of One Health, it is important to consider how resistance can disseminate in different settings (12), and specially what role humans and companion animals play in it.

1.2 Carbapenems – a fading hope

Antibiotics are chemical compounds responsible for inhibiting bacteria, thus stopping the further development of an active infection (13). There are multiple classes of antibiotics, each with a specific target for action. Nonetheless, antibiotic resistance has been increasing due to bacterial evolution, causing changes in the bacteria that lead to the drug of choice being ineffective.

Among the most frequently used antibiotic classes, both in human and small animal veterinary medicine, are β-lactams (14,15). This class of antibiotics inhibits bacteria growth by covalently binding to penicillin-binding proteins (PBP), thus preventing the synthesis of the peptidoglycan, which is the main component of bacterial cell wall (13).

β-lactams antibiotics are divided in four different categories: penicillins, cephalosporins, monobactams and carbapenems (13). The main resistance mechanism in Gram-negative bacteria against the β-lactams is the production of β-lactamases. The most frequently encountered are ESBL, usually associated with mobile genetic elements, which contribute to the fast rise in resistance and continuous dissemination (16).

With the emergence of ESBL genes, carbapenems became last-line antibiotics for the treatment of human patients suffering from infections caused by ESBL-producing Enterobacterales (17). To protect their efficacy and prevent the spread of resistant strains, the WHO released an updated list of critically important antimicrobials in 2024 (11). This list took into consideration the increasing trend of resistance against carbapenems and how important it is to preserve their use for human medicine. Thus, carbapenems are classified as of restricted use to human medicine, while prohibiting their use in veterinary medicine. The purpose of this classification is to preserve, for as long as possible, the use of carbapenems in treating severe infections while trying to prevent further resistance to spread.

Carbapenems first came to use in 1985 with the introduction of imipenem – this necessity came after an increasing resistance to 3rd generation cephalosporins (18). Similar to other β-lactams, carbapenems function as mechanism-based inhibitors of the peptidase present in PBP. They can bind to multiple PBPs while evading degradation by most β-lactamases, thus preventing the peptidoglycan from being structurally sound and causing cell lysis.

There are seven carbapenems: biapenem, doripenem, ertapenem, faropenem, imipenem, meropenem and panipenem (19). In the European Union, only ertapenem, imipenem and meropenem have commercial formulations approved by European Medicines Agency (EMA) for human use.

All formulations have to be intravenously administered and excreted via kidney (18,19), present little side-effects when compared to other current last-resort antibiotics against Gram-negative bacteria such as polymyxins (20). Due to their mode of action, carbapenems have been grouped: i) low-activity carbapenems which possess limited activity against Gram-negative agents, being suitable only for community-acquired infections, such as ertapenem; ii) broad-spectrum carbapenems which are active against Gram-negative bacteria, including non-fermentative agents (*Pseudomonas* spp; *Acinetobacter* spp. and *Stenotrophomonas* spp.) – imipenem and meropenem (19,21).

Despite the wide spectrum of action and the mostly reserved use for life-threatening ESBL infections(20), there has been a growing trend of increased carbapenem-resistance in recent years.

1.2.1 Evolution and epidemiology of carbapenem resistance

The annual epidemiological report of 2022 from the ECDC (22) revealed there's been a rise in the isolation of carbapenem-resistant *K. pneumoniae* in European countries, increasing to a prevalence of 10.9% (as oppose to the 9.0% in 2019), with a 50% rise in the incidence of bloodstream infections between 2019-2022. The same report also concluded that carbapenem resistance was common in multidrug-resistant (MDR) *Klebsiella* spp., which limits the therapeutic options available. It is of importance to note that, similar to previous years, *K. pneumoniae* carbapenem resistance is higher in eastern and southern Europe (Figure 1A). Conversely, when looking at the *E. coli* data, no changes were detected in carbapenem resistance during the same period of time. Moreover, across all participating European countries, the prevalence of carbapenem-resistant *E. coli* is lower than 1% (Figure 1B).

Although no changes overtime were detected for non-fermenting Gram-negative bacteria, carbapenem-resistant *Acinetobacter* spp. were recovered in 36.3% of isolates, while carbapenem-resistant *Pseudomonas* spp. accounted for 18.6% of isolates (Figure 1 C and D).

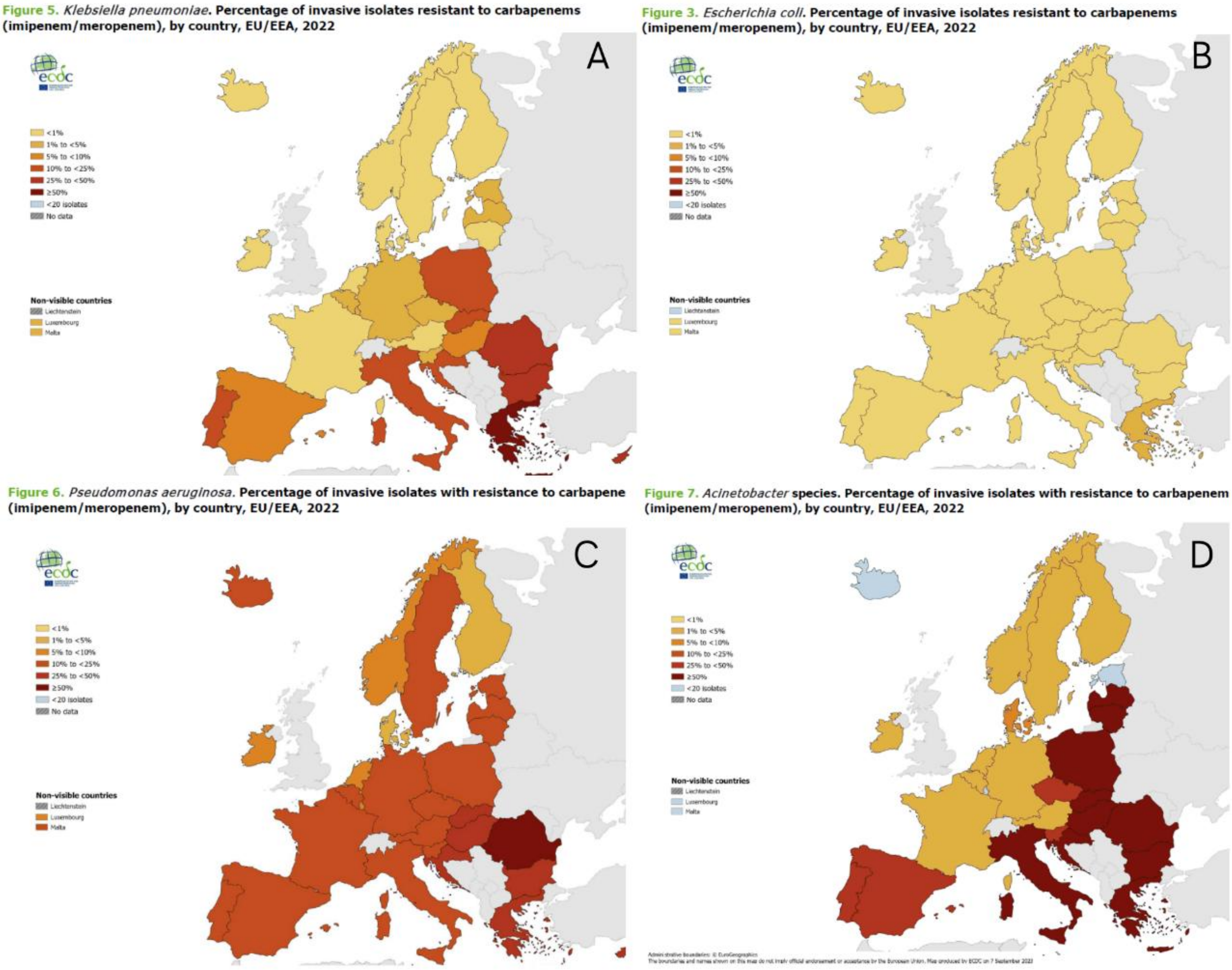

Figure 1 – Pattern of carbapenem-resistant *K. pneumoniae* (A); *E. coli* (B); *P. aeruginosa* (C) and *Acinetobacter* spp. (D) in Europe. Reprinted from European Centre for Disease Prevention and Control. Antimicrobial resistance in the EU/EEA (EARS-Net) -Annual Epidemiological Report 2022. Stockholm: ECDC; 2023 (22).

Part of the issue regarding the dissemination of carbapenem-resistant pathogens lies in controlling patient-to-patient direct and indirect transmission. A risk factor for the appearance of HAI is prior colonization by one of the causative agents (23,24), even in settings where the prevalence of HAI is low. Studies have shown that patients who are colonized or have active clinical infections by carbapenem-resistant bacteria can be reservoirs for dissemination to other patients (25). In an attempt to mitigate transmission and curtail the increase in reported resistance, European guidelines recommend patients to be actively screened for carbapenem-resistant Enterobacterales (CRE) colonization if they have a history of hospital stays in the last year, a known history of CRE carriage or contact with a known CRE carrier (25–27).

Carbapenem resistance can be acquired by several bacterial genera and be associated with multiple mechanisms of resistance (28). The most commonly described is the acquisition of carbapenemases (β-lactamases with versatile hydrolytic capacities) followed by porin loss and increased efflux pump activity (29), which will all be described further ahead. Carbapenemases, in particular, are frequently coded in mobile genetic elements, disseminated by horizontal gene transfer, which justifies its spreading and increasing levels of prevalence.

1.2.2 Horizontal gene transfer

Horizontal gene transfer (HGT) occurs between bacteria by the dissemination of mobile genetic elements that carry antibiotic resistance genes. These mobile genetic elements are plasmids, transposons and insertion sequences (IS) (Figure 2).

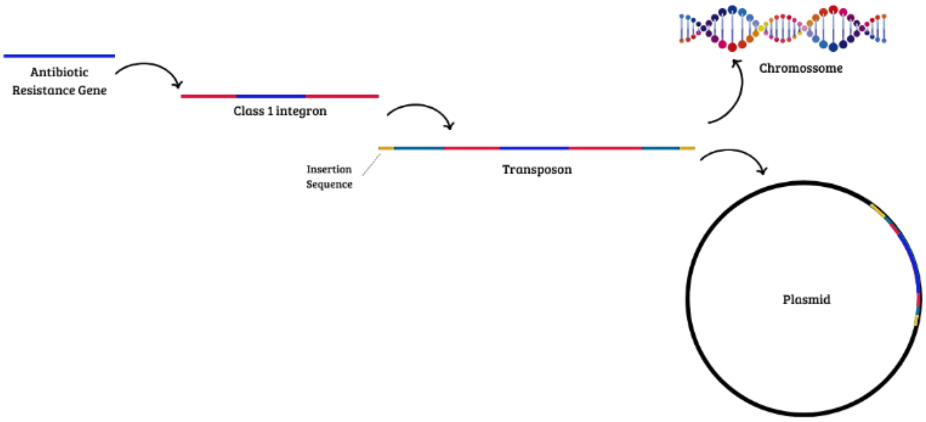

Figure 2 – Mobile Genetic elements and their composition. Gene cassettes (where the antibiotic resistance gene is located) is inserted into integrons by integrase-mediated site-specific recombination. Then, integrons are inserted into composite transposons, flanked by insertion sequences. Lastly, the transposon is inserted into a conjugative plasmid, which becomes the main vehicle of dissemination for AMR resistance**.**

Plasmids are the most described and studied mobile genetic element and have long been associated with HGT (30,31). There are three types of plasmids, depending on the machinery linked to DNA transfer: conjugative, mobilizable and non-mobilizable. The former not only possess the machinery to easily disseminate between different bacterial hosts, but are, generally, also rich in AMR genes pertaining to different antibiotic classes, leading to MDR phenotypes (31). Moreover, conjugative plasmids enable the transfer of AMR genes across far-apart phylogenetic bacterial lineages, while connecting human-pathogenic bacteria worldwide (30,31). Thus, it should come as no surprise that the spread of resistance is occurring at a fast pace and on a global scale for certain resistance determinants, with the same AMR genes being described on different bacterial species (30,32).

Transposons are transposable elements, whose transfer is mediated by the enzyme transposase. They can be divided in composite- and non-composite transposons. The former are delimited by IS, while the latter are not (33). As for IS, they are flanked by inverted repeats and similar to transposons, can be disseminated across different bacterial genomes by means of a transposase. Both of these genetic elements are strongly linked to AMR genes and their transfer within plasmids (30,31,34).

As for integrons, although these are not mobile genetic elements, they allow cells to capture and express exogenous gene cassettes with antibiotic resistance genes (35,36). This ability to acquire multiple gene cassettes confers genomic plasticity and advantages to its bacterial host to thrive in a certain environment. Considering a hospital setting, where antibiotics and detergents/disinfectants use is common, pathogenic bacteria that secure gene cassettes in integrons, allowing them to be more virulent or resistant, will be at a survival advantage (35,37). Of the different existing integron classes (classes 1, 2, 3, 4), the most studied are class 1 integrons as these are commonly found in pathogenic bacteria (37).

1.2.3 Carbapenemases

Following the Ambler classification, carbapenemases are divided into three classes: i) Ambler class A; ii) Ambler class B; iii) Ambler class D (28).

Of all the previously mentioned Gram-negative bacteria, *S. maltophilia* chromosomally harbours two carbapenemases, L1 and L2. L1 is a serine β-lactamase, while L2 is a metallo-β-lactamase (38,39). Additionally, a mutation can occur in the regulatory genes of these proteins, leading to an overexpression of these enzymes (39).

1.2.3.1 Class A

Starting with Ambler Class A carbapenemases , these correspond to KPC, IMI/NMC, SFC and GES enzymes due to their hydrolytic mechanism, which involves a serine at the active site position. These confer resistance to penicillin's, first-, second- and third-generation cephalosporins, plus meropenem and imipenem (40). Additionally, they are inhibited by β-lactamases inhibitors (41).

KPC-like enzymes are the most disseminated carbapenemases worldwide, having been associated with multiple *K. pneumoniae* resistant clonal lineages (42). In Europe, KPC-2- and KPC-3- (single nucleotide difference) producing *K. pneumoniae* are the most described, with high-risk clonal group (CG) 258 being dominant in the early 2010s. This CG comprises three sequence types (ST): ST258, ST11 and ST512 (42) – for example, in Greece and Poland, it is associated with the carriage of $bla_{\text{KPC-2}}$, while in Italy and Israel it harbours $bla_{\text{KPC-3}}$ (43,44). Lately, other CGs have been emerging such as CG147 [which comprises ST147, ST392 and ST273 (45)], CG307, CG15, among others (42,46–48).

Different genetic environments for $bla_{\text{KPC-like}}$ genes have been described. These genes are usually associated with transposon Tn*4401*, which is delimited by two inverted repeated sequences which harbour the carbapenemase gene, transposase and resolvase genes, with insertion sequence ISKpn7 upstream of $bla_{\text{KPC-like}}$ gene and ISKpn6 downstream of $bla_{\text{KPC-like}}$ gene (49). So far, four isoforms (*Tn4401a – d*) have been identified, depending on alterations observed between ISKpn7 and $bla_{\text{KPC-like}}$ gene, yielding different promoter regions to the carbapenemase gene and consequently, leading to different expression levels (50). Additionally, carriage of $bla_{\text{KPC-like}}$ genes in plasmids from different incompatibility groups has been described. Briefly, IncFIA, IncX3, IncN, ColE, among others, have been described in different settings as harbouring the $bla_{\text{KPC-like}}$ gene (44,47,48), together with the different isoforms of the transposons. This genetic plasticity and mobility has been championed for intra and inter-species dissemination, which turned the carbapenem-resistant *K. pneumoniae* into a WHO top priority pathogen (8).

In Portugal, the first description of KPC-3-producing *K. pneumoniae* ST11 human nosocomial isolate was reported in 2009 (51). Since then, KPC-3 carbapenemase is the most common cause of carbapenem-resistance in *K. pneumoniae* in Portugal, and is usually linked to non-CG258 strains, namely ST147, ST15, ST307 (46,52–54). Regarding the genetic environment of $bla_{\text{KPC-3}}$ gene, very little data exists from Portuguese isolates. A study conducted in 2016 showed that all the analysed *K. pneumoniae* strains harboured $bla_{\text{KPC-3}}$ gene in Tn*4401d,* associated with different incompatibility group plasmids, namely IncFIA, IncFII, and ColE (48).

Descriptions of KPC-like-producing *P. aeruginosa* and KPC-like-producing *A. baumannii* have been made. However, this type of resistance mechanism is more frequent in South America and Asia, opposed to Europe (55,56).

1.2.3.2 Class B

Class B carbapenemases (also known as metallo-β-lactamases) include IMP, VIM, GIM and NDM enzymes, which require zinc as a cofactor. A large proportion of encoding genes for IMP and VIM are found on gene cassettes inserted on class 1 integrons (57). Like KPC-encoding genes, most metallo-β-lactamases are disseminated by conjugative plasmids (57) and unlike serine carbapenemases, they are not inhibited by β-lactamases inhibitors (41). Moreover, they possess other characteristics granting high concern: i) absence of clinically relevant metallo-β-lactamase inhibitors; ii) increasing number of new variants; iii) transferability of encoding genes; iv) ubiquity in the environment (41,58).

In Europe, the prevalence of IMP-producing Enterobacterales accounted for 0.2% of carbapenem-resistant isolates observed (42). Additionally, a reference laboratory in Spain had a prevalence of 0.4% of IMP-producing Enterobacterales, with a *bla*$_{\text{IMP-8}}$ carbapenemase gene accounting for the most prevalent (59) in *K. pneumoniae*, whereas reports in the rest of Europe are scarce.

On the other hand, NDM-like-producing Enterobacterales and VIM-like-producing Enterobacterales are slightly more predominant, with a prevalence of 4.8% and 3.4%, respectively (42). An epidemiological study conducted in the Netherlands had a 16% prevalence of *bla*$_{\text{NDM-1}}$ gene (29), while in Germany there was a rise in the number of NDM-5-producing *E. coli*, becoming the third most prevalent carbapenemase (60). An outbreak in Italy between 2018-2019 was caused by NDM-1-producing *K. pneumoniae* ST147, of which 77.2% of cases were intestinal carriage, resulting from rectal screening surveillance (61). Data from Asian studies demonstrate the plasticity of NDM-like carbapenemases, being found in plasmids with different incompatibility groups such as IncX3, IncF, IncC (62,63).

Lastly, VIM-producing Enterobacterales are probably the less reported in Europe, being responsible for sporadic outbreaks and having a low epidemiological expression (29,42,64,65). Nonetheless, a few European countries have high rates of VIM-producing Enterobacterales, namely Poland, Hungary and Greece, and similar to other carbapenemase genes, it is found in association with variable genomic environments, from different transposons to plasmids' of varying incompatibility groups (66–68).

Contrary to the landscape of serine carbapenemases, there is a higher prevalence of non-fermenting Gram-negative bacteria harbouring metallo-β-lactamases. VIM-2-producing *P. aeruginosa* has a prevalence of 53.6%, followed by *bla*$_{\text{VIM-1}}$ gene (14.3%) in Germany, followed by an equal proportion of IMP-7 and NDM-like carbapenemase (7.1%) (69). Yet, in a European multicentric study, only 5.1% of the collected *P. aeruginosa* were carbapenemase producers, despite 27.4% of all *P. aeruginosa* presenting a phenotype of meropenem resistance (70). Conversely, the prevalence of *A. baumannii* harbouring metallo-β-lactamases is rare in Europe, with only one report of NDM-1-producing *A. baumannii* in France, Germany, Slovenia and Switzerland (71).

Similarly, in Portugal, reports on Gram-negative bacteria harbouring metallo-β-lactamases are rare, with the most recent case being a NDM-1-producing *K. pneumoniae* outbreak in a tertiary hospital in the Lisbon area (72). VIM-2-producing *P. aeruginosa* have also been described, as well as IMP-5-producing *A. baumannii*, both events in the central region of Portugal (73,74).

1.2.3.3 Class D

Class D β-lactamases are also called oxacillinases. Not all oxacillinases are capable of hydrolysing carbapenems – the ones that do are also called carbapenem-hydrolysing class D β-lactamases (CHDL) (75). The most predominant are OXA-48-like carbapenemases, which include the variant OXA-181 (four amino-acid substitutions). These carbapenemases have been reported in different Enterobacterales, with a higher association with *K. pneumoniae* and *E. coli* (42,75,76).

Multiple genomic environments have been associated with these carbapenemases. OXA-48-producing *E. coli* reports are common worldwide (75,77), having been disseminated across different STs (78). Despite being usually associated with plasmid transmission, reports of chromosomal insertion are emerging (76,79). The same pattern is observed in *K. pneumoniae*, although it was possible to pin-point *bla*$_{\text{OXA-48-like}}$ genes spreading to a single plasmid – pOXA-48 (Inc L/M) (80). However, other studies have also reported the occurrence of *bla*$_{\text{OXA-48-like}}$ genes in IncX3 (81,82).

The first report of an OXA-48-producing *E. coli* and *Enterobacter cloacae* in Portugal was published in 2014 (83). After 10 years, OXA-181-producing *K. pneumoniae* is commonly found in Portuguese hospitals, together with KPC-3, with some strains harbouring both carbapenemase genes (46,84). As previously mentioned, OXA-181-producing *K. pneumoniae* are usually linked to non-CG258 clones (46,84). As for the genetic environment of *bla*$_{\text{OXA-181}}$ gene, very little data exists from Portuguese isolates, with multiple plasmid types having been associated with it (46).

In the case of *Pseudomonas* spp. and *Acinetobacter* spp., these intrinsically possess CHDLs, which are used as markers for species identification. *P. aeruginosa* carry *bla*$_{\text{OXA-50-like}}$ genes (85), while *A. baumanni* carry *bla*$_{\text{OXA-51-like}}$ genes and other *Acinetobacter* spp. harbour other variants (86,87). Yet, the genetic environment of these CHDLs needs to be adequate for them to be expressed and hydrolyse carbapenems – the best example is the occurrence of OXA-23 in *Acinetobacter radioresistens*, which is the naturally occurring CHDL for this species. Yet, *A. radioresistens* are commonly susceptible to carbapenems since *bla*$_{\text{OXA-23}}$ is not expressed due to the lack of ISAbA1. This carbapenemase gene is only expressed in the presence of two IS, like is the case of OXA-23-producing *A. baumannii* (88–90).

1.2.4 Efflux pumps

Efflux pumps are transport proteins that expel toxic substances from inside the cell to the surrounding environment, including clinically important antibiotics (91). Alterations to the structure of these efflux pumps, can cause antibiotic resistance, being a recognized resistance mechanism. These efflux systems fall into five classes – multidrug and toxin extrusion (MATE), small multidrug resistance (SMR), major facilitator superfamily (MFS), ATP binding cassette (ABC), and the resistance nodulation-division (RND) (Figure 3). For the purposes of this dissertation, focus will be put on RND efflux pumps (91,92).

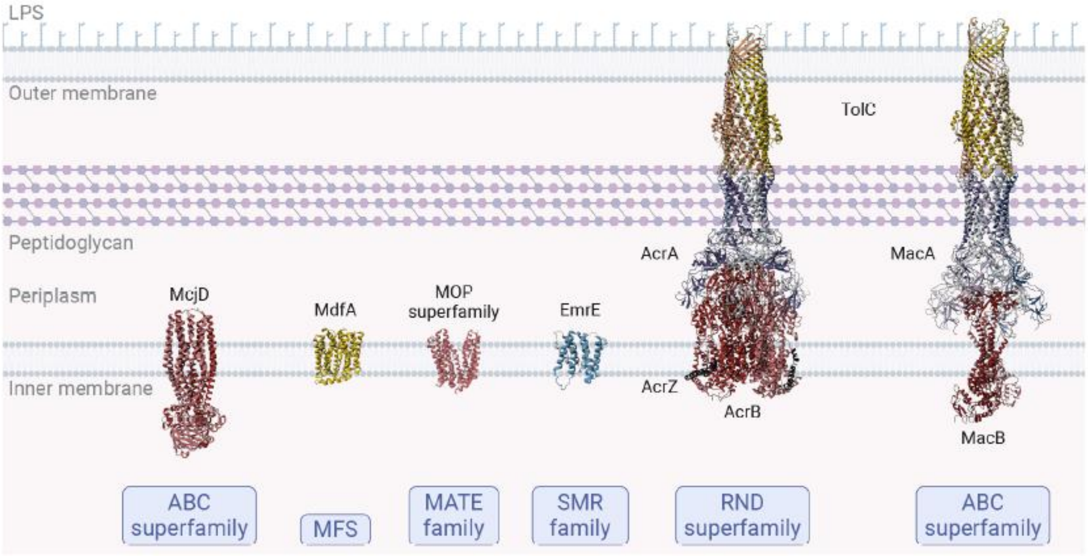

Figure 3 – Representative efflux pumps and respective structures for the main MDR efflux pump families in Gram-negative bacteria. ATP-binding cassette (ABC), major facilitator (MFS) superfamilies, multidrug and toxin extrusion (MATE), small multidrug resistance (SMR), and resistance–nodulation–cell division (RND) families. Reproduced from Duffey *et al*., 2024 (92), under the license CC-BY-NC-ND 4.0**.**

RND efflux pumps are exclusive to Gram-negative bacteria and have a tripartite structure, which consists of an inner membrane element, an adaptor protein in the periplasm and outer membrane component. Similar to other efflux pumps, it has many different substrates, including different classes of antibiotics such as beta-lactams (e.g carbapenems). The best characterized RND efflux systems are those found in *E. coli* and *P. aeruginosa* - AcrAB-TolC and MexAB-OprM, respectively (93).

The AcrAB-TolC system plays a crucial role in carbapenem resistance. Studies have revealed that this system's efflux pump expression is contingent upon the concentration of carbapenems present (94,95). Specifically, when exposed to carbapenems, the *acrA* (membrane fusion gene) and *acrB* gene expression increases, contributing to carbapenem resistance. Additionally, mutations in the *tolC* gene lead to its overexpression and further antibiotic resistance.

P. aeruginosa possesses multiple RND efflux pumps, of which only MexAB-OprM and MexXY-OprM are expressed on a basal level even on wild-type strains (96) (Table 1).

Table 1 – RND efflux systems present in *P. aeruginosa* and respective substrates. Adapted from Scoffone et al. 2021 (96).

RND Efflux Pump	Identified Regulators	Substrates
MexAB-OprM	MexR, repressor (MarR-type regulator)	β-lactams except imipenem; β-lactams inhibitors; fluoroquinolones; tetracycline; chloramphenicol; macrolides
MexCD-OprJ	NfxB, repressor (TetR/AcrR-type regulator)	β-lactams; macrolides; trimethoprim; fluoroquinolones; tetracycline; chloramphenicol
MexEF-OprN	MexT, activator (LysR-type regulator)	Trimethoprim; fluoroquinolones; chloramphenicol
MexXY	MexZ, repressor (TetR -type regulator)	Fluoroquinolones; aminoglycosides; tetracycline; erythromycin
MexJK	MexL, repressor (TetR/AcrR-type regulator)	Tetracycline; erythromycin
MexVW	Not determined	Norfloxacin; ofloxacin; chloramphenicol; tetracycline; cefpirome

Focusing on the MexAB-OprM system, carbapenems are one of its substrates apart from imipenem (97,98). The genes encoding the proteins of this tripartite system form an operon, which is controlled by regulatory genes – *mexR*, *nalD* and *nalC* (99,100). Despite all of them acting as repressors of the operon, *nalC* gene is an indirect repressor since it influences the expression of ArmR protein, which in turn is an *mexR* anti-repressor. Thus, if *nalC* is mutated, a suppression of *armR* expression will occur. In turn, *mexR* will be expressed, leading to an overexpression of the efflux pump (100). Multiple mutations have been reported for *nalC*, with G71E being the most common (99).

Simultaneous overexpression of multiple efflux system might confer a MDR phenotype to bacteria, such as what happens to *P. aeruginosa* due to its multiple efflux pumps with different antibiotic classes as substrates (Table 1).

S. maltophilia also harbours multiple efflux pumps belonging to different families. The existence of these pumps contributes to the clinical MDR phenotype commonly associated with *S. maltophilia.* Similar to what has been described regarding MexAB-OprM, mutations on repressor genes of *S. maltophilia* efflux pumps have been associated to its overexpression (39,101).

1.2.5 Porins

Another transport system located in bacterial membranes is the influx of substances, which is mainly controlled by porins. Porins allow the passive entrance of hydrophilic molecules (102).

They were firstly described in *E. coli* (OmpF, OmpC and PhoE), with OmpF being the larger channel porins, and OmpC the smaller channel porin. Homologues of these porins were described on other Gram-negative bacteria, like *K. pneumoniae* OmpK35 (homologue to *E. coli* OmpF) and OmpK36 (homologue to *E. coli* OmpC) (103).

As the majority of carbapenem-resistant *E. coli* harbour carbapenemase genes, there is a knowledge gap on the role of porins and its link to carbapenem resistance. Yet, a recent study compared the response levels/expression of *ompF* and *ompC* to different concentrations of meropenem and imipenem, concluding that OmpF porin expression diminished in the presence of meropenem (104). However, more studies are necessary.

On the other hand, the role of *K. pneumoniae* OmpK35/36 has been widely studied. The absence or deficiency of OmpK35/36 is known to play a role in carbapenem resistance of ESBL-producing strains (103). Furthermore, loss of OmpK36 has a higher impact in the occurrence of carbapenem resistance compared to OmpK35. This is probably due to the fact that OmpK36 has a smaller channel and with the alterations, it becomes even more constrictive (105).

As for *P. aeruginosa*, the most described porin associated with carbapenem resistance is OprD which is mostly responsible for imipenem resistance. Insertion of IS (106), point mutations (107,108) and premature stop codons (107,109) are commonly associated with resistance to imipenem by OprD modification, with minimal inhibitory concentration (MIC) ranging from 8 to ≥ 32 mg/L (109).

1.3 Epidemiology within One Health

The One Health concept is an integrated, unifying approach which aims to balance and optimize the health of people, animals, and ecosystems by recognizing the interconnection between human health, animal health, and the environment (110). Thus, a disease affecting one group will also affect the other two contexts, or at least, have some repercussions. The latest example is the COVID-19 pandemic, whose origin is a zoonotic virus, in which the close contact with carcasses in wet markets scaled up to a pandemic leading to, approximately, 6 million deaths worldwide (111).

As of 2022, there were 352 million pets in Europe, which translated into 166 million households with at least one companion animal (112). Looking at the data from Portugal, there are 1785 veterinary practices and approximately 6 million pets (112,113).

In the last decades, the relationship between tutors and companion animals has drastically evolved, to animals being considered part of the family and living in close contact. To accompany this evolution, the number of veterinary practices and hospitals has increased as well as the demand for better standard veterinary healthcare. Consequently, the number of animal patients subjected to invasive procedures and in need of intensive care, thus susceptible to bacterial infections and antibiotic treatment is rising (9,114,115).

Given this proximity, bacteria once thought to be exclusive of humans started appearing in companion animals, with the most resonating examples being *E. coli* and *K. pneumoniae* as causative agents of urinary tract infections (UTI) (116–118). ESBL-producing *E. coli* are frequently associated with UTIs, both in humans and companion animals, with the presence of high-risk clone ST131 present in both contexts (118). Additionally, ESBL-producing *K. pneumoniae* causing urinary tract infections in companion animals has also been linked to human pandemic clones, such as ST15 and ST147 (117).

Moreover, as evidenced by the ESBL-carrying lineages stated above, these pathogenic bacteria are also exhibiting antibiotic resistance patterns equal to those found in human medicine (119), likely because of the overlapping antibiotic classes in use in human and veterinary medicine, namely from the highly important (i.e. penicillins, penicillins with β-lactamase inhibitors, 1st and 2nd generation cephalosporins), critically important (i.e. aminoglycosides) and highest priority critically important antimicrobials (i.e 3rd cephalosporins, quinolones and polymyxins) (11). Coincidently, penicillins (including those with β-lactamase inhibitors) represent 48.8% of antibiotics sales for companion animals, followed by 1st and 2nd generation cephalosporins with 19.8% (120).

Studies on the sharing of bacterial strains between companion animals and their tutors have been undertaken in the past years, as an attempt to understand the directionality of transmission (121,122), as well as studies on prevalence of carriage of ESBL-producing Enterobacterales in households (123). Sharing of susceptible *K. pneumoniae* between healthy humans and their companion animals has been reported, with the *K. pneumoniae* strains shared belonging to pandemic CG11 (121), while other study also reported the sharing ESBL-producing *E. coli* human pandemic lineages between healthy companion animals and tutors (122).

As previously mentioned, carbapenems are of exclusive use of human medicine for both their preservation as therapeutic choices for life-threatening infections and to limit the chance of undue bacterial exposure that may lead to further dissemination resistance However, resistance to carbapenems is appearing in companion animals at an alarming rate. In Europe, carbapenem-resistant Enterobacterales are sporadically but consistently reported in companion animals (Figure 4), usually harbouring OXA-48-like carbapenemase (124–126).

It is interesting to observe that the types of carbapenemases identified on companion animals carbapenem-resistant strains often reflect the human epidemiology within the same country. For instance, VIM-1 has been reported in a dog in Spain, where this enzyme is frequently found in hospitals (127,128), with Portugal following the European tendency (124) (Figure 4).

Furthermore, the mobile genetic elements associated with the reported carbapenemase genes are also the same as those described in human strains, with an OXA-181-producing *E. coli* from a dog's infection harbouring the carbapenemase gene on a IncX3 plasmid (82,114,124) while an OXA-48-producing *K. pneumoniae* carried the gene on IncL plasmids (129). Moreover, the identified STs belonged to the same lineages linked to MDR strains in human medicine such as *K. pneumoniae* ST307 (114,130).

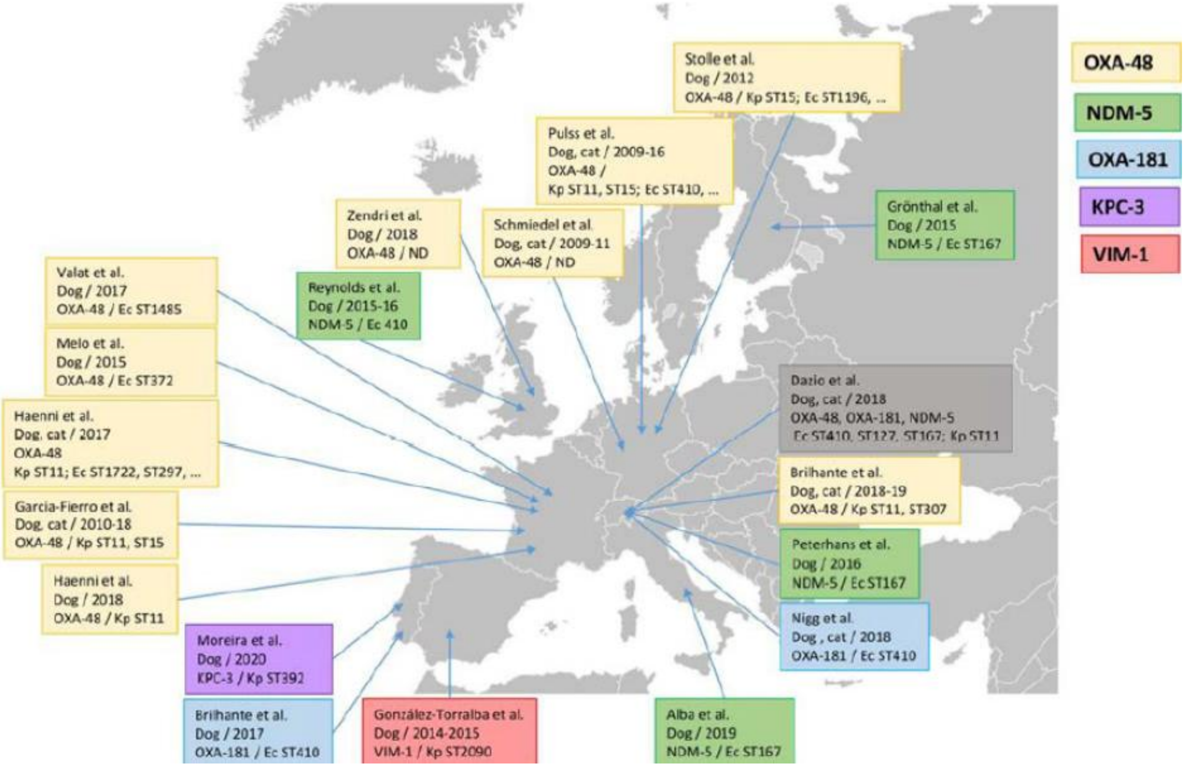

Figure 4 – Map of Europe with the carbapenemase-producing Enterobacterales described so far in companion animals. Reprinted from European Food Safety Authority report 2021-2022 (131).

Although still rare, cases of direct transmission of MDR carbapenemase producing bacteria between companion animals and their tutors have been reported, such as an NDM-5-producing *E. coli* ST167 (132). As for non-fermenting Gram-negative, a case of OXA-23-producing *A. baumannii* causing an urinary tract infection in a cat from Portugal has been reported, as well as other sporadic cases in Europe (133,134).

All of these instances point to an easiness of spreading of carbapenemases, likely related to the fact they are present in conjugative plasmids, possibly following the ESBL example of global spread through plasmids (135). Additionally, carbapenemase presence is usually associated with an MDR phenotype, which can have dire consequences to human and animal health by leaving a limited choice of antibiotics available for treatment, particularly in animal health since the pool of antibiotics available is already smaller .

1.4 Carbapenem resistance diagnosis and One Health

Diagnosis plays a crucial role in the One Health framework, with accurate and timely diagnosis being the cornerstone of effective disease management and control. In human diagnostic laboratories, detection methods for carbapenem resistance and carbapenemase production are common (136,137). However, with the emergence of carbapenem-resistant bacteria causing infections in companion animals, it should be expected that veterinary diagnostic laboratories start investing in such methods as a tool to inform clinicians and prevent further dissemination of carbapenem-resistant bacteria.

The following manuscript not only highlights the global threat of antibiotics, but also reports increasing instances of carbapenem-resistant strains in companion animals worldwide, together with the most commonly associated bacteria found. Moreover, this manuscript also reflects on the importance of effective monitoring and diagnosis of carbapenem-resistant bacteria in veterinary diagnostic laboratories, safeguarding animal health and preventing further dissemination to human health and the environment.

The section 1.4 is based on the review article:

Moreira da Silva, J., Menezes, J., Marques, C., & Pomba, C. F. (2022). Companion Animals—An Overlooked and Misdiagnosed Reservoir of Carbapenem Resistance. Antibiotics, 11(4), 533. https://doi.org/10.3390/antibiotics11040533

Supplementary material to this peer-reviewed paper can be found on Annex A.

