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TITOLO TESI

Surveillance of antimicrobial resistance and healthcare-associated infections in veterinary teaching hospitals

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Summary

ABSTRACT

The role of small animal veterinary hospitals in the onset and dissemination of antimicrobial-resistant organisms (AMROs) is an emerging research field, and the implementation of internal surveillance programs is a cost-effective tool to better understand their impact. The aim of this PhD project was to develop a surveillance program in the Veterinary Teaching Hospital (VTH) of the Department of Veterinary Medical Sciences, University of Bologna, to obtain, analyze and compare data on AMROs and healthcare-associated infections (HAIs). The program was composed by passive surveillance, active surveillance on patients, active surveillance on the environment, outbreak surveillance and the communication system. Results from passive surveillance on 1342 clinical isolates highlighted a multi-drug resistance (MDR) percentage of 41.6%, with worrying non-susceptibility percentages detected for clindamycin (58.4%), amoxicillin-clavulanate (20.3%), piperacillin-tazobactam (15%) and enrofloxacin (45.8%). Whole genome sequencing on some selected MDR Enterobacterales isolates revealed the presence of multiple worrisome resistance genes, including the carbapenemase-encoding gene $bla_{\text{NDM-5}}$. Considering only isolates from suspected HAIs (13.9% of the total), MDR percentage was considerably higher (71.7%). Active surveillance on patients showed a high in-hospital acquisition rate (25.6%) of commensal carbapenem-resistant gram-negative bacteria (CR-GNB) with multiple resistance genes, including bla_{NDM}. Active surveillance on the environment detected high prevalence of methicillin- resistant Staphylococci (MRS, 19.5%). Active surveillance was also executed in a Spanish VTH, showing a lower AMROs frequency of detection at admission and in-hospital acquisition. Outbreak surveillance allowed to early detect and manage two outbreaks both caused by the *Enterobacter cloacae* complex. The communication system involved professionals from different fields and assisted the redaction of a new departmental biosecurity manual. Data collected from surveillance can serve as a basis to develop a tailored Infection Control Programs and underline the potential role of VTHs in the maintenance and dissemination of AMROs, including some considered highest priority for human medicine.

Chapter 1

Introduction and literature review

1A. The silent pandemic

When sir Alexander Fleming discovered penicillin in 1928, humanity just entered in a new era. An era of revolution, in which bacterial infections became treatable: the antibiotic era. The introduction of antibiotics in medicine can be considered a milestone for the whole humanity, at the same level (if not more impactful) of vaccines, or the invention of water purification systems. Since that, a lot of lives, both between humans and animals, have been cured from diseases that had been considered lethal for a long time. Antibiotics are clearly one of the most important reasons of the exponential increase of both life expectancy and world population that humanity experienced in the last century. They are crucial not only for the direct impact they have on bacterial disease, but also in terms of prevention. Indeed, they protect from complications from viral infections, and their use made possible some advanced and complex medical procedures, such as invasive surgeries and chemotherapy. Furthermore, antibiotics introduction resulted in an important improvement of the health and welfare also in animals, including livestock, wildlife, and companion animals (1), and allowed, in the case of livestock animals, the implementation of intensive industrialized production. Considering all the progresses made, a world without antibiotics seemed to be impossible to imagine. As reported by Jawtetz in 1956, bacterial infections were considered by some authors to be a subject not worthy of further extensive research:

"*on the whole, the position of antimicrobial agents in medical therapy is highly satisfactory. The majority of bacterial infections can be cured simply, effectively and cheaply. The mortality and morbidity from bacterial diseases have fallen so low that they are no longer among the important unsolved problems of medicine. These accomplishments are widely known and appreciated*".(2)

But nowadays things are deeply changed, and the idea of a post-antibiotic era, in which antibiotics are no more effective, is becoming a concrete threat. Antibiotic resistance, also known as "the silent pandemic", is hunting the world like a specter of marxist memory. Fleming himself was aware of such threat. In 1945, when he received the Nobel Prize, he predicted it:

> "*It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body."*

But how is this happening? And why the threat is becoming more and more important?

To answer these two fundamental questions, we need to summarize antibiotic resistance and its role in the modern era in four key points.