Abstract: The dissemination of antimicrobial-resistance is a major global threat affecting both human and animal health. Carbapenems are human use β-lactams of last resort, thus the dissemination of carbapenemase-producing (CP) bacteria creates severe limitations for the treatment of multidrug-resistant bacteria in hospitalized patients. Even though carbapenems are not routinely used in veterinary medicine, reports of infection or colonization by carbapenemase-producing Enterobacterales in companion animals are being reported. NDM-5 and OXA-48-like carbapenemases are among the most frequently reported in companion animals. Like in humans, *Escherichia coli* and *Klebsiella pneumoniae* are the most represented CP Enterobacterales found in companion animals, alongside with *Acinetobacter baumannii*. Considering that the detection of carbapenemase-producing Enterobacterales presents several difficulties, misdiagnosis of CP bacteria in companion animals may lead to important animal and public-health consequences. It is of the upmost importance to ensure an adequate monitoring and detection of CP bacteria in veterinary microbiology in order to safeguard animal health and minimise its dissemination to humans and the environment. This review encompasses an overview of the carbapenemase detection methods currently available, aiming to guide veterinary microbiologists on the best practices to improve its detection for clinical or research purposes.

Keywords: Companion animals, Enterobacterales, carbapenemase detection methods

1.4.1 Introduction

Carbapenems are β-lactam antibiotics with broad antimicrobial spectrum. With the emergence of Extended Spectrum β-lactamases (ESBLs), carbapenems became the antibiotics of last resort for treatment of human patients with ESBL-producing Enterobacterales infections (138). Although carbapenems are not hydrolysed by most β-lactamases, their effectiveness was seriously compromised by the emergency of carbapenem-hydrolysing enzymes, the carbapenemases (138,139). The most important carbapenemases belong to three different Amber classes (139) (i) class A, including the KPC, IMI/NMC, SFC, GES type enzymes (138,139); (ii) class B, including VIM, IMP, and NDM metallo-β-lactamases (MBL) (140); and (iii) class D, including OXA-48-like type enzymes (141).

Regulation on the use of carbapenems in animals varies worldwide and they do not belong to the OIE List of Antimicrobial Agents of Veterinary Importance (142). According to the European Medicine Agency categorization of antibiotics for animal use, carbapenems are included in category A (“Avoid”), meaning they are not authorized for use in veterinary medicine in the European Union (EU), except in exceptional clinical cases in companion animals, under the cascade according to Article 112 of the veterinary medicinal products Regulation 2019 of the European Union Legislation (143). Reports of carbapenemase-producing (CP) and carbapenem-resistant Enterobacterales (CRE) detection among companion animals are emerging worldwide (Table 2). The identification of CP bacteria in companion animals, that have significant direct contact with humans, has raised public health concern as animals may constitute an important reservoir of carbapenems resistance genes and contribute to its dissemination (144). Very recently, the building of an European Antimicrobial Resistance Surveillance network in veterinary medicine (EARS-Vet) has been reported (145). Yet, carbapenem resistance epidemiology remains quite unknow, as, unlike in human medicine, no global surveillance protocol is currently in place for companion animal veterinary medicine. Furthermore, the detection of CP bacteria relying on antimicrobial susceptibility testing alone (AST) presents several pitfalls leading to its possible miss-detection in veterinary medicine. Appropriate monitoring and detection of antimicrobial resistance against these critically important antimicrobials in veterinary medicine is of the utmost importance to avoid treatment failure and prevent its dissemination to humans and the environment. However, there is a lack of recommendations directed specifically to the veterinary medicine needs in the published literature.

In this review, an updated overview of the current methods available for the detection of CP bacteria directed at veterinary medicine will be made aiming to guide veterinary microbiologists on the best practices to improve carbapenemase detection for clinical AST reports or even research purposes.

1.4.2 Carbapenemase producing bacteria in companion animals

To our best knowledge, more than 25 reports of CP bacteria in dogs and cats have been published worldwide. These include, both infection and colonization CP isolates harbouring KPC, VIM, IMP, NDM or OXA β-lactamases (Table 2).

Briefly, three studies detected KPC-producing *Escherichia coli* and *Klebsiella pneumoniae* from dogs in Brazil and in *Enterobacter xiangfangensis* from a dog in the United States (146–148). A IMP-4-enzyme in *Salmonella* isolates was recovered from cat's faecal samples in Australia (149), VIM-2 in *Pseudomonas aeruginosa* from dogs with pyoderma and otitis in South Korea (150) and VIM-1 in *K. pneumoniae* from dogs in Spain (151). A number of NDM-5-producing *E. coli* have been found in dogs and cats (152–159), one NDM-1-producing *Acinetobacter radioresistens* was detected in a dog, six NDM-1-producing *E. coli* from dogs and cats in the United States, two NDM-1-producing *E. coli* from a dog in China, and finally one NDM-9 from a farm dog in China (160–162). Several OXA-48-like carbapenemase-producing *E. coli*, *K. pneumoniae, Klebsiella oxytoca* and *Enterobacter cloacae* isolates were recovered from dogs, cats and horses, representing one of the most frequent carbapenemases detected in companion animals alongside with NDM-5 (Table 2) (154,163–169). In addition, OXA-23- and OXA-66-producing *Acinetobacter baumannii* were isolated from clinical samples from dogs and cats (160,170,171).

Interestingly, although the detection of CP bacteria in companion animals dates to at least 2009, detection methods vary widely between studies, with the use of selective culture media being the most frequent for the detection of commensal CP isolates, while antimicrobial susceptibility testing alone (AST) is the main method used for the detection of CP isolates in infection cases (Table 2). Another important finding is that most CP bacterial species isolated from companion animals belong to the priority 1 ("critical") category within the WHO priority pathogens list (172), thus highlighting the importance of properly monitoring and effectively detecting these carbapenem resistance mechanisms in companion animals.

Table 2 - Carbapenemases found in companion animals across the world.

Enzyme	Year	Country	Host	Source	Bacterial species	Detection methods	Ref.
IMP-4	2016	Australia	Cats	Commensal	*Salmonella enterica* serovar Typhimurium	AST	(149)
KPC-2	2018	Brazil	Dog	Infection (UTI)	*Escherichia coli*	Imipenem synergy test, modified Hodge testing, PCR	(146)
KPC-2	2021	Brazil	Dog	Infection (UTI)	*Klebsiella pneumoniae*	Imipenem synergy test, AST	(147)
KPC-4	2018	USA	Dog	Infection (UTI, SSTI)	*Enterobacter xiangfangensis*	Biochemical Tests	(148)
NDM-1	2013	United States	Dogs, Cats	Infection (SSTI, UTI)	*Escherichia coli*	AST	(161)
NDM-1	2017	China	Dogs	Commensal	*Escherichia coli*	Selective culture media	(153,162)
NDM−1	2018	Italy	Dog	Commensal	*Acinetobacter radioresistens*	Selective culture media	(160)
NDM-5	2016	Algeria	Dogs	Commensal	*Escherichia coli*	PCR	(154)
NDM-5	2017	China	Dogs	Commensal	*Escherichia coli*	Selective culture media	(153)
NDM-5	2019	United Kingdom	Dog	Infection (SSTI)	*Escherichia coli*	AST	(156)
NDM-5	2018	Finland	Dogs	Infection (Otitis externa)	*Escherichia coli*	AST followed by modified Hodge testing, UV spectrometric detection of imipenem hydrolysis	(155)
NDM-5	2021	Italy	Dog	Infection (UTI)	*Escherichia coli*	Meropenem synergy test	(152)
NDM-5	2018	United States	Dog	Infection (URTI)	*Escherichia coli*	AST	(157)
NDM-5	2018	United States	Dogs, Cats	Infection (UTI, URTI)	*Escherichia coli*	AST	(159)
NDM-5	2018	South Korea	Dog, Cat	Commensal	*Escherichia coli*	AST, PCR	(158)
NDM-9	2017	China	Dog	Commensal	*Escherichia coli*	Selective culture media	(153)

Enzyme	Year	Country	Host	Source	Bacterial species	Detection methods	Ref.
OXA-48	2009-2010	Germany	Dogs, Cats, Horses	Infection	*Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae*	Selective culture media for cephalosporin resistance, PCR	(173)
OXA-48	2013	Germany	Dog	Commensal, Infection (UTI, SSTI, URTI, CRBSI)	*Klebsiella pneumoniae, Escherichia coli*	AST	(166)
OXA-48	2016	United States	Dogs, Cats	Infection (UTI, SSTI, Genital tract)	*Escherichia coli*	AST	(168)
OXA-48	2016	Algeria	Dogs	Commensal	*Escherichia coli*	PCR	(154)
OXA-48	2017	Algeria	Dogs, Cat, Horses, Pet birds	Commensal	*Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae*	Selective culture media	(169)
OXA-48	2017	France	Dog	Commensal	*Escherichia coli*	Selective culture media	(167)
OXA-48	2018	Germany	Dogs, Cats, Horses	Infection (UTI, SSTI, genital tract, otitis, URTI)	*Klebsiella pneumoniae, Enterobacter cloacae, Escherichia coli, Klebsiella oxytoca*	Selective culture media	(165)
OXA-181	2018	Switzerland	Dogs, Cats	Commensal	*Escherichia coli*	Selective culture media	(163)
OXA-181	2020	Portugal	Dog	Commensal	*Escherichia coli*	Selective culture media and AST	(164)
OXA-181	2021	Portugal	Cat	Infection (SSTI)	*Klebsiella pneumoniae*	Selective culture media and AST	(174)
OXA-23	2014	Portugal	Cat	Infection (UTI)	*Acinetobacter baumannii*	AST	(170)

Enzyme	Year	Country	Host	Source	Bacterial species	Detection methods	Ref.
OXA-23	2017	Germany	Dogs, Cats	Infection (UTI, suppurate inflammation)	*Acinetobacter baumannii*	Selective culture media	(171)
OXA−23	2018	Italy	Dogs, Cats	Commensal	*Acinetobacter baumanni*	Selective culture media	(160)
OXA-66	2017	Germany	Dogs, Cats	Infection (UTI, SSTI, URTI, CRBSI, suppurate inflammation)	*Acinetobacter baumannii*	Selective culture media	(171)
VIM-1	2016	Spain	Dog	Commensal	*Klebsiella pneumoniae*	Selective culture media, Meropenem synergy test	(151)
VIM-2	2018	South Korea	Dog	Infection (SSTI)	*Pseudomonas aeruginosa*	AST	(150)

AST, antimicrobial susceptibility testing; CRBSI, catheter-related bloodstream infection; SSTI, skin soft tissue infection; URTI, upper respiratory tract infections; UTI, urinary tract infection.

1.4.3 . Phenotypic characteristics of carbapenemases and its genetic background in isolates from companion animals.

The β-lactam resistance phenotype of CP isolates can vary depending on the type of carbapenemase and its hydrolysing activity.

1.4.3.1 Serine carbapenemases

Serine carbapenemases of molecular (Ambler) class A corresponds to the KPC, IMI/NMC, SFC, GES enzymes that have a hydrolytic mechanism involving an active site serine at position 70 (Ambler numbering of class A β-lactamases), conferring resistance to first-, second- and third-generation cephalosporins, imipenem and meropenem (139). Class A carbapenemases have been rarely detected in companion animals, being the KPC enzyme the only one reported until now from dogs with UTI and SSTI (Table 2). In *K. pneumoniae* and *E. coli* from dogs, the *bla*$_{\text{KPC-2}}$ gene was found in Tn*4401* transposons contained in IncN plasmids (146,147) and the *bla*$_{\text{KPC-4}}$ gene was detected in an IncHI2 plasmid in the context of Tn*4401b* transposon in *E. xiangfangensis* isolated from a dog's clinical samples (148).

Table 3 - Common β-lactam hydrolysis profile of carbapenemases

Amber Class	Representative Carbapenemase type	Hydrolysis profile				Ref.
		Narrow spectrum cephalosporins	Extended spectrum cephalosporins	Imipenem*	Meropenem*	
Class A	KPC	+	+	+	+	(139,146)
Class B	IMP, VIM, NDM,	+	+	+	+	(140)
Class D	OXA-48-like	+	-	Variable [1]	-	(141,175,176)
	OXA-23-like	+	+	+	+	(177)

* Imipenem and meropenem representative MIC values for carbapenemase-producing isolates from companion animals are listed in Table S1.

[1] Imipenem susceptible in OXA-48-like has been reported.

1.4.3.2 Metallo-β-lactamases

Class B carbapenemases have a critical clinical significance due to their ability to hydrolyse all β-lactams (Table 3) (178,179). So far, more than 50 allelic β-lactamase-conferring imipenem resistance (IMP) variants are listed at GenBank DNA sequence database. But only IMP-4 has been reported among companion animals, namely cats, in *Salmonella enterica* serovar Typhimurium (Table 2) (149). The IMP-4 coding gene was located on a gene cassette (*bla*$_{\text{IMP-4}}$-*qacG-aacA4-catB3*) in a class 1 integron, associated with a conjugative plasmid IncHI2, also carrying other resistance genes, such as *tetA* (mediating resistance to tetracycline), *aac* (resistance to aminoglycosides), *cat* (chloramphenicol resistance), *sul* (sulphonamide resistance), *bla*$_{\text{OXA}}$ (different serine oxacillinases) and *bla*$_{\text{TEM-1}}$ (narrow-spectrum β-lactamases) (149).

Verona Integron–encoded Metallo-β-Lactamase (VIM) enzymes are the second most common Class B carbapenemase detected in companion animals (Table 2). VIM-1 and VIM-2 were described in *K. pneumoniae* and *P. aeruginosa* isolates from dogs, respectively; both located in class 1 integrons incorporated on untyped plasmids (150,151).

The *bla*$_{\text{NDM}}$ genes pose a serious public health concern, since most common plasmids associated with its spread often have various antibiotic resistance genes resulting in multidrug resistance phenotypes (155,156,180). Until now, 28 variants have been described, with resistance against all β-lactams expect monobactams (181). In companion animals only NDM-1 and NDM-5 have been described so far (Table 2), the latter being more frequent. For one metallo-β-lactamase NDM-1, the encoding gene was located in a transposon Tn*125* (composed by *bla*$_{\text{NDM-1}}$-*ble*$_{MBL}$-*trpF-TAT-cutA1-groES-groEL-insE-Δpac* genes between a pair of IS*Aba125*), integrated in the chromosome of an *A. radioresistens* isolated from a dog in Italy (160). This Tn*125* transposon usually encompasses *bla*$_{\text{NDM}}$ genes with two flanking IS*Aba125* elements, and in companion animals it was also found in *bla*$_{\text{NDM-5}}$ carrying strains (153,182). A NDM-1-producing *E. coli* isolate harboured *bla*$_{\text{NDM-1}}$ in another genetic region, which was not flanked by IS*Aba125* elements downstream of the resistance gene (162). NDM-5 metallo-β-lactamase differs from NDM-1 by four amino acids and has been found in the chromosome of an integrated IncF plasmid, from an *E.coli* isolate causing skin and soft tissue infection on a dog in the United Kingdom (156). In the United States, *bla*$_{\text{NDM-5}}$ encoding gene has been found on IncFII-type plasmids (157,159), whereas in South Korea it was described in an IncX3-type plasmid (158) with the surrounding genetic environment of IS*Aba125*-*bla*$_{\text{NDM-5}}$-*ble*$_{MBL}$-*trpF-TAT*-IS*CR26*.

1.4.3.3 Oxacillinases

The class D, carbapenem hydrolysing OXA-48 and its variants, namely OXA-181, are one of the most common in veterinary settings (Table 2). The OXA-181 variant weakly hydrolyses both carbapenem and extended-spectrum cephalosporins and differs from OXA-48 at four amino acid substitution, yet its kinetic properties appear broadly similar to OXA-48 (183–185). These enzymes can be associated with different β-lactam hydrolysis profiles than the other serine-metallo-β-lactamases, making its accurate detection difficult. By possibly being susceptible *in vitro* to meropenem and imipenem (Table 3), two widely used surrogates to identify carbapenem resistance in clinical microbiology, carbapenem resistant bacteria harbouring OXA-48 like carbapenemases may easily be misdiagnosed as ESBL-producers which may lead to treatment failure. OXA-48 coding genes in CP isolates have been associated with no other resistance genes; or with extended-spectrum β-lactamases coding genes, thus conferring either low or high MIC against carbapenems (186). High-level resistance to carbapenems has also been observed (168) that may be associated with the combination of these carbapenemases with outer membrane lack of permeability (187). Importantly, regardless of the carbapenem susceptibility profile detected *in vitro*, carbapenem therapy is not reliable against OXA-48-like producing bacteria (188).

In companion animals, the *bla*$_{\text{OXA-48}}$ gene has been commonly observed on pOXA-48a plasmid, a self-conjugative IncL/M plasmid (165,189,190). This plasmid has a high conjugation rate, therefore, it can be transferred at a very high frequency across Gram-negative bacteria (191,192). Flanking the *bla*$_{\text{OXA-48}}$ gene is the Tn*1999* composite transposon, which cooperates in mobilizing pOXA-48a or closely related plasmids (193,194).

The *bla*$_{\text{OXA-181}}$ gene was found to be part of the transposon Tn*2013,* inserted at the downstream region of IS*Ecp1,* which is a very efficient genetic vehicle for spreading ESBL genes, namely the *bla*$_{\text{CTX-M-15}}$ gene (195). The *bla*$_{\text{OXA-181}}$ gene has been frequently identified in IncX3 plasmids (163,196).

The frequency of OXA-48-like producing bacteria in companion animals (**Table 2**) and its frequent association with mobile genetic determinants that facilitate its dissemination, highlight the importance of monitoring this resistance mechanism in companion animals. Furthermore, the possible misdiagnose of OXA-48-like producing bacteria when using meropenem and imipenem as surrogates may lead to underestimating its frequency and the epidemiological role of companion animals as reservoirs.

The *bla*$_{\text{OXA-23}}$ gene has been reported coming from *A. baumannii* isolates (**Table 2**). This gene is often located on transposon Tn*2006*, but has also been identified in transposon Tn*2008* in animals isolates (160,171). The *bla*$_{\text{OXA-23}}$ is usually flanked between IS*Aba1* insertion sequences, known to promote the expression of *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-51-like}}$ genes in *A. baumannii* for an elevated level sufficient to display carbapenem resistance (182,197). In addition to carbapenems, the OXA-23 enzymes can hydrolyse cephalosporins, aminopenicillins, piperacillin, oxacillin, and aztreonam (Table 3) (177).

1.4.4 Methods for detection and identification of carbapenemases

Detection of CP bacteria has proven to be a difficult task, as it cannot solely be based on the resistance profile observed during AST (198). Usually, an elevated MIC against a carbapenem is a marker for testing for carbapenemase production. Yet, some CP isolates have low carbapenem MICs, being susceptible according to EUCAST and CLSI guidelines, such as OXA-type carbapenem-hydrolysing class D β-lactamases (163,164,166,167).

For such reason, it is important for veterinary diagnostic laboratories to employ specific tests to correctly identify CP bacteria during routine microbiology procedures. The accurate detection of carbapenem resistance is key to improve animal health; and to minimize its dissemination to humans and the environment. A variety of methodologies and tests are available for this purpose, that vary in its practicality and in the technical expertise required.

1.4.4.1 Selective culture media

Several different selective culture media are available for the detection of CP isolates. The most common ones are: SUPERCARBA Medium (CHROMagar™, Paris, France); CRE Agar (Brilliance™ Oxoid, Thermofisher Scientific Illkirch, France); ChromID CARBA Smart (Biomerieux, Marcy l'Etoile, France) and CHROMagar™ KPC/OXA-48 (CHROMagar™) (Table 4) (199–201). All these culture media have chromogenic molecules in their composition, allowing for a rapid presumptive species identification after overnight incubation. Several studies have been done, comparing and evaluating the performance of these selective culture media. The SUPERCARBA medium seems to have the higher sensitivity of all, ranging from 95.6%-96.5%, with 100% sensitivity for KPC and OXA-48 producers (136,202). However, its specificity decreases to 60.7%, as it also detects non-carbapenemase isolates that are carbapenem-resistant due to ESBL/AmpC overexpression in combination with porin loss (203). Regarding the CRE Agar medium, it has sensitivity of 78% and a specificity that variates from 60-66% (199). A study conducted in Germany has shown that ChromID CARBA Smart fails to detect CP isolates with low carbapenem resistance, when comparing the same isolates plated on MacConkey agar supplemented with 1 mg/L of cefotaxime and 0.125 mg/L of meropenem (204). This culture medium presents a sensitivity of 91% and a specificity that variates from 76-89% (199). The CHROMagar™ KPC medium presents a sensitivity of 100% (200), with a positive predictive value (PPV) of 100% for KPC producers and negative predictive value (NPV) of 98.8%, whereas in the same comparison study, MacConkey agar supplemented with 1mg/L of imipenem yielded 94.7% PPV and 88.6% NPV, having failed to detect 10 positive isolates (205). Another specific carbapenemase selective medium is CHROMagar™ OXA-48, however its sensitivity is suboptimal (75.8%) in direct sampling, only increasing to 90.9% when performed after an enrichment method. Nonetheless, its specificity is of 99.3% (206).

Apart from CHROMagar™ KPC/OXA-48, none of the other media can accurately identify the specific *bla* gene responsible for causing resistance against carbapenems. Regardless of it, all isolates grown in these selective media must be confirmed as CP with subsequent molecular testing (207).

Table 4 - Characteristics of selective culture media and biochemical tests for detection of carbapenemase-producing bacteria.

Technique	Sensitivity (%)	Specificity (%)	Turnaround time (h)	Advantages	Disadvantages
Selective Culture Medium					
SUPERCARBA	95.6-96.5	60.7	18-24	Colour identification of bacterial species	Extensive turnaround time; possible growth of non-carbapenemase producing bacteria; positive control needed.
CRE Agar	78	60-66			
ChromID CARBA Smart	91	76-89			
CHROMagar™ KPC	100	NDA			Only detects KPC-producing bacteria
CHROMagar™ OXA-48	75.8	99.3			Only detects OXA-48-producing bacteria
Biochemical Tests					
Rapidec® CarbaNP	100	100	2	Rapid Detection of carbapenemase-producing bacteria	Non-specific detection; colour interpretation; expensive
CIM	NDA	NDA	8	Affordable; no commercial kit necessary	Non-specific detection; negative control strain needed; non-standardized
BlueCarba	100	100	2	Rapid Detection of carbapenemase-producing bacteria	Non-specific detection; positive control needed; expensive
β CARBA Test™	84.9	95.6	0.5	Rapid Detection of carbapenemase-producing bacteria	Non-specific detection; expensive

NDA, no data available

1.4.4.2 Biochemical tests

Biochemical tests are relatively quick and easy to use (**Table 4**). To the best of our knowledge, the Rapidec® CarbaNP (Biomérieux, Marcy l'Etoile, France) was the first commercial kit of its kind offering a positive result under two hours. Positive results occur due to colour shifting from pH alteration as consequence of carbapenem hydrolysis (208). It has 100% sensibility and specificity for Enterobacterales (209,210). A study conducted by Tijet et al. reported a decreased sensibility (72.5%) on account of mucoid isolates and/or isolates harbouring low carbapenemase activity genes, such as OXA-48 and GES-5 (211). When using the commercial version of the CarbaNP test, some difficulties in results interpretation due to colour shifting have been reported (212).

As a cheaper alternative to CarbaNP test, the carbapenem inactivation method (CIM) is available. CIM is also based on carbapenem hydrolysis. In this test, a disc of meropenem 10μg is emerged in a bacterial suspension of the isolate to be tested. A Mueller-Hinton Agar plate is inoculated with a known susceptible *E. coli* strain prior to disc placement (213). The turnaround time is approximately eight hours and if positive, growth is observed until the disc of meropenem due to its previous hydrolysis by the CP isolate being tested.

The Blue Carba test (BTC) is another test that also gives results within 2h. Similar to CarbaNP, it is based on imipenem hydrolysis by CP bacteria, which leads to colour changes due to pH alteration in case of a positive result (214). It has a sensibility and specificity of 100%, with additional advantages: use of colonies grown on Mueller-Hinton Agar; it is cheaper as it doesn’t use imipenem monohydrate but Tienam® (imipenem/cilastatin, Merck Sharp & Dohme, Campinas, Brazil); and it was validated against OXA-type carbapenemase (214). However, in a study conducted by Pasteran et al., the OXA-type carbapenemase detection using this method had a sensibility and specificity of 97% and 96%, respectively. On the other hand, isolates with low imipenem MICs were correctly identified (215).

The β CARBA Test™ (Bio-Rad, Marne la Coquette, France) also relies on colour changing for result interpretation. It has a sensibility of 84.9% and specificity of 95.6%, having failed to detect non-KPC Ambler class A carbapenemases (216).

Besides allowing a short turnaround time, another advantage of these biochemical tests is the fact that, unlike PCR, these are not targeted to any specific carbapenemase groups.

Therefore, it allows the detection of carbapenemase activity of yet undiscovered resistance genes.

1.4.4.3 Disc Diffusion Methods

This method requires the use of meropenem discs and meropenem discs supplemented with different inhibitors to detect and identify carbapenemases (Figure 5) (217).

Meropenem synergy with phenyl boronic acid is indicative of the presence of Ambler class A KPC. Meropenem synergy with EDTA plus dipicolinic acid is indicative of the presence of an MBL. Detection of a positive synergy with a disc of cloxacillin plus phenyl boronic acid indicates carbapenem resistance due to porin loss or AmpC overexpression (218). A zone diameter increase of ≥4 mm around the discs containing inhibitors, in comparison with the disc with meropenem alone, is considered to be a positive synergy result for phenyl boronic acid, whereas an increase of ≥5 mm is considered to be a positive synergy result for EDTA/dipicolinic acid and cloxacillin/phenyl boronic acid (Figure 5) (217–219).

To detect OXA-48-like CP bacteria, it is recommended to add a temocillin disc (30µg) to the group of synergy tests (Figure 5), due to its weak meropenem hydrolysis (141,175,217,219). Temocillin lacks activity against Gram-positive bacteria as well as non-fermenters (220). Temocillin high-level resistance phenotype is proposed as a marker for OXA-48-like producers. However, this marker is not specific for OXA-48-like carbapenemases, as other resistance mechanisms may confer this phenotype, therefore, the presence of carbapenemases must be confirmed using complementary tests in all isolates showing a zone diameter ≤10 mm (217,219). In a study conducted by van Dijk et al., this detection method had a 100% sensibility and specificity, with all positive isolates having a zone diameter ≤10 mm (221). Nevertheless, the authors alert to its incapability of detecting MBL in combination with OXA-48 producers.

The main disadvantage in using disc diffusion methods for carbapenemase screening is the turnaround time of approximately 18h of incubation, whereas biochemical tests have a turnaround time of 2h. Yet, they are low cost compared with biochemical testing.

The Hodge modified test used to be an option as a phenotypic method, but due to its dubious results and low sensibility/specificity, it has since been advised against using by EUCAST and CLSI (217,222).

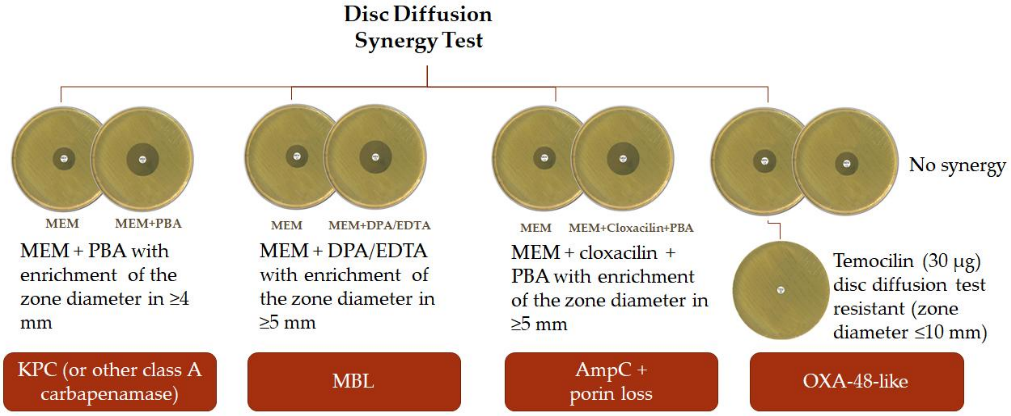

Figure 5 - Interpretation of phenyl boronic acid (PBA), dipicolinic acid (DPA) and cloxacillin synergy tests and temocillin disc diffusion in comparation with meropenem (MEM) disc diffusion alone.

1.4.4.4 Lateral Flow Assays

Some immunochromatographic assays are available to readily identify suspecting colonies grown in non-specific media. Currently, there are numerous options for lateral flow assays, yet the most commonly used seems to be the OXA-48 K-set, KPC K-set, Resist-3 O.K.N K-set and Resist-4 O.K.N.V. (CorisBio Concept, Gembloux, Belgium). Resist-3 O.K.N K-set is a multiplex assay, detecting OXA-48, KPC and NDM-like enzymes (223). The sensitivity and specificity is of 100% for all cassettes (224–226). Although these tests were designed to be used with bacterial inoculum, studies have evaluated the multiplex efficacy in positive blood culture bottles (BacT/ALERT, Biomérieux). The multiplex system is compatible with blood culture bottles, albeit it showed weak signal bands for NDM-like enzymes positive isolates; and the positive signal was also influenced by the blood volume used (227). The fourth version of the test, the Resist-4 O.K.N.V., added the detection of VIM-like enzymes to the previous Resist-3 O.K.N K-set. The test maintains its 100% sensitivity to OXA-48 like and KPC enzymes, as well as VIM, but it decreases to 83.3% regarding NDM producers (228). Previous reports have shown 100% sensitivity for NDM producers detection using Resist-4 O.K.N.V., however this could be as in some of those studies only Enterobacterales were evaluated (229,230). Another possible explanation is the impact that the morphological characteristics of the colony used in the test can have in its accuracy. The NG-Test® CARBA 5 (Hardy Diagnostics, Santa Maria, CA, US) is another available commercial kit, which can additionally detect IMP enzymes. Similar to the previously described tests, NG-Test® CARBA 5 has 100% sensitivity and sensibility (231).

Recently, it was also launched the Resist-5 O.K.N.V.I. cassette, but compared to its homologous, it has sensitivity of 98.4% and specificity of 100% (232), however not many comparative studies have been conducted and none has compared both rapid tests. Overall, these immunochromatographic assays are useful to be used as a screening method in routine microbiology when the isolated bacteria is suspected to be a carbapenemase producer. Nonetheless, positive results should always be confirmed with PCR targeting for the most common carbapenemase genes (207).

1.4.4.5 Molecular Testing

Molecular techniques are mostly based on PCR, and may be followed by sequencing if needed for precise identification of a specific carbapenemase, rather than just its group (e.g. KPC-type, IMP-type, VIM-type, NDM-type, and OXA-type) (207).

Nowadays PCR assays are becoming a routine method in many veterinary clinical diagnostic laboratories. This molecular testing remains the reference standard for the identification and differentiation of carbapenemases, recommended by guidelines and expert groups (217,233).

PCR assays performed on genomic DNA for the detection of carbapenemase genes are easily available in the literature, including multiplex PCRs, and can give results within 4–6 h (207,234).

Nevertheless, these PCRs require the acquisition and manipulation of CP control strains to be used as DNA positive controls. Nowadays there are real time-PCR fully automated systems that allow the detection of bla_{VIM}, bla_{NDM}, bla_{IMP}, $bla_{\text{OXA-48}}$, bla_{KPC}, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-58}}$, $bla_{\text{OXA-24}}$, and ISAba1 associated $bla_{\text{OXA-51}}$ carbapenemase genes and colistin resistance *mcr-1* gene, such as the Novodiag®CarbaR+ (Mobidiag, Espoo, Finland). The Novodiag®CarbaR+ test can be applied to fresh bacterium isolates or directly from rectal swabs, having a sensitivity and specificity of 98.2% and 99.7%, respectively (235). Results are available after 1h approximately, with only 5 minutes hands-on preparation of samples which might be crucial when dealing with critically ill patients. When rectal swabs are directly analysed, the sensitivity decreases only slightly to 97.8% and specificity to 98.6%, revealing its usefulness to rapidly detect colonized patients. Compared to other already available tests within the same category, the main advantage of Novodiag®CarbaR+ is that the panel tested is comprised of resistance genes associated to carbapenem resistance in *Pseudomonas* spp. and *Acinetobacter* spp.. An alternative to this automated system is the Xpert® Carba-R (Cepheid, Sunnyvale, CA, US), which is quite similar in terms of functioning but only tests for the main 5 carbapenemase groups - *bla*$_{\text{VIM}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC}}$. PCR and real-time PCR are standard techniques, widely used by the scientific community.

Similarly commercial PCR kits are also available, like the Check-MDR CT103XL (Check-Points Health, Wageningen, The Netherlands) DNA microarray assay, capable of detecting a wide range of carbapenemase (KPC, GES, IMP, VIM, NDM, OXA-23-like; OXA-24-like, OXA-48-like and OXA-58-like) with an accuracy of 94.2% (236). The principle of the Check-Points diagnostic system is based on DNA amplification followed by amplicon detection in a tube microarray (236).

Another type of molecular commercial kit is the eazyplex® SuperBug CRE (AmplexDiagnostics, Gars-Bahnhof, Germany), a loop-mediated isothermal amplification (LAMP) method that can be used for direct screening of KPC, VIM, NDM and OXA-48-like carbapenemases on rectal swab and urine samples in 20 minutes as well as confirmation from positive blood culture and culture plate in 15 minutes. This LAMP assay has shown a sensitivity from 95.2% to 100% with a specificity of 97.9% (237).

An alternative promising molecular commercial methodology is the hybridization technology by Luminex xMAP (Multi-Analyte Profiling, Austin, Texas, US) that although it does not have a specific panel for carbapenemase detection available, one can create their own personalised panel. In a study by Bilozor et al., this system had a sensitivity >95% in detecting KPC, IMP, VIM, NDM, OXA-48-like carbapenemases when using a tailored panel (238).

Nonetheless, specific equipment and experienced staff are required for theses molecular-based technologies which might be seen as a disadvantage to smaller microbiology laboratories. Furthermore, it should be noted that only the carbapenemases targeted by each specific assay will be detected.

Whole genome sequencing (WGS) is a state of the art methodology with promising applications for medical microbiology (239). WGS allows fast and accurate identification and typing of pathogens with the highest possible discriminatory power currently available for effective surveillance and outbreak detection. In addition, WGS of bacterial genomes provides information regarding antimicrobial resistance determinants; virulence and pathogenicity determinants; in addition to providing data for the discovery of new genetic determinants (240). Thus, efforts are being made by the scientific community to use WGS in the routine laboratory workflow to improve the diagnostic turnover and retrieve information that might replace older routine procedures that are time-consuming and expensive. Furthermore, the use of untargeted metagenomic next-generation sequencing (mNGS) from clinical samples is also considered very promising. Although still being improved, mNGS may revolutionize the diagnosis of infectious disease in the future, since, once optimized, it may allow the simultaneous identification of viruses, bacteria, fungi and parasites in a single assay (241). The main disadvantage in using these techniques nowadays lies in the need of a multidisciplinary team of personnel specialized in WGS/NGS and bioinformatics (240). Data analyses should be performed by staff members that have been trained to use commercial or open-source software tools to extract the appropriate information from the large amount of sequence data that is generated, and ultimately deliver clinically relevant information to the clinicians. To become truly accessible as a future everyday routine diagnostic tool, new user-friendly software platforms need to be developed so that information may be retrieved easily without the need of extensive technical and bioinformatic skills.

1.4.4.6 Mass Spectrometry Analysis

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is an analysis method where the material is ionized within a high vacuum chamber, accelerated in an electric field. Being widely used for species identification, it can also be used to detect carbapenemase production through enzyme detection. Prior to testing, it is necessary to establish the mass spectrum of a pure carbapenem (242). Some carbapenemases have fast carbapenem hydrolysis activity, compared to others which are slower, making it necessary to have several runs and specs to achieve a reliable result. Several protocols for detection have been described, with the lack of standardization being a problem when trying to implement this method (243). MALDI-TOF MS requires an even higher level of staff expertise for result interpretation when compared to most of the previously described methods. It offers a fast response compared to molecular and disc diffusion methods, but it is expensive to buy the necessary equipment if not already in use and it doesn’t detect other carbapenem resistance mechanisms such as porin loss.

1.4.5 Transmission potential

The regular and close contact between companion animals and humans provides excellent opportunities for interspecies transmission of resistant bacteria and their resistance genes in either direction (155,244–246). Hence, the increasing trends and prevalence of carbapenem-resistant bacteria observed in many companion animals is of major public health concern as companion animals could be reservoirs of CP bacteria, thus acting as direct players in the transmission of these resistant bacteria to humans (144).

The European Medicine Agency and its Antimicrobial Working Party have already warned for the indirect hazard associated with carbapenem-resistant bacteria from companion animals to public health in its reflection paper (144). Since then, sharing of clinical NDM-5-producing multidrug-resistant ST167 *E. coli* between dogs and co-habiting human was reported in a Finland study (155). Moreover, in a Chinese study across farming sectors, common NDM-positive *E. coli* strains were identified among farms, flies, dogs and farmers (247), providing additional scientific support regarding concerns not only about the transfer of resistance between companion animals and humans, but also about their potential role as reservoirs for environmental contamination (155,247,248). Furthermore, the similarity of carbapenem-resistant clonal lineages isolated from companion animals and humans worldwide, and its genetic features, suggests an interspecies exchange of resistant-bacteria or resistance genes located at mobile genetic elements (163,165,189,196).

There is a big concern towards carbapenemases following the same exponential spread as ESBL-producing bacteria, where reports of transmission between companion animals and humans are numerous worldwide (245,246,249,250). ESBL-producing Enterobacterales can serve as a model for the spread of CP bacteria because the same bacterial species are involved, and the resistance genes are also carried on plasmids (251). Studies have shown that bacteria causing infection in dogs and cats, were increasingly resistant to the antimicrobials most widely used for animal treatment (250,252,253). Although the use of carbapenems is not currently licensed for companion animals, it has been reported and it is regulated in the EU under the cascade prescribing (143,254). Nevertheless, the dissemination of CP bacteria to companion animals is likely one of anthropogenic nature, since carbapenems are essentially used in human medicine. Once colonizing companion animals, one must keep in mind that the exposure to systematic broad-spectrum antimicrobials approved for veterinary use, including β-lactams, are likely to co-select and facilitate the propagation of CP bacteria within the companion-animal population, thus further highlighting the relevance of monitoring these resistance mechanisms in veterinary microbiology.

To foster antimicrobial stewardship and consequently, the reduction of the emergence of resistance, a prudent use of critically important antimicrobials for human medicine, such as fluoroquinolones, aminoglycosides and third generation cephalosporins is needed. Furthermore, the risk of increasing selection pressure for the maintenance of CP bacteria in companion animals gut and their potential transfer to humans, needs a severe restriction or elimination of carbapenems use in veterinary medicine worldwide (10,255). Not only are pet owners at risk of acquiring these resistance strains by interspecies transmission due to direct contact and /or indirectly via the common environment, but also, veterinary personnel, veterinary students, or trainees are a professional hazard group. As important, CP bacteria also present animal health risk due to treatment failure. Therefore, it is vital to implement systematic monitoring programs in veterinary laboratories to screen for carbapenem resistance in a One Health perspective. The screening of CR and CP bacteria should be conducted in all companion animal samples submitted to culture and AST regardless of clinical presentation and animal species. The most reliable detection methods should be preferred according to each laboratory technical and financial availability. Figure 6 summarizes a possible workflow that can be adapted to veterinary diagnostic laboratories.

A pressing action is required to reduce the public and animal health hazard posed by the emergence of carbapenem-resistant bacteria isolated from companion animals. In summary, these safety measures should be taken in consideration (10,256):

Achieving the principles of prudent use of antibiotics in veterinary practice to ensure that carbapenems are used only in the very few cases that lack other suitable alternatives based on culture and AST;

Include the systematic screening for carbapenem resistance in veterinary microbiology laboratories;

Surveillance and monitoring for the presence of genes encoding resistance to critically important antimicrobials, such as carbapenems;

Appropriate hygiene practices after handling animals both in domestic and health care settings;

Infection control measures when dealing with companion animals with infections caused by carbapenem-resistant strains that include isolation of infected animals.

Communication between all the specialists involved in human and veterinary medicine should be established in a One Health approach to develop a universal strategy that scientific and non-scientific audiences could follow.

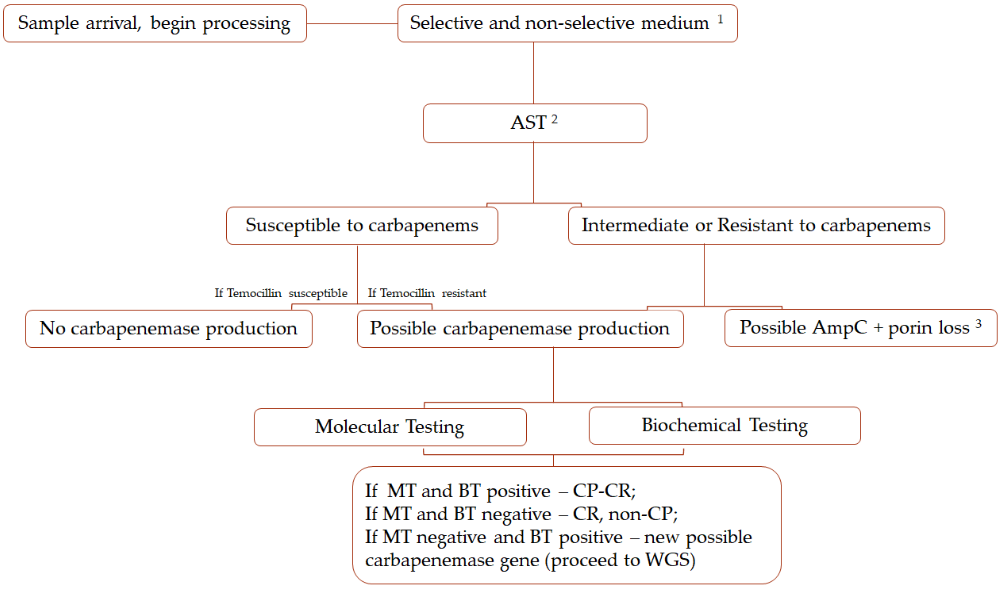

Figure 6 - Suggested diagnostic routine for carbapenemase detection. AST, antimicrobial susceptibility testing; BT, biochemical testing; CP, carbapenemases-producing; CR, carbapenem resistant; MT, molecular testing; WGS, whole genome sequencing.

[1] If possible, include commercially available selective media to carbapenemase-producing Enterobacterales.

[2] AST including meropenem (10ug) and/or imipenem (10ug) plus temocillin (30ug).

[3] May be identified as described in “1.4.4.3. Disc Diffusion Methods”.

1.4.6 Conclusions and final remarks for veterinary medicine

In veterinary medicine, screening for carbapenemase producing bacteria is not usually performed, and frequently relies on the use of meropenem and imipenem as surrogates in AST. However, in past years, many reports of the presence of genes encoding for carbapenemases in companion animals have been made, as well as its direct transmission to humans. Accurate detection of CP bacteria is essential for infection control purposes and particularly to minimize the spread of its resistant determinants that are known to cause a major health impact by limiting antimicrobial therapy.

Even though all the detection methods presented here are applied and have been evaluated in Human Medicine, not all are useful in Veterinary Medicine. For example, investing in automated PCR machines or a Mass Spectrometer is not yet viable for most laboratories, whether due to the expected low volume of positive samples; or the need for experienced staff; or the required financial investment in expensive equipment and consumables.

Although molecular identification of carbapenemase encoding genes is the gold standard, the phenotypic detection of carbapenem resistance is a feasible alternative for routine diagnosis. Screening of CP bacteria on clinical veterinary laboratories can be made quite easy and affordable, by implementing commercially available CP selective culture media in the veterinary microbiology routine workflow. Ideally, all Enterobacterales isolates should be plated despite AST results. Furthermore, the high frequency of OXA-48-like CP bacteria reported in companion animals and the pitfalls in its detection should prompt the inclusion of temocillin in the routine AST of samples from companion animals or the use of selective culture media with high sensibility and specificity for OXA-48-like carbapenemases. Other methods, such as the biochemical test or immunochromatographic lateral flow assays, may be useful in laboratories with a high case load of suspected CR infections, which is currently not yet the case of veterinary microbiology laboratories.

Regardless of the method/methods that are chosen, the following aspects should be taken into consideration: 1) the method chosen has to have high sensitivity and specificity, as the probability of having a positive result is low; 2) The fact that reports of CP bacteria in companion animals are still scarce does not make the inclusion of this method unnecessary, on the contrary, as prevalence may be underestimated and misdiagnosis of CP bacteria may have important animal and public-health consequences; 3) in the presence of a positive result, microbiologists should follow-up the positive isolate in a specialized laboratory and advise clinicians to implement infection control procedures.

Finally, surveillance on CP bacteria in companion animals, is key to add knowledge and aid predicting the interplay of the human-environment-animal triad on the increase of carbapenem resistance, as a mean to fight the burden of AMR following the One Health concept.

The section 1.4 is based on the review article: Moreira da Silva, J., Menezes, J., Marques, C., & Pomba, C. F. (2022). Companion Animals—An Overlooked and

Misdiagnosed Reservoir of Carbapenem Resistance. Antibiotics, 11(4), 533. https://doi.org/10.3390/antibiotics11040533

1.5 The importance of infection, prevention and control programmes

Infection, prevention and control (IPC) is a crucial part of public health strategies to prevent the dissemination of infectious diseases and AMR, especially in healthcare settings . Additionally, IPC protocols aim to protect patients and healthcare workers by ensuring a safe and hygienic environment. There are multiple core components that must be taken into consideration when implementing IPC in a healthcare facility (Table 5).

The primary goal of IPC is to reduce HAIs and, consequently, control the spread of AMR within a health care facility (257). As previously mentioned, HAI and AMR have a negative impact in healthcare systems economically, and having an efficient IPC programme will ease the global economic burden . Additionally, IPC measures are also designed to not only protect patients and workers, but also visitors, from epidemic and pandemic pathogens (258).

In human medicine, hospitals are aware of which bacterial clones are circulating in their wards by passive surveillance and are controlled by their infectious disease committee as part of their core components determined by WHO (**Table 5**). European and national guidelines are in place to be followed and help mitigate the occurrence of outbreaks (259). Some studies have been conducted to evaluate the impact that IPC interventions have in the reduction of HAI, specifically in carbapenem-resistant Gram-negative bacteria.

A review paper in 2022 condensed all the available information, concluding that the exiting data favour the active screening culture of patients as part of IPC interventions, however more studies are necessary to increase the quality of existing evidence (260). Nonetheless, a study conducted in a neonatal unit showed reduction on the incidence of bacteraemia caused by ESBL-producing *K. pneumoniae* ST307 after reinforcement of hand hygiene and contact precautions amongst healthcare workers, more frequent cleaning and disinfection of fomites and active surveillance cultures in patients without clinical symptoms (261). The same interventions were effective in the control of carbapenem-resistant *K. pneumoniae,* together with staff training and patient isolation

(262). Another study reported reduction on the incidence of carbapenem-resistant *K. pneumoniae* in intensive care units following an outbreak and active screening culture and a team of healthcare workers responsible for monitoring IPC interventions (263).

In veterinary medicine of companion animals, there is some documentation on how to effectively apply IPC protocols directed at the unique characteristics of this healthcare setting (264,265). Yet, they are very simple and not all professionals may be aware of them.

In the following sections, a closer look in the interventions applied to human medicine are discussed.

Table 5 – Infection, prevention and control core components and main remarks. Adapted from World Health Organization guidelines 2016 (266).

Core component	Main remark
IPC programmes	Dedicated and trained team for the purpose of combating HAI and AMR The programme must have clear objectives based on local epidemiology Reliable quality microbiological support is crucial for an effective IPC programme
IPC guidelines	Development of evidence-based guidelines for implementation to reduce HAI and AMR It is essential to adapt the guidelines to the local epidemiology of the healthcare facility Training of the team on the guidelines is fundamental for a successful performance
IPC education and training	Workshops and simulation activities to all healthcare workers Training contents should be tailored to the level of seniority and type of human resource. Three categories have been identified – IPC specialists; workers directly involved in patient care/service delivery and personnel who support health delivery (for example, administrative staff and housekeeping)
Surveillance	Be aware of commonly-associated microorganisms to HAI and AMR resistance patterns by prevalence and incidence data Active and passive surveillance systems Data compilation to drive personalized guidelines
Multimodal strategies	Cultural change in the working environment as IPC reflects good patient care and safety Campaigns promoting behavioural changes Accreditation bodies and interventions to present results to stakeholders

Core component	Main remark
Monitoring IPC practices and feedback	Behavioural changes and relevant alterations to improve patient safety Behavioural changes to facilitate the compliance with IPC programmes Evaluate the impact IPC programme has on the reduction of HAI and AMR Hand hygiene monitorization
Workload, staff and bed occupancy	Bed occupancy should not exceed the facility capacity Number of staff must be adequate to expected workload

AMR – Antimicrobial resistance; HAI – Healthcare-associated infection; IPC – Infection, prevention and control

1.5.1 Surveillance

The surveillance component of IPC can be achieved by active and/or passive surveillance. Active surveillance engages hospital staff in acquiring data on reported cases and sometimes, identifying them specifically for the intent of hospital surveillance or to curate local epidemiology data. Conversely, passive surveillance is performed using administrative data available and is normally reported to the national public health agency, which then compiles and analyses it (267,268). Although they often work independently, which can lead to different results at times, passive surveillance data can enhance and complement data from active surveillance (267).

When the local epidemiology of a hospital facility is known, regarding its HAI and AMR patterns, it is easier to detect an emerging outbreak. Despite the fact that outbreak preparedness and response is a central point of IPC, the COVID-19 pandemic highlighted the existing gaps in IPC worldwide, such as hand hygiene and accessible personal protective equipment (PPE) (269).

1.5.2 Staff education and training

Another important element of a cost-effective IPC is staff training, particularly in hand hygiene. The use of hand hygiene practices to prevent and reduce HAI, cross-infections and diminish the spread of AMR has been a routine practice for some time (270). WHO established the moments when hand hygiene should occur during patient clinical care, yet studies have shown that compliance remains low and it is dependent on the resources available at the healthcare facility (270–272). Another important aspect in staff education is the use of gloves. It is imperative for staff members to understand that the use of gloves mustn't replace hand hygiene and that this is warranted after removal of gloves, since contamination from the environment or after patient contact can also occur (272).

Nasal colonization is also to be considered in IPC for the use of PPE, mostly for the carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) of healthcare workers (273,274). The presence of MDR Gram-negative bacteria in the nose is rarer, although one study has shown a higher rate of nasal colonization in intensive care units workers (275).

As for faecal colonization, there are a few studies which show the colonization of workers with MDR bacteria (276,277).

Within health care facilities, pathogens are typically transmitted from a patient carrying the infection to a vulnerable individual through healthcare workers' hands (278). However, hospital environments and medical devices can also play a role in spreading infections, albeit less frequently than water and air (279). The routes of such transmissions have been mapped out, and these diagrams (Figure 7) serve as a foundation for creating strategies aimed at halting the spread of pathogens.

Patient colonized with bacteria Occurrence of the same bacteria on hospital surfaces, possibly disseminated by the patient.

Cross-transmission of the bacteria through contact between patient and doctor.

Cross-contamination between patient A and patient B by the hands of the doctor.

Failure to cleanse hands during patient care results in within-patient cross-transmission

Figure 7 – Example of a transmission route for pathogens by hands in human hospital setting. Reprinted from Pittet *et al.*, 2006 (280) with permission from Elsevier (license number 5900151366500).

1.5.3 Environmental and staff screening

A crucial aspect that always needs to be considered in IPC programmes is the environmental cleaning and disinfection procedures, mostly due to the formation of biofilms. Biofilms are a community of microorganisms growing as a layer on inanimate surfaces, commonly associated to agents responsible for HAI such as *P. aeruginosa*, *A. baumannii* and *S. maltophilia* (272). High-touch surfaces and fomites play an important role in the spread of AMR bacteria within hospital facilities, with a possible correlation between environmental cleaning and prevalence of MDR organisms in the environment (281), with a link between ESKAPE pathogens and hospital environmental contamination (130,282–286).

Depending on the organisms, its ability to survive in inanimate surfaces varies, ranging from a few days to months. Nonetheless, most Gram-negative MDR organisms (such as *Pseudomonas* spp. and *Klebsiella* spp.) are capable of persisting for months (279,287).

Thus, within the core IPC component of education and training, it is necessary for staff to learn how to clean and disinfect surfaces correctly, minimizing the spread of MDR organisms, especially as the disinfectant effectiveness and choice may vary according to the bacteria (278).

The frequency of environmental contamination screening in hospitals can vary based on several factors, including the type of facility, the patient population, and the prevalence of infections (including HAIs), as well as data collected from the IPC surveillance component.

There is no universally established frequency for environmental contamination screening, so hospitals decide when to audit the efficacy of their IPC programme (as part of core-component). However, these screenings usually become more frequent when trying to identify the environmental source of an outbreak (288).

Regardless of when screening is performed, there are breakpoints established for how many colony forming units (CFU) per cm^2 microorganisms are expected to grow on surfaces whose clean and disinfecting protocol is working appropriately (288,289).

Environmental sample collection can be done in at least two different ways: (i) by using surface swabs on high-touch surfaces, followed by plating on non- and selective culture media for MDR organisms; (ii) by using direct non-selective agar contact plates on high-touch surfaces. On both instances, it is possible to evaluate the bacterial burden of surfaces, although surface swabs are more laborious, mainly used to identify specific pathogens during outbreaks, or on research studies.

As for staff screening, data show that intestinal carriage by hospital healthcare workers offers limited contribution to the dissemination of MDR (277) and European guidelines do not support rectal screening in hospital staff (27). For hand hygiene, the majority of studies evaluating the impact hand hygiene had on MDR dissemination by staff's screening focus on MRSA carriage (290,291), while the existing guidelines for nasal screening are also for MRSA and recommend for workforce not to be screened unless there is an epidemiological reason (292).

1.5.4 Transmission routes and where to tackle

A possible route of dissemination of bacteria within healthcare facilities has been established, from which four key-points are highlighted and represent where to intervene in order to prevent further spreading (**Figure 8**).

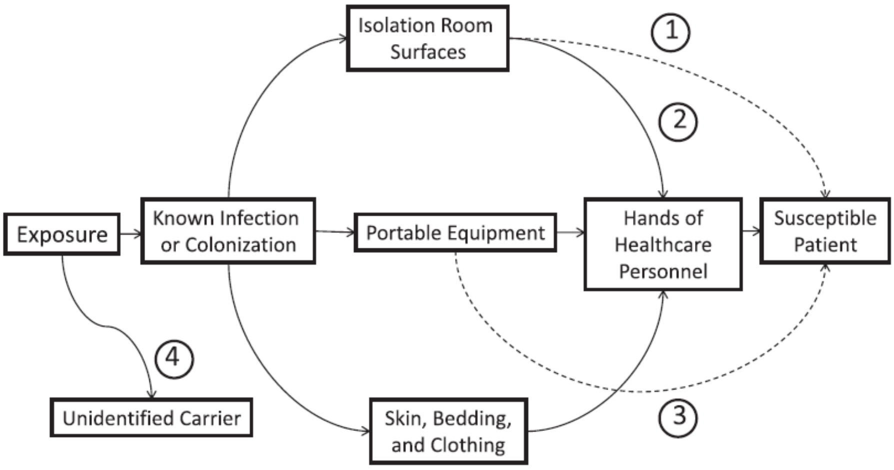

Figure 8 - Outline of common routes of dissemination and key disinfection points to prevent the spread of multidrug resistant (MDR) organisms. The primary step is thorough room cleaning post-patient discharge to avoid infecting the next occupant (1). Secondly, staff hand hygiene and surface disinfection are vital to stop them from becoming transmission vectors. Equipment must also be regularly disinfected (2,3). Lastly, undetected infected patients can spread HAIs without consistent disinfection practices (4). Reprinted from Donskey 2013 (278) with permission from Elvesier (license number 5899320248809).

After a patient is identified as infected or carrying a microorganism, isolation measures are implemented to prevent the spread of the infection. Surfaces and materials in the patient's room, along with any equipment used, become potential contamination points. Therefore, adherence to daily cleaning and disinfection routines is essential (287).

Healthcare workers' hands may also become contaminated through contact with these surfaces. As they continue their duties, there is a risk of transmitting the microorganism to other patients who are at risk of infection (**Figure 8**). Additionally, susceptible patients may acquire infections through direct contact with contaminated surfaces or equipment (279,293).

As such, disinfection has to be an integrated part of the IPC protocol, without forgetting the use of appropriate standards. It is important to establish principles for cleaning and disinfection which must cover routines, type of disinfectants used and how to use them. Additionally, fulfilment of these procedures has to be audited and monitored from time to time as to understand if any changes are necessary (294).

The successful implementation of IPC programmes is fundamental to maintain a safe, effective, and trustworthy healthcare system. For that reason, the European Society of Clinical Microbiology and Infectious Disease (ESCMID) has guidelines on which interventions to follow in order to mitigate the transmission of MDR Gram-negative bacteria in hospital settings (27). In them, endemic and outbreak setting are defined since recommendations vary according to the event. Some of the recommendations include hand hygiene educational programmes for both settings, while isolation of patients colonized or infected is only recommended in outbreak setting. Furthermore, contact precautions and an alert code to rapidly identify patients colonized is required in both settings. On the other hand, active surveillance culture must be implemented in an outbreak situation, while in an endemic situation, there is not enough evidence to force implementation as part of the normal IPC programme of these healthcare facilities. A new version of these guidelines will be released in 2025.

1.5.5 IPC in veterinary medicine and One Health

IPC programmes are indispensable in safeguarding public health, especially within healthcare environments. They play a crucial role in reducing HAI, controlling AMR, protecting healthcare workers, enhancing patient safety, managing outbreaks, providing economic benefits, ensuring regulatory compliance, and fostering continuous education (257). All of these standards are rare in Veterinary medicine, despite being covered in the documents available (264).

Taking into consideration the high number of companion animals per household that exist nowadays, the high expectations of small animal caretakers regarding veterinary healthcare, and the growing tendency of carbapenem-resistant bacteria causing infections in companion animals (115,130,286), it is not surprising that small animal veterinary healthcare mirrors human healthcare, showing an increase of HAI and AMR over the last decades (295–297). In this regard, the role of companion animals and of the veterinary healthcare setting in the dissemination of carbapenem-resistant bacteria might become a public health concern in the near future.

IPC documentations and its importance directed at veterinary medicine are publicly available, highlighting some of the components to be considered and offering guidance on how to apply them in this setting (264,265). Yet, there is still a long way to go regarding application of IPC programmes in small animal veterinary practices. First, it is important to establish which MDR bacteria can be found in the veterinary healthcare context. In this regard, studies started to be conducted in the past four years (130,286,297,298). Only then can interventions be applied and measure how effective they are in reducing MDR bacteria in the veterinary context.

Studies regarding IPC implementation and the role of veterinary healthcare facilities in the dissemination of AMR bacteria in admitted animal patients and to the humans in the community are still scarce. Highlighting the importance of the veterinary healthcare setting under a One Health concept, one study has documented the carriage of OXA-181-producing *E. coli* and NDM-5-producing *E. coli* by two employees in different veterinary practices (298). Interestingly, both these strains were closely related to environmental and clinical strains of the studied veterinary practice, respectively (298).

Regarding carbapenem resistance, in addition to environmental sampling, adequate support from diagnostic laboratories will also be necessary, since accurate detection methods are still being optimized as shown in section 1.4 of this chapter.

Still, there is a knowledge gap on what is circulating in the environment of small animal veterinary practices regarding MDR and how these resonate within clinical strains and possible outbreaks in Portugal, as geographic differences are expected, following the pattern of carbapenem-resistant bacteria in human medicine.

1.6 Dissertation goals and workflow outline

As shown throughout the introduction, carbapenem-resistant Gram-negative bacteria are emerging in Veterinary Medicine of companion animals with the same genetic characteristics as those described in Human Medicine, with the absence of IPC protocols to further prevent their spreading to other players in the same context. Thus, the work of this thesis focused on IPC core component of surveillance and monitoring IPC practices and feedback (Table 5).

The main goal of this dissertation was to evaluate which MDR bacteria can be found in the veterinary healthcare setting and how do they relate to previously described Human strains. The specific goals were as follows: (i) characterize a group of clinical strains from companion animals and characterize the carbapenemase-producing Enterobacterales (CPE) through whole-genome sequencing (WGS); (ii) describe the basal level of environmental contamination of small animal veterinary practices; (iii) explore the spreading of carbapenem-resistant bacteria within environmental surfaces and veterinary staff.

Therefore, this dissertation is organized as follows:

Carbapenemase-producing Enterobacterales from sick companion animals

- Chapters 2 & 3 depict the prevalence of carbapenemase-producing Enterobacterales from sick companion animals and how similar to nosocomial human strains they are.

Environmental contamination of small animal veterinary practices across Portugal

- Chapters 4 & 5 focus on the environmental contamination of small animal veterinary practices by OXA-23-producing *Acinetobacter* spp. and other carbapenem-resistant Gram-negative bacteria.

General discussion and conclusion

- Chapter 6 presents a general discussion of all the data presented in this dissertation and their relevance within literature. Chapter 7 reflects on the conclusions drawn by these studies and future work.

A graphical representation of the work can be seen on Figure 9.

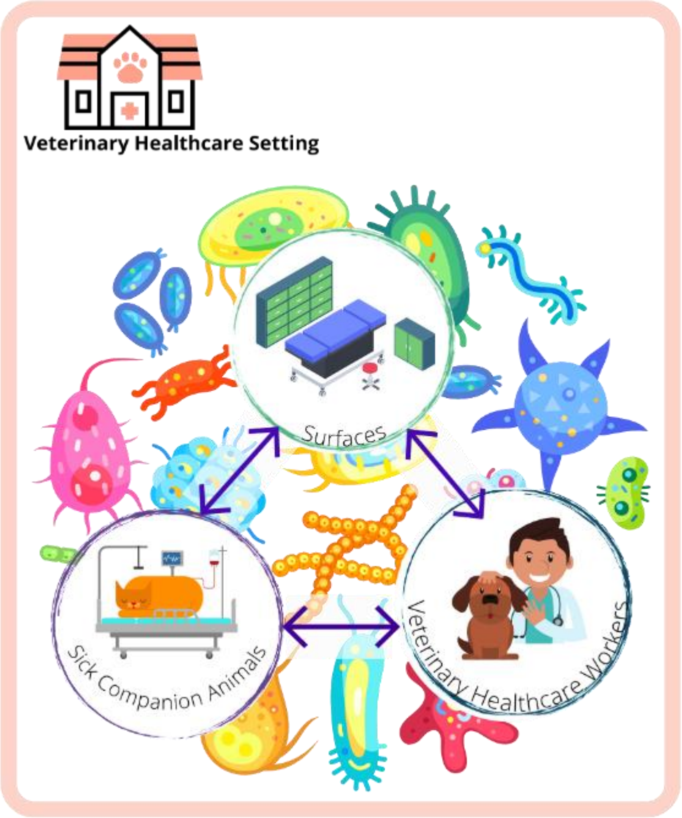

Figure 9 – Visual representation of the dissertation.

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Carbapenemase -producing Enterobacterales from sick companion animals

Chapter 2 KPC-3-producing *Klebsiella pneumoniae* ST392

This chapter is based on the published peer-reviewed paper:

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LETTER TO THE EDITOR

KPC-3-Producing *Klebsiella pneumoniae* Sequence Type 392 from a Dog's Clinical Isolate in Portugal

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KEYWORDS Carbapenemases, companion animals, multidrug-resistant *Klebsiella pneumoniae*

Resistance to carbapenems in Enterobacterales poses a threat to health care systems worldwide since those infections are associated with high mortality and limited treatment options. The latest European reports show that some southern European countries are almost endemic for carbapenemase-producing Enterobacterales (CPEs) (1, 2). Mirroring the European tendency, Portugal has been observing a steady increase in the occurrence of carbapenem-resistant *Klebsiella pneumoniae*, particularly KPC-3-producing *K. pneumoniae* ST147 lineage (Clonal Group 147) strains in health care settings (3, 4).

To assess the relevance of CPEs in veterinary health care, 977 Enterobacterales isolates obtained at the veterinary molecular diagnostic laboratory (Genevet) during 2020 were screened for carbapenem resistance. Phenotypic disc testing with 10 μg meropenem, 30 μg temocillin, and MAST CAT-ID discs (Mast Group, UK) and genotypic confirmatory tests

TABLE 1 Antimicrobial MICs and resistance genes identified during WGS analysis on KPC-3-producing *Klebsiella pneumoniae* ST392[a]

Antimicrobials tested	MIC (mg/L)	Susceptibility phenotype[b]	AMR genes
Amikacin	≤8	S	*aac(6')-Ib-cr*
Ampicillin	>16	R	$bla_{\text{TEM-1B;}}$ $bla_{\text{SHV-11;}}$ $bla_{\text{CTX-M-15;}}$ $bla_{\text{KPC-3}}$
Amoxicillin-clavulanic acid	>16/8	R[c]	$bla_{\text{CTX-M-15;}}$ $bla_{\text{KPC-3}}$
Aztreonam	>16	R	$bla_{\text{CTX-M-15;}}$ $bla_{\text{KPC-3}}$
Cefotaxime	>32	R	$bla_{\text{CTX-M-15;}}$ $bla_{\text{KPC-3}}$
Ceftazidime	>16	R	$bla_{\text{CTX-M-15;}}$ $bla_{\text{KPC-3}}$
Ceftazidime-avibactam	2	S	NA
Ciprofloxacin	>2	R	*aac(6')-Ib-cr; OqxB/A; qnrB1;*
Colistin	≤2	S	NA
Ertapenem	>1	R	$bla_{\text{KPC-3}}$
Gentamicin	≤2	S	*aac(6')-Ib-cr*
Imipenem	>8	R	$bla_{\text{KPC-3}}$
Meropenem	>8	R	$bla_{\text{KPC-3}}$
Tetracycline	>8	R[c]	*tet(A)*
Trimethoprim/sulfamethoxazole	≤2/73	S[c]	*sul2; OqxB/A*

[a]S, susceptible; R, resistant; NA, not applicable; AMR, antimicrobial resistance; WGS, whole genome sequencing.
[b]Susceptibility phenotype was determined according to EUCAST breakpoint guidelines.
[c]Susceptibility phenotype was determined according to Clinical and Laboratory Standards Institute guidelines (9).

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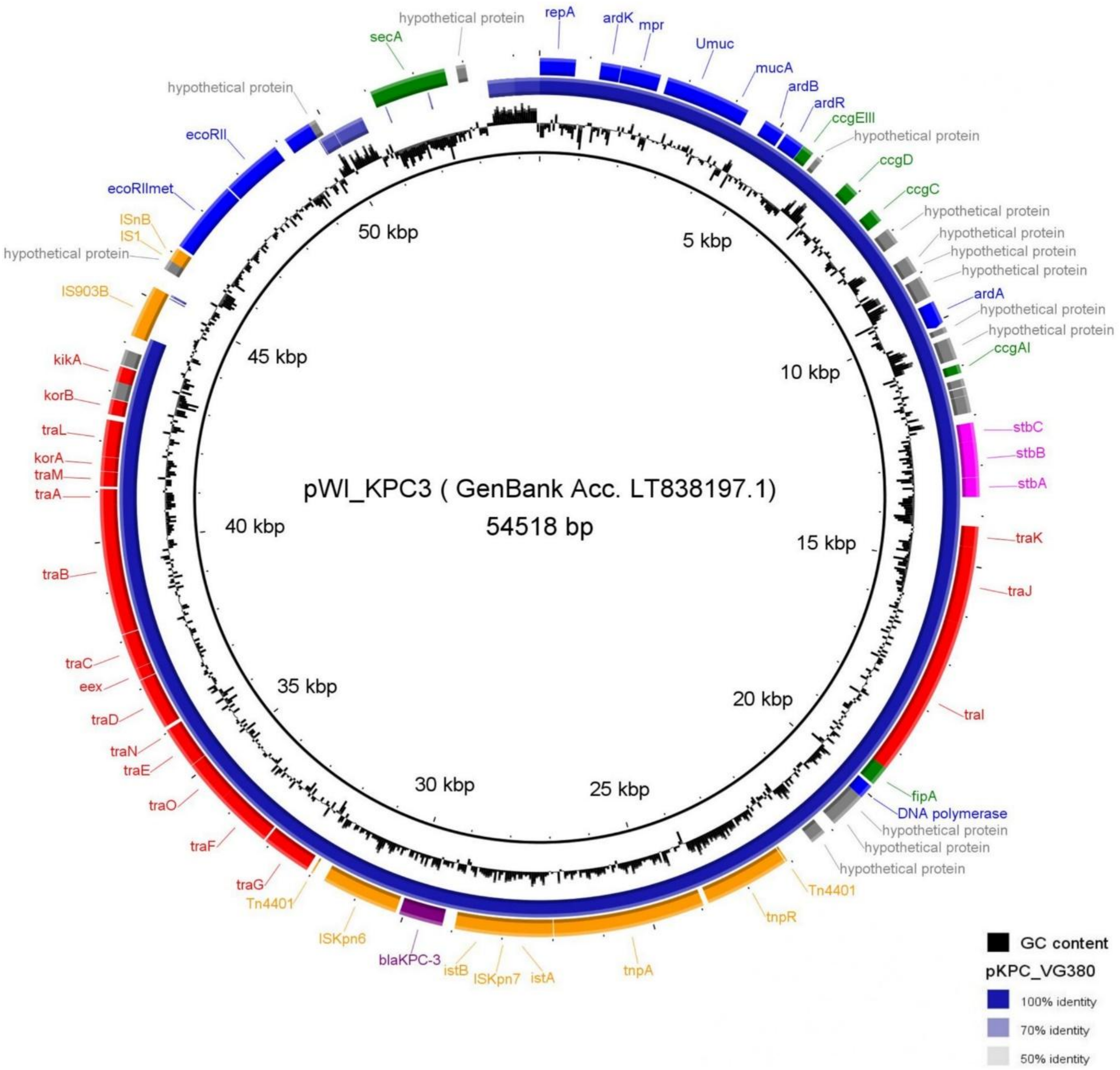

FIG 1 Plasmid alignment comparison between *de novo* assembled contig pKPC_VG380 (GenBank accession number PRJNA808048) found on the strain KPC-3-producing *Klebsiella pneumoniae* taken from a dog's with an upper respiratory tract infection (in blue) and plasmid pWI_KPC3 (GenBank accession number LT838197.1), used as backbone plasmid reference, previously described on a French nosocomial isolate (11). Genes are represented by colored blocks: purple, resistance gene; blue, DNA replication, regulation, and restriction systems; red, conjugation-association genes; fuchsia, genes associated with partition and stability systems; orange, transposons, insertion sequences (IS), and transposase genes; green, other genes; gray, hypothetical proteins. Image generated using BRIG 0.95, available at http://brig.sourceforge.net/.

were performed to detect plasmid-mediated AmpC β-lactamase and ESBL- and carbapenemase-producing Enterobacterales (5, 6).

Interestingly, a carbapenem-resistant and carbapenemase-producing *K. pneumoniae* isolate (VG380) was recovered, being positive for the $bla_{\text{KPC-type}}$ gene. It was recovered from a nasal swab collected from a 10-year-old dog with an upper respiratory tract infection. Cytology of the nasal exudate displayed an abundance of degenerated neutrophils, bacilli, and cocci. Biopsy of the nasal tissue displayed an ulceration showing signs of mixed inflammation, with an abundance of neutrophils, lymphocytes, macrophages, and dense fibrovascular proliferation, which was compatible with the diagnosis of chronic ulcerative rhinitis. A 1-month course of treatment with trimethoprim-sulfamethoxazole was followed and the dog made a full recovery.

K. pneumoniae strain VG380 was resistant to ertapenem, meropenem, and imipenem (MIC >1 mg/L for ertapenem and >8 mg/L for imipenem and meropenem; determined by MicroSan NEG44 plates [Beckman Coulter, USA]). It was susceptible to ceftazidime-avibactam (MIC = 2 mg/L; determined by Etest [bioMérieux, France]). Interpretation of MIC determinations followed EUCAST breakpoint tables (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_12.0_Breakpoint_Tables.pdf).

Whole Genome Sequencing (WGS) analysis of strain VG380 was performed using Illumina NovaSeq platform with 2 × 150-bp paired-end reads. The quality of the resulting raw reads was evaluated using FastQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). *De novo* genomes were assembled using SPAdes v3.14.1 (7), following two rounds of polishing with Pilon v1.24 (8). The generated assemblies were used to screen for antimicrobial resistance genes and mobile genetic elements resorting to tools available at Centre for Genomic Epidemiology (http://genomicepidemiology.org/), ResFinder 4.1 and Mobile Element Finder v1.0.3, respectively. MLST 2.0 and pMLST 2.0 were also performed.

WGS analysis showed that VG380 possessed a KPC-3-encoding gene and belonged to Sequence Type ST392, a single-locus variant of ST147, also belonging to Clonal Group 147. Therefore, this canine strain was related to the KPC-3-producing *K. pneumoniae* ST147 lineage responsible for the majority of nosocomial infections reported so far in Portugal (3, 4). Virulence factors *traT* and *iutA* were identified, with the former being located on a IncFII(K)-type plasmid. IncFIB(K)- and IncN- type plasmids were also identified. Noteworthy, strain VG380 harbored a series of resistance genes leading to a quite unusual multi-drug resistance profile for a canine isolate (Table 1).

The bla_{KPC-3} gene was located within transposon Tn*4401d* (10) on an ~50-kb IncN-type plasmid (pKPC_VG380, pMLST ST15) (GenBank accession number PRJNA808048) (Fig. 1). When using a previously described IncN plasmid carrying the bla_{KPC-3} gene in Tn*4401* (pWI_KPC3, pMLST ST15) of clinical human origin (11) as a reference for BLAST, it was possible to demonstrate that one of the contigs for the KPC-3-producing *K. pneumoniae* ST392 VG380 had a large extent of homology with the reference plasmid (Fig. 1).

We report here, to the best of our knowledge, the first characterization of a KPC-3-producing *K. pneumoniae* strain, belonging to the human high-risk clonal group 147 isolated from a dog in Europe. Noteworthy, isolates belonging to the high-risk emerging lineage ST392 from CG147 and carrying the bla_{KPC-3} gene have been reported in a single Portuguese central hospital during 2020 (4).

To prevent spreading of carbapenem resistance, a one-health surveillance approach is urgent. The study of the epidemiology of plasmid-mediated carbapenem resistance seems to be relevant for the identification of possible pathways for antimicrobial resistance transmission.

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Chapter 3 CPE strains causing infections in companion animals

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Supplementary material to this peer-reviewed paper can be found on Annex B.

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| Clinical Microbiology | Research Article

Carbapenemase-producing Enterobacterales strains causing infections in companion animals—Portugal

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ABSTRACT An increase in *Klebsiella pneumoniae* carbapenem-resistant human nosocomial strains is occurring in Europe, namely with the *bla*$_{\text{OXA-48-like}}$ and *bla*$_{\text{KPC-like}}$ genes. We determined the prevalence of carbapenemase-producing Enterobacterales clinical strains in companion animals in Portugal and characterized their mobile genetic elements. Susceptibility data of a consecutive collection of 977 Enterobacterales clinical strains from a Portuguese private veterinary diagnostic laboratory were evaluated (January–December 2020). Additional phenotypical and genotypical assays were performed in a subset of 261 strains with a resistant phenotype. Whole-genome sequencing was performed for carbapenemase-producing strains. The frequency of carbapenemase-producing Enterobacterales clinical strains in companion animals in Portugal was 0.51% (*n* = 5/977). Thus, five strains were characterized: (i) one OXA-181-producing *K. pneumoniae* ST273, (ii) two KPC-3-producing *K. pneumoniae* ST147; (iii) one KPC-3-producing *K. pneumoniae* ST392; and (iv) one OXA-48-producing *E. coli* ST127. The *bla*$_{\text{KPC-3}}$ gene was located on transposon Tn*4401d* on IncFIA type plasmid for the *K. pneumoniae* ST147 strains and on a IncN-type plasmid for the *K. pneumoniae* ST392 strain, while *bla*$_{\text{OXA-181}}$ gene was located on an IncX3 plasmid. All *de novo* assembled plasmids and plasmid-encoded transposons harboring carbapenemase genes were homologous to those previously described in the human healthcare. No plasmid replicons were detected on the OXA-48-producing *E. coli* ST127. The dissemination of carbapenem resistance is occurring horizontally *via* plasmid spreading from the human high burden carbapenem resistance setting to the companion animal sector. Furthermore, companion animals may act as reservoirs of carbapenem resistance. Implementation of carbapenemase detection methods in routine clinical veterinary microbiology is urgently needed.

IMPORTANCE This is the first study on the prevalence of carbapenemase-producing Enterobacterales (CPE) clinical strains from companion animals in Portugal. Despite the generally low prevalence of CPE in companion animals, it is imperative for veterinary diagnostic laboratories to employ diagnostic methods for carbapenemase detection. The resemblance found in the mobile genetic elements transporting carbapenemase genes between veterinary medicine and human medicine implies a potential circulation within a One Health framework.

KEYWORDS veterinary, diagnostics, KPC-3, *Klebsiella pneumoniae*, OXA-48, *E. coli*, OXA-181

Resistance to carbapenems by Enterobacterales poses a threat to healthcare systems worldwide. Such infections are associated with high mortality, with limited options for treatment available (1). The 2021 European Centre for Disease Prevention and Control (ECDC) report demonstrates an increase in carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* strains. While the prevalence of *E. coli* strains resistant to

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carbapenems remains low, a quarter of European countries report a prevalence above 10% in *K. pneumoniae* carbapenem-resistant strains (2).