1) **Bacteria are hard to die.**

Antimicrobial resistance (AMR) is a natural phenomenon that derives from the high capacity of adaptation of bacterial populations. Bacteria are estimated to be on earth for more than a billion years (3), and their unique evolutionary path gave them the ability to survive in almost every environment, including volcanos and the space (4,5). They can adapt to everything, including to substances made to kill them, such as antibiotics and detergents. As humankind, we discovered antibiotics less than one century ago, so

expect to steal a march on bacterial adaptation looks difficult, if not pretentious. To win the battle against AMR, we must be aware of this evolutionary gap.

This bacterial adaptation can be achieved in several ways, that have been summarized in table 1, including the degradation of antibiotics with enzymes, the change of the target or the alteration of the membrane pumps (see Table 1). There is not uniformity in the resistance pattern, and resistance rates may be enormously variable even within related bacterial populations (6). Susceptibility and resistance are usually measured with quantitative methods such as minimum inhibitory concentration (MIC), or semi-quantitative such as the disc diffusion test (also known as Kirby-bauer method).

Table 1. Bacterial resistance mechanisms. Modified from (5).

Drug	Drug Uptake Limitation	Drug Target Modification	Drug Inactivation	Efflux Pumps
β-Lactams	Decreased numbers of porins, no outer cell wall	Gram pos—alterations in PBPs	Gram pos, gram neg—β-lactamases	RND
Carbapenems	Changed selectivity of porin			
Cephalosporins	Changed selectivity of porin			
Monobactams				
Penicillins				
Glycopeptides	Thickened cell wall, no outer cell wall	Modified peptidoglycan		
Lipopeptides		Modified net cell surface charge		
Aminoglycosides	Cell wall polarity	Ribosomal mutation, methylation	Aminoglycoside modifying enzymes, acetylation, phosphorylation, adenylation	RND
Tetracyclines	Decreased numbers of porins	Ribosomal protection	Antibiotic modification, oxidation	MFS, RND
Chloramphenicol		Ribosomal methylation	Acetylation of drug	MFS, RND
Lincosamides		Gram pos—ribosomal methylation		ABC, RND
Macrolides		Ribosomal mutation, methylation		ABC, MFS, RND
Oxazolidinones		Ribosomal methylation		RND
Streptogramins				ABC
Fluoroquinolones		Gram neg—DNA gyrase modification Gram pos—topoisomerase IV	Acetylation of drug	MATE, MFS, RND
Sulfonamides		DHPS reduced binding, overproduction of resistant DHPS		RND
Trimethoprim		DHFR reduced binding, overproduction of DHFR		RND

ABC—ATP binding cassette family, DHFR—dihydrofolate reductase, DHPS—dihydropteroate synthase, MATE—multidrug and toxic compound extrusion family, MFS—major facilitator superfamily, PBP—penicillin-binding protein, RND—resistance-nodulation-cell division family.

From a genetic point of view, resistance may be of two types:

- **Natural resistance:** when resistance mechanisms are naturally present in the bacterial DNA, becoming structural and typical of some bacterial species. Natural resistance can be **intrinsic** (when it is always expressed in the species) or **induced** (the genes are always present but are expressed to resistance levels only after exposure to an antibiotic). Some examples are species-specific natural resistances are given in Table 2. Usually, an intrinsic resistance is defined when the average MICs for a bacterial species is in the resistant part of the range (5).

Table 2. Examples of bacteria with intrinsic resistances. Adapted from (5).