The most predominant genes across Europe are the $bla_{\text{OXA-48-like}}$ and $bla_{\text{KPC-like}}$ associated with *K. pneumoniae* strains (3). Similarly, in Portugal, a rise in the number of studies reporting *K. pneumoniae* strains resistant to carbapenems in the healthcare setting is observed, with $bla_{\text{OXA-181}}$ and $bla_{\text{KPC-3}}$ being the primary genes detected (4, 5).

In 2020, The European Medicines Agency (EMA) performed an update on the categorization of antibiotics available for use in Veterinary medicine which impact public health, where carbapenems are classified as category A ("Avoid use") (6). Yet, carbapenem-resistant bacteria have been described either as causative agents of infection or commensal in dogs and cats across the world (7–10). Regardless of the type of studies, most reported bacteria are identified as OXA-48-like-producing Enterobacterales (7–9). In Portugal, three reports of carbapenem-resistant bacteria in companion animals have, so far, been described: (i) an OXA-23-producing *Acinetobacter baumannii* causing a urinary tract infection in a cat (11); (ii) an OXA-181-producing *E. coli* was found as a commensal strain on a dog (12); and (iii) a KPC-3-producing *K. pneumoniae* causing an upper respiratory tract infection in a dog (13), which was previously characterized in-depth by the authors of this study. Thus, a comprehensive screening for the prevalence of carbapenem-resistant bacteria in companion animals is lacking.

The purpose of this retrospective study is to determine the frequency of carbapenem-resistant bacteria causing infection in companion animals and to evaluate the possible transmission of mobile genetic elements carrying carbapenemase genes within the One Health context.

In partnership with a veterinary diagnostic laboratory, a consecutive collection of Enterobacterales strains collected during 2020 was evaluated and whole-genome sequencing (WGS) was used to further characterize carbapenemase-producing strains.

MATERIALS AND METHODS

Bacterial strains

Samples for microbiology analysis were sent from different veterinary practices in Portugal to the Genevet Molecular Diagnostic Laboratory. Enterobacterales clinical strains (*E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., and *Proteus* spp.) were identified using MacConkey and chromogenic agar UriSelect™ (Bio-Rad, US). Antibiogram results for a consecutive collection of 977 Enterobacterales clinical strains of 2020 were interpreted according to EUCAST 2023 and Veterinary CLSI breakpoint guidelines (14, 15). Minimum inhibitory concentrations (MICs) were determined using MicroSan NEG44 plates (Beckman Coulter, US) when requested by clinicians. Pure bacterial strains were stored in Brain Heart Infusion liquid media with 20% glycerol at −20°C.

Enterobacterales strain collection

Within the initial collection, 21 strains were unaccounted for or did not grow when defrosted, thus being excluded from further analyses. Four groups were observed: (i) clinical strains with an ESBL phenotype (n = 204); (ii) clinical strains with a possible OXA-48-like phenotype (resistance toward amoxicillin in combination with clavulanic acid while susceptible to cefoxitin and cefotaxime) (n = 34) (16); (iii) clinical strains with a multidrug-resistant (MDR) profile as per criteria established by Magiorakos et al., 2012 (17) (resistance to enrofloxacin, gentamicin, tetracycline, and trimethoprim/sulfamethoxazole) (17) (n = 23); and (iv) susceptible clinical strains (susceptibility to ampicillin/amoxicillin, amoxicillin in association with clavulanic acid, cephalothin, cefotaxime, ceftazidime, cefoxitin, trimethoprim/sulfamethoxazole, gentamicin, tetracycline, enrofloxacin, and amikacin) (n = 692). Due to the observed resistant phenotype, only study groups i, ii, and iii were included. This inclusion criteria resulted in a subset of 261 strains that were additionally phenotypically and genotypically evaluated.

Phenotypic screening

Additional phenotypic antimicrobial susceptibility testing for the Enterobacterales strains was carried out against meropenem (10 µg), imipenem (10 µg), temocillin (TMC, 30 µg), and MAST CAT-ID discs (Mast Group, United Kingdom). KPC-3-producing strains were also tested against Ceftazidime-Avibactam (CAZ, 14 µg) by disc diffusion method. Results were interpreted in accordance with EUCAST breakpoint guidelines 2023 (14). TMC discs and MAST CAT-ID discs were used as previously described in Moreira da Silva et al., 2022 (8). For all carbapenemase-producing strains, MIC panel testing was performed and interpreted according to Veterinary CLSI breakpoints or EUCAST breakpoints (14, 15).

Genotypic screening via PCR assays and sequencing

DNA was extracted from pure cultures using a boiling extraction method, and a series of multiplex PCRs were performed, as previously described for the detection of extended-spectrum β-lactamase, plasmid-encoded AmpC β-lactamase and carbapenemase genes (18). Sanger sequencing was performed to identify the amplified β-lactamase and carbapenemase genes. Confirmation of species identification was performed by sequencing 16S rRNA as previously described (19).

WGS analysis

Strains harboring carbapenemase genes were selected for whole-genome sequencing. Whole DNA was extracted from RNase-treated lysates *via* NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal). All libraries for WGS were prepared using a TruSeq DNA PCR-Free preparation kit (Illumina, San Diego, California, USA). DNA sequencing was performed using the Illumina NovaSeq platform with 2 × 150 bp paired-end reads. The quality of the resulting raw reads was evaluated using FastQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), read quality filtering was performed using PRINSEQ v0.20.4 (20) using the following criteria, mean base quality score ≥20 and minimum read length of 90 nt. *De novo* genomes were assembled using SPAdes v3.14.1 (21), following two rounds of polishing using Pilon v1.24 (22). Finally, genome annotation was performed using Prokka v1.14.6 (23). Resfinder 4.1 was used to screen the novel generated assemblies for antimicrobial resistance genes, while Mobile Element Finder v1.0.3 and PlasmidFinder v2.1 were used to locate the antimicrobial resistance genes in the generated assemblies and plasmid's incompatibility group, respectively. MLST 2.0 and plasmid MLST 2.0 (pMLST) were also performed as typing tools for the *de novo* generated genomes and plasmids identified, respectively. In addition, for the *E. coli* isolate, the tool VirulenceFinder 2.0 was also used to identify the existing virulence factors. All tools are available at the Centre of Genomic Epidemiology (https://www.genomicepidemiology.org/). Pathogenwatch online platform was used to search for outer-membrane protein alteration on the *K. pneumoniae* strains (24).

Phylogenetic analysis

Parsnp v1.2 was used to create a multiple sequence alignment (MSA) of the generated assemblies plus the reference genome *K. pneumoniae* ST258 NJST258_2 (GCF_000597905.1) and publicly available *K. pneumoniae* sequences Clonal Group (CG) 147 isolated in Europe of human origin (Table S2: Data used to generate *Klebsiella pneumoniae* Clonal Group 147 phylogenetic tree) (25). The obtained MSA was used as an input by Gubbins to generate a phylogeny of corrected for recombination events with bootstrapping (100 replicates) (26). The obtained tree and MSA corrected for recombination were submitted to Raxml-NG to infer a phylogeny with bootstrapping support (27). iTOL software was used to visualize the obtained tree (28).

Plasmid comparison

Using the BRIG analysis tool (29), obtained contigs that contained antimicrobial resistance genes were aligned to a reference plasmid on the NCBI database (Table S3: Accession numbers and relevant information on plasmids used for the study's contigs circularization).

RESULTS

Antimicrobial susceptibility of the initial collection by the veterinary microbiology diagnostic laboratory

The first approach to search for possible carbapenemase-producing Enterobacterales (CPE) strains was to assess the susceptibility patterns in a collection of consecutive samples from 2020, which resulted in 977 strains. The phenotypic evaluation of all these strains is described in Table S1. It was possible to observe resistance to critically important antimicrobials for human medicine, according to the WHO classification (30), particularly the high prevalence of resistance to third-generation cephalosporins (204 clinical strains with an ESBL phenotype, 20.8%), followed by resistance to fluoroquinolones. Of importance, resistance to trimethoprim/sulfamethoxazole (classified as category D by EMA) (6) was also high (Table S1).

Some strains (n = 62, 6.4%) had extended susceptibility information on their MIC including the evaluation of carbapenems. Results are present in Table S4 (Table S4: Routine carbapenem minimal inhibitory concentration results for clinical strains; n = 62). Only one possible CPE was identified—a *Klebsiella* spp. strain resistant to ertapenem (MIC >1 mg/L).

Antimicrobial susceptibility and molecular carbapenemase confirmatory testing

Resistant clinical strains according to origin and type of infection are reported in Table S5. The most prevalent β-lactamase gene found was the $bla_{\text{TEM-1}}$ gene (n = 152; 58.2%). Within the ESBL group (n = 204), 23.5% of *K. pneumoniae* and 11.3% of *E. coli* strains harbored the $bla_{\text{CTX-M-15}}$ genes. All found β-lactamase genes are reported in Table S6 (Table S6: β-lactamase genes detected in clinical strains from companion animals; n = 261).

Results from the phenotypic screening for carbapenem resistance are depicted in Table 1. Genotypic confirmatory assays showed that within *K. pneumoniae* strains resistant to carbapenems, three were positive for the $bla_{\text{KPC-3}}$ gene (VG313, VG314, and VG380 strains). The same strains exhibited growth up to the MAST CAT-ID disc. Furthermore, among the TMC-resistant strains, one was an OXA-181-producing *K. pneumoniae* strain (VG117 strain) and the other was an OXA-48-producing *E. coli* strain (VG204 strain).

TABLE 1 Phenotypic carbapenem screening of Enterobacterales clinical strains from study groups i to iii (n = 261)

Bacterial species	Resistance to Meropenem[a] (10 µg)	Resistance to Imipenem[a] (10 µg)	Resistance to Temocillin[b] (30 µg)	MAST CAT-ID[b]
Escherichia coli	1	1	4	1
Klebsiella pneumoniae	5	6	2	10
Enterobacter cloacae complex	0	0	2	2
Bacterial species other than *Proteus* spp.	2	2	0	0

[a] Carbapenem resistance was determined in accordance with EUCAST breakpoint guidelines 2023 (14). Imipenem resistance R < 19 mm; Meropenem resistance <16 mm.
[b] Resistance was defined when the bacterial growth was in contact with the disc.

As expected, due to the ertapenem resistance, the OXA-181-producing *K. pneumoniae* strain had been previously identified at the veterinary diagnostic laboratory as a possible carbapenemase-producing strain.

Both VG204 and VG313 strains were the causative agents of urinary tract infection in a cat and a dog, respectively. VG314 strain was isolated from a dog suffering from skin and soft tissue infection, while the VG117 strain was isolated from an oesophagostomy tube infection site in a cat. VG380 strain was the causative agent of an upper respiratory tract infection in a dog.

In this study, we have found that the frequency of carbapenemase-producing Enterobacterales clinical strains in companion animals in Portugal was 0.51% (*n* = 5/977). These strains were further selected for WGS analysis.

Carbapenemase-producing-Enterobacterales characterization using whole-genome sequencing

Properties of the obtained assemblies are described in Table S7 (Table S7: Assemblies properties following WGS analysis).

OXA-181-producing *K. pneumoniae* strain belonged to the sequence type 273, while two of the KPC-3-producing *K. pneumoniae* strains belonged to ST147 (*K. pneumoniae* VG313 and VG314 strains) and the KPC-3-producing *K. pneumoniae* VG380 strain belonged to ST392. Both ST273 and ST392 are a single locus variant of ST147 (31); thus, all strains belong to Clonal Group (CG) 147, sublineage 147 (32). The obtained *K. pneumoniae* assemblies were investigated for the presence of resistance genes, with results showing that all harbored a set of resistance genes, which contributes to their MDR profiles, including $bla_{\text{CTX-M-15}}$ and *oqxB/A* genes (Table 2; Table S8: antimicrobial MICs and resistance genes identified during WGS analysis on carbapenemase-producing *K. pneumoniae* strains). All KPC-3-positive strains were susceptible to the combination disc of ceftazidime-avibactam. Both KPC-3-producing *K. pneumoniae* ST147 strains carried the *ybt16* gene on the integrative conjugative element *ICEKp12*, while the *K. pneumoniae* ST273 strain carried the *ybt9* gene on the *ICEKp3* element (Table 2).

TABLE 2 Carbapenemase resistance profile, MLST, resistance genes, virulence determinants, and plasmid replicons for carbapenemase-producing Enterobacterales strains

Strain	Month of Isolation	Location of clinic	Carbapenem resistance[a]	MLST	Resistance genes	Mutations	Plasmid replicons	Virulence factors[b]	Yersiniabactin
OXA-181-producing *K. pneumoniae* (VG117)	April	Lisbon	ERT	273	*aac(6')-lb-cr*,$bla_{\text{SHV-1}}$, $bla_{\text{CTX-M-15}}$,$bla_{\text{OXA-181}}$, *oqxB/A*, *qnrS1*, *tetD*, *sul1*, *dfrA27*	GyrA-S83I, ParC-S80I	IncFIB (K),IncF(K), Col440I,Col440II, IncX3	*iutA; fyuA irp2; traT*	*ybt 9;ICEKp3*
KPC-3-producing *K. pneumoniae* (VG313)	January	Lisbon	MEM IPM ERT	147	$bla_{\text{TEM-1A}}$; $bla_{\text{SHV-11}}$; $bla_{\text{KPC-3}}$, *sul2;oqxB/A; drfA14*	GyrA-S83I, ParC-S80I	IncFIB, IncFIA	*iutA; fyuA ;irp2*	*ybt 16;ICEKp12*
KPC-3-producing *K. pneumoniae* (VG314)	January	Coimbra	MEM IPM ERT	147	$bla_{\text{TEM-1A}}$; $bla_{\text{SHV-11}}$; $bla_{\text{KPC-3}}$, *sul2; oqxB/A; drfA14*	GyrA-S83I, ParC-S80I	IncFIB, IncFIA	*iutA; fyuA;irp2*	*ybt 16;ICEKp12*
KPC-3-producing *K. pneumoniae* (VG380)	June	Porto	MEM IPM ERT	392	*aac(6')-lb-cr*, $bla_{\text{TEM-1B}}$; $bla_{\text{SHV-11}}$; $bla_{\text{CTX-M-15}}$; $bla_{\text{KPC-3}}$*oqxB/A; qnrB1;tet(A), sul2*	GyrA-S83I, ParC-S80I	IncFIB(K), IncFII(K), IncN	*iutA*	NA[c]
OXA-48-producing *E. coli* (VG204)	March	Lisbon		127	$bla_{\text{OXA-48}}$		NA	*chuA; fyuA;vat; yfcV*	NA

[a] Carbapenem resistance was determined in accordance with EUCAST breakpoint guidelines 2023 (14). Ertapenem resistance MIC >0.5 mg/L; Imipenem resistance MIC >4 mg/L; Meropenem resistance MIC >8 mg/L.

[b] The virulence factors present in this table are the ones associated with uropathogenic lineages. The complete list of virulence factors for strain VG204 is described in Table S9.

[c] NA—not applicable.

The only resistance gene harbored by the carbapenemase-producing *E. coli* was the $bla_{OXA\text{-}48}$ gene (VG204 strain) (Table 2). MIC determination revealed resistance only to ampicillin (MIC >16 mg/L) and amoxicillin in combination with clavulanic acid (MIC >8/4 mg/L), being fully susceptible to third-generation cephalosporins (MIC ≤1 mg/L) and reduced susceptibility to carbapenems. This *E. coli* strain belonged to the ST127 uropathogenic pathotype (UPEC). In addition to having the virulence factors (VF) encoding genes characteristic of a UPEC (*chuA*, *fyuA*, *vat,* and *yfcV*) (33), it also has other VF-encoding genes which contribute to its virulent profile. The complete list of VF-encoding genes is present in Table S9 (Table S9: Complete list of virulence factors encoding genes found on the OXA-48-producing *E. coli* ST127 strain).

Phylogenetic analysis

Phylogenetic analysis showed that our strains are related to other *K. pneumoniae* belonging to the CG 147, previously described in European countries and of human origin. Figure 1 shows that the OXA-181-producing *K. pneumoniae* VG117 strain clusters with a Norwegian VIM-1-producing strain, which might indicate the capacity of the ST273 lineage to acquire different antimicrobial resistance genes. Similarly, the KPC-3-producing *K. pneumoniae* VG380 strain is clustering with strains that do not harbor any carbapenemase gene. This analysis also revealed that KPC-3-producing *K. pneumoniae* ST147 VG313 and VG314 strains belonged to the same clone, with only four single nucleotide polymorphisms (SNPs) difference, which concurs with the breakpoint established of the intra-clade relationship of 10 SNPs difference (34).

No phylogenetic analysis was performed for the OXA-48-producing *E. coli* ST127 strain as it is a commonly described lineage across the globe, both in humans and animals (35).

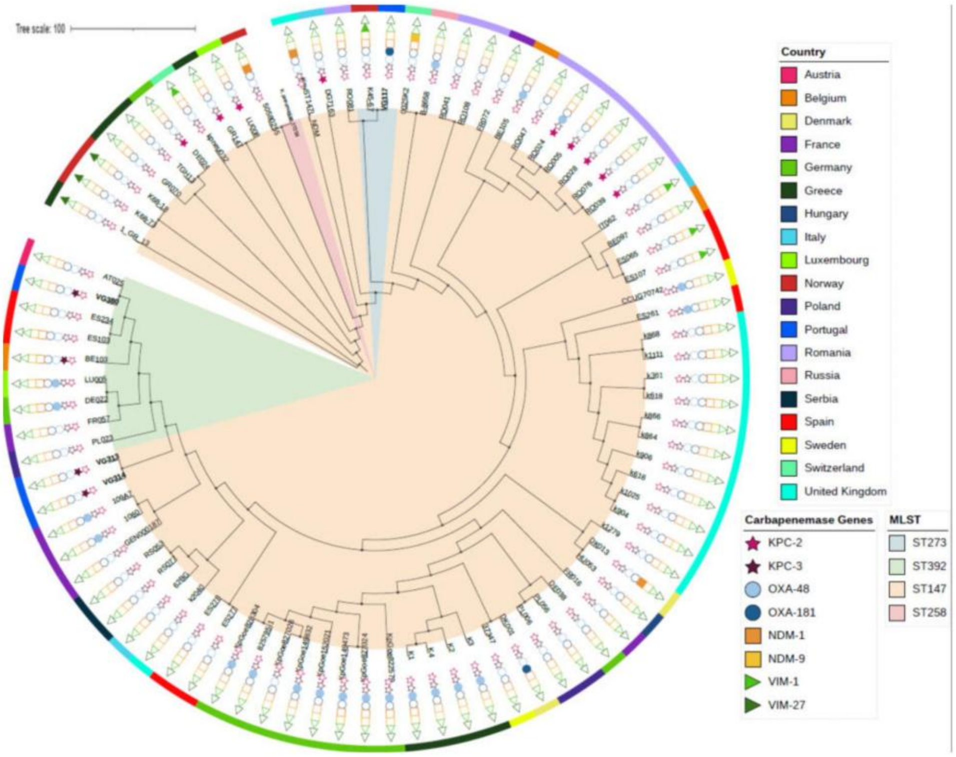

FIG 1 SNP-based phylogenetic tree for *Klebsiella pneumoniae* Clonal Group 147 in Europe from human hosts, characterized according to their carbapenemase genes and country of isolation. Blank symbols represent non-carbapenemase-producing strains. The different clades are colored according to their sequence type. The strains described in this study are in bold.

Detection of plasmid derived contigs

The $bla_{OXA-181}$ gene was located on insertion sequence IS*Kpn19* on a ~50 kb IncX3-type plasmid (pOXA_VG117), as previously described (12, 36). When using a formerly described IncX3 plasmid of clinical human origin containing the $bla_{OXA-181}$ gene as a reference for BLAST (pBC947-OXA-181) (37), several contigs of OXA-181-producing *K. pneumoniae* VG117 strain showed a large extent of homology with the reference plasmid (Fig. 2A). Both resistance genes $bla_{OXA-181}$ and *qnrS1* were part of a composite transposon flanked at both ends by IS26. When comparing our plasmid to the previously described pLB_OXA-181_PT109 (GenBank Acc. CP041033), a complete alignment of the plasmids was observed. Plasmid pLB_OXA-181_PT109 was found on a commensal OXA-181-producing *E. coli* strain from a Portuguese dog (12).

For all strains, the bla_{KPC-3} gene was located on transposon Tn*4401d*—an isoform of Tn*4401* with 68 bp deletion between ISKpn7 and bla_{KPC-3} (38, 39). This isoform has been described in different plasmid types and, similarly to previous reports, VG313 and VG314 strains contained Tn*4401d* in a ~140 kb IncFIA type plasmid (pKPC_VG313 and pKPC_VG314), whereas in VG380, the transposon was located on a ~ 50 kb IncN-type plasmid (pKPC_VG380, pMLST ST15).

A previously described IncFIA plasmid carrying the bla_{KPC-3} gene in Tn*4401d* (pBK30661) from a human nosocomial strain (40) was used as a reference for the BLAST analysis. Several contigs for the KPC-3-producing *K. pneumoniae* ST147 VG313 and VG314 strains shared a 100% identical IncFIA plasmid between them, with a high percentage of homology to the reference (Fig. 2B).

For the KPC-3-producing *K. pneumoniae* ST392 VG380 strain, its plasmid-derived contig has been previously described by us in an IncN plasmid from a human nosocomial strain (Fig. 2C) (13).

No plasmid replicons were detected for the OXA-48-producing *E. coli* strain.

DISCUSSION

In this retrospective study, we found a frequency of 0.51% carbapenem-resistant Enterobacterales strains causing infections in companion animals in Portugal in 2020. Furthermore, we established the horizontal transmission of mobile genetic elements carrying carbapenemase genes in *K. pneumoniae* strains in human and animal settings. Our study is in agreement with the hypothesized high probability for the cross-transmission of resistant bacteria within the One Health context (8, 10, 41).

With the analysis of the antimicrobial susceptibility data, we observed a prevalence of 20.8% for ESBL-producing Enterobacterales among the initial collection of clinical strains. Studies evaluating a heterogeneous collection of Enterobacterales isolates are scarce. In a Swiss study using data from 2012 to 2016 (42), the same prevalence as ours was reported. In another study, only on clinical *E. coli* strains from the UK, 18.7% of the ESBL-producing strains were reported (43). The resemblance observed is of concern since it demonstrates that, despite efforts to decrease the usage of third-generation cephalosporin in veterinary medicine in Europe (44), resistance to critically important antimicrobials for human medicine is still high (30).

In the present study, five CPE strains (0.51%) were found (three KPC-3-producing *K. pneumoniae,* one OXA-181-producing *K. pneumoniae,* and one OXA-48-producing *E. coli* strains). Our result is equivalent to a German study of a large collection of Enterobacterales strains obtained between 2009 and 2016, which reported a prevalence of 0.64% of CPE, all of which were OXA-48-producing strains (7).

To the best of our findings, none of the affected animals received treatment with carbapenems—the oeasophasgotmy tube was removed and surgical cleaning of the wound was performed, followed by antimicrobial treatment with metronidazole, enrofloxacin, and amoxicillin-clavulanic acid. For the dog with an upper respiratory tract infection, treatment with trimethoprim-sulfamethoxazole was effective (13). The dog with a urinary tract infection died before the diagnostic laboratory completed the analysis. In the two other instances, the small animal veterinary practices, where the

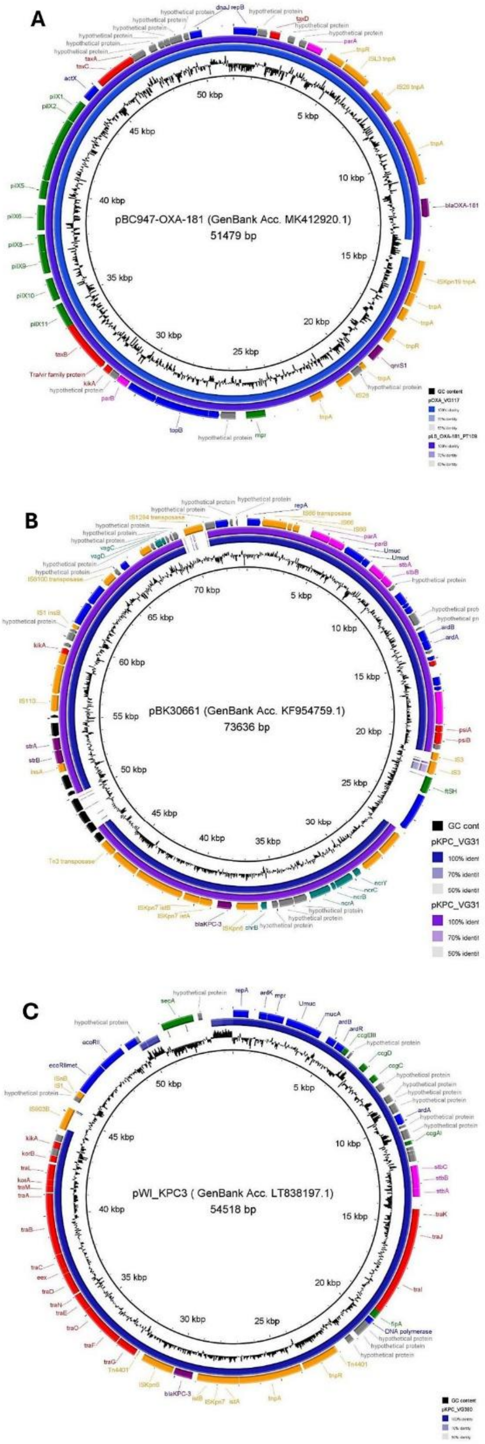

FIG 2 Plasmid alignment comparison between *de novo* assembled plasmids and respective references. (A) pOXA_VG117 (GenBank accession number PRJNA808048) (blue) against pBC947-OXA-181 (GenBank Acc. MK412920.1) and pLB_OXA-181_PT109 (GenBank Acc. CP041033) (purple); (B) pKPC_VG313 and

(Continued on next page)

FIG 2 (Continued)

pKPC_VG314 against pBK30661 (GenBank Acc. KF954759.1); (C) – pKPC_VG380 (13) against pWI_KPC3 (GenBank Acc. LT838197.1). Genes are represented by colored blocks: purple, resistance genes; blue, DNA replication, regulation, and restriction systems; red, conjugation-association genes; fuchsia, genes associated with partition and stability systems; orange, transposons, insertion sequences (IS) and transposase genes; teal, genes associated with resistance to heavy metals; black, sulfonamide resistance pathway genes; green, other genes; gray, hypothetical proteins. Image generated using BRIG 0.95, available at http://brig.sourceforge.net/.

animals were being monitored, did not reply to our inquiries. Overall, three animals were not treated with carbapenems. Thus, no selective pressure from these antibiotics was exerted. As animals can be reservoirs for MDR bacteria, particularly ESBL and CPE Enterobacterales (41, 45), active surveillance by veterinary diagnostic laboratories is essential.

Of the carbapenemase-producing *K. pneumoniae* strains, only one was identified as a possible CPE by the diagnostic laboratory. This identification was accidental since MIC evaluation was only performed upon the clinician's request. The evaluation of carbapenem susceptibility in clinical microbiology veterinary diagnostic laboratories is not mandatory since these antibiotics are not for use in veterinary medicine (6). Despite the low frequency observed, summarized in Table 1, our results emphasize the need for implementing detection methods for carbapenemase-producing strains in veterinary medicine (45). The main purpose of screening for carbapenemase-producing strains would be to minimize its spread, which ultimately impacts human health as it challenges the use of carbapenems as a human therapeutic option. Such implementation can be quite easy and affordable to accomplish in the veterinary microbiology routine workflow, as previously described (8). Resistance to carbapenems was observed on all our KPC-3-producing *K. pneumoniae* strains. Yet, they remain susceptible to the combination disc of ceftazidime-avibactam, which is the therapeutic choice for infections caused by carbapenem-resistant strains (46). Furthermore, all of them were ESBL-producing strains, harboring *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{SHV-11}}$ genes. In addition, resistance genes to other antibiotic classes were identified during WGS characterization, which further highlights their MDR profile. MLST analysis also showed that the OXA-181-producing *K. pneumoniae* ST273 strain, KPC-3-producing *K. pneumoniae* strains ST147 strains, and the KPC-3-producing *K. pneumoniae* ST392 strain are all part of the clonal group 147.

The Human hospital setting in Portugal exhibits a high prevalence of *K. pneumoniae* CG147 strains in circulation (5, 47). *K. pneumoniae* ST147 is internationally disseminated with an increasing report of carbapenemase carriage, particularly of *bla*$_{\text{OXA-48}}$-like genes (Fig. 1) (31). In Portugal, reports of ST147 *K. pneumoniae* strains harboring the *bla*$_{\text{OXA-181}}$ or *bla*$_{\text{KPC-3}}$ genes are most frequent in human clinical strains (5, 34, 47). This lineage is classified as a high-risk clone, playing a major role in the spread of resistance (48). Hence, its detection in companion animals is of great public health concern. As for ST392, there are still few reports in Europe—this might be as it was the last lineage to diverge from ST147 (31). The fact that our KPC-3-producing *K. pneumoniae* is closely related to the human Spanish and Austrian strains, despite neither carrying a carbapenemase gene, demonstrates how this ST392 is distributed across Europe. Lastly, the genetic background of the *K. pneumoniae* ST273 lineage has been described in Italy, Russia, Norway, and the United Kingdom, where it was associated with *bla*$_{\text{KPC-like}}$, *bla*$_{\text{VIM-1}}$, and *bla*$_{\text{NDM-1}}$, respectively (49–52). Although the epidemiology of carbapenemase-producing *K. pneumoniae* is changing across Europe (53), ST273 is still found in low numbers. Thus, the association of the ST273 genetic background with different carbapenemase genes highlights the need for continued monitoring (Fig. 1).

Concerning plasmids carrying carbapenemase genes, both KPC-3-producing *K. pneumoniae* ST147 strains harbored the *bla*$_{\text{KPC-3}}$ gene on an IncFIIA type plasmid, while KPC-3-producing *K. pneumoniae* ST392 strain harbored the *bla*$_{\text{KPC-3}}$ gene on an IncN type plasmid. On these three strains, the *bla*$_{\text{KPC-3}}$ gene was located on Tn*4401d*. Portuguese studies on human nosocomial strains have shown that KPC-3-producing *K. pneumoniae* ST147 can carry multiple plasmid replicons, including the IncFIIA and IncN-types, with the $bla_{\text{KPC-3}}$ gene always located on transposon Tn*4401d* (5, 34, 39, 47). Similarly, our strains harbor the carbapenemase gene in different types of plasmids but in the same genetic environment. These results are worrisome as they might indicate the dissemination/transmission of carbapenemase genes through transposon Tn*4401d* in Veterinary medicine may be fast, thus following the same path as human nosocomial strains.

As for the OXA-181-producing *K. pneumoniae* strain, the $bla_{\text{OXA-181}}$ gene was located on an IncX3-type plasmid. Moreover, our group had previously described an OXA-181-producing *E. coli* strain from a dog's feces (12). In both instances, the two strains shared an identical composition of the mobile genetic elements—the transposon where the carbapenemase gene is located, and the plasmid type (Fig. 2A).

Conjugative plasmids have been associated with the horizontal gene transfer of antibiotic resistance genes (54). All carbapenemase-carrying plasmids identified in this study are conjugative plasmids as they have all the characteristic genetic machinery present (55) (Fig. 2). Such results are alarming as they may point to the dissemination and or horizontal transmission of carbapenemase genes in veterinary medicine.

The OXA-48-producing *E. coli* ST127 VG204 strain was a virulent UPEC pathotype, albeit very susceptible to the third-generation cephalosporins, it presented a reduced susceptibility to carbapenems, which underrates its epidemiological value (16, 56). In 2013, an OXA-48-producing *E. coli* ST127 strain was found to have the resistance gene inserted into its chromosome (57). The lack of plasmid replicon identification on our OXA-48-producing *E. coli* strain during WGS analysis may probably mean that the $bla_{\text{OXA-48}}$ gene is inserted on the chromosome as well.

We recognize that our study has some limitations. First, long-read sequencing needs to be performed to complement the information obtained during the WGS analysis—particularly, to better evaluate the genetic environment surrounding the $bla_{\text{OXA-48}}$ gene. In addition, an underestimation of carbapenem-resistant strains is possible since we excluded from the study other resistant mechanisms to carbapenems, such as loss/alteration of porins.

This is the first study on the prevalence of carbapenemase-producing Enterobacterales clinical strains from companion animals in Portugal. Although the overall prevalence of CPE in companion animals is quite low, it is important for veterinary diagnostic laboratories to perform phenotypic and/or genotypic carbapenemase detection methods. The similitude observed between the mobile genetic elements carrying carbapenemase genes in veterinary medicine and Human Medicine suggests that circulation in a One Health context is occurring.

Furthermore, infection prevention and control measures should be implemented in small animal veterinary practices to prevent the dissemination of resistance to this high priority critically important antimicrobials in human medicine to the community environment.

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AUTHOR CONTRIBUTIONS

Joana Moreira da Silva, Conceptualization, Data curation, Investigation, Supervision, Writing – original draft, Writing – review and editing | Juliana Menezes, Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review and editing | Laura Fernandes, Investigation, Writing – review and editing | Sofia Santos Costa, Methodology, Writing – review and editing | Andreia Amaral, Methodology, Writing – review and editing | Constança Pomba, Conceptualization, Supervision, Writing – review and editing

DATA AVAILABILITY

The genomes generated and analyzed during this study are available at the NCBI GenBank under the BioProject Acc. Number PRJNA808048.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Tables S1 to S9 (Spectrum03416-23-s0001.docx). Supplemental tables with data characterization and reference sequences used for phylogenetic tree and plasmid reconstruction.
Supplemental File 10 (Spectrum03416-23-s0002.xlsx). SNP matrix used for phylogenetic tree generation of *K. pneumoniae* strains.

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Carbapenem-resistant bacteria in veterinary medicine healthcare

Chapter 4 OXA-23-producing *Acinetobacter* spp. in a veterinary practice

This chapter is based on the published peer-reviewed paper:

Moreira da Silva, J., Menezes, J., Fernandes, L., Marques, C., Costa, S. S., Timofte, D., Amaral, A., & Pomba, C. (2024). Dynamics of *bla*$_{\mathrm{OXA\text{-}23}}$ gene transmission in *Acinetobacter* spp. from contaminated veterinary environmental surfaces: an emerging One Health threat? *Journal of Hospital Infection*, *146*, 116–124. https://doi.org/10.1016/j.jhin.2024.02.001

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Dynamics of $bla_{\mathrm{OXA-23}}$ gene transmission in *Acinetobacter* spp. from contaminated veterinary environmental surfaces: an emerging One Health threat?

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SUMMARY

Background: Carbapenem-resistant *Acinetobacter baumannii* is a common pathogen associated with healthcare-acquired infections, and robust infection prevention and control protocols exist in human healthcare settings. In contrast, infection prevention and control (IPC) standards are limited in veterinary medicine, necessitating further investigation.

Aim: Examine the possible transmission of carbapenem-resistant *Acinetobacter* spp. in a veterinary practice where a cat was diagnosed with an OXA-23-producing *A. baumannii* ST2 strain.

Methods: Environmental samples together with nasal and hand swabs from the veterinary personnel were collected. All swabs were screened for the presence of extended-spectrum-β-lactamase- and carbapenemase-producing Enterobacterales, meticillin-resistant staphylococcus and multi-drug-resistant *Acinetobacter* spp. Whole-genome sequencing was performed for carbapenemase-producing strains.

Results: Of the veterinary staff, 60% carried meticillin-resistant *Staphylococcus epidermidis*. Environmental evaluation showed that 40% (N=6/15) of the surfaces analysed by contact plates and 40% (N=8/20) by swabs failed the hygiene criteria. Assessment of the surfaces revealed contamination with five OXA-23-producing *Acinetobacter* spp. strains: an OXA-23-producing *Acinetobacter schindleri* on the weight scale in the waiting room;

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and four OXA-23-producing *Acinetobacter lwoffii* strains, on different surfaces of the treatment room. The *bla*$_{\text{OXA-23}}$ gene was located on the same plasmid-carrying Tn*2008* across the different *Acinetobacter* spp. strains. These plasmids closely resemble a previously described OXA-23-encoding plasmid from a human Portuguese nosocomial *Acinetobacter pittii* isolate. Distinctly, the OXA-23-producing *A. baumannii* ST2 clinical strain had the resistant gene located on Tn*2006*, possibly inserted on the chromosome.

Conclusion: The detection of an OXA-23-producing *A. baumannii* ST2 veterinary clinical strain is of concern for companion animal health and infection, prevention and control. This study established the dynamic of transmission of the plasmid-mediated *bla*$_{\text{OXA-23}}$ gene on critical surfaces of a small animal veterinary practice. The genetic resemblance to a plasmid found in human nosocomial settings suggests a potential One Health link.

Introduction

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) infections are of great concern in healthcare human setting, with mortality surpassing 40% in critically ill patients [1]. According to the latest European Centre for Disease Prevention and Control report on healthcare-associated infections (HAIs) acquired in intensive care units [2], carbapenem resistance was observed on 82% of *A. baumannii* recovered. Additionally, *Acinetobacter schindleri* and *Acinetobacter lwoffii* have also been described as causative agents of nosocomial infections, regardless of their carbapenem resistance status [3,4]. The need for infection, prevention and control (IPC) policies in human medicine is clear and is mandatory in preventing outbreak situations and in controlling healthcare-associated infections caused by multi-drug-resistant (MDR) and biofilm-producing organisms, such as meticillin-resistant *Staphylococcus aureus* and *Acinetobacter* spp. [5—7].

In the past decade, the number of companion animals per family has risen, which consequently leads to the increasing number of veterinary hospitals and practices. This has led to an increased development around small-animal intensive care facilities [8]. Furthermore, the number of animal patients subjected to invasive procedures is expected to increase, and consequently a rise in the preventive use of antibiotics.

In veterinary medicine, carbapenemase-producing Enterobacterales and *Acinetobacter* spp. clinical strains from companion animals are emerging in Portugal and other European countries [9—11]. Subsequently, colonization of staff with carbapenemase-producing bacteria is occurring and spread of OXA-48-producing Enterobacterales inside a veterinary practice is, nowadays, a reality [12,13]. These recent studies draw a parallel between human and veterinary medicine, indicating that it is necessary to comprehend the level of contamination by MDR bacteria in veterinary practices to better guide antimicrobial stewardship practices and IPC programmes.

In the last week of April 2022, in a medium-size veterinary practice in the central region of Portugal, a hospitalized cat was diagnosed with a skin and soft tissue infection, secondary to a post-traumatic lesion in the posterior aspect of its body, caused by OXA-23-producing *A. baumannii*. To evaluate the possible environmental contamination by carbapenem-resistant *Acinetobacter* spp. and other bacteria, samples from different surfaces of the clinic were taken one week after the cat's stay. Nasal and hand carriage of MDR bacteria were also evaluated in the veterinary personnel. Possible transmission of carbapenem-resistance was identified by whole-genome bacterial sequencing.