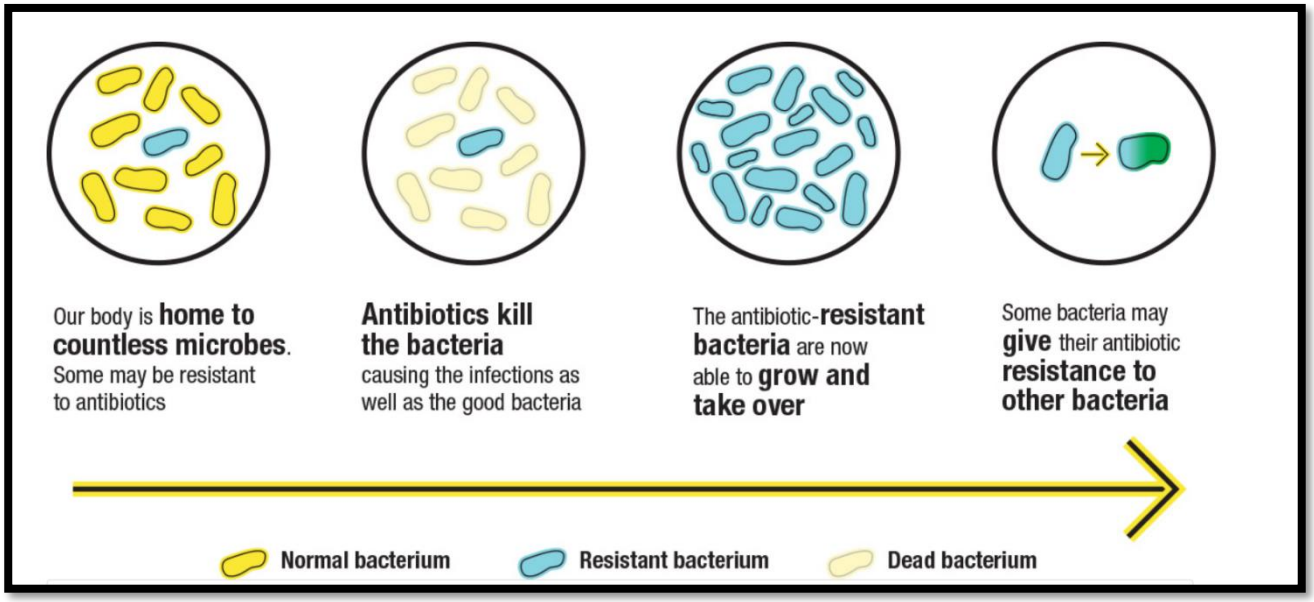
Table 2.

Examples of bacteria with intrinsic resistance.

Organism	Intrinsic resistance
Bacteroides (anaerobes)	aminoglycosides, many β-lactams, quinolones
All gram positives	aztreonam
Enterococci	aminoglycosides, cephalosporins, lincosamides
Listeria monocytogenes	cephalosporins
All gram negatives	glycopeptides, lipopeptides
Escherichia coli	macrolides
Klebsiella spp.	ampicillin
Serratia marcescens	macrolides
Pseudomonas aeruginosa	sulfonamides, ampicillin, 1st and 2nd generation cephalosporins, chloramphenicol, tetracycline
Stenotrophomonas maltophilia	aminoglycosides, β-lactams, carbapenems, quinolones
Acinetobacter spp.	ampicillin, glycopeptides

- **Acquired resistance**: when resistance mechanisms are acquired from all of the main routes used by bacteria to acquire genetic material (conjugation, transposition or transformation) or from spontaneous mutations on their own DNA. Internally, mutations naturally happen averagely with a rate of 1 for every 10^6 to 10^9 cell divisions (6), and are triggered by stressors (UV radiation, chemicals, starvation..). Most of them are deleterious for the bacteria, but in some cases these mutations can lead to resistance, even if they can be an additional metabolic cost for the organism, resulting a decrease of the growth rate (5). Under the selective pressure exerted by antibiotics, only bacteria that developed such mutations are able to survive, and consequently to multiply unopposed and transmit resistance genes to others (see Figure 1). Acquisition from transposition is usually a rare event. Certain species (*Acinetobacter* spp.) are naturally able to acquire genetic material from the environment through transformation, but from an epidemiologic point of view the most common and concerning acquisition way is plasmid-mediated acquisition (conjugation), especially in gram-negatives bacteria. In any case, acquired resistance can be transitory or permanent, and is more complicated to manage, because of its rapid spread directly linked with antibiotics use. Indeed, even the use of sub-inhibitory concentrations of antibiotics may select not only for resistance itself, but also for hyper-mutability and for the increase in the movement of mobile genetic elements, such as plasmids (8).