Methods

Clinical isolate

A clinical swab collected from a cat with skin and soft tissue infection was sent to a diagnostic laboratory for microbiological culture analysis and minimum inhibitory concentration (MIC) determination. The swab was plated on Columbia Blood agar (Thermofisher Scientific), MacConkey agar, Brilliance™ ESBL agar and Brilliance™ MRSA 2 agar (Thermofisher Scientific) for phenotypic identification of the causative agent of infection. MIC panel testing for 22 antibiotics (nalidixic acid, amikacin, ampicillin, amoxicillin in combination with clavulanic acid, aztreonam, cefoxitin, cephalothin, ceftazidime, ciprofloxacin, colistin, doripenem, ertapenem, gentamicin, imipenem, levofloxacin, meropenem, nitrofurantoin, norfloxacin, piperacillin, tetracycline, tobramycin, trimethoprim — sulfamethoxazole) using MicroSan NEG44 plates (Beckman Coulter, USA) was performed and interpreted according EUCAST breakpoints [14]. Species identification was performed using 16S rRNA [15] and detection of *bla*$_{\text{OXA-23}}$ gene was carried out by polymerase chain reaction (PCR) detection [16].

On-site collection

Contact plates (with 28.26 cm^2 of Plate Count Agar area) and surface swabs (TS/5—42 with 10 mL neutralizing buffer, TSC Ltd) were taken from different critical surfaces of the clinic. Samples using surface swabs were limited to an area of 100 cm^2 using a template square of 10 × 10 cm. Flat surfaces were sampled by both methods, while irregular ones were only sampled by surface swabs. Samples were taken from locations as they were, i.e., no cleaning procedure was specifically performed prior to sampling. Additionally, surfaces where an animal has just been in contact with or that was currently in use, were not considered for collection as this would lead to biased results.

The veterinary practice had no record of the locations where the infected cat had passed, apart from the treatment room table. As such, we decided to analyse all critical surfaces — areas which are critically important in a veterinary practice such as high-touch and high-contact surfaces. Regarding detergents and disinfectants in use at the clinic, the detergents routinely used were purchased at the supermarket and the only disinfectant used was bleach diluted with water at an unknown concentration. Supplementary Table S1 summarizes all locations sampled and by which method.

Two nasal swabs (one per nostril) and one swab sampling both hands were taken from each member of staff (five veterinary doctors, three nurses and two support staff), with a written consent form having been signed prior to collection. A questionnaire was used to assess demographic and general human health data, professional situation and previous antibiotic treatment.

To reduce potential bias, hand swabs were taken during the daily procedures, when the workload permitted. All samples were carefully coded and placed in a cooler until processing.

Sample analysis

Contact plates were placed directly at 37 °C and colony forming units (cfu) were counted at 24 and 48 h of incubation. Evaluation of efficacy of hygiene and disinfection protocols was interpreted in accordance with the criteria established by Mulvey *et al.* [17] — a growth >2.5 cfu/cm^2 fails the efficacy cleaning criteria for aerobic colony count (ACC).

To evaluate the efficacy of cleaning regimen by surface swabs, a criterion of >1 cfu/cm^2 was applied [18] for the growth observed on non-selective media Brain Heart Agar (BHA) (Biokar Diagnostics, France). Following an enrichment step on Brain Heart Infusion broth (Biokar Diagnostics, France) overnight at 37 °C, samples were plated on MacConkey agar supplement with 1.5 mg/mL of cefotaxime, MacConkey agar supplemented with 1.5 mg/mL of meropenem (Thermofisher Scientific), CHROMagar™ *Acinetobacter* supplemented with CHROMagar™ MDR Selective (CR102, Chromagar) and Brilliance™ MRSA 2 agar (Thermofisher Scientific).

One randomly selected nasal swab was placed overnight on buffered peptone water (Biokar Diagnostics, France) at 37 °C for the enrichment procedure, and then plated on MacConkey agar supplemented with 1.5 mg/mL of cefotaxime for the selective growth of extended-spectrum-β-lactamase-producing bacteria, on MacConkey agar supplemented with 1.5 mg/mL of meropenem for carbapenem-resistant bacteria and on CHROMagar™ *Acinetobacter* supplemented with CHROMagar™ MDR Selective (CR102, Chromagar). The other nasal swab was placed overnight on sodium chloride supplement with 13% tryptone soy broth and plated on Mannitol Salt Agar (Biokar Diagnostics, France) and Brilliance™ MRSA 2 agar for the selective growth of meticillin-resistant staphylococci.

Hand swabs followed an enrichment procedure with peptone water, followed by plating on the non-selective (Brain Heart Agar and Mannitol Salt Agar) and the selective media mentioned previously for nasal swab analysis.

In all cases, up to three isolates with similar phenotypical appearance were further selected for analysis. MIC testing was performed as described previously [13].

Resistance gene identification and sequencing

DNA was extracted from pure cultures using a boiling extraction method, and a series of multiplex PCRs was performed, as previously described for detection of β-lactamase and carbapenemase genes [19]. Sanger sequencing was performed to identify the amplified β-lactamase and carbapenemase genes. For staphylococci strains, presence of the *mecA* gene was evaluated [20]. Confirmation of species identification was performed by sequencing 16S rRNA as previously described [15]. Multi-locus sequence typing (MLST) was performed for meticillin-resistant staphylococci isolates according to the scheme published by Thomas *et al.* [21,22].

Whole-genome sequencing analysis

One representative resistant strain from each surface harbouring carbapenemase genes was selected for whole-genome sequencing (WGS). Whole DNA was extracted from RNase-treated lysates via NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal). All libraries for WGS were prepared using TruSeq DNA PCR-Free preparation kit (Illumina, San Diego, CA, USA). DNA sequencing was performed using Illumina NovaSeq platform with 2×150 bp paired-end reads. *De novo* assembled genomes were obtained using a previously described pipeline [19]. ResFinder 4.1 (available at the Centre of Genomic Epidemiology — https://www.genomicepidemiology.org/) and CARD database (available at https://card.mcmaster.ca/home [23]) were used for screening the novel generated assemblies for the identification of antimicrobial resistance genes. Single-nucleotide polymorphism (SNP) analysis was conducted using Parsnp v1.2 for multiple sequence alignment of generated assemblies plus reference genome for each acinetobacter species (reference *A. baumannii* — Genbank Acc. GCF_003264275.1; reference *A. lwoffii* — Genbank Acc. GCF_019787625.1). All *de novo* assemblies have been submitted to NCBI under the accession number PRJNA1000421.

ISAba1 amplification

A set of primers was designed using NCBI Primer Blast server (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify the nucleotide sequence of ISABA1 (ISABA1_Forward — 5′-TCCTATCAGGGTTCTGCCTTC-3′; ISABA1_Reverse — 5′-ACGGGTGAATGGCAACATGA-3′). The reaction mix contained 25 μL Supreme NZYTaq II 2× Green Master Mix (Nzytech, Portugal), 10 pmol/μL of each primer for a volume of 1 μL and 5 μL of DNA in a final volume of 50 μL. Initial denaturation (94 °C for 4 min) was followed by 35 cycles of amplification. Each cycle consisted of 90 °C at 30 s, 60 °C for 40 s and 72 °C for 1 min. A last extension step (72 °C for 5 min) completed the amplification. PCR product was amplified by Sanger sequencing to complete the missing gaps in WGS.

Plasmid comparison

Using BRIG analysis tool [24], obtained contigs which contained antimicrobial resistance genes were aligned to a reference plasmid on NCBI database (GenBank Acc. MF078634).

Ethics statement

Ethical approval for the study was obtained (CEBEA011/2021) at the Faculty of Veterinary Medicine, University of Lisbon.

Results

Surface hygiene evaluation by direct culture

A total of 20 surfaces were analysed throughout the clinic, 15 with contact plates and surface swabs, five with surface swabs alone (Supplementary Table S1). According to contact plate evaluation, 40% of the surfaces (N=6/15) failed the cleaning efficacy assessment when interpreted in accordance with the Mulvey *et al.* criterion of >2.5 cfu/cm^2 [17] (Table I). Yet, when evaluating the total surfaces by swabs, 40% (N=8/20) failed the criterion established by Dancer *et al.* of >1 cfu/cm^2 [18]. Incidentally, a susceptible *Acinetobacter radioresistens* strain was identified based on its peculiar phenotypical appearance on the BHA plate from the ultrasound's keyboard.

Environmental contamination with MDR bacteria after enrichment

Of the 20 surfaces analysed, five (25%) also showed environmental contamination with MDR organisms (Table 2), namely *Acinetobacter* spp. (N=4) and an ST554 meticillin-resistant *Staphylococcus epidermidis* (MRSE) (N=1), that were recovered from acinetobacter- and meticillin-resistant-staphylococcus-selective plates, respectively. Four of these surfaces had failed the cleaning efficacy criteria (Table I).

Further analysis of these *Acinetobacter* spp. MDR strains classified them as *A. schindleri* (B1E8A1, on the weight scale waiting room) and *A. lwoffii* on different surfaces of the treatment room (B4Z4A1, B4Z8A1, B11Z4A1 and B12Z8A1) (Table II). Moreover, MIC determination confirmed their resistance to fluoroquinolones and carbapenems (Table III). Molecular analysis confirmed that all *Acinetobacter* spp. strains harboured the *bla*$_{\text{OXA-23}}$ carbapenemase gene.

Veterinary personnel carriage with MDR bacteria after enrichment

All team members (N=10) worked exclusively in the clinic, except for one veterinarian. Three members had taken antibiotics in the last six months prior to sampling, albeit only two classes were labelled — one took the combination of amoxicillin with clavulanic acid, while the other took a fluoroquinolone (not specified). The third member did not fill out which antibiotic had been taken.

Of the nasal samples collected, 20% were negative (N=2/10). Among the positive samples, no *Acinetobacter* spp. strains were recovered and 60% (N=6/10) of veterinary staff carried meticillin-resistant *S. epidermidis* (MRSE) (ST32, ST487, ST54 and ST59), recovered from MRS selective plates. The remaining employees only carried meticillin-susceptible *S. aureus* (MSSA), recovered from mannitol plates.

Table I
Results of surface evaluation based on contact plates and environmental swabs on non-selective media

Surfaces	Contact plates	Criterion <2.5 cfu/cm^2 [a]	Non-selective media	Criterion <1 cfu/cm^2 [b]
Surgery room				
Surgery table — window side	1.77	Passed	—	Passed
Wood stool	Uncountable	Failed	0.01	Passed
Stainless-steel supporting table	Uncountable	Failed	0.01	Passed
Thermal blanket	0.50	Passed	Uncountable	Failed
Surgery bed	0.88	Passed	Uncountable	Failed
Surgery table — door side	0.88	Passed	0.02	Passed
Anaesthetic device buttons	NA	NA	—	Passed
Oxygen balloon	NA	NA	—	Passed
Treatment room				
Stainless-steel tray	Uncountable	Failed	Uncountable	Failed
Black plastic tapete on treatment table	0.07	Passed	—	Passed
Treatment table grills	0.04	Passed	—	Passed
Weight scale treatment room	Uncountable	Failed	Uncountable	Failed
Keyboard computer	NA	NA	Uncountable	Failed
Isolation unit				
Cage 01	0.78	Passed	—	Passed
Cage 02	0.00	Passed	0.05	Passed
Ultrasound room				
Ultrasound screen	Uncountable	Failed	0.5	Passed
Ultrasound table	NA	NA	0.5	Passed
Ultrasound keyboard	NA	NA	Uncountable	Failed
Others				
Consultation room — table	0.28	Passed	Uncountable	Failed
Waiting room weight scale	Uncountable	Failed	Uncountable	Failed

cfu were considered uncountable if >300. NA, not applicable.

[a] Cut-off value defined by Mulvey et al., 2011 [17].

[b] Cut-off value defined by Dancer 2004 [18].

Table II
Multi-drug-resistant bacteria found at the surfaces analysed and number of cfu/cm2

Surfaces	Isolates	Selective growth media	Cfu/100 cm^2	Bacterial strains
Waiting room weight scale	B1E8A1	CHROMagar™ *Acinetobacter*	50	MDR *Acinetobacter schindleri*
Weight scale treatment room	B4Z4A1	MCK + 1.5 mg/mL of MEM	50	MDR *Acinetobacter lwoffii*
	B4Z8A1	CHROMagar™ *Acinetobacter*	8	MDR *A. lwoffii*
Stainless steel tray	B11Z4A1	MCK + 1.5 mg/mL of MEM	25	MDR *A. lwoffii*
Keyboard computer	B12Z8A1	CHROMagar™ *Acinetobacter*	>100	MDR *A. lwoffii*
Ultrasound table	B19Z9S1	Brilliance™ MRSA 2 agar	50	Meticillin-resistant *Staphylococcus epidermidis*

As for hand swab analysis, MRSE was also detected on two veterinarians, however the clones were different from those colonizing the nares (ST278 and ST510). No *Acinetobacter* spp. strains were found.

Acinetobacter spp. whole-genome analysis

WGS was performed to evaluate the core- and accessory genome of the MDR *Acinetobacter* strains, namely the genetic environment of the *bla*$_{\text{OXA-23}}$ carbapenemase genes. Properties on the whole-genome-sequenced strains are listed in Supplementary Table S2.

All environmental strains (B1E8A1, B4Z4A1, B4Z8A1, B11Z4A1 and B12Z8A1) possessed the operon *czc*, which encodes proteins responsible for resistance to cobalt, zinc and cadmium. However, only *A. lwoffii* strains possessed the *mer* operon, that encodes for mercury resistance.

Intrinsically carbapenem-hydrolysing class D β-lactamase *bla*$_{\text{OXA-134-like}}$ non-expressed genes were identified on *A. lwoffii* and *A. schindleri* species, as previously described [25,26]. Specifically, the *bla*$_{\text{OXA-276}}$ gene was present on strain B1E8A1 (*A. schindleri*) and the *bla*$_{\text{OXA-285}}$ gene was present on three *A. lwoffii* strains (B4Z4A1, B11Z4A1 and B12Z8A1). Strain B4Z8A1 harboured the *bla*$_{\text{OXA-362}}$ gene. In addition, all strains

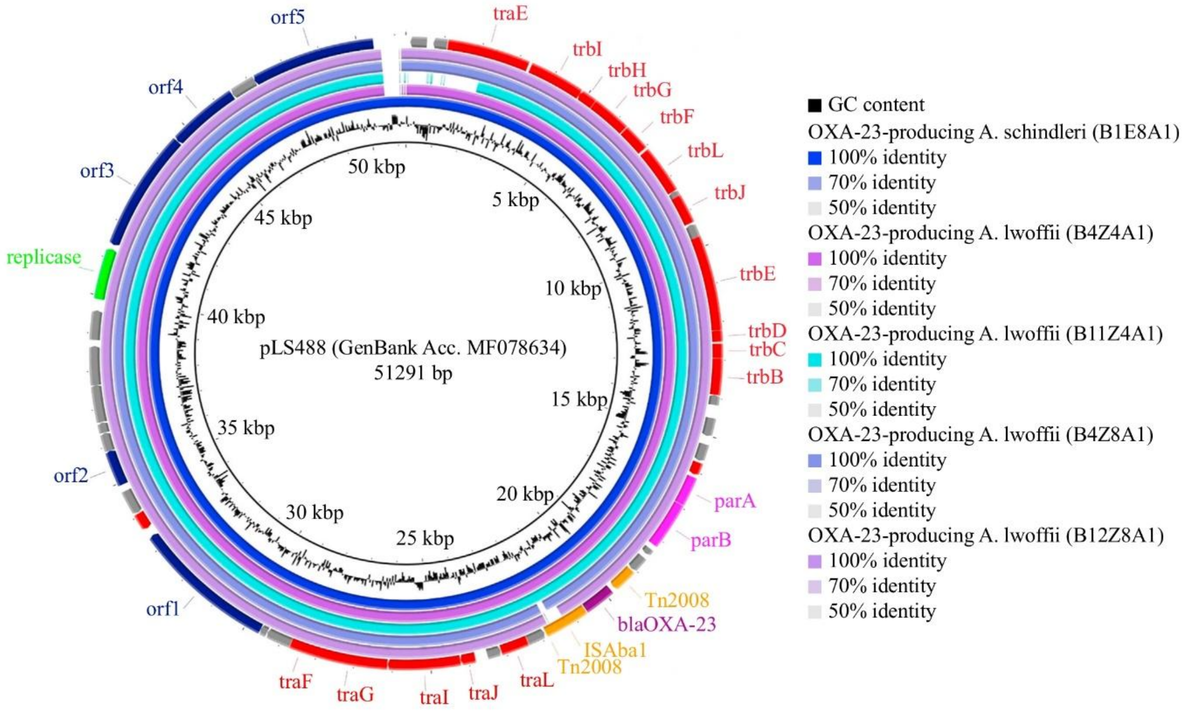

Figure 1. Plasmid alignment comparison between de-novo assembled plasmids and Portuguese nosocomial reference (GenBank Acc. MF078634). From inner ring to outer ring: p_B1E8A1 (dark blue, weight scale waiting room); p_B4Z4A1 (pink, weight scale treatment room); p_B4Z8A1 (purple, weight scale treatment room); p_B11Z4A1 (turquoise, stainless steel tray in the treatment room); p_B12Z8A1 (lilac, computer's keyboard treatment room). Genes are represented by coloured blocks: purple, resistance genes; blue and green, DNA replication, regulation, and restriction systems; red, conjugation-association genes; fuchsia, genes associated with partition and stability systems; orange, transposons, insertion sequences (ISs) and transposase genes; grey, hypothetical proteins. Image generated using BRIG 0.95, available at http://brig.sourceforge.net/.

Table III
OXA-23-producing *Acinetobacter* spp. molecular characteristics and susceptibility profile

					Antibiotic resistance profile [a]					
Environmental surface isolation	Bacterial species	Representative strain	Resistance genes	Mutations	Amikacin (R>8)	Gentamicin (R>4)	Ciprofloxacin (R<1)	Doripenem (R>2)	Imipenem (R>4)	Meropenem (R>8)
Waiting room weight scale	*Acinetobacter schindleri*	B1E8A1	*bla*$_{OXA-23}$ *sul2* *aph(3″)-Ib* *aph(6)-Id*		≤8	≤2	>2	4	8	4
Weight scale (treatment room)	*Acinetobacter* lwoffii	B4Z4A1	*bla*$_{OXA-23}$ *tet39*		≤8	≤2	2	4	8	4
	A. lwoffii	B4Z8A1	*bla*$_{OXA-23}$		≤8	≤2	>2	4	8	8
Stainless steel tray (treatment room)	*A.* lwoffii	B11Z4A1	*bla*$_{OXA-23}$ *tet39*		≤8	≤2	2	2	8	4
Keyboard computer (treatment room)	*A.* lwoffii	B12Z8A1	*bla*$_{OXA-23}$ *tet39*		≤8	≤2	2	2	4	4
Infection	*Acinetobacter baumannii*	10854	*bla*$_{TEM-1;}$ *bla*$_{OXA-23;}$ *sul2; aph(3″)-Ib aph(6)–Id; tet(B)*	*gyrA* (S81L) *parC* (S84L, V104I, D105E)	≤8	>2	>2	>2	>8	>8

[a] Breakpoints determined in accordance with EUCAST breakpoint guidelines 2023.

produced OXA-23 oxacillinases, which were responsible for the carbapenem resistance observed phenotype (Table III). As for additional antibiotic resistance genes, strains B4Z4A1, B11Z4A1 and B12Z8A1 possessed the *tet39* gene and B1E8A1 harboured the *sul2*, *aph(3″)–Ib* and *aph(6)–Id* genes (Table III).

Three of the four MDR *A. lwoffii* strains had a range from 12 to 22 different SNPs (Supplementary Table S3), while the *A. lwoffii* B4Z8A1 strain had a difference greater than >6000 SNPs. Thus, two different clones of MDR *A. lwoffii* strains were circulating in the veterinary practice environment.

For the OXA-23-producing *A. baumannii*, the intrinsically *bla*$_{OXA-66}$ gene was identified, as well as *tet*(B), *sul*2 and *bla*$_{TEM-1}$. Mutations conferring resistance to fluroquinolones were also identified on *gyrA* (S81L) and *parC* (S84L, V104I, D105E). In-depth analysis did not yield any environmental heavy metal resistance genes (Table III). This strain was classified as OXA-23-producing *A. baumannii* ST2, belonging to the Global Clone 2.

WGS analysis on *A. radioresistens* only confirmed the presence of intrinsically *bla*$_{OXA-23}$ non-expressed gene and the operon *czc*.

Dynamics of plasmid-mediated transmission of the bla$_{OXA-23}$ genes

On all studied environmental strains (B1E8A1, B4Z4A1, B4Z8A1, B11Z4A1 and B12Z8A), the *bla*$_{OXA-23}$ gene was located on transposon Tn*2008*, which is characterized by the presence of a single copy of ISAba1 and absence of seven base pairs between ISAba1 and the beginning of the *bla*$_{OXA-23}$ gene [27].

When using a formerly described plasmid containing the *bla*$_{OXA-23}$ gene (GenBank Acc. MF078634) from an *Acinetobacter pittii* Portuguese human nosocomial strain [28], a high degree of homology was observed with p_B1E8A1, p_B4Z4A1, p_ B4Z8A1, p_ B11Z4A1 and p_B12Z8A from the present study. Figure 1 depicts the complete alignment of the plasmids found on the studied strains and the reference plasmid.

The cat's clinical strain *A. baumannii*, *bla*$_{OXA-23}$ was present on transposon Tn*2006*. This transposon is characterized by the additional seven base pairs before the beginning of the *bla*$_{OXA-23}$ gene and two copies of the ISAba1 [27]. An in-depth analysis of the bacterial genome confirmed the absence of genes commonly associated with the plasmid conjugation system, which would suggest that the *bla*$_{OXA-23}$ gene was located on the chromosome.

Data availability

All *de novo* assemblies have been deposited on Bioproject PRJNA1000421 in GenBank (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1000421/).

Discussion

Following the stay of a cat infected with an OXA-23-producing *A. baumannii* human Global Clone 2, evaluation of critical surfaces from the veterinary practice revealed contamination with five OXA-23-producing *Acinetobacter* spp. strains. Furthermore, the evaluation of the genetic environment of the *bla*$_{OXA-23}$ gene showed that it was located on the same transposon Tn*2008* and on the same plasmid in the different *Acinetobacter* spp. found in the veterinary environment. Additionally, these plasmids were homologous with a carbapenemase-encoding plasmid also harbouring the *bla*$_{\text{OXA-23}}$ gene from a clinical human emergent *A. pittii* strain isolated in a Portuguese hospital [28].

Our study investigated the hygiene of clinical surfaces by direct culture and found that some failed the hygiene criteria established for contact plates, despite meeting the criteria established for surface swabs. This fact may be explained because contact plates are designed to permit the growth of any type of micro-organism (e.g., fungi and yeast), whereas surfaces swabs with a universal neutralizing liquid buffer are specific for bacterial growth. Surface swab evaluation revealed that eight surfaces were unclean, having failed the criteria established by Dancer [18]. Furthermore, 25% of the surfaces were contaminated with carbapenem-resistant *Acinetobacter* spp.: *A. schindleri* (on the weight scale waiting room) and *A. lwoffii* on different surfaces of the treatment room (weight scale of the treatment room, stainless-steel tray and keyboard computer). In contrast, a Swiss study [8] reported, for a veterinary practice of the same size as our work, that 33% of the analysed surfaces were contaminated with meticillin-resistant staphylococci. Yet, the same study described carbapenem-resistant bacteria only in large veterinary hospitals. As such, according to our research, the criteria to establish IPC guidelines cannot be based on case numbers because carbapenem-resistant bacteria may be contaminating the environment regardless of the veterinary practice dimension.

Regarding veterinary personnel carriage with MDR bacteria after enrichment, we found that MRSE was far more common (60%) than MSSA carriage. Despite the absence of meticillin-resistant *S. aureus*, susceptible *S. aureus* can acquire resistance to β-lactams by acquisition of *mecA* gene through horizontal gene transfer by MRSE [29]. Thus, the screening of veterinary personnel for MRSE carriage may be useful to in the prevention of *mecA* dissemination and in the resistance to spreading.

All five OXA-23-producing *Acinetobacter* spp. strains (one *A. schindleri* and four *A. lwoffii*) from the veterinary practice environment underwent WGS. *A. lwoffii* and *A. schindleri* are commonly found in the environment [30,31], but they are also opportunistic pathogens, with cases of New Delhi metallo-beta-lactamase-1 (NDM-1)-producing *A. lwoffii* and *A. schindleri* infections in immunocompromised patients being reported [3,32]. To the best of our knowledge, only one report of OXA-23-producing *A. lwoffii* has been made in an inpatient [32]. Conversely, in veterinary medicine, infections caused by *A. lwoffii* are rarely reported [33] and none caused by *A. schindleri* have been described.

The incidental finding of a susceptible *A. radioresistens* strain on the ultrasound keyboard is relevant, as this species is considered the source of *bla*$_{\text{OXA-23}}$ gene [16,27]. However, the gene is not expressed in this species as it lacks the promoter region of ISAba1. Reports of infection in humans and animals have occurred [33,34]. Thus far, no human *A. radioresistens* nosocomial strains harbouring carbapenemase genes have been reported. However, in veterinary medicine, a hospitalized dog was colonized with NDM-1-producing *A. radioresistens* in Italy [10]. Although the authors of this study did not evaluate the environmental contamination, this result demonstrates the importance of applying IPC guidelines to veterinary healthcare facilities and of performing active surveillance screening for this *Acinetobacter* species.

Both in *A. lwoffii* strains and *A. schindleri*, the *bla*$_{\text{OXA-23}}$ gene was located on Tn*2008*. An in-depth analysis of the plasmid revealed that the same plasmid was present on all strains found on the contaminated surfaces. The plasmid used as reference was previously described in A. *pittii* Portuguese human nosocomial strain [28]. Also, the plasmids here described are conjugative which contributes to easy horizontal gene transfer across different species [35]. These results are worrisome as they show that a probable dissemination of the *bla*$_{\text{OXA-23}}$ gene across the different surfaces of the veterinary practice was occurring through plasmid dissemination. Moreover, they also suggest the transmission of this carbapenemase-encoding gene in the transposon Tn*2008* through a homologous plasmid from the human hospital healthcare setting to the veterinary healthcare setting in Portugal. Further studies are needed to establish this important One Health link. Hospital—human environmental and human clinical strains from *A. baumannii* which carried *bla*$_{\text{OXA-23}}$ gene on the Tn*2008* have also been reported, showing the ability of bacteria carrying this tranposon to colonize different settings [36].

An OXA-23-producing *A. baumannii* ST2 strain was identified as the causative agent of a skin and soft tissue infection in a cat. ST2 lineage (which is part of Global Clone 2) has been described worldwide and is associated to *bla*$_{\text{OXA-23}}$ carbapenemase gene [37]. A previous 2009 report in Portugal also characterized an OXA-23-producing *A. baumannii* ST2 clinical strain with the *bla*$_{\text{OXA-23}}$ of feline origin in the veterinary healthcare setting [38]. While this clone was prevalent in Portugal in 2010 [39], lack of updated information of circulating MLST lineages of clinical *A. baumannii* strains and their resistance genes make it difficult to ascertain whether this clone is still in circulation in the human healthcare setting in Portugal, as it has been shown that the dominant clone tends to evolve with time [40]. The *bla*$_{\text{OXA-23}}$ gene is commonly associated to Tn*2006* in the *A. baumannii* ST2 lineage, whether it is chromosome or plasmid inserted [41]. In our strain, *Tn*2006 is possibly located on the chromosome as no conjugative elements from plasmids were identified. This is in agreement with the previously described Portuguese veterinarian clinical strain [38], in which Tn*2006* is located on the chromosome.

It would have been interesting to evaluate clinical samples from the animals hospitalized at the time of the environmental sampling, but owner consent would have had to be obtained. Thus, this study has the limitation of being unable to establish whether any of the strains described here were linked to a possible outbreak.

The detection of an infection in an admitted cat to a veterinary practice with an OXA-23-producing *A. baumannii* ST2 strain is of concern for companion animal health and IPC programmes. The whole-genome sequencing performed on the environmental carbapenem-resistant *Acinetobacter* spp. strains allowed us to observe the occurrence of equal mobile genetic elements — transposon- and plasmid-carrying *bla*$_{\text{OXA-23}}$ gene on different *Acinetobacter* spp. strains found on distinguished surfaces of the small-animal veterinary practice. Additionally, the homology with a plasmid of nosocomial human origin in Portugal is worrisome as it might suggest the transmission between the human hospital healthcare and the veterinary healthcare settings. Additional studies are required to comprehend this important One Health link. The need for implementation of IPC guidelines directed at antimicrobial-resistance in veterinary medicine is urgent. Regular surveillance, IPC protocols and antimicrobial stewardship are key to preventing the dissemination of these MDR bacteria on to humans and pets.

Author contributions

J.M.S. and C.P. designed the study. J.M.S., J.M. and L.F. collected and analysed the data. J.M.S. and A.A. analysed W.G.S. data. J.M.S., C.M., D.T., S.C.C. and C.P. wrote, revised and approved the manuscript.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2024.02.001.

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Chapter 5 Evaluation of MDR bacteria in veterinary practices in Portugal

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Evaluation of multidrug-resistant bacteria and their molecular mechanisms found in small animal veterinary practices in Portugal

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Introduction: Intensive medical care provided in companion animal practices carries the potential risk of selecting and disseminating multidrug-resistant organisms (MDROs). However, data on infection, prevention and control standards specific to small animal veterinary practices (SAVPs) remains limited. The goal of our work was to evaluate the environmental contamination and staff carriage by MDROs in veterinary practices across Portugal.

Methods: Fourteen SAVPs were enrolled. Environmental samples were collected from critical areas such as operating room, wards and pre-operative area. Veterinary team members voluntarily gave nasal, hand and rectal swabs. All samples were screened for the presence of, including extended-spectrum β-lactamases (ESBL)- and carbapenemase-producing Gram-negative bacteria and methicillin-resistant *Staphylococcus* spp. (MRS). Whole-genome sequencing was performed for carbapenem resistant strains.

Results: Environmental evaluation by surface swabs revealed that 6.5% (n=32/490) were contaminated with multidrug-resistant Gram-negative bacteria. OXA-23-producing *Acinetobacter* spp. (n=5) and IMP-8-producing *Pseudomonas juntendi* (n=2) strains were described on different locations of different SAVPs. Moreover, *Stenotrophomonas maltophilia* (n=12) and *Pseudomonas aeruginosa* (n=3) strains were also found on multiple surfaces of different SAVPs. Three human samples (two rectal, one hand) had carbapenem-resistant *P. aeruginosa* strains by OprD mutations, while *S. maltophilia* strains were recovered from four

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samples (two rectal, two hands). One nasal swab was positive for carbapenem-resistant *Klebsiella pneumoniae* ST11. Only one SAVP surface was positive for the newly typed for methicillin-resistant *Staphylococcus aureus* (MRSA) ST9220-II. MRSA nasal carriage was found in 14% of samples (n=9/64), with an equal prevalence of ST22-IV and ST8-VI. As for hand samples, MRSA was present in 10.7% (n=4/38), with a predominance of ST8-VI.

Discussion: These emerging data indicate that SAVPs may significantly contribute to the dissemination of MDROs. To address this, rigorous infection, prevention and control (IPC) measures should be implemented, alongside educational workshops directed to all veterinary staff as well as to veterinary and nursing students.

KEYWORDS

small animal veterinary practices, multidrug-resistant organisms, environmental contamination IPC, carbapenem resistance, MRSA, veterinary staff carriage

1 Introduction

In recent years, antimicrobial resistance has led to approximately 1.27 million global deaths (Murray et al., 2022). The rise in antimicrobial resistance is driven by multiple mechanisms, notably the acquisition of resistance genes, such as *mec*A or $bla_{\text{CTX-M-15}}$ and $bla_{\text{KPC-like}}$, through plasmids (Dulon et al., 2011; Mathers et al., 2015). Key culprits include *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. These microorganisms are linked to community- and to healthcare-associated infections, posing challenges due to extended treatment periods, increased mortality and higher economic costs. The European Centre for Disease Prevention and Control (ECDC) reported that 4.3 million patients in Europe experienced at least one healthcare-associated infections in 2022-2023 (European Centre for Disease Prevention and Control, 2024). The World Health Organization updated the list of medically important antibiotics, classifying which antibiotics can be used in veterinary medicine, following the prescribing cascade. As expected, last-line antibiotics, such as carbapenems and oxazolidinones, are only to be used in human medicine (European Centre for Disease Prevention and Control, 2024), while 3rd generation cephalosporins, high-priority critically important antimicrobials, are authorized for both human and veterinary medicine (Volkov, 2024). This classification is aligned with European Medicines Agency guidelines, released in 2019 (EMA, 2019).

In the last years, the bond between humans and companion animals has significantly evolved, with animals now being regarded as integral members of the family. This shift has led to an increase not only in the number of veterinary practices, as well as in rapid advancements in small animal care and animals' lifespan, pressuring veterinarians towards higher standards of healthcare (Eurodev, 2024). Although carbapenems are not approved for veterinary use (EMA, 2019), carbapenem-resistant Enterobacterales, *Acinetobacter* spp. and *P. aeruginosa* are sporadically but consistently reported in companion animals in Europe (Gentilini et al., 2018; Moreira da Silva et al., 2024b). Furthermore, cases of multidrug-resistant organisms (MDROs) such extended-spectrum β-lactamases (ESBL) - producing Enterobacterales and methicillin-resistant *Staphylococcus* spp. (MRS) sharing between humans and companion animals have been reported (Sing et al., 2008; Nienhoff et al., 2009; Grönthal et al., 2018; Hemeg, 2021; Menezes et al., 2022; Menezes et al., 2023), showing the need for a One Health and integrative approach to mitigate the dissemination of MDROs.

The implementation of infection, prevention and control (IPC) programs has long been recognized as a cornerstone to minimize the spread of MDROs within human hospital environments. Not only do they protect hospital staff and patients, IPC also helps to minimize the spillover of resistant bacteria into the community (World Health Organization, 2023). In veterinary medicine, antimicrobial stewardship and IPC programs are rising concepts; notwithstanding, a lack of studies characterizing veterinary IPC programs or which MDROs are circulating within small animal veterinary practices (SAVP) still exists. According to ECDC (European Centre for Disease Prevention and Control, 2016), only genomic-level investigations, such as Whole Genome Sequencing (WGS), can provide the resolution needed to track the distribution of resistance genes across hosts, time, and space. In this way, this study aimed to assess the standard cleanliness of high-touch surfaces in SAVP and to characterize the environmental contamination and staff transient carriage by relevant bacterial pathogens, particularly carbapenem-resistant Gram-negative bacteria and MRS, together with determining bacterial transmission between humans, companion animals, and hospital surfaces through WGS. The outcomes of this study clarified how effective standard IPC protocols in SAVPs are, as well as elucidate on the role SAVPs have in the dissemination of MDROs within a One Health context.

2 Materials and methods

2.1 Hospitals and clinics characteristics

Fourteen SAVP were enrolled between March 2021 and November 2023, and coded from A to N. SAVP-A to SAVP-G were hospitals and SAVP-H to SAVP-N were clinics. According to the Portuguese legislation, it is mandatory for hospitals to have emergency care available 24/7, as opposed to clinics. SAVPs were located in different regions of Portugal. A previously published self-assessment form was adapted and provided to each SAVP covering different aspects of each facility's functioning characteristics and IPC practices (Schmidt et al., 2020). After the results evaluation, all SAVP received a written report of the findings and suggestions for improvement.

2.2 On-site collection

Contact plates (Plate Count Agar, 28.26 cm²) and surface swabs (TS/5–42 with 10 mL neutralizing buffer, TSC Ltd.) were used to sample critical surfaces in the practice environment. Surface swab samples were collected from a defined area of 100 cm² using a sterile template frame. Both methods were applied to flat surfaces, while only surface swabs were used for irregular surfaces. Samples were taken from the locations as they were found, without any previous cleaning and disinfecting procedure. Surfaces that had been immediately used before sampling were excluded to avoid biased results.

Critical areas for sampling were defined as high-touch and high-rotation surfaces within the practice. Due to their importance for veterinary activities and potential to act as transmission hubs, three locations were always sampled: operating room, wards and pre-operative area. Other locations such as examination rooms and treatment areas were included in the study depending on availability at the time of sampling (if the areas were not in use and were cleaned) and the individual concerns pertaining to each SAVP. Supplementary Tables 1, 2 describe the sampling techniques and the surfaces considered in each SAVP.

The disinfection protocols and products in use were defined by each SAVP, and as such, these varied across practices. This information was recorded in the general SAVP self-assessment form previously mentioned.

2.3 Human sampling

Sampling was voluntary and written informed consent was obtained. Three swabs (one per nostril and one for both hands) were collected from each member of the working force per SAVP (89 veterinary doctors, 32 nurses, 35 technicians and 13 administrative staff, n=169). Rectal swabs were sampled from 30 people (17.7%, n= 30/169). To reduce potential bias, hand swabs were taken randomly during daily procedures, to avoid unusual hygiene prior to collection. All samples were carefully coded and placed in a cooler until processing. Ethical approval for the study was obtained (CEBEA011/2021) and performed in accordance to relevant guidelines.

2.4 Sample analysis

Contact plates were incubated at 37°C and colony forming units (CFU) were counted at 24 and 48 hours of incubation. Following previously established efficacy criteria for aerobic colony count (ACC), a growth >2.5 CFU/cm² indicated failure of the cleaning protocol (Mulvey et al., 2011).

For surface swabs, a cleaning criterion of >1 CFU/cm² was applied (Dancer, 2004) for direct plating growth in non-selective media Brain Heart Agar (Biokar Diagnostics, France) after 24 and 48 hours incubation at 37°C. Following an overnight enrichment step on Brain Heart Infusion broth (Biokar Diagnostics, France) at 37°C, samples were plated onto MacConkey agar supplemented with 1.5 mg/mL cefotaxime or 1.5 mg/mL of meropenem (Thermofisher Scientific, United States) (to select for ESBL-producing or carbapenem-resistant bacteria, respectively), CHROMagar™ *Acinetobacter* supplemented with CHROMagar™ MDR Selective (CR102, Chromagar, Japan), and Brilliance™ MRSA 2 agar (Thermofisher Scientific, United States).

One randomly selected nasal swab was placed overnight on buffered peptone water (Biokar Diagnostics, France) at 37°C, and then plated onto MacConkey agar plates supplemented with 1.5 mg/mL cefotaxime or 1.5 mg/mL meropenem, and on CHROMagar™ *Acinetobacter* supplemented with CHROMagar™ MDR Selective. The other nasal swab was placed overnight on sodium chloride supplemented with 13% tryptone soy broth at 37°C and then plated onto Mannitol Salt Agar (Biokar Diagnostics, France) and Brilliance™ MRSA 2 agar (Thermofisher Scientific, United States).

Rectal and hand swabs were incubated overnight at 37°C in peptone water, followed by plating onto the non-selective and selective media described above.

In all cases, up to three isolates with similar phenotypical appearance were isolated for further characterization. Microdilution antibiotic susceptibility testing was performed for Gram-negative bacteria isolated from MacConkey agar with cefotaxime and meropenem supplementation following EUCAST guidelines (European Committee on Antimicrobial Susceptibility Testing, 2024), to further confirm their resistant phenotype.

2.5 Resistance genes detection

DNA was extracted from pure cultures using a boiling extraction method (Dashti et al., 2009). Multiplex PCRs followed by Sanger sequencing were performed as previously described for the detection of β-lactamase genes in Gram-negative bacteria, including ESBL and carbapenemase genes (Menezes et al., 2022). Gram-negative bacteria species identification was performed by sequencing 16S rRNA (Srinivasan et al., 2015).

Staphylococci species identification and *mecA* gene detection was performed as previously described (Couto et al., 2015; Rodrigues et al., 2018). MLST and SCC*mec* identification (Kondo et al., 2007) was performed for methicillin-resistant *Staphylococcus aureus* (MRSA), and one new sequence type (ST) was assigned in accordance to PubMLST (Jolley et al., 2018).

2.6 Whole-genome sequencing analysis

One representative resistant strain from each surface was selected for WGS. Genomic DNA was extracted from RNase-treated lysates via NZY Tissue gDNA Isolation kit (NZYTech, Portugal). All libraries for WGS were prepared using TruSeq DNA PCR-Free preparation kit (Illumina, United States). DNA sequencing was performed using Illumina NovaSeq platform with 2×150 bp paired-end reads. *De novo* assembled genomes were obtained using a previously described pipeline (Menezes et al., 2022). ResFinder 4.1 (available at the Centre of Genomic Epidemiology – https://www.genomicepidemiology.org/) and CARD database (available at https://card.mcmaster.ca/home (Alcock et al., 2023),) were used for the screening of the novel generated assemblies for identification of antimicrobial resistance genes. Single-nucleotide polymorphism (SNP) analysis was conducted for each bacterial species using Parsnp v1.2 for multiple sequence alignment of the generated assemblies plus a reference genome. *De novo* assemblies were submitted to NCBI under the bioprojects accession numbers PRJNA1131754 and PRJNA1000421 (OXA-23-producing *Acinetobacter* spp.). Newly identified STs were assigned and submitted to the PubMLST database.

3 Results

3.1 Surface hygiene evaluation by direct culture

According to contact plate evaluation, 28% of the flat surfaces (n=74/264) failed the cleaning efficacy assessment when interpreted with the criteria of >2.5 CFU/cm^2, in accordance to Mulvey et al., 2011 (Mulvey et al., 2011) (Supplementary File 1). On the other hand, flat and irregular surface evaluation using swabs revealed that 17.7% of surfaces (n=87/490) failed the criteria established by Dancer 2004 of >1 CFU/cm^2 (Dancer, 2004) (Supplementary File 1). Contact plates and swab results were discordant in 27.6% (n=72/264) of flat surfaces. Considering both evaluation methods, flat surfaces cleaning efficacy failure increased to 36.8% (n=96/261). All SAVP had at least one unclean surface (Supplementary File 1) with frequencies varying from 3.3% (n=1/30) in SAVP-C to 72% (n=18/25) in SAVP-G.

Surfaces that frequently failed cleaning efficacy assessment included weight scales (60.9%, n=14/23), sinks and taps (44.1%, n=15/34), animal cages (40.5%, n=17/42), shearing blades (41.7%, n=10/24) and keyboards (30.3%, n=10/33).

The average results on the self-assessment form were 37/60 points (25–51 points). The highest scoring practice was SAVP-G; yet, SAVP-G showed the highest level of environmental contamination in the different areas of the hospital, with 72% of surfaces (n=18/25) failing the cleaning efficacy assessment (Supplementary File 1).

3.2 Surface contamination with Gram-negative bacteria

Considering all surfaces, 6.5% (n=32/490) had positive growth in the selective culture media for Gram-negative bacteria, namely, *Acinetobacter* spp. (3.1%, n=15/490), *Stenotrophomonas* spp. (2.5%, n=12/490), *Pseudomonas* spp. (1.0%, n=5/490), *Enterobacter* spp. (0.4%, n=2/490), *Escherichia* spp. (0.2%, n=1/490), *Klebsiella* spp. (0.2%, n=1/490), and *Leclercia* spp. (0.2%, n=1/490). Table 1 summarizes all the different bacterial species found on each SAVP.

Carbapenem-resistant non-fermenting Gram-negative bacteria were also recovered on 58.9% (n=20/32) of contaminated surfaces. Overall, 57.1% (n=8/14) of SAVP had at least one positive surface for carbapenem-resistant Gram-negative bacteria – three hospitals and five clinics (Table 1).

Considering veterinary hospitals, *Stenotrophomonas maltophilia*, which is intrinsically resistant to multiple classes of antibiotics, including carbapenems (due to intrinsic carbapenemases L1 and L2 (Brooke, 2021)), was identified on two surfaces of the cat ward of SAVP-E, and on the tap of the washing area of SAVP-F. In SAVP-G, *P. aeruginosa* and *S. maltophilia* were present on different surfaces (Table 1). Additionally, carbapenem-resistant *Pseudomonas juntendi* was found on a plastic mat on the countertop and inside one dog cage (Table 1).

Regarding veterinary clinics, carbapenem-resistant *Acinetobacter* spp. was found on four surfaces of SAVP-H. Carbapenem-resistant *P. aeruginosa* (A6E4P2) was found on the cat examination room sink of SAVP-I. *S. maltophilia* were found on the examination room sinks of SAVP-K) and SAVPL. For SAVP-N, it was present, on the post-surgery cage the practice's mobile phone and a hand cloth (Table 1).

All *S. maltophilia* were susceptible to trimethoprim/sulfamethoxazole (MIC < 0,001 mg/L). All carbapenem-resistant isolates were characterized using WGS.

3.2.1 Whole-genome sequence analysis of carbapenem-resistant Gram-negative bacteria from surfaces

The two *S. maltophilia* isolated from SAVP-E belonged to different STs, namely, ST4 in a cat cage (HVD1E4P1), and the newly assigned ST1185 on a cage handle from the same cat ward (HVD4E4P1). The *S. maltophilia* from SAVP-F belonged to ST94 (EP3G8P3).

On SAVP-H, all the carbapenem-resistant *Acinetobacter* spp. (n=5) harbored $bla_{\text{OXA-23}}$ carbapenemase gene in the same plasmid

TABLE 1 Gram-negative bacteria identified upon environmental evaluation of SAVPs A-N.

SAVP	Location	Area	Identified organism	Sequence Type (ST)	NCBI strain ID	Carbapenem Resistance*	
						Imipenem	Meropenem
C	Pre-operative area	Plastic mat table 02	*Acinetobacter calcoaceticus-baumannii* complex	NA	NA	≤2 (S)	≤2 (S)
		Plastic mat table 03					
		Weight scale					
		Oxygen balloon					
		Anaesthetic device buttons					
		Tap					
	Wash room	Countertop					
	Operating room 02	Blanket					
D	Cat ward	Big cage	*Acinetobacter baumannii*	NA	NA	≤2 (S)	≤2 (S)
		Small cage		NA	NA		
E	Pre-operative area	Tap	CTX-M-15-producing *Enterobacter kobei*	NA	NA	≤2 (S)	≤2 (S)
	Cat ward	Cage	CTX-M-15-producing *Escherichia hermannii*	NA	NA	≤2 (S)	≤2 (S)
			Stenotrophomonas maltophilia	4	HVD1E4P1	NA	NA
		Cage handle	*Stenotrophomonas maltophilia*	1185 (new ST)	HVD4E4P1	NA	NA
F	Wash room	Tap	*Stenotrophomonas maltophilia*	94	EP3G8P3	NA	NA
G	Dog ward	Tap	*Pseudomonas aeruginosa*	253	ER3C4P3	>16 (R)	>32 (R)
			CTX-M-3-producing *Klebsiella pneumoniae*	11	ER3C8K2	≤2 (S)	≤2 (S)
	Dog ward	Treatment table plastic mat	IMP-8-producing *Pseudomonas juntendi*	NA	ER1C4P2	16 (R)	32 (R)
		Cage	IMP-8-producing *Pseudomonas juntendi*	NA	ER4C8A1	16 (R)	32 (R)
		Treatment table grids	GES-7-producing *Leclercia adecarboxylata*	NA	ER5C2K1	≤2 (S)	≤2 (S)
	Isolation unit	Cage	*Stenotrophomonas maltophilia*	120	ER3F8A1	NA	NA
		Plastic mat on countertop	*Stenotrophomonas maltophilia*	115	ER2F4P2		
			Pseudomonas aeruginosa	253	ER2F8P2	16 (R)	>32 (R)
	Cat ward	Tap	*Stenotrophomonas maltophilia*	120	ER3D8P2	NA	NA
			CTX-M-15- producing *Enterobacter hormaechei*	NA	ER3D2K1	≤2 (S)	≤2 (S)
H	Treatment area	Weight scale	OXA-23-producing *Acinetobacter lwoffii*	NA	B4Z4A1 B4Z8A1	8 (R)	4 (S)
	Treatment room	Stainless steel supporting tray	OXA-23-producing *Acinetobacter lwoffii*	NA	B11Z4A1	8 (R)	8 (R)
		Keyboard computer	OXA-23-producing *Acinetobacter lwoffii*	NA	B12Z8A1	4 (I)	4 (S)
	Waiting room	Weight scale	OXA-23-producing *Acinetobacter schindleri*	NA	B1E8A1	8 (R)	4 (S)

(Continued)

TABLE 1 Continued

SAVP	Location	Area	Identified organism	Sequence Type (ST)	NCBI strain ID	Carbapenem Resistance*	
						Imipenem	Meropenem
I	Cat examination room	Sink	*Pseudomonas aeruginosa*	267	A6E4P2	8 (R)	4 (I)
K	Dog examination room	Sink	*Stenotrophomonas maltophilia*	967 (new type)	C1DE4P2	NA	NA
	Cat examination room	Sink	*Stenotrophomonas maltophilia*	5	C6EE4A1	NA	NA
L	Examination room 01	Sink	*Stenotrophomonas maltophilia*	39	B4EE4A2	NA	NA
N	Recovery ward	Cage	*Stenotrophomonas maltophilia*	27	EX2C4P3	NA	NA
	Fomites	Practice's mobile phone	*Stenotrophomonas maltophilia*	115	EX3I8A1	NA	NA
		Hand cloth	*Stenotrophomonas maltophilia*	115	EX2I4A1	NA	NA

NA, Not applicable; S, Susceptible; I, Susceptible to increased exposure; R, Resistant.

*EUCAST 2024 breakpoints (European Committee on Antimicrobial Susceptibility Testing, 2024) – for Enterobacterales, breakpoints are as follows: imipenem (S ≤2; R > 4) and meropenem (S ≤2; R > 4); for *Acinetobacter* spp., imipenem (S ≤2; R > 4) and meropenem (S ≤2; R > 2); and for *Pseudomonas* spp., imipenem (S ≤0.001; R > 4) and meropenem (S ≤2; R > 8). There are no breakpoints for *S. maltophilia* due to their naturally occurring carbapenemases, making them intrinsically resistant.

across different sub-species, as previously described by our group (Moreira da Silva et al., 2024a).

The two *P. aeruginosa* strains from SAVP-G belonged to ST253 and were unrelated (single nucleotide polymorphism (SNP) difference > 14, Supplementary Table 4) (Schürch et al., 2018). Both strains were carbapenem-resistant, having the same mutations on OprD porin channel, and on *nalC* and *mexR* genes (MexAB-OprM efflux pump repressor and regulator, respectively) (Table 2). Both *P. juntendi* were IMP-8-producers (ER1C4P2 and ER4C8A1) and were considered unrelated by using the SNP relatedness cut-off of *P. aeruginosa* (SNP difference >14, Supplementary Table 5), as no cut-of has been proposed for this sub-species (Schürch et al., 2018). The *bla*$_{\text{IMP-8}}$ carbapenemase gene was possibly chromosomally inserted as no plasmids were detected (Aytan-Aktug et al., 2022).

Finally, one *S. maltophilia* ST115 strain (ER2F4P2) and two ST120 strains (ER3F8A1 and ER3D8P2) were identified. Interestingly, the two ST120 were closely related (5 SNP difference) showing its likely dissemination between the isolation unit and cat ward of SAVP-G (Supplementary Table 6).

SAVP-I, SAVP-K and SAVP-L are veterinary clinics from the same business group (i.e. some staff alternate between veterinary practices); however, none of the isolated strains were shared between practices. The SAVP-I carbapenem-resistant *P. aeruginosa* ST267 strain (A6E4P2) had one mutation on OprD porin channel and a wild-type MexAb-OprM efflux pump (Table 2). As for *S. maltophilia* strains, multiple STs were identified, namely ST5 (C6EE4A1) and a newly assigned ST967 (C1DE8A3) in SAVP-K; and ST39 strain (B4EE4A2) in SAVP-L.

Lastly, on SAVP-N, there was one *S. maltophilia* ST27 strain (EX2C4P3) and two ST115 strain (EX3I8A1 and EX2I4A1; 10 SNP difference).

3.3 Carbapenem-resistant Gram-negative bacteria transient carriage by veterinary staff

Diverse and normal microbial flora was observed across the 30 fecal samples available. Carbapenem-resistant non-fermenting Gram-negative bacteria were isolated from 16.7% (n=5/30) participants, including *P. aeruginosa* (n=3) and *S. maltophilia* (n=2).

The three *P. aeruginosa* strains were isolated from participants of different SAVP, namely a ST27 (HVD5R4P1) in one veterinarian from SAVP-E; a ST244 (A5R4P1) in one veterinarian from SAVP-I; and a ST274 (C8R8P1) in one nurse from SAVP-K.

P. aeruginosa ST244 strain was carbapenem-susceptible and was found to have a wild-type OprD porin channel and MexAB-OprM efflux pump. *P. aeruginosa* ST27 strain had an early stop codon on *oprD* together with mutations on *nalC* and *mexR; and P. aeruginosa* ST274 had several mutations on *oprD*, thus explaining their carbapenem-resistant phenotype (Table 2).

One *S. maltophilia* ST317 strain (B3R4P1) was detected in a nurse from SAVP-L, and one *S. maltophilia* ST84 strain (C10R8A1) in a technician from SAVP-K, both susceptible to trimethoprim/sulfamethoxazole.

Gram-negatives were rarely isolated from nasal swabs (1.2%, n=2/169) using selective media. One veterinarian from SAVP-G was positive for an imipenem-resistant CTX-M-3-producing *K. pneumoniae* ST11 (R11Np4K1; MIC= 8 mg/mL), albeit susceptible to meropenem, showing a 15% truncated Omp36K in the WGS analysis (Supplementary Figure 1). One veterinarian from SAVP-I was positive for a carbapenem-susceptible *P. aeruginosa* ST244 (A6Np4P1) that was related to the ST244 (A5R4P1, SNP difference ≤ 3, Supplementary Table 3) isolated from the rectal swab of a distinct veterinarian from the same veterinary practice.

TABLE 2 Mutations on OprD porin channel and MexAB-OprM efflux pump of *P. aeruginosa* strains (n=8).

SAVP	Type of Sample	NCBI strain ID	ST	Serotype	OprD	MexAB-OprM	Carbapenem resistance*	
							Imipenem	Meropenem
G	Environmental	ER3C4P3	253	O10	T103S; K115T; F170L; E185Q; P186G; V189T; R310E; A315G; A425G	*nalC* (G71E; A145V; S209R); *mexR* (V126E)	>16 (R)	>32 (R)
	Environmental	ER2F8P2	253	O10	T103S; K115T; F170L; E185Q; P186G; V189T; R310E; A315G; A425G	*nalC* (G71E; A145V; S209R); *mexR* (V126E)	>16 (R)	>32 (R)
I	Environmental	A6E4P2	267	O2	S278P	Wild-type	>8 (R)	4 (I)
E	Rectal swab	HVD5R4P1	27	O1	Stop Codon (TAG) at position 94	*nalC* (G71E; S209R); *mexR* (V126E; V132A)	>16 (R)	>32 (R)
I	Rectal swab	A5R4P1	244	O2	Wild-type	Wild-type	≤1 (S)	≤1 (S)
K	Rectal swab	C8R8P1	274	O3	D43N; S57E; S59R; E202Q; I210A; E230K; S240T; N262T; A267S; S278P; A281G; K296Q; Q301E; R310G; V359L; M372V; S373D; D374S; N375S; N376S; V377S; G378S; K380A; N381G; Y382L	*nalC* (S209R, G71E)	>8 (R)	8 (R)
I	Nasal swab	A6Np4P1	244	O2	Wild-type	Wild-type	≤1 (S)	≤1 (S)
K	Hand swab	C11Hp4P1	274	O3	Stop Codon (TAG) at position 378; filled with deletions	*nalC* (G71E, S209R)	>16 (R)	>32 (R)

S, Susceptible; I, Susceptible to increased exposure; R, Resistant. *EUCAST 2024 breakpoints (European Committee on Antimicrobial Susceptibility Testing, 2024) –for *Pseudomonas* spp., imipenem (S ≤0.001; R > 4) and meropenem (S ≤2; R > 8).

Only 1.8% (n=3/169) hand swabs were positive for Gram-negative bacteria. One *P. aeruginosa* ST274 strain (C11Hp4P1) was found on a technician from SAVP-K, showing an early stop codon on *oprD*, leading to failure in of the protein expression; and mutations on *nalC* (Table 2). In SAVP-N, two related *S. maltophilia* ST115 (6 SNP difference, Supplementary Table 7) were detected on the hands of a veterinarian (X4Hp4P2) and a technician (X3Hp4P1). These were closely related to the strains present in the fomites (≤ 10 SNPs difference, Supplementary Table 7), showing likely dissemination across the practice fomites and staff.

3.4 Surface contamination with methicillin-resistant *Staphylococcus* spp.

Nearly five percent (n=23/489) of surfaces were contaminated with methicillin-resistant *Staphylococcus* spp. (MRS). Two veterinary hospitals (SAVP-A; and SAVP-C) and one clinic (SAVP-N) had surfaces contaminated with MR *Staphylococcus pseudintermedius* (MRSP) (n=4) (Table 3). Overall, coagulase-negative MRS isolates predominated (78.3%, n=18/23), especially MR *Staphylococcus epidermidis* (72%, n=13/18) (Table 3). One newly typed ST9220 (clonal complex, or CC, 5) MRSA, harboring SCC*mec*II, was isolated from a cage at the cat ward of hospital SAVP-E.

3.5 Methicillin-resistant *Staphylococcus* spp. transient carriage by staff

A total of 169 nasal swabs were collected, of which 38% were positive for MRS (n=64/169). Amongst these, 14% were MRSA (n=9/64). Further characterization identified them as belonging to different CCs: ST22-IV (also known as EMRSA-15) (n=2) (SAVP-A, n=1; SAVP-B, n=1) and ST974-IV (SAVP-I, n=1) from CC22; ST8-VI (n=3) (SAVP-B, n=2; SAVP-L, n=1) from CC8; ST30-V (SAVP-C, n=1) from CC30; ST9220-II (SAVP-E, n=1) and ST125-IV (SAVP-F, n=1) from CC5. MRSP was not detected.

Considering hand swabs, 22.4% (n=38/169) were positive for MRS, and again, none was identified as MRSP. MLST and SSC*mec* cassette characterization of MRSA (10.5%, n=4/38) yielded the following classifications: ST8-VI (n=2) (SAVP-I, n=1; SAVP-L,

TABLE 3 Methicillin resistant *Staphylococcus* spp. on environmental evaluation of SAVPs A-N.

SAVP	Location	Area	Identified organism
A	Waiting room	Weight scale	MRSP
	Operating room	Stainless steel supporting tray	MRSE
B	Treatment area	Treatment table 02	MRSE
		Computer keyboard	
	Cat ward	Cage 06 (group on the left)	MRS
	Operating room 02	Computer keyboard	MRSE
C	Pre-operative area	Tap	MRSP
		Anaesthetic device buttons	
H	Ultrasound room	Ultrasound keyboard	MRSE
J	Examination room/Treatment room	Computer keyboard	MRS
	Examination room/Treatment room	Desk	MRSE
K	Pre-operative/Treatment area	Fridge handle	MRSE
	Operating room	Anesthesia tent - Outside	MRSE
		Operating table - Head	MRSE
		Blanket	MR *Staphylococcus hominis*
	Ward	Inside knob	MRSE
		Drawer handles	
		Pink blanket	
		Tap	
		Shearing blade	*MR Staphylococcus warneri*
E	Operating room	Detergent dispenser	MRS
	Cat ward	Cage left top	MRSA
N	Others	Keyboard personal computer	MRSP

MRSA, Methicillin resistant *S. aureus*; MRSE, Methicillin resistant *S. epidermidis*; MRS, Methicillin resistant *Staphylococcus* spp.; MRSP, Methicillin resistant *S. pseudintermedius*.

n=1) from CC8, ST9220-II (SAVP-E, n=1) from CC5 and non-typable ST from CC45 (SAVP-B, n=1) – it was not possible to type this strain of MRSA, albeit it carried SCC*mec*V.

Both MRSA ST8-VI strains belong to different clinics, despite being part of the same business group, with these members rotating between the different practices.

Interestingly, the new MRSA ST9220-II was only identified on SAVP-E in one surface, one nasal swab from a nurse, and one hand swab from a technician, pointing to an ongoing dissemination within this veterinary hospital.

4 Discussion

This study depicts the environmental contamination and veterinary staff carriage by resistant bacteria towards medically important antimicrobials within veterinary clinics and hospitals in Portugal. It was found that 6.5% (n=32/490) surfaces analyzed were positive for MDROs, amongst which were detected carbapenem-resistant bacteria, namely OXA-23-producing *Acinetobacter* spp., (n=5) and IMP-8 *P. juntendi* (n=2). Veterinary carriage analysis revealed that 38% and 22.4% were positive for MRS in their nasal cavities and hand swabs, respectively. For rectal swabs, only 16.7% yielded carbapenem-resistant bacteria carriage.

Notably, carbapenem-resistant Gram-negative bacteria (*Acinetobacter* spp., *Pseudomonas* spp. and *S. maltophilia*) and MRSA, belonging to epidemiologically relevant clones such as EMRSA-15, were found in several high-touch surfaces and fomites of SAVPs. The SAVP-G is a particularly interesting case, since, despite having a high self-assessment score about ongoing IPC procedures, it showed the highest level of environmental contamination, together with the isolation of the highest diversity of resistant bacteria. These results highlight that inadequate/insufficient IPC programs or the lack of compliance to them, may facilitate environmental contamination by MDROs, increasing the chance of their dissemination within SAVP and into the environment and community (e.g., staff, tutors, animal patients).

The hygiene evaluation of high-touch surfaces was conducted by two distinct methods, using contact plates and/or surface swabs.

The observed discrepancies between results could be attributed to the design of the contact plates, which support the growth of all types of microorganisms (such as fungi and yeast), while surface swabs are loaded with a universal neutralizing liquid buffer specifically geared towards bacterial growth. Conversely, swabs can be used to evaluate irregular surfaces, supporting the use of both techniques for a more complete evaluation of environmental contamination. Considering both methods, 96 surfaces failed the cleaning efficacy assessment (Dancer, 2004). Coincidently, most of these were high-rotation surfaces such as weight scales, sinks and taps, animal cages and computer keyboards.

The worldwide ongoing rising in carbapenem-resistance is worrisome, as these are last-line antibiotics (EMA, 2019). In this study carbapenem-resistant bacteria and possible dissemination events within some SAPV were detected. On SAVP-H, OXA-23-producing *Acinetobacter* spp. strains were found on different surfaces of the same room, indicating a possible transfer (previously published (Moreira da Silva et al., 2024a)).

The detection of various clones of carbapenem-resistant *P. aeruginosa* strains on high-touch surfaces is worrisome. Between 2022-2023, 7.9% of healthcare-associated infections reported in Europe in the human healthcare setting were caused by *P. aeruginosa*, of which 29.7% were resistant to carbapenems (European Centre for Disease Prevention and Control, 2024). *P. aeruginosa* ST244, ST27 and ST253 clonal lineages are commonly associated with outbreaks and nosocomial infections in humans (del Barrio-Tofiño et al., 2020). A previous study conducted in a veterinary teaching hospital reported the presence of *P. aeruginosa* ST244 on sinks (Soonthornsit et al., 2023), while *P. aeruginosa* ST27 has higher affinity towards cystic-fibrosis patients (Weimann et al., 2024). There aren't any guidelines that support rectal sampling on healthy healthcare-workers in human medicine, as studies have shown that in low prevalence settings of MDROs, the transient carriage by healthcare-workers will also be low (Bassyouni et al., 2015; Decker et al., 2018). Since in this study's context, the reality of MDROs carriage by healthcare-workers is unknown, we decided to evaluate it. The low percentage of rectal samples positive for carbapenem-resistant bacteria (n=5/30, 16.7%) of veterinary healthcare-workers aligned with what is already known in human medicine as they work in a low-exposure setting to MDROs.

Strains reported on our study were from healthy human samples (*P. aeruginosa* ST244 from one nasal and one rectal swab from unrelated individuals; carbapenem-resistant *P. aeruginosa* ST27 from one rectal swab). Although SAVP-I and SAVP-K belong to the same business group, it was interesting to perceive that each SAVP had its own associated clone shared between staff members, namely, *P. aeruginosa* ST244 on SAVP-I, and carbapenem-resistant *P. aeruginosa* ST274 on SAVP-K (rectal swab and hand swab from unrelated individuals). Although none of these clones were recovered from the environment, their sharing among team members points to their potential for dissemination. These results highlight the importance of screening veterinary staff as part of an effective IPC protocol to identify potential carriers, thereby preventing transmission to patients, staff, and the community. Moreover, a carbapenem-resistant *P. aeruginosa* ST253 was present on a plastic mat on a countertop inside an isolation unit (SAVP-G), an area with specific cleaning and disinfection protocols (Weese, 2004), and although it is not expected for this area to be completely sterile, potentially nosocomial microorganisms should not be present.

Genetically unrelated IMP-8-producing *P. juntendi* strains were present on the inside of an empty cage and on the plastic cover of the treatment table in the dog ward of SAVP-G. To the best of our knowledge, this is the first description of IMP-8-producing *P. juntendi*. So far, only one description of carbapenem-resistant *P. juntendi*, harboring an IMP-1, has been made in a Chinese human patient (Zheng et al., 2022). This data highlights the capacity of this species to acquire and disseminate resistance genetic elements, making it a pathogen to be considered in epidemiological surveillance schemes.

S. maltophilia is an opportunistic pathogen with intrinsic resistance to many antibiotics, including carbapenem, posing major challenges in clinical settings (Mojica et al., 2022). In our study, multiple clones (including the new ST967 and ST1185) of *S. maltophilia* were described in the environment of various SAVPs, as well as colonizing staff members. These bacteria are ubiquitous in the environment, yet they have also been associated with nosocomial and community-acquired infections (Gröschel et al., 2020; Mojica et al., 2022; European Centre for Disease Prevention and Control, 2024) The relatedness between environmental and pathogenic *S. maltophilia* strains has been described, indicating that the environment may be a source of human contamination – including from sinks and taps (Mojica et al., 2022). As expected, the majority of the contaminated surfaces in our study were water-related. The spread of *S. maltophilia* through fomites ultimately causing human infection has also been described (Gideskog et al., 2020). The occurrence of *S. maltophilia* ST115 strains on the handcloth, the practice's mobile phone and on the hands of two staff members of SAVP-N demonstrate that such objects likely acted as fomite and contributed to dissemination of this clone (Gideskog et al., 2020). Yet, in veterinary medicine, reports of infections caused by this agent are rare. Nonetheless, *S. maltophilia* ST115 clonal lineage has been associated with infections in cats (Shimizu et al., 2021), underscoring its disease-causing capability and the importance of closely monitoring it.

In Europe and according to the 2022–2023 ECDC report, 23.7% of *S. aureus* causing healthcare-associated infections were MRSA, showing a 6.3% decrease from the previous report (European Centre for Disease Prevention and Control, 2024). EMRSA-15 is a major clone found in hospitals and in the community in Portugal (Tavares et al., 2013). This epidemic clone has also been described on clinical strains from pets (Couto et al., 2015; Costa et al., 2022), with studies showing that working in close contact with companion animals is a risk factor for MRSA carriage (Weiß et al., 2013; Bal et al., 2016; Feßler et al., 2018; Rodrigues et al., 2018). The newly described MRSA ST9220-II, belonging to CC5, was found on SAVP-E. The presence on a surface as well as on a nasal swab from a nurse and a hand swab from a technician suggests that this clone might be spreading within this veterinary practice, possibly through contaminated surfaces.

A Portuguese study identified 61% of nasal carriage of MRS among veterinary professionals (Rodrigues et al., 2018), which is higher than what was found in the present study. However, this same study reported 14% carriage of MRSA, comparable to our findings, with EMRSA-15 also being the most prevalent clone (Rodrigues et al., 2018). The frequent colonization by MRS and MRSA reported in these two studies showcases that veterinary healthcare providers may contribute to the transmission cycle of these pathogens into the community. The frequency of MRSA recovered from hand swabs was lower. It is known that hand hygiene is a pivotal measure in IPC programs, with studies showing that improvements in the healthcare workers' hand hygiene protocols cause a direct decrease in the carriage of MRSA (Marimuthu et al., 2014).

Overall, the detection of MRS and carbapenem-resistant Gram-negative bacteria on high-touch surfaces in SAVPs underscores the need for strict IPC procedures. These measures are essential not only to protect patients but also to address the ongoing antimicrobial resistance crisis.

5 Conclusion

The current study depicts varying levels of environmental contamination and staff carriage of carbapenem-resistant Gram-negative and MRS strains in SAVP across Portugal. These findings question the effectiveness of ongoing IPC protocols and highlight the risk of environment/staff cross-contamination through high-touch surfaces. This data suggests that SAVP may play an active role in the spread of priority pathogens resistant to medically important antimicrobials, emphasizing the need for targeted educational workshops for veterinary healthcare students and professionals. In the long run, implementing and monitoring evidence-based IPC protocols and staff training should be mandatory to ensure strict compliance in SAVP.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA1131754 https://www.ncbi.nlm.nih.gov/, PRJNA1000421.

Ethics statement

The studies involving humans were approved by the Comissão de ética e bem-estar animal (CEBEA) of Veterinary Faculty of the University of Lisbon - CEBEA011/2021. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JMS: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing. JM: Investigation, Writing – review & editing. LF: Investigation, Writing – review & editing. CM: Writing – original draft, Writing – review & editing. SC: Writing – review & editing. DT: Writing – review & editing. AA: Writing – review & editing. CP: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

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General discussion, conclusion & future perspectives

Chapter 6 General discussion

The increase in antibiotic resistance, coupled with the lack of development of new antibiotics, is becoming a silent pandemic. It is a longstanding recognized problem in human medicine, in which occurrence of AMR among the ESKAPE pathogens is common (1,2).

HAIs are becoming more prevalent in human healthcare systems (3), with dire effects on economic costs and impact on the lives of patients and, indirectly, their families.

Carbapenems were once thought to be a promising therapeutic option against the increasing levels of MDR ESBL-producing bacteria (4). Nowadays, carbapenem resistance patterns are reaching increasingly concerning levels, making it necessary to develop new antibiotics, but also to invest in antimicrobial stewardship programmes and alternatives to antibiotics (5,6).

Early in 2024, the WHO updated the list of medically important antibiotics, classifying which antibiotics can be used in veterinary medicine, following the prescribing cascade. As expected, carbapenems are only to be used in human medicine (7). This classification is aligned with EMA's, released in 2019 (8). Despite the restriction, carbapenem-resistant bacteria are emerging in clinical strains of companion animals, particularly *E. coli* and *K. pneumoniae*, across the world (9–11).

As such, the main goal of this dissertation was to evaluate the occurrence of carbapenem-resistant bacteria in veterinary medicine healthcare systems and how homologous these are to those described in human medicine. Firstly, it was important to establish the prevalence of carbapenem-resistant Enterobacterales from clinical samples from companion animals. With these data, it was observed that causative agents of disease in companion animals were homologous to those associated with human nosocomial infections, which in turn, can also be found in hospital environments. Thus, an evaluation of surface contamination of small animal veterinary practices, as well as colonization of veterinary healthcare workers, was conducted, to check if the same bacteria (or others) were present in this environment. The finding of carbapenem-resistant Gram-negative bacteria in various surfaces of different veterinary practices, together with the colonization of healthcare workers, give force to the need to implement specifically designed IPC programmes for veterinary medicine since these MDR bacteria may arise and disseminate from this context.

6.1 Carbapenem resistance in companion animal clinical strains

Firstly, evaluation of clinical strains from companion animals was performed, with the aim of estimating the frequency of CPE. Despite the low frequency found (0.51%), this study described three KPC-3-producing *K. pneumoniae*, one OXA-181-producing *K. pneumoniae* and one OXA-48-producing *E. coli* strains.

This result is similar to the one obtained in a German study of a large collection of Enterobacterales strains obtained between 2009-2016 from companion animals, which reported a prevalence of 0.64% of CPE, all of which were OXA-48-producing strains (11).

OXA-181-producing *K. pneumoniae* was the only probable CPE strain accidentally identified at the diagnostic laboratory. Information gathered by diagnostic laboratories through the clinical specimens sent to analysis is an important source of AMR resistance data at a population level of companion animals, since there is no formal surveillance system of resistance for companion animals, as oppose to what occurs in livestock (12,13). Carbapenem susceptibility screening by veterinary diagnostic laboratories is not compulsory since this antibiotic class is of prohibited use in veterinary medicine (8).

Yet, screening is easy and affordable, with different techniques available and suitable for different laboratory sizes (14). Given the panorama of growing resistance and a need to preserve the use of carbapenems for human medicine, active screening of carbapenem resistance could possibly minimize its spreading onto the community, together with a gain of information which might help design antimicrobial stewardship strategies for veterinary medicine (14).

The antibiotic prescribing cascade for companion animals starts by using an antibiotic that has been approved in Portugal, if not possible, the next step is to use an authorized one in human medicine (15). Of the five clinical cases of companion animals infected by carbapenem-resistant strains, it was not possible to retrieve any treatment data from two animals, with a third one dying after sample collection. For KPC-3-producing *K. pneumoniae* ST392, the dog was treated with trimethoprim-sulfamethoxazole and made a full recovery, while for OXA-181-producing *K. pneumoniae* ST273, the cat's wound was surgically cleaned and the animal started an antibiotic course of trimethoprim-sulfamethoxazole, enrofloxacin and amoxicillin-clavulanate a full recovery was achieved. In both instances, the veterinary approved antibiotics were effective in the treatment of these MDR infections (8), yet these classes of antibiotics are highly important antimicrobials according to the WHO (7). These two cases are examples of how important laboratory diagnoses are to guide treatment, thus preventing the inadequate use of higher classes of antibiotics.

All KPC-3-producing *K. pneumoniae* described in Chapters 2&3 were ESBL producers, as well as harbouring other resistance genes (*tet* genes and *sul2* genes), conferring them an MDR profile. Furthermore, they were also susceptible to ceftazidime-avibactam, a combination antibiotic used in human medicine against carbapenem-resistant infections (16).

Additionally, all these strains belong to Clonal Group 147 (OXA-181-producing *K. pneumoniae* ST273 strain, KPC-3-producing *K. pneumoniae* ST147 strains and the KPC-3-producing *K. pneumoniae* ST392). CG147 is globally distributed, having been associated with *bla*$_{\text{OXA-48-like}}$ genes (17). In Europe, the epidemiological landscape of carbapenem-resistant *K. pneumoniae* varies from region to region – although the ST258/512 is mostly frequently encountered, other high-risk clones are dominant – still, CG147 only represent 6% of isolates (18).

In Portugal, there is an elevated presence of *K. pneumoniae* CG147 strains in circulation (19–21). The majority of these strains is associated with *bla*$_{\text{OXA-48-like}}$ or *bla*$_{\text{KPC-3}}$ genes (19,20,22), with IncX3-type plasmids and IncF/IncN -type plasmids associated with the carbapenemase genes, respectively (19,20,22,23).

As such, the discovery of *K. pneumoniae* strains in companion animals with similar characteristics to those in human medicine is an indication that spillover from human hospital strains into the community could be occurring, mirroring other countries (21,24). In spite of reports of KPC-3-producing *K. pneumoniae* being scarce in companion animals, reports for OXA-48-like-producing Enterobacterales are commonly found in veterinary medicine (11,25–28).

A common aspect amongst our strains is the presence of carbapenemase genes in conjugative plasmids. Conjugative plasmids have been associated with horizontal gene transfer of antibiotic resistance genes and are commonly linked to MDR resistance (29).

They play a role in augmenting AMR since they possess all the necessary mechanisms to self-replicate and be stable within a cell-host (30,31). Furthermore, Orlek et al., found a direct association between carbapenem resistance genes and conjugative plasmids, with the possibility of plasmids encoding carbapenem resistance genes to acquire additional virulence factors, which will strengthen plasmid carriage through co-selection (32). Altogether, these factors point to an AMR dissemination occurring via mobile genetic elements (plasmids), in veterinary medicine, making it necessary to reinforce surveillance on these epidemiological markers.

As for OXA-48-producing *E. coli*, no plasmid replicons were detected. Such observation probably indicates that the carbapenemase gene was inserted into the chromosome. This event has also been described in a human nosocomial isolate (33). Moreover, the pattern of susceptibility to third-generation cephalosporins combined with lower susceptibility to carbapenems, may cause strains with these susceptibility pattern to be overlooked during laboratory diagnosis, thus misjudging their epidemiological worth (34,35).

There are some limitations to the work developed in the initial chapters. Since the method of WGS used generated short reads of 150 base-pairs at length, the accuracy the achieved assemblies is lower than if a third generation sequencing method had also been used, such as long-read sequencing. Without this supplemental sequencing method, the genetic environment of OXA-48 was not fully described, and complete reconstruction of the plasmids of KPC-3 carbapenemases was not possible. Furthermore, carbapenem-resistant strains causing infections in companion animals might be undervalued as other resistance mechanism (Chapter 1, Section 1.2) were excluded from analysis.

Nevertheless, these results provide evidence that carbapenem resistance screening is important in veterinary diagnostic laboratories, either by phenotypic or genotypic methods since the likeness of the mobile genetic elements harbouring carbapenemase genes between veterinary medicine and human medicine imply a One Health dissemination. This screening is even more important for OXA-48-producing strains due to their uncommon susceptibility patterns to carbapenems.