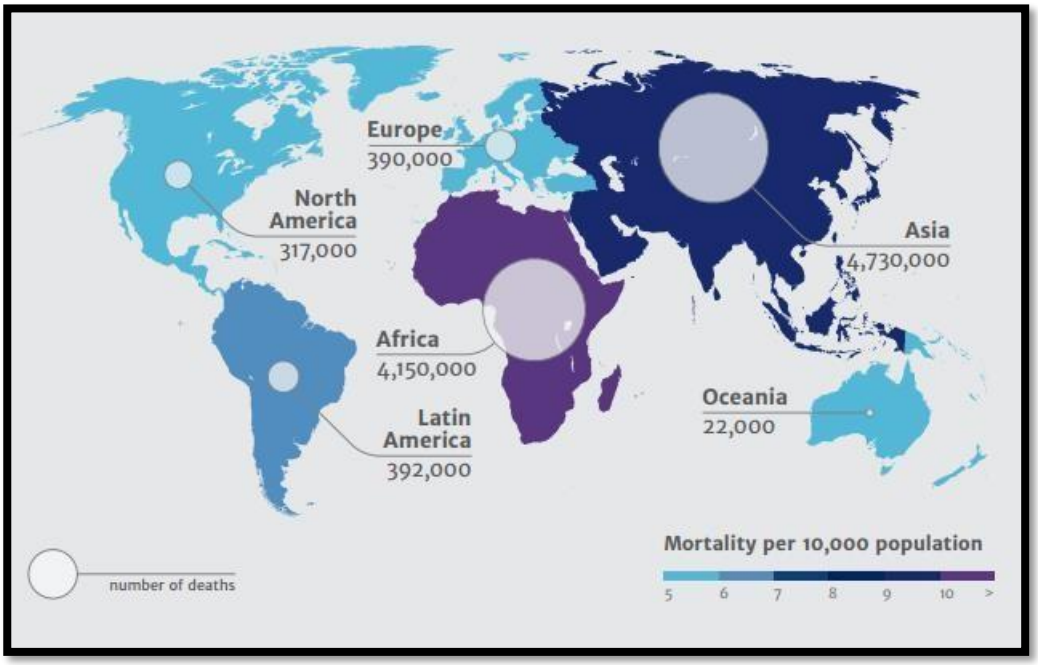
Figure 1. How Antibiotic Resistance happens. From: https://www.canada.ca/en/public-health/corporate/publications/chief-public-health-officer-reports-state-public-health-canada/preserving-antibiotics/about-antibiotic-resistance.html



2) Use, misuse, overuse.

The real issue that makes AMR so urgent is that this natural phenomenon is undergoing a growing acceleration. Since Fleming pronounced those prophetic words, it has become one of the major global sanitary threats. According to the World Health Organization (https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance), in 2019 AMR was directly responsible for 1.27 million global deaths and contributed to 4.95 million deaths, 75% of them attributable to six pathogens; and these numbers are destined to grow. . Only in Europe, a 2019 systematic analysis calculated 133,000 deaths attributable to AMR, and 541,000 associated with AMR (9). A 2016 review commissioned by the UK Government (10) estimated 10 million deaths by 2050 caused by AMR (see Figure 2), and although this forecast, as every kind of long-term prediction, has been disputed (11), the urgency of a global, coordinated intervention on AMR is well-recognized by the whole scientific community

Figure 2. Deaths attributable to AMR by 2050 according with (10)



The reasons behind the acceleration of AMR are attributable in the first place to antibiotics use: the more antibiotics are used, the more AMR will be induced. With the increase of the world population, and consequently of antibiotics consumption, also AMR levels are growing: this topic is especially important in low- and middle-income countries, that are recently experiencing a large-scale availability of antibiotics in line with their economic development**.** But if antibiotics use is often legitimate, if not essential, in some cases it is not (antibiotics misuse) or it is excessive (antibiotics overuse). Both antibiotics misuse and overuse are a leading cause of AMR exponential acceleration. Some examples:

a) **Using antibiotics to treat other diseases**: this can be due to insufficient or absent diagnostics, more frequent in low- and middle-income countries. But also in high-income countries, such as the United States, overprescription is present: a 2017 report of the Centers for Disease Control and Prevention, estimated that 20-50% of total antibiotic prescriptions are made even if the bacterial nature of the infection is not confirmed (https:// www.cdc.gov