6.2 AMR and infection, prevention and control strategies in veterinary medicine

The development of large and specialized companion animal veterinary hospitals performing high-standard animal care, involving complex surgeries, intensive care facilities and greater dependence on antibiotic therapy, has created conditions for the emergence of MDR bacteria mimicking those that exist in human hospitals.

The data found on early chapters of this dissertation demonstrated that carbapenem-resistant strains can be the cause of infection in companion animals, despite the prohibition of usage of carbapenems in veterinary medicine. Considering that the same bacteria species are known HAI in human healthcare systems and are also found in hospital environment, it was hypothesized the same could be happening in veterinary practices.

Consequently, the work from Chapters 2&3 evolved to evaluate the level of environmental contamination by MDR bacteria, particularly with carbapenem resistance. In the second part of this dissertation, both studies (Chapters 4&5) depict the evaluation of environmental contamination and the role of veterinary staff as potential vectors of transmission of resistant bacteria in veterinary settings as well as to perform an evaluation of IPC protocols in veterinary practices (7). These results mirror those described in Human medicine, where outbreaks caused by MDR bacteria are usually traced back to an environmental contamination (36–38).

Starting with the work presented on Chapter 4, evaluation of the high-touch surfaces of this clinic showed contamination with OXA-23-producing *Acinetobacter* spp. strains on five different surfaces. A cat with a soft skin and tissue infection caused by an OXA-23-producing *A. baumannii* human Global Clone 2 had been hospitalized there one week prior to environmental sampling. As for data presented in Chapter 5, carbapenem-resistant *P. aeruginosa* and *S. maltophilia* were also found to be environmental contaminants in veterinary practices. Furthermore, IMP-8-producing *Pseudomonas juntendi* was here firstly described.

Overall, a high level of environmental contamination was found, with high-touch surfaces being contaminated with resistant bacteria. Incidentally, most of these were high-rotation surfaces such as weight scales, sinks and taps, animal cages and computer keyboards. Sinks and keyboards are also a pivotal point of IPC programmes in human medicine, recognized for facilitating the dissemination of CPE and carbapenem-resistant *P. aeruginosa* within the hospital environment (37,39–42). As for cages, these are known potential sources of contamination, despite little research having been done on the subject (43). Nonetheless, the importance of cages in the dissemination of MDR bacteria should be the same as the one given in human hospital beds (44).

The level of resistance to carbapenems is increasing in Europe (Chapter 1, Figure 1). As previously mentioned, these antibiotics are of prohibited use in veterinary medicine so that their mode of action can be preserved. Yet, the occurrence of carbapenem-resistant bacteria in different veterinary practices raises a concern (45).

Out of all the SAVPs analysed, eight had at least one surface contaminated with a carbapenem-resistant strain. Of these, five were clinics and the remaining were hospitals. This is contrary to a Swiss study, in which carbapenem-resistant bacteria were only detected in large hospitals (46). Hence, the design of IPC protocols for veterinary practices not only need to reflect the size of the practice, but also consider an adaptation to the functioning of the practice.

Acinetobacter spp., *P. aeruginosa* and *S. maltophilia* are ubiquitous to the environment, yet they are also opportunistic pathogens (36,47–50). Notably, the vast majority of HAI reported in Europe were caused by one of these agents (3).

The occurrence of carbapenem-resistant *Acinetobacter* spp. is not uncommon in human healthcare. According to the latest EARS-NET surveillance report, of all *Acinetobacter* spp. reported in clinical routine surveillance, 36.3% were resistant to carbapenems (51). Consequently, in the same time frame, approximately 80% of the stated HAI caused by *Acinetobacter* spp. were carbapenem-resistant (3). In veterinary medicine, research has shown the isolation of *Acinetobacter* spp. as a colonizer of the skin from healthy dogs (52) and reports of carbapenem-resistant *A. baumannii* are increasing, being associated with multiple types of infections (9,53,54). On the other hand, infections caused by *A. lwoffii* are rarely reported (55) and none caused by *A. schindleri* have been described.

As such, the findings of non-traditional *Acinetobacter* spp. harbouring a carbapenemase gene showed the importance of environmental surveillance on veterinary practice, especially when an animal with a clinical resistant strain has been hospitalized. In this instance, the veterinary practice was only evaluated for the level of environmental contamination because the assisting veterinary diagnostic laboratory screened for carbapenem resistance of the clinical strain, which led to further assessment of the practice since the cat stayed hospitalized for treatment. Although the clinical strain was not detected in the environment, other carbapenem-resistant *Acinetobacter* spp. strains were found, highlighting the importance of environmental screening, with or without a clinical cause behind it. Furthermore, this underscores the role that diagnostic laboratories play in effective and custom made IPC strategies.

In the absence of an MLST scheme for *A. lwoffii*, a SNP analysis was assess the isolates relatedness. Although no SNP cut-off has been proposed for this species, the low number of differences between each strain (an average 16 SNPs differences), pointed to a likely dissemination within the different fomites of the veterinary practice ward.

The *bla*$_{\text{OXA-23}}$ carbapenemase gene was present in the same mobile genetic elements – transposon and plasmid, across the different *Acinetobacter* species identified in the different locations of the veterinary practice. As priorly discussed, conjugative plasmids are commonly associated with MDR resistance patterns and play a role in augmenting AMR disseminations (30–32). Similar to what is occurring in clinical strains, environmental dissemination of AMR is also arising from mobile genetic elements, making it important to study these elements within surveillance programmes.

P. aeruginosa is also commonly linked to HAI, particularly with high-level resistance to carbapenems (3). In human medicine, reports of *P. aeruginosa* outbreak associated with original contamination of sinks is widespread (40,41). Mirroring this data, most *P. aeruginosa* showing high-levels of resistance to carbapenems in Chapter 5 were detected on water sources or near water sources (*P. aeruginosa* ST253 on tap and on countertop; *P. aeruginosa* ST267 on a sink). Furthermore, they also showed high-levels of resistance to carbapenems.

None of the *P. aeruginosa* isolates presented in Chapter 5 harboured a carbapenemase gene, which is not uncommon as Proteobacteria (except Enterobacterales) are less likely to harbour carbapenems resistance genes (32). Nevertheless, *P. aeruginosa* are known to harbour all classes of carbapenemases (56,57). All the described *P.* aeruginosa environmental strains were carbapenem-resistant due to OprD channel alteration and/or efflux pump MexAB-OprM overexpression, causing imipenem and meropenem resistant phenotypes, respectively. Multiple types of mutations in *oprD* porin channel have been connected to the imipenem resistance phenotype, ranging from single nucleotide to frameshift mutations (58,59). Moreover, SNPs differences have also been previously identified on repressor genes of efflux pump, increasing resistance patterns on nosocomial strains of *P. aeruginosa* compared to environmental strains (60).

It was interesting to observe that only *P. aeruginosa* ST253 had mutations both in porin channel and efflux pump. This data highlighted the possible nosocomial origin of this strain ,with this clone being associated with Spanish nosocomial infections (61), albeit not being a globally disseminated clone.

In Chapter 5, IMP-8-producing *P. juntendi* was isolated from two surfaces of the dog ward. This species belongs to the *Pseudomonas putida* group, which is a low-incidence group of opportunistic pathogens, despite its association to metallo-β-lactamases genes. The discovery of *P. juntendi* in association with a *bla*$_{\text{IMP-8}}$ gene is a novelty, demonstrating the possibility of new strains arising from veterinary healthcare systems, and also highlighting the need for AMR surveillance continuous of veterinary practices as a mean to prevent further dissemination into the community.

Multiple clones of *S. maltophilia* were found, particularly near water-related environments (Chapter 5, Table 1). This bacteria is not uncommon in nosocomial environments and has been associated with HAI (50), specifically in cystic-fibrosis patients and skin soft tissue infections (62). This bacterium is intrinsically resistant to multiple classes of antibiotics due to its low membrane permeability, leading to infections caused by it to be difficult to treat (50). Additionally, the dissemination of *S. maltophilia* through fomites ultimately causing human infection has also been described (63). In veterinary medicine, infections caused by *S. maltophilia* are still rarely reported (64). However, this may change due to the increasing use of invasive procedures, technological medical devices (e.g.endoscopic surgery) or the use of immunosuppressant therapies in small animals (65).

Within the veterinary healthcare workforce, a variety of *P. aeruginosa* and *S. maltophilia* clones were found amongst the hand, nasal and rectal swabs. Only one case of human colonization by S. *maltophilia* ST115 could be attributed to environmental contamination, with fomites acting as vectors. Although rare, the dissemination of *S. maltophilia* through fomites, eventually causing human infection have been described (63,66). As for the remaining occurrences of colonization, the same clones were found on different sampling sites of the workforce, despite not being recovered from the environment. Two possible explanations arise. : i) due to their wide environmental distribution of these pathogens and without the occurrence of an outbreak, different clones are normal to be found (67,68); ii) clone dissemination leading to staff colonization may be through contact with a fomite or surface that was not analysed.

In Europe, human healthcare professionals should be aware of which clones are circulating in their hospitals due to ongoing IPC surveillance (Chapter 1, Table 5) and as required by European and national guidelines (69). Regarding hospital staff, ESCMID does not support hospital screening in case of an outbreak (70), as data shows that intestinal bacterial carriage by hospital healthcare workers offers limited contribution to the dissemination of MDR (71). Nonetheless, rectal swabs from the veterinary healthcare workers were collected since there has been a report on companion animals clinical strains also colonizing veterinary staff (72), and the same could possibly occur with environmental carbapenem-resistant strains.

The importance of hand hygiene as a key component of IPC programmes has long been recognized. Numerous guidelines outline the correct moments to wash or disinfect hands and highlight the significant impact this practice has on the spread of MDR bacteria (73–75). However, most studies assessing the direct impact of proper hand hygiene on MDR bacteria dissemination have focused on MRSA carriage (76–78), in spite of the high prevalence of carbapenem-resistant Gram-negative bacteria as causative agents of HAIs in humans. In veterinary medicine, available guidelines also emphasize the importance of good hygiene (79). Yet, only one report has evaluated the impact of hand hygiene measures in the veterinary setting, and it was also focused on MRSA (80). The discovery of carbapenem-resistant Gram-negative bacteria on hand samples will help address this knowledge gap, allowing these bacteria to be considered when evaluating the impact of hand hygiene in the future.

As for nasal screening, the existing guidelines directed at human medicine are also focused on MRSA and recommend for the workforce not to be screened unless there is an epidemiological reason (81). In veterinary medicine, it is also known that veterinary workers are nasally colonized with MRSA (82). The observation of colonization of veterinary staff with Gram-negative bacteria, including with strains resistant to carbapenems, highlights the need to expand nasal screening guidelines. This would ensure a more comprehensive approach to preventing the spread of various MDR species in both human and veterinary healthcare settings, as well as into the community.

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Chapter 7 Conclusions & future perspectives

Throughout this dissertation, it has been shown that bacterial strains once thought to only occur in human healthcare settings, are also appearing in Veterinary Medicine, in unforeseen contexts. Despite the appearance of clinical strains from pets harbouring carbapenemase genes, mirroring the country's epidemiological data from human medicine, being increasingly reported, the occurrence of such strains in the veterinary healthcare environment has been underreported. The project developed during this dissertation contributes to the knowledge that carbapenem-resistant clinical strains in companion animals is a reality in Portugal, with the current IPC programmes being insufficient to minimize the occurrence of carbapenem-resistant bacteria not only in the environment of veterinary healthcare systems, but also in the workforce.

As for future perspectives, it is still necessary for more veterinary specific data regarding clinical strains to be generated in order to develop adequate policies for infection, prevention and control in veterinary medicine healthcare setting. An example could be a study looking at the possibility of an hospitalized animal to contaminate the home environment and its tutors with an HAI.

A simple step to implement as part of an IPC programme is passive surveillance of diagnostic data as a means to rapidly identify outbreaks and to know which type of organisms are more commonly found in the practice.

Similar to the public data available for the epidemiological patterns of bacteria found in livestock, it would be important to develop a similar effort for companion animals. By knowing the true prevalence of carbapenem-resistant bacteria in this setting, the design of IPC protocols could be more efficient as it would take this data into consideration.

As for veterinary practices, promotion of educational workshops on how and where to start implementing IPC protocols is important.

Education of tutors on what AMR is and what can be done to slow down its dissemination should also be an important part of any IPC protocol of a veterinary practice. Veterinary healthcare workers play a crucial role in One Health, ensuring the health of animals while safeguarding public health.

Ultimately, the data presented in this dissertation contributes to give strength that One Health approaches are crucial and how important it is not only to consider companion animals, but also the environment that surrounds them when questioning their role in the spread of AMR.

Annexes

Annex A – Supplementary material for section 1.4, Carbapenem resistance diagnosis and One Health

Table S1. Representative MIC vales for Imipenem, Meropenem and Ertapenem from companion animals Carbapenemase-producing isolates

Carbapenemase	Year	Country	Host	Source	Bacterial species	Minimum inhibitory concentration (mg/L)			Ref.
						IMP	MEM	ETP	
KPC-2	2018	Brazil	Dog	Infection (UTI)	*Escherichia coli*	4	4	4	(146)
KPC-2	2021	Brazil	Dog	Infection (UTI)	*Klebsiella pneumoniae*	>32	>32	NA	(147)
KPC-4	2018	USA	Dog	Infection (UTI, SSTI)	*Enterobacter xiangfangensis*	≤1	2	NA	(148)
KPC-4	2018	USA	Dog	Infection (UTI, SSTI)	*Enterobacter xiangfangensis*	≤1	1	NA	(148)
NDM-1	2013	United States	Dogs and Cats	Infection (SSTI)	*Escherichia coli*	NA	1	NA	(161)
NDM-1	2013	United States	Dogs and Cats	Infection (UTI)	*Escherichia coli*	NA	0.5	NA	(161)

Carbapenemase	**Year**	**Country**	**Host**	**Source**	**Bacterial species**	**Minimum inhibitory concentration (mg/L)**			**Ref.**
						IMP	**MEM**	**ETP**	
NDM-1	2013	United States	Dogs and Cats	Infection (UTI)	*Escherichia coli*	NA	4	NA	(161)
NDM-1	2013	United States	Dogs and Cats	Infection (UTI)	*Escherichia coli*	NA	16	NA	(161)
NDM-1	2017	China	Dog	Commensal	*Escherichia coli*	64	64	256	(162)
NDM-1	2017	China	Dog	Comensal	*Escherichia coli*	32	64	NA	(153)
NDM−1	2018	Italy	Dog	Comensal	*Acinetobacter radioresistens*	>32	>32	NA	(160)
NDM-5	2017	China	Dogs	Comensal	*Escherichia coli*	32	32	NA	(153)
NDM-5	2017	China	Dogs	Comensal	*Escherichia coli*	16	64	NA	(153)
NDM-5	2017	China	Dogs	Comensal	*Escherichia coli*	64	128	NA	(153)
NDM-5	2017	China	Dogs	Comensal	*Escherichia coli*	8	32	NA	(153)
NDM-5	2017	China	Dogs	Comensal	*Escherichia coli*	16	32	NA	(153)
NDM-5	2017	China	Dogs	Comensal	*Escherichia coli*	8	16	NA	(153)

Carbapenemase	**Year**	**Country**	**Host**	**Source**	**Bacterial species**	**Minimum inhibitory concentration (mg/L)**			**Ref.**
						IMP	**MEM**	**ETP**	
NDM-5	2018	United States	Dog	Infection (URTI)	*Escherichia coli*	4	NA	NA	(157)
NDM-5	2019	South Korea	Dog	Commensal	*Escherichia coli*	4	4	16	(158)
NDM-5	2019	South Korea	Cats	Commensal	*Escherichia coli*	16	32	>32	(158)
NDM-5	2019	United Kingdom	Dog	Infection (SSTI)	*Escherichia coli*	4	4	NA	(156)
NDM-5	2021	Italy	Dog	Infection (UTI)	*Escherichia coli*	>16	>16	>2	(152)
NDM-9	2017	China	Dogs	Comensal	*Escherichia coli*	8	64	NA	(153)
OXA-48	2017	France	Dog	Commensal	*Escherichia coli*	1.5	0.75	0.75	(167)
OXA-181	2018	Switzerland	Dogs	Commensal	*Escherichia coli*	1	0.5	>2	(163)
OXA-181	2018	Switzerland	Dogs and Cats	Commensal	*Escherichia coli*	0.5	0.5	>2	(163)

Carbapenemase	**Year**	**Country**	**Host**	**Source**	**Bacterial species**	**Minimum inhibitory concentration (mg/L)**			**Ref.**
						IMP	**MEM**	**ETP**	
OXA-181	2018	Switzerland	Dogs and Cats	Commensal	*Escherichia coli*	0.5	0.5	2	(163)
OXA-181	2018	Switzerland	Dogs and Cats	Commensal	*Escherichia coli*	0.5	0.25	2	(163)
OXA-181	2018	Switzerland	Dogs	Commensal	*Escherichia coli*	0.5	1	>2	(163)
OXA-181	2018	Switzerland	Cat	Commensal	*Escherichia coli*	0.25	0.25	2	(163)
OXA-181	2020	Portugal	Dog	Commensal	*Escherichia coli*	≤1	≤1	1	(164)
OXA-23	2014	Portugal	Cat	Infection (UTI)	*Acinetobacter baumannii*	>8	>8	NA	(170)
OXA-23	2017	Germany	Dogs and Cats	Infection (UTI, SSTI, URTI, CRBSI, suppurate inflammation)	*Acinetobacter baumannii*	>16	NA	NA	(171)

Carbapenemase	**Year**	**Country**	**Host**	**Source**	**Bacterial species**	**Minimum inhibitory concentration (mg/L)**			**Ref.**
						IMP	**MEM**	**ETP**	
OXA−23	2018	Italy	Dogs and Cats	Comensal	*Acinetobacter baumanni*	>32	>32	NA	(160)
OXA−23	2018	Italy	Dogs and Cats	Comensal	*Acinetobacter baumanni*	13	>32	NA	(160)
OXA-23	2014	Portugal	Cat	Infection (UTI)	*Acinetobacter baumannii*	>8	>8	NA	(170)
VIM-1	2016	Spain	Dog	Commensal	*Klebsiella pneumoniae*	4	4	≤2	(151)

ETP, Ertapenem; IMP, Imipenem; MEM, Meropenem; NA, not applicable.

Annex B – Supplementary material for Chapter 3, CPE strains causing infections in companion animals

The SNP matrix table can be found on the following link: https://journals.asm.org/doi/10.1128/spectrum.03416-23#:~:text=Supplemental%20File%2010%20%2D%20spectrum.03416%2D23%2Ds0002.xlsx

Supplementary Table 1. Antimicrobial susceptibility results of the clinical Enterobacterales strains isolated from companion animals (n=977) human origin.

Antibiotic Tested	Percentage of susceptible clinical strains	Percentage of susceptible to increase exposure clinical strains	Percentage of resistant clinical strains
Amikacin (30µg)	94.9	3.6	1.0
Ampicillin (10µg)	36.3	8.9	54.4
Amoxicillin in association with clavulanic acid (30µg)	65.5	8.4	25.3
Cefoxitin (30µg)	80.6	4.2	14.5
Cephalothin (30µg)	36.1	20.2	43.4
Cefotaxime (30µg)	73.2	3.7	22.7
Ceftazidime (30µg)	81.3	3.6	14.4
Enrofloxacin (5µg)	64.2	7.6	27.9
Gentamicin (10µg)	83.2	1.8	14.5
Tetracycline (30µg)	36.1	5.9	57.4
Trimethoprim/sulfamethoxazole (25µg)	62.0	1.6	35.8

Enterobacterales strains include *E. coli*, *Klebsiella spp*., *Enterobacter spp*., *Citrobacter spp*., *Serratia spp*. and *Proteus spp*.

Supplementary Table 2. Data used to generate *Klebsiella pneumoniae* Clonal Group 147 phylogenetic tree. All *K. pneumoniae* strains are of human origin

Strain Identification	GenBank Accession Number	Country	ST	Infection Type	Carbapenemase gene
K45-67	GCA_001483725.1	Norway	273	NA	*bla*$_{\text{VIM-1}}$
DE022	GCA_900512335.1	Germany	392	infection (NS)	*bla*$_{\text{OXA-48}}$
LU005	GCA_900503635.1	Luxembourg	392	infection (NS)	*bla*$_{\text{OXA-48}}$
BE103	GCA_900507095.1	Belgium	392	infection (NS)	*bla*$_{\text{KPC-3}}$
AT025	GCA_900508275.1	Austria	392	infection (NS)	NA
ES103	GCA_900516645.1	Spain	392	infection (NS)	NA
ES234	GCA_900517365.1	Spain	392	infection (NS)	NA
FR057	GCA_900511745.1	France	392	infection (NS)	NA
PL023	GCA_900511445.1	Poland	392	infection (NS)	NA
002SK2	GCA_002848605.1	Switzerland	147	NA	*bla*$_{\text{NDM-9}}$
B-8658	GCA_004310675.1	Russia	147	NA	*bla*$_{\text{OXA-48}}$
LU008	GCA_900503645.1	Luxembourg	147	infection (NS)	*bla*$_{\text{KPC-2}}$
GR147	GCA_900502395.1	Greece	147	infection (NS)	*bla*$_{\text{KPC-2}}$
1_GR_13	GCA_001701425.2	Greece	147	NA	*bla*$_{\text{VIM-27}}$
K68-18	GCA_001462825.1	Norway	147	NA	*bla*$_{\text{VIM-27}}$
K68-73	GCA_001462845.1	Norway	147	NA	*bla*$_{\text{VIM-27}}$
kpneu032	GCA_900608075.1	Switzerland	147	infection (NS)	*bla*$_{\text{VIM-1}}$
TGH13	GCA_001746535.1	Greece	147	NA	NA
GR070	GCA_900500845.1	Greece	147	infection (NS)	NA
DE024	GCA_900512345.2	Germany	147	NA	*bla*$_{\text{KPC-2}}$

Strain Identification	**GenBank Accession Number**	**Country**	**ST**	**Infection Type**	**Carbapenemase gene**
RO005	GCA_900504855.1	Romania	147	infection (NS)	*bla*$_{\text{KPC-2}}$
RO028	GCA_900504585.1	Romania	147	NA	*bla*$_{\text{KPC-2}}$
RO039	GCA_900504685.1	Romania	147	NA	*bla*$_{\text{KPC-2}}$
RO076	GCA_900504225.1	Romania	147	infection (NS)	*bla*$_{\text{KPC-2}}$
DG7163	GCA_003227055.1	Italy	147	colonization	*bla*$_{\text{KPC-2}}$
ES065	GCA_900516415.1	Spain	147	infection (NS)	*bla*$_{\text{VIM-1}}$
ES107	GCA_900501685.1	Spain	147	infection (NS)	*bla*$_{\text{VIM-1}}$
IT062	GCA_900513215.1	Italy	147	infection (NS)	*bla*$_{\text{VIM-1}}$
RS053	GCA_900505345.1	Serbia	147	infection (NS)	NA
RS077	GCA_900505425.1	Serbia	147	infection (NS)	NA
ES261	GCA_900517095.1	Spain	147	infection (NS)	*bla*$_{\text{OXA-48}}$
ES261	GCA_900517095.1	Spain	147	infection (NS)	*bla*$_{\text{OXA-48}}$
RO047	GCA_900504015.1	Romania	147	infection (NS)	NA
BE097	GCA_900507055.1	Belgium	147	infection (NS)	NA
FR016	GCA_900510435.1	France	147	infection (NS)	NA
HU053	GCA_900517045.1	Hungary	147	infection (NS)	NA
62BG	GCA_000822405.1	Italy	147	NA	NA
k1025	GCA_900085785.1	United Kingdom	147	bacteraemia	NA
k1111	GCA_900086215.1	United Kingdom	147	infection (NS)	NA
k1279	GCA_900084235.1	United Kingdom	147	infection (NS)	NA
k2040	GCA_900086515.1	United Kingdom	147	infection (NS)	NA
k361	GCA_900086065.1	United Kingdom	147	infection (NS)	NA
k616	GCA_900084905.1	United Kingdom	147	infection (NS)	NA
k864	GCA_900085105.1	United Kingdom	147	infection (NS)	NA
k866	GCA_900085095.1	United Kingdom	147	infection (NS)	NA

Strain Identification	GenBank Accession Number	Country	ST	Infection Type	Carbapenemase gene
k868	GCA_900085115.1	United Kingdom	147	infection (NS)	NA
k906	GCA_900085145.1	United Kingdom	147	infection (NS)	NA
k618	GCA_900086085.1	United Kingdom	147	infection (NS)	NA
k904	GCA_900086155.1	United Kingdom	147	infection (NS)	NA
RO081	GCA_900504315.1	Romania	147	infection (NS)	NA
ES277	GCA_900517665.1	Spain	147	infection (NS)	NA
ES278	GCA_900517655.1	Spain	147	infection (NS)	NA
GEN000187	GCA_004145685.1	France	147	NA	NA
DK013	GCA_900502585.1	Denmark	147	infection (NS)	*bla*$_{\text{NDM-1}}$
BE105	GCA_900507565.1	Belgium	147	infection (NS)	*bla*$_{\text{OXA-48}}$
RO024	GCA_900504755.1	Romania	147	infection (NS)	*bla*$_{\text{OXA-48;}}$ *bla*$_{\text{KPC-2}}$
1060	GCA_004005735.1	France	147	NA	*bla*$_{\text{OXA-48}}$
109A7	GCA_004005185.1	France	147	NA	*bla*$_{\text{OXA-48}}$
DE038	GCA_900512445.1	Germany	147	infection (NS)	NA
PL006	GCA_900511365.1	Poland	147	colonization	NA
PL056	GCA_900511505.1	Poland	147	infection (NS)	NA
PL056	GCA_900511505.1	Poland	147	infection (NS)	NA
37347	GCA_003856595.1	Sweden	147	colonization	NA
DK001	GCA_900502525.1	Denmark	147	infection (NS)	*bla*$_{\text{OXA-181}}$
K1	GCA_003034565.1	Greece	147	bacteraemia	*bla*$_{\text{OXA-48}}$
K2	GCA_003034485.1	Greece	147	bacteraemia	*bla*$_{\text{OXA-48}}$
K3	GCA_003034435.1	Greece	147	bacteraemia	*bla*$_{\text{OXA-48}}$
K4	GCA_003034385.1	Greece	147	bacteraemia	*bla*$_{\text{OXA-48}}$
825795-1	GCA_001956965.1	Germany	147	colonization	*bla*$_{\text{OXA-48}}$
KpGoe149473	GCA_001908595.1	Germany	147	pneumonia	*bla*$_{\text{OXA-48}}$

Strain Identification	GenBank Accession Number	Country	ST	Infection Type	Carbapenemase gene
KpGoe149832	GCA_001908875.1	Germany	147	peritonitis	*bla*$_{\text{OXA-48}}$
KpGoe828304	GCA_001908695.1	Germany	147	colonization	*bla*$_{\text{OXA-48}}$
KpGoe152021	GCA_001908675.1	Germany	147	peritonitis	*bla*$_{\text{OXA-48}}$

NA – Not applicable; NS – Not Specified

Supplementary Table 3. Accession numbers and relevant information on plasmids used for the study's contigs circularization

Plasmid Identification	GenBank Accession Number	Plasmid Replicon	Carbapenemase Gene	Country	Origin
pBC947-OXA-181	MK412920.1	IncX3	*bla*$_{\text{OXA-181}}$	United Arab Emirates	Nosocomial Infection - bacteremia
pBK30661	KF954759.1	IncFIA	*bla*$_{\text{KPC-3}}$	United States	Nosocomial Infection – urinary tract infection
pWI_KPC3	LT838197.1	IncN	*bla*$_{\text{KPC-3}}$	France	Nosocomial Infection - Not specified

Supplementary Table 4. Routine carbapenem minimal inhibitory concentration results for clinical strains (n=62)

Strains (n=62)	**MIC[a] range for Imipenem**	**Number of strains resistant to Imipenem**	**MIC[a] range for Meropenem**	**Number of strains resistant to Meropenem**	**MIC[a] range for Ertapenem**	**Number of strains resistant to Ertapenem**
Klebsiella spp. (n=19)	≤1- 4 mg/L	0	≤1	0	≤0,5- >1 mg/L	1
Proteus spp. (n=15)	≤1- >8 mg/L	2[b]	≤1- >4 mg/L	0	≤0,5- >1 mg/L	2
E. coli (n=13)	≤1mg/L	0	≤1 mg/L	0	≤0,5 mg/L	0
Serratia spp. (n=5)	≤1mg/L	0	≤1 mg/L	0	≤0,5 mg/L	0
Enterobacter spp. (n=10)	≤1mg/L	0	≤1 mg/L	0	≤0,5- 1 mg/L	0

[a] Carbapenem resistance was determined in accordance to EUCAST breakpoint guidelines 2023 (14) . Ertapenem resistance MIC >0.5 mg/L; Imipenem resistance MIC > 4 mg/L; Meropenem resistance MIC >8 mg/L.

[b] *Morganellaceae*. are intrinsically resistant to low concentrations of imipenem, thus requiring exposure to high doses of imipenem (14) .

Supplementary Table 5. Distribution of strains amongst the study groups, according to the animal species and type of infection

	Clinical strains with an ESBL phenotype (n = 204)	Clinical strains with a possible OXA-48-like phenotype (n = 34)	Clinical strains with an MDR profile (n = 23)	Total
Animal Species (%)				
Dog	64.7% (n =132)	64.7 % (n =22)	65.2% (n =15)	169
Cat	28.9% (n =59)	26.5 % (n =9)	26.1% (n =6)	74
Other	6.4 % (n =13)	8.8% (n =3)	8.7% (n =2)	18
Infection Type (%)	a	b		
UTI	42.1 % (n =86)	38.2% (n =13)	60.9% (n =14)	113
SSTI	34.3 % (n =70)	50.0 % (n =17)	13.0% (n =3)	90
URTI	13.2 % (n =27)	2.9% (n =1)	8.7% (n =2)	30
Otitis externa	9.8 % (n =20)	5.9% (n =2)	17.4% (n =4)	26

ESBL – Extended Spectrum β-Lactamases; MDR – Multidrug Resistant; SSTI – Skin and soft tissue infections; URTI- Upper respiratory tract infection; UTI – Urinary tract infection.

[a] Infection characterized as SSTI/UTI (n =1); [b] Haemoculture (n =1)

Supplementary Table 6. β-lactamase genes detected in clinical strains from companion animal (n=261)

β-lactamases genes detected	Clinical strains with an ESBL phenotype	Clinical strains with a possible OXA-48-like phenotype	Clinical strains with an MDR profile
bla$_{TEM-1}$	*K. pneumoniae* (n=49) *E. coli* (n=34) *E. cloacae* complex (n=23) *P. mirabilis* (n=10) *P. vulgaris* (n=5) *K. variicola* (n=2) *M. morganni* (n=2) *K. quasipneumoniae* (n=1)	*E. coli* (n=7) *P. mirabilis* (n=1)	*E. coli* (n=5) *P. mirabilis* (n=8) *P. vulgaris* (n=2) *K. pneumoniae* (n=1) *K. quasipneumoniae* (n=1)
bla$_{TEM-135}$	*E. coli* (n=1)		*E. coli* (n=1)
bla$_{TEM-156}$	*P. mirabilis* (n=1)		
bla$_{TEM-32}$			*E. coli* (n=1)
bla$_{TEM-35}$		*E. coli* (n=1)	
bla$_{SHV-28}$	*K. pneumoniae* (n=6) *E. cloacae* complex (n=1)		*K. pneumoniae* (n=1)
bla$_{SHV-11}$	*K. pneumoniae* (n=4)		
bla$_{SHV-12}$	*E. cloacae* complex (n=3) *E. coli* (n=3) *K. quasipneumoniae* (n=1)		
bla$_{SHV-1}$	*K. pneumoniae* (n=1)		
bla$_{SHV-76}$	*K. pneumoniae* (n=1)		
bla$_{SHV-2}$	*K. pneumoniae* (n=1)		

β-lactamases genes detected	**Clinical strains with an ESBL phenotype**	**Clinical strains with a possible OXA-48-like phenotype**	**Clinical strains with an MDR profile**
$\mathit{bla}_{\text{CTX-M-15}}$	*K. pneumoniae* (n=48) *E. coli* (n=23) *E. cloacae* complex (n=21) *K. variicola* (n=3) *P. mirabilis* (n=3) *M. morganni* (n=2) *K. quasipneumoniae* (n=1) *P. vulgaris* (n=1)		
$\mathit{bla}_{\text{CTX-M-1}}$	*E. coli* (n=8) *K. pneumoniae* (n=2) *E. cloacae* complex (n=1) *P. mirabilis* (n=1)		
$\mathit{bla}_{\text{CTX-M-14}}$	*P. mirabilis* (n=5) *E. coli* (n=4) *P. vulgaris* (n=3) *E. cloacae* complex (n=1) *K. quasipneumoniae* (n=1)		
$\mathit{bla}_{\text{CTX-M-65}}$	*E. coli* (n=6) *P. vulgaris* (n=1)		
$\mathit{bla}_{\text{CTX-M-9}}$	*E. cloacae* complex (n=1) *K. quasipneumoniae* (n=1)		
$\mathit{bla}_{\text{CTX-M-55}}$	*E. coli* (n=6)		
$\mathit{bla}_{\text{CTX-M-156}}$	*E. cloacae* complex (n=1)		
$\mathit{bla}_{\text{CTX-M-28}}$	*E. coli* (n=1)		
$\mathit{bla}_{\text{CTX-M-3}}$	*K. pneumoniae* (n=1)		

β-lactamases genes detected	Clinical strains with an ESBL phenotype	Clinical strains with a possible OXA-48-like phenotype	Clinical strains with an MDR profile
bla$_{\text{CTX-M-32}}$	*E. coli* (n=1)		
bla$_{\text{CMY-2}}$	*E. coli* (n=10) *P. mirabilis* (n=3) *P. vulgaris* (n=2) *E. cloacae* complex (n=1) *K. pneumoniae* (n=2) *C. murliniae* (n=1)		
bla$_{\text{DHA-1}}$	*K. pneumoniae* (n=18) *P. mirabilis* (n=3) *E. cloacae* complex (n=2) *E. coli* (n=1) *M. morganni* (n=1)	*E. coli* (n=1)	
bla$_{\text{DHA-16}}$	*K. pneumoniae* (n=1)		
bla$_{\text{DHA-17}}$	*M. morganni* (n=1)	*P. mirabilis* (n=1)	
bla$_{\text{DHA-5}}$	*M. morganni* (n=2) *E. coli* (n=1)		
bla$_{\text{MIR-like}}$	*E. cloacae* complex (n=1) *K. pneumoniae* (n=1)		

ESBL – Extended Spectrum β-Lactamases; MDR – Multidrug Resistant

Supplementary Table 7. Assemblies properties following WGS analysis

Strains tested	N50	Total Assembly Length	Reference Length	GC Content (%)	GC Content Reference (%)	Nº of contigs	L50	Depth Coverage
OXA-181-producing *Klebsiella pneumoniae* (VG117)	166995	5669435	5279178 (GenBank Acc. CP102077.1)	57.12	57.17	340	13	105x (84-119x)
OXA-48-producing *Escherichia coli* (VG204)	159909	5347507	5184627 (GenBank Acc. HG994856.1)	50.44	50.43	1233	10	105x (77-119x)
KPC-3-producing *Klebsiella pneumoniae* (VG313)	278948	5544925	5438016 (GenBank Acc. CP023839.1)	57.08	57.03	151	6	250x (216-270x)
KPC-3-producing *Klebsiella pneumoniae* (VG314)	323447	5553936	5438016 (GenBank Acc. CP023839.1)	57.08	57.03	185	6	250x (231-294x)
KPC-3-producing *Klebsiella pneumoniae* (VG380)	168573	5625432	5325495 (GenBank Acc. CP08370.1)	57.18	57.24	319	11	98x (84-108x)

Supplementary Table 8. Antimicrobial MICs and resistance genes identified during WGS analysis on carbapenemase-producing *Klebsiella pneumoniae* strains

Strain	Antimicrobials Tested	MIC mg/L	Susceptibility Phenotype[§]	AMR genes
OXA-181 *K. pneumoniae* VG117 ST273	Amikacin	≤8	S	*aac(6’)-Ib-cr*
	Ampicillin	>16	R	*bla*$_{\text{SHV-1;}}$ *bla*$_{\text{CTX-M-15;}}$ *bla*$_{\text{OXA-181}}$
	Amoxicillin- Clavulanic Acid	>16/8	R[†]	*bla*$_{\text{CTX-M-15;}}$ *bla*$_{\text{OXA-181}}$
	Aztreonam	>16	R	*bla*$_{\text{CTX-M-15;}}$ *bla*$_{\text{OXA-181}}$
	Cefotaxime	>32	R	*bla*$_{\text{CTX-M-15;}}$ *bla*$_{\text{OXA-181}}$
	Ceftazidime	>16	R	*bla*$_{\text{CTX-M-15;}}$ *bla*$_{\text{OXA-181}}$
	Ciprofloxacin	>2	R	*OqxB/A; qnrS1;* GyrA-S83I; ParC-S80I
	Colistin	≤2	S	NA
	Ertapenem	>1	R	*bla*$_{\text{OXA-181}}$
	Gentamicin	≤2	S	NA
	Imipenem	≤1	S	*bla*$_{\text{OXA-181}}$
	Meropenem	≤1	S	*bla*$_{\text{OXA-181}}$
	Tetracycline	>8	R[†]	*tetD*
	Trimethoprim/Sulfamethoxazole	>4/76	R[†]	*sul1; OqxB/A; dfrA27*
KPC-3 *K. pneumoniae* VG313 ST147	Amikacin	≤8	S	NA
	Ampicillin	>16	R	*bla*$_{\text{TEM-1A;}}$ *bla*$_{\text{SHV-11;}}$ *bla*$_{\text{KPC-3}}$
	Amoxicillin- Clavulanic Acid	>16/8	R[†]	*bla*$_{\text{KPC-3}}$
	Aztreonam	>16	R	*bla*$_{\text{KPC-3}}$
	Cefotaxime	>32	R	*bla*$_{\text{KPC-3}}$

Strain	Antimicrobials Tested	MIC mg/L	Susceptibility Phenotype[§]	AMR genes
KPC-3 *K. pneumoniae* VG313 ST147	Ceftazidime	>16	R	$bla_{\text{KPC-3}}$
	Ciprofloxacin	>2	R	GyrA-S83I; ParC-S80I
	Colistin	≤2	S	NA
	Ertapenem	>1	R	$bla_{\text{KPC-3}}$
	Gentamicin	≤2	S	NA
	Imipenem	>8	R	$bla_{\text{KPC-3}}$
	Meropenem	>8	R	$bla_{\text{KPC-3}}$
	Tetracycline	≤4	S[†]	NA
	Trimethoprim/Sulfamethoxazole	>4/76	R[†]	*sul2; OqxB/A; drfA14*
KPC-3 *K. pneumoniae* VG314 ST147	Amikacin	≤8	S	NA
	Ampicillin	>16	R	$bla_{\text{TEM-1A;}}$ $bla_{\text{SHV-11;}}$ $bla_{\text{KPC-3}}$
	Amoxicillin- Clavulanic Acid	>16/8	R[†]	$bla_{\text{KPC-3}}$
	Aztreonam	>16	R	$bla_{\text{KPC-3}}$
	Cefotaxime	>32	R	$bla_{\text{KPC-3}}$
	Ceftazidime	>16	R	$bla_{\text{KPC-3}}$
	Ciprofloxacin	>2	R	GyrA-S83I; ParC-S80I
	Colistin	≤2	S	NA
	Ertapenem	>1	R	$bla_{\text{KPC-3}}$
	Gentamicin	≤2	S	NA
	Imipenem	>8	R	$bla_{\text{KPC-3}}$
	Meropenem	>8	R	$bla_{\text{KPC-3}}$
	Tetracycline	≤4	S[†]	NA
	Trimethoprim/Sulfamethoxazole	>4/76	R[†]	*sul2; OqxB/A; drfA14*

Strain	**Antimicrobials Tested**	**MIC mg/L**	**Susceptibility Phenotype**[§]	**AMR genes**
KPC-3-*K. pneumoniae* VG380 ST392	Amikacin	≤8	S	*aac(6')-Ib-cr*
	Ampicillin	>16	R	$bla_{\text{TEM-1B};}$ $bla_{\text{SHV-11};}$ $bla_{\text{CTX-M-15};}$ $bla_{\text{KPC-3}}$
	Amoxicillin- Clavulanic Acid	>16/8	R[†]	$bla_{\text{CTX-M-15};}$ $bla_{\text{KPC-3}}$
	Aztreonam	>16	R	$bla_{\text{CTX-M-15};}$ $bla_{\text{KPC-3}}$
	Cefotaxime	>32	R	$bla_{\text{CTX-M-15};}$ $bla_{\text{KPC-3}}$
	Ceftazidime	>16	R	$bla_{\text{CTX-M-15};}$ $bla_{\text{KPC-3}}$
	Ciprofloxacin	>2	R	*aac(6')-Ib-cr ; OqxB/A; qnrB1*; GyrA-S83I; ParC-S80I
	Colistin	≤2	S	NA
	Ertapenem	>1	R	$bla_{\text{KPC-3}}$
	Gentamicin	≤2	S	*aac(6')-Ib-cr*
	Imipenem	>8	R	$bla_{\text{KPC-3}}$
	Meropenem	>8	R	$bla_{\text{KPC-3}}$
	Tetracycline	>8	R[†]	*tet(A)*
	Trimethoprim/Sulfamethoxazole	≤2/73	S[†]	*sul2; OqxB/A*

S – Susceptible; R – Resistant; NA – Not applicable

[§] Susceptibility phenotype was determined according to EUCAST breakpoint guidelines (14)

[†] Susceptibility phenotype was determined according to CLSI guidelines (15)

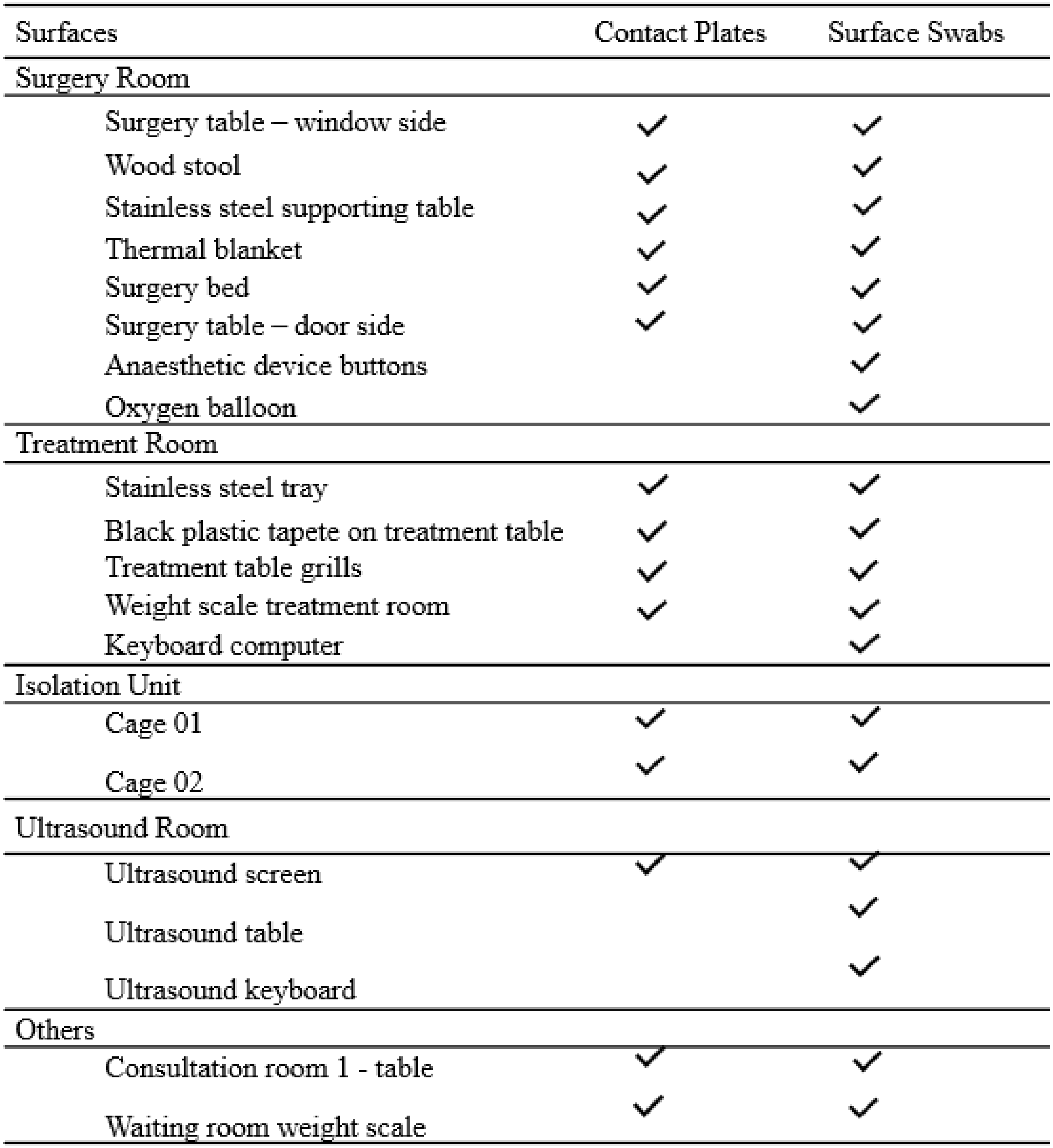
Supplementary Table 9. Complete list of virulence factors encoding genes found on the OXA-48-producing *E. coli* ST127 strain*

Encoding Gene	Virulence Factor
afaD	Afimbrial Adhesion
astA	EAST-1 heat stable toxin
chuA	**Outer membrane hemin receptor**
clbB	Hybrid non-ribosomal peptide /polyketidemegasynthase
cnf1	Cytotoxic necrotizing factor
fyuA	**Siderophore receptor**
gad	Glutamate decarboxylase
hra	Heat resistant agglutinin
iroN	Enterobactin siderophore receptor protein
irp2	Non-ribosomal peptide synthetase
iss	Increased serum survival
kpsE	Capsule polysaccharide export inner membrane protein
kpsMII	Polysialic acid transport protein
mcmA	Microcin M
ompT	Outer membrane protease
papA_F48	Major pilin subunit F48
papC	Outer membrane usher P
sfaD/ sfaE	S fimbrial/F1C minor subunit
sfaS	S-fimbriae minor subunit
sitA	Iron transport protein
tcpC	Tir domain containing protein
terC	Tellurium ion resistance protein
usp	Uropathogenic specific protein
vat	**Vacuolatin autotransporter toxin**
yfcV	**Fimbrial protein**

* Bold indicates the VF-encoding genes associated to uropathogenic lineages.

Annex C – Supplementary material for Chapter 4, OXA-23-producing *Acinetobacter* spp. in a veterinary practice

Supplementary Table I. List of surfaces from veterinary practice where samples were taken from and by which method



Surfaces	Contact Plates	Surface Swabs
Surgery Room		
Surgery table – window side	✓	✓
Wood stool	✓	✓
Stainless steel supporting table	✓	✓
Thermal blanket	✓	✓
Surgery bed	✓	✓
Surgery table – door side	✓	✓
Anaesthetic device buttons		✓
Oxygen balloon		✓
Treatment Room		
Stainless steel tray	✓	✓
Black plastic tapete on treatment table	✓	✓
Treatment table grills	✓	✓
Weight scale treatment room	✓	✓
Keyboard computer		✓
Isolation Unit		
Cage 01	✓	✓
Cage 02	✓	✓
Ultrasound Room		
Ultrasound screen	✓	✓
Ultrasound table		✓
Ultrasound keyboard		✓
Others		
Consultation room 1 - table	✓	✓
Waiting room weight scale	✓	✓

Supplementary Table II. Assembly properties following WGS analysis

Strains tested	N50	Total Assembly Length	Reference Length	GC Content (%)	GC Content Reference (%)	Nº of contigs	L50	Depth Coverage
OXA-23-producing-*Acinetobacter schindleri* (B1E8A1)	102818	3134297	3244663 (GenBank Acc. GCF_003072585.2)	42.51	42.59	267	9	450x
OXA-23-producing-*Acinetobacter lwoffii* (B4Z4A1)	36614	3307782	3515733 (GenBank Acc. GCF_019787625.1)	42.90	42.58	1002	31	370x
OXA-23-producing-*Acinetobacter lwoffii* (B4Z8A1)	50485	3418997	3515733 (GenBank Acc. GCF_019787625.1)	42.84	42.58	497	23	430x
OXA-23-producing-*Acinetobacter lwoffii* (B11Z4A1)	36614	3453014	3515733 (GenBank Acc. GCF_019787625.1)	42.85	42.58	761	31	330x
OXA-23-producing-*Acinetobacter lwoffii* (B12Z8A1)	36754	3415128	3515733 (GenBank Acc. GCF_019787625.1)	42.91	42.58	732	30	310x
Acinetobacter radioresistens (B7Z9X1)	182574	4734185	3132860 (GenBank Acc. GCF_002993105.1)	41.62	41.61	4529	6	490x
OXA-23-producing-*Acinetobacter baumannii* (10854)	113705	4057149	4199500 (GenBank Acc. GCF_001026965.1)	39.01	39.13	174	14	260x

Supplementary Table III. SNP matrix on *Acinetobacter lwoffii* environmental strains

SNP distance	**Reference Genome *A. lwoffii* (Genbank Acc. GCF_019787625.1)**	**OXA-23-producing-*A. lwoffii* (B12Z8A1)**	**OXA-23-producing-*A. lwoffii* (B4Z4A1)**	**OXA-23-producing-*A. lwoffii* (B11Z4A1)**	**OXA-23-producing-*A.lwoffii* (B4Z8A1)**
Reference Genome *Acinetobacter lwoffii* (Genbank Acc. GCF_019787625.1)	0	63759	63753	63755	72257
OXA-23-producing-*Acinetobacter lwoffii* (B12Z8A1)	63759	0	22	14	62127
OXA-23-producing-*Acinetobacter lwoffii* (B4Z4A1)	63753	22	0	12	62123
OXA-23-producing-*Acinetobacter lwoffii* (B11Z4A1)	63755	14	12	0	62125
OXA-23-producing-*Acinetobacter lwoffii* (B4Z8A1)	72257	62127	62123	62125	0

Annex D – Supplementary material for Chapter 5, Evaluation of MDR bacteria in veterinary practices in Portugal

An additional table with the degree of environmental contamination of each SAVP, on each surfaces collected, by contact plates and surface swabs, can be found on the following external link: https://docs.google.com/spreadsheets/d/1jtBXuqxP6YvXvLPNEsGpf1_JDcrVmDv5/edit?usp=sharing&ouid=100549588577922073306&rtpof=true&sd=true

Supplementary Table 1 – Environmental sampling sites in hospitals A-G

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
March 2021	SAVP-A	Operating room	Anaesthetic device	SS
			Ultrasound keyboard	SS
			Stainless steel supporting table	CP+SS
			Operating table	CP+SS
		Cat ward	Small cage	CP+SS
			Large cage	CP+SS
			Computer keyboard	SS
		Examination room 01	Examination table	CP+SS
		Examination room 02	Examination table	CP+SS
		Locker room	WC latch	SS
			WC cistern	SS
		Waiting room	Weight scale	CP+SS
April 2021	SAVP-B	Operating room 01	Operating table	CP+SS
		Operating room 02	Operating table	CP+SS
			X-ray table	CP+SS
			Ultrasound keyboard	SS
			Ultrasound table	SS
		Treatment area	Treatment table 01	CP+SS
			Treatment table 02	CP+SS
			Computer keyboard	SS
		Examination room 02	Examination table	CP+SS
		Examination room 03	Examination table	CP+SS
		Locker room	WC latch	SS
		Laboratory	Microscopy countertop	CP+SS
February 2022	SAVP-C	Operating room 01	Operating table - Head	CP+SS
			Operating table - Toes	CP+SS
			Anaesthetic device keyboard	SS
			Anaesthetic device	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
February 2022	SAVP-C	Operating room 01	Vital sign monitor buttons	SS
			Anaesthetic device buttons	SS
			Oxygen balloon	SS
			Peroxide hydrogen machine	SS
			Stainless steel supporting tray	SS
		Operating room 02	Operating table -Head	CP+SS
			Operating table - Toes	CP+SS
			Blanket	CP+SS
			Anaesthetic device	CP+SS
			Anaesthetic device buttons	SS
			Microscope cover	SS
			Buttons on respiratory device	SS
			Stainless steel supporting tray	CP+SS
		Pre-operative area	Plastic mat table 01	SS
			Plastic mat table 02	SS
			Table grids 01	SS
			Table grids 02	SS
			Plastic mat table 03	SS
			Tap	SS
			Weight scale	CP+SS
			Oxygen balloon	SS
			Anaesthetic device buttons	SS
			Disinfectant dispenser	SS
			Shearing blade	CP+SS
			Soap dispenser	SS
		Wash room	Countertop	CP+SS
April 2022	SAVP-B	Operating room 02	Stainless steel supporting tray 01	CP+SS
			Stainless steel supporting tray 02	CP+SS
			Stainless steel supporting tray 03	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
April 2022	SAVP-B	Operating room 02	Oxygen balloon	SS
			Anaesthetic device	SS
			Operating table	CP+SS
		Cat ward	Cage 06	CP+SS
			Cage 08	CP+SS
			Cage 06 (group on the left)	CP+SS
			Infusion pump	SS
		Ultrasound room	Ultrasound table	CP+SS
			Ultrasound bed	CP+SS
			Ultrasound keyboard	SS
May 2022	SAVP-D	Operating room 01	Cabinet	CP+SS
			Oxygen balloon	SS
			Stainless steel supporting tray	CP+SS
			Anaesthetic device buttons	SS
			Anaesthetic device	CP+SS
			Blanket	CP+SS
			Thermal blanket	SS
			Incubator table	CP+SS
		Operating room 02	Operating table - Head	CP+SS
			Operating table - Toes	CP+SS
			X-ray keyboard	SS
			X-ray table	CP+SS
		Cat ward	Stainless steel supporting tray	CP+SS
			Blanket	CP+ SS
			Big cage	CP+SS
			Small cage	CP+SS
May 2022	SAVP-D	Waiting room	Weight scale	CP+SS
		Wash area	Grids washing table	CP+SS
February 2023	SAVP-B	Operating room 01	Glass wall operating table	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
February 2023	SAVP-B	Operating room 01	Wall operating table	CP+SS
			Stainless steel supporting tray	CP+SS
			Stainless steel supporting tray (against the wall)	CP+SS
			Table with support material (e.g. compresses)	CP+SS
			Anaesthetic device	CP+SS
			Wood shelf	CP+SS
			Operating light handle 01	SS
			Operating light handle 02	SS
			Computer keyboard	SS
			Computer mouse	SS
			Blankets	CP+SS
			Oxygen balloon	SS
		Operating room 02	Door-side operating table	CP+SS
			Wall -side operating table	CP+SS
			Thermal blanket	CP+SS
			Anaesthetic device	CP+SS
			Computer desk	CP+SS
			Stainless steel supporting tray	CP+SS
			Operating light handle	SS
			Anaesthetic device buttons	SS
			Oxygen balloon	SS
			Computer keyboard	SS
			Computer mouse	SS
			Infusion pump	SS
			Drain	SS
		Treatment area	Stainless steel supporting table	CP+SS
			Table with washing grids	CP+SS
			Hallway table	CP+SS
			Computer desk	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
February 2023	SAVP-B	Treatment area	Computer keyboard	SS
			Computer mouse	SS
			Shearing blade	SS
			Ward side table	SS
			Tap	SS
			Liquid soap dispenser	SS
		Dog ward	Stainless steel supporting tray	SS
			Grids cage	CP+SS
			Weight scale	CP+SS
			Empty cage 01	CP+SS
			Empty cage 02	CP+SS
		Cat ward	Stainless steel supporting tray	CP+SS
			Empty cage	CP+SS
			Infusion pump	SS
			Sink	SS
		Isolation unit	Weight scale	CP+SS
			Table with support material (e.g. syringes)	CP+SS
			Empty cage 01	CP+SS
			Empty cage 02	CP+SS
			Empty cage mat	CP+SS
			Sink grids	CP+SS
			Tap	SS
			Antiseptic solution dispenser	SS
			Single cage	CP+SS
September 2023	SAVP-E	Operating room	Operating table - Head	CP+SS
			Operating table - Toes	CP+SS
			Thermal blanket	CP+SS
			Anaesthetic device	CP+SS
			Stainless steel stool on the toes of operating table	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
September 2023	SAVP-E	Operating room	Sink	SS
			Operating light handle	SS
			Sink countertop	CP+SS
			Chlorohexidine dispenser	SS
			Incubator	CP+SS
			Incubator hatch	SS
			Tap	SS
			Detergent dispenser	SS
			Internal valve anaesthetic device	SS
			Internal valve tubes anaesthetic device	SS
			Oxygen balloon	SS
			Operating table handle	SS
		Pre-operative area	Treatment table	CP+SS
			Weight scale	CP+SS
			Tap	SS
			Computer keyboard	SS
			Computer mouse	SS
			Grids	SS
			Catheter saline solution	SS
			Shearing blade	SS
		Dog ward	Large left cage	CP+SS
			Large right cage	CP+SS
			Small left cage	CP+SS
		Cat ward	Cage handle	SS
			Cage left top	CP+SS
			Cage left down	CP+SS
			Cage right down	CP+SS
			Shearing blade	SS
November 2023	SAVP-F	Operating room 01	Operating table -Head	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
November 2023	SAVP-F	Operating room 01	Operating table - Toes	CP+SS
			Stainless steel supporting tray	CP+SS
			Buttons anaesthetic device	SS
			Oxygen balloon	SS
			Countertop with alcohol	CP+S
			Operating light handle	SS
			Wood stool	CP+SS
		Operating room 03	Operating table -Head	CP+SS
			Operating table -Toes	CP+SS
			Operating light handle	SS
			Endoscopy table -Head	CP+SS
			Endoscope table -Toes	CP+SS
			Siemens keyboard	SS
			Countertop with material	CP+SS
		Dog ward	Treatment table with plastic mat	CP+SS
			Small cage	CP+SS
			Computer keyboard	SS
			Black shearing blade	SS
			Light handle 01	SS
		Dog ward	Light handle 02	SS
			Red shearing blade	SS
			Weight scale	CP+SS
		Cat ward	Double cage	CP+SS
		Intensive care unit	Countertop	CP+SS
		Wash room	Tap	SS
			Sink	SS
			Drain	SS
November 2023	SAVP-G	Operating room 01	Operating table - Head	CP+SS
			Operating table - Toes	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
November 2023	SAVP-G	Operating room 01	Blanket	CP+SS
			Anaesthetic device	SS
			Operating light handle	SS
			Computer keyboard	SS
			Oxygen balloon	SS
		Recovery ward	Treatment table plastic mat	CP+SS
			Small cage	CP+SS
			Large cage	CP+SS
			Plastic mat	CP+SS
			Shearing blade	CP+SS
		Dog ward	Treatment table plastic mat	CP+SS
			Computer keyboard	SS
			Tap	SS
			Cage	CP+SS
			Treatment table grids	SS
		Cat ward	Rough table top	CP+SS
			Stainless steel supporting tray	CP+SS
			Tap	SS
			Cage	CP+SS
		Isolation unit	Grids	CP+SS
			Plastic mat on countertop	CP+SS
			Cage	CP+SS
			Tap	SS

Supplementary Table 2 – Environmental sampling sites in clinics H-N

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
May 2022	SAVP-H	Operating room	Anaesthetic device buttons	SS
			Oxygen balloon	SS
			Stainless steel supporting tray	CP+SS
			Bed	CP+SS
			Wood stool	CP+SS
			Operating Table – Window side	CP+SS
			Thermal blanket	CP+SS
			Operating Table – Door side	CP+SS
		Treatment area	Weight scale	CP+SS
			Black mat	CP+SS
			Grids	CP+SS
			Stainless steel supporting tray	CP+SS
			Computer keyboard	SS
		Isolation unit	Cage 01	CP+SS
			Cage 02	CP+SS
		Ultrasound room	Ultrasound keyboard	SS
			Ultrasound screen	CP+SS
			Ultrasound Table	SS
		Waiting room	Weight Scale	CP+SS
		Examination room	Examination Table	CP+SS
June 2022	SAVP-I	Operating room	Operating table_Head	CP+SS
			Operating table_Toes	CP+SS
			Stainless steel supporting Tray	CP
			Electric scalpel	SS
			Operating light handle	SS
			Vital signs monitor	SS
			Medicine cabinet	CP+SS
June 2022	SAVP-I	Operating room	Oxygen balloon	SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
June 2022	SAVP-I	Operating room	Anaesthetic device buttons	SS
			Door knob (inside the operating room)	SS
			Door knob (corridor)	SS
		Pre-operative/Treatment area	Fridge handle	SS
			Tap	SS
			Sink grids	CP+SS
			Shearing blade 01	CP+SS
			Treatment table plastic mat	CP+SS
			Microscope countertop	CP+SS
			Handle cabinet 01	SS
			Handle cabinet 03	CP
			Handle cabinet 05	CP
			Door knob outside	SS
			Brush of shearing blade	SS
		Ward	Stainless steel supporting tray	CP+SS
			Cage 01	CP+SS
			Cage 02	CP+SS
July 2022	SAVP-J	Operating room	Operating table - Head	CP+SS
			Operating table - Toes	CP+SS
		Examination/Treatment room	Weight scale	CP+SS
			Stainless steel supporting tray 01	CP+SS
			Stainless steel supporting tray 2	CP+SS
			Shearing blade	CP+SS
			Brush of shearing blade	CP+SS
			Otoscope	SS
			Macrometric/micrometric microscope	SS
			Platinum microscope	SS
			Microscope countertop	CP+SS
			Computer keyboard	CP+SS
			Computer mouse	SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
July 2022	SAVP-J	Examination/Treatment room	Desk	CP+SS
			Door knob 01	SS
			Door knob 02	SS
			Handle cabinet 01	SS
			Handle cabinet 02	SS
			Fridge handle	SS
			Trash	CP+SS
			Tap	SS
September 2022	SAVP-K	Operating room	Anaesthetic device buttons	SS
			Oxygen balloon	SS
			Upper cabinet	SS
			Door knob - Inside	SS
			Shearing blade cleaning brush	CP+SS
			Anaesthesia Tent - Inside	CP+SS
			Thermal mat	CP+SS
			Anaesthesia tent - Outside	CP+SS
			Door knob - Exterior	SS
			Operating table - Head	CP+SS
			Operating table - Toes	CP+SS
			Drawer handle	SS
			Door knob (operating room to hallway)	SS
			Blanket	CP+SS
			Light handle	SS
			White countertop	CP+SS
			Stainless steel supporting tray	CP+SS
September 2022	SAVP-K	Pre-operative/Treatment area	Pre-operating knob - Inside	SS
			Fridge handle	SS
			Sink grids	SS
			Tap	SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
September 2022	SAVP-K	Pre-operative/Treatment area	Sink	SS
			Microwave table	SS
			Pre-operative area cabinet handle	SS
			Pre-operative area knob - Outside	SS
		Ward	Small cage	CP+SS
			Outside knob	SS
			Drawer handles	SS
			Pink blanket	CP+SS
		Ward	Inside knob	SS
			Large cage	CP+SS
			Bench	CP+SS
			Tap	SS
			Shearing blade	SS
March 2023	SAVP-K	Operating room	Operating table - Toes	CP+SS
			Operating table - Head	CP+SS
			Stainless steel supporting tray	CP+SS
			Thermal blanket	CP+SS
			Shearing blade	CP+SS
			Anaesthetic device buttons	SS
			Infusion pump	SS
			Cabinet handle	SS
			Operating table (Inside handle)	SS
			Light switch	SS
		Pre-operative/Treatment area	Grey plastic mat	CP+SS
March 2023	SAVP-K	Pre-operative/Treatment area	Bathtub grids	CP+SS
			Fluid appliance	SS
			Cabinet handles	SS
			Shearing blade	CP+SS
		Ward	Cage	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
March 2023	SAVP-K	Ward	Supporting countertop	CP+SS
			Cabinet handles	SS
			Infusion pump	SS
		Dog examination room	Sink	SS
			Plastic mat table	CP+SS
			Computer keyboard	SS
			Weight scale	CP+SS
			Cabinet handles	SS
			Computer mouse	SS
		Cat examination room	Plastic mat	CP+SS
			Computer keyboard	SS
			Computer mouse	SS
			Weight scale	CP+SS
			Cabinet handles	SS
			Sink	SS
		Exotic animals examination room	Sink	SS
			Weight scale	CP+SS
			Plastic mat	CP+SS
			Computer keyboard	SS
			Computer mouse	SS
			Cabinet handles	SS
			Round table - Lunch table	CP+SS
		Ultrasound room	Ultrasound table	CP+SS
		Ultrasound room	Ultrasound buttons	SS
			Ultrasound keyboard	SS
			Ultrasound mouse	SS
		Waiting room	Weight scale	CP+SS
March 2023	SAVP-I	Operating room	Operating table - Toes	CP+SS
			Operating table - Head	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
March 2023	SAVP-I	Operating room	Stainless steel supporting tray	CP+SS
			Anaesthetic device buttons	SS
			Light handle	SS
			Mindray buttons	SS
			Operating table protection	CP+SS
			Shearing blade	CP+SS
			Shearing blade cleaning brush	CP+SS
			Clean countertop	CP+SS
		Pre-operative/Treatment area	Plastic table mat	CP+SS
			Bathtub grids	CP+SS
			Shearing blade	CP+SS
			Shearing blade cleaning brush	CP+SS
			Outside knob	SS
			Microscope countertop	CP+SS
		Ward 01	Cage upper left corner	CP+SS
			Sink	CP+SS
			Countertop	SS
			Cabinet handles	SS
		Ward 02	Lower cage	CP+SS
			Countertop	CP+SS
			Sink	SS
		Dog examination room	Examination table	CP+SS
		Dog examination room	Countertop	CP+SS
			Computer keyboard	SS
			Computer mouse	SS
			Weight scale	CP+SS
			Cabinet handles	SS
			Sink	SS
		Cat examination room	Examination table	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
March 2023	SAVP-I	Cat examination room	Countertop	CP+SS
			Computer keyboard	SS
			Computer mouse	SS
			Cabinet handles	SS
			Sink	SS
			Weight Scale	CP+SS
		Exotic animals examination room	Examination table	CP+SS
			Countertop	CP+SS
			Computer keyboard	SS
			Computer mouse	SS
			Cabinet handles	SS
			Sink	SS
		Waiting room	Weight scale	CP+SS
March 2023	SAVP-L	Operating room	Operating table -Toes	CP+SS
			Operating table -Head	CP+SS
			Stainless steel supporting tray	CP+SS
		Ward	Infusion pump	SS
			Large shearing blade	CP+SS
			Small shearing blade	CP+SS
			Anaesthetic device buttons	SS
			Sink	SS
			Cage 04	CP+SS
		Dog examination rom	Examination table	CP+SS
			Computer keyboard	SS
			Computer mouse	SS
			Sink	SS
		Cat examination room	Examination table	CP+SS
			Computer keyboard	SS
			Computer mouse	SS

Date of collection	**Small animal veterinary practices**	**Areas**	**Specific areas**	**Sampling methods**
March 2023	SAVP-L	Cat examination room	Sink	SS
		Examination room 02	Countertop	CP+SS
			Computer keyboard	SS
			Computer mouse	SS
			Sink	SS
			Weight scale	CP+SS
		Examination room 03	Weight scale	CP+SS
			Computer keyboard	SS
			Computer mouse	SS
			Sink	SS
			Countertop	CP+SS
		Waiting room 01	Weight scale	CP+SS
		Waiting room 02	Weight scale	CP+SS
March 2023	SAVP-M	Operating room	Operating table - Toes	CP+SS
			Operating table - Head	CP+SS
			Stainless steel supporting tray	CP+SS
			Large shearing blade	CP+SS
			Small shearing blade	CP+SS
		Operating room	Thermal blanket	CP+SS
			Cabinet handles	SS
			Anaesthetic device buttons	SS
			Light handle	SS
			Clean countertop	CP+SS
		Pre-operative area	Countertop	CP+SS
			Sink	SS
			Cabinet handles	SS
		Ward	Cage 06	CP+SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
March 2023	SAVP-M	Ward	Countertop	CP+SS
			Cabinet handles	SS
			Sink	SS
		Examination room 01	Examination table	CP+SS
			Sink	SS
			Computer keyboard	SS
			Computer mouse	SS
		Examination room 02	Examination table	CP+SS
			Weight scale	CP+SS
			Sink	SS
			Computer keyboard	SS
			Computer mouse	SS
			Cabinet handles	SS
		Waiting room	Weight Scale	CP+SS
November 2023	SAVP-N	Operating room 01	Operating table - Head	CP+SS
			Operating table - Toes	CP+SS
			Anesthetic device	CP+SS
			Operating light handle	SS
			Oxygen balloon	SS
			Thermal mat	CP+SS
		Operating room 02	Operating table -Head	CP+SS
			Operating table - Toes	CP+SS
			Stainless steel supporting tray	CP+SS
			Light handle	SS
			Computer keyboard	SS
			Anaesthetic device	SS
		Treatment area	Treatment table	CP+SS
			Shearing blade	CP+SS
			Treatment table grids	SS

Date of collection	Small animal veterinary practices	Areas	Specific areas	Sampling methods
November 2023	SAVP-N	Treatment area	Light handle	SS
			Disinfection dispenser	SS
			Tap	SS
		Recovery ward	Small cage 06	CP+SS
			Large cage 02	CP+SS
		Fomites	Veterinarian mobile phone	SS
			Hand cloth	CP+SS
			Practice’s mobile phone	SS
			Keyboard personal computer	SS

Supplementary Table 3 – SNP matrix distance between *Pseudomonas aeruginosa* strains found on SAVPs I, K, and L

SNP-distance	***P. aeruginosa* H07 (NCBI ASM2257023v1)**	A6E4P2	A5R4P1	A6Np4P1
***P. aeruginosa* H07 (NCBI ASM2257023v1)**	0	56132	56049	56046
A6E4P2	56132	0	14081	14078
A5R4P1	56049	14081	0	3
A6Np4P1	56046	14078	3	0

Supplementary Table 4 – SNP matrix distance between *Pseudomonas aeruginosa* strains found on different surfaces of SAVP-G.

SNP-distance	***P. aeruginosa* H02 (NCBI ASM2257047v1)**	ER2F8P2	ER3C4P3
***P. aeruginosa* H02 (NCBI ASM2257047v1)**	0	1738	1738
ER2F8P2	1738	0	0
ER3C4P3	1738	0	0

Supplementary Table 5 – SNP matrix distance between *Pseudomonas juntendi* strains found on SAVP-G.

SNP-distance	***P. juntendi* K37 (NCBI ASM2526364v1)**	**ER4C8A1**	**ER1C4P2a**
***P. juntendi* K37 (NCBI ASM2526364v1)**	0	2098	2098
ER4C8A1	2098	0	36
ER1C4P2	2098	36	0

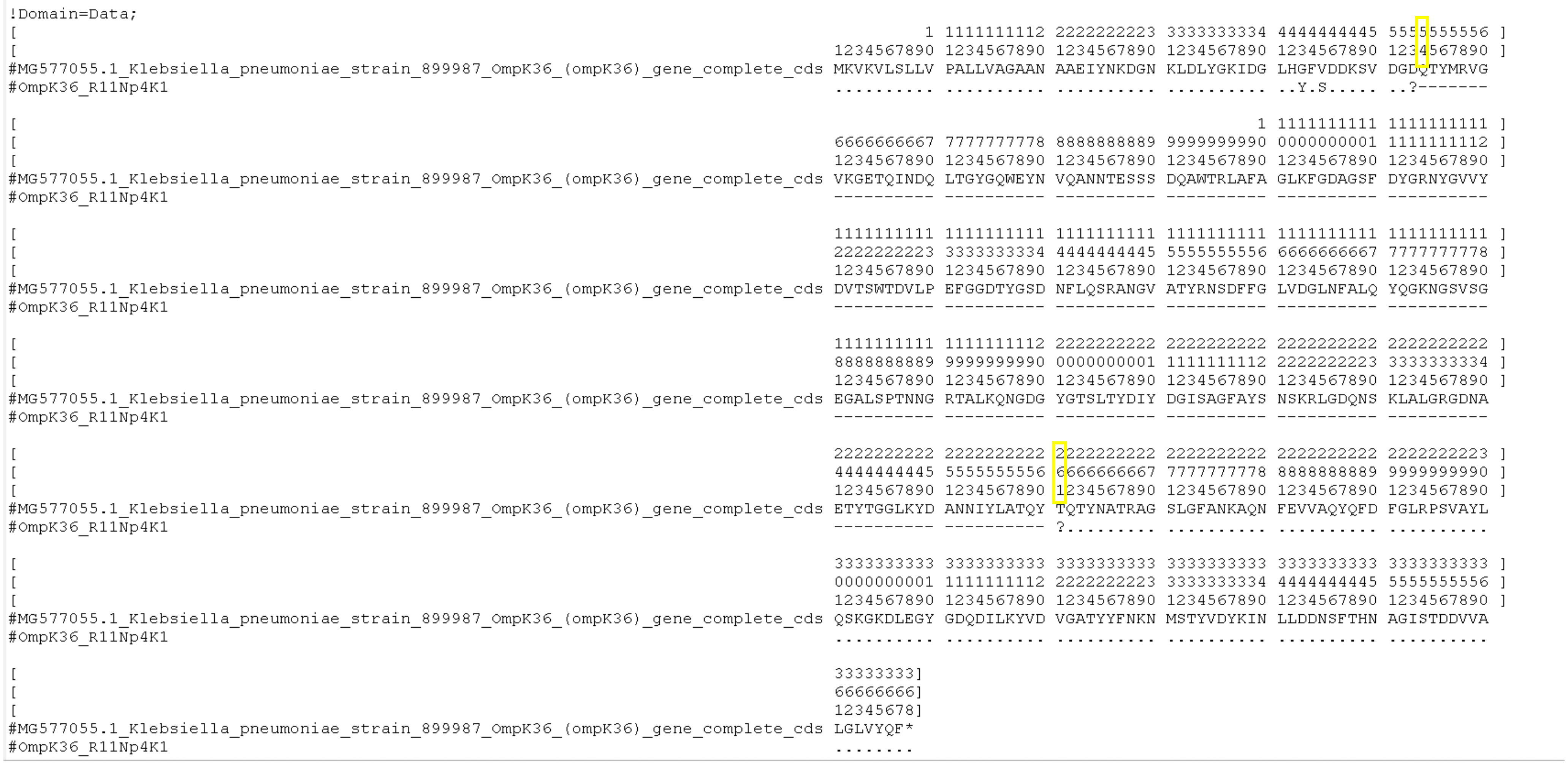
Supplementary Table 6 – SNP matrix distance between *Stenotrophomonas maltophilia* strains found on different surfaces and team of SAVP-G.

SNP-distance	***S.maltophilia* NCTC10498 (NCBI ASM1138692v1)**	**ER3D8P2**	**ER2F4P2**	**ER3F8A1**
***S.maltophilia* NCTC10498 (NCBI ASM1138692v1)**	0	52315	51413	52318
ER3D8P2	52315	0	57170	5
ER2F4P2	51413	57170	0	57173
ER3F8A1	52318	5	57173	0

Supplementary Table 7 – SNP matrix distance between *Stenotrophomonas maltophilia* strains found on different surfaces and team of SAVP-N.

SNP-distance	***S.maltophilia* SM15 (NCBI ASM2111719v1)**	**X3Hp4P1**	**X4Hp4P2**	**EX3I8A1**	**EX2I4A1**	**EX2C4P3**
***S.maltophilia* SM15 (NCBI ASM2111719v1)**	0	848	844	849	849	57099
X3Hp4P1	848	0	6	9	9	56684
X4Hp4P2	844	6	0	7	7	56680
EX3I8A1	849	9	7	0	10	56683
EX2IAP1	849	9	7	10	0	56684
EX2C4P3	57099	56684	56680	56683	56684	0

Supplementary Figure 1 – *Klebsiella pneumoniae* OmpK36 from R11Np4K1 and OmpK36 refence (GenBank: MG577055.1) from position 54-261 (highlighted in yellow)



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[                                                                                 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 ]
#MG577055.1_Klebsiella_pneumoniae_strain_899987_OmpK36_(ompK36)_gene_complete_cds ETYTGGLKYD ANNIYLATQY TQTYNATRAG SLGFANKAQN FEVVAQYQFD FGLRPSVAYL
#OmpK36_R11Np4K1                                                                  ---------- ---------- ?......... .......... .......... ..........

[                                                                                 3333333333 3333333333 3333333333 3333333333 3333333333 3333333333 ]
[                                                                                 0000000001 1111111112 2222222223 3333333334 4444444445 5555555556 ]
[                                                                                 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 ]
#MG577055.1_Klebsiella_pneumoniae_strain_899987_OmpK36_(ompK36)_gene_complete_cds QSKGKDLEGY GDQDILKYVD VGATYYFNKN MSTYVDYKIN LLDDNSFTHN AGISTDDVVA
#OmpK36_R11Np4K1                                                                  .......... .......... .......... .......... .......... ..........

[                                                                                 33333333]
[                                                                                 66666666]
[                                                                                 12345678]
#MG577055.1_Klebsiella_pneumoniae_strain_899987_OmpK36_(ompK36)_gene_complete_cds LGLVYQF*
#OmpK36_R11Np4K1                                                                  ........
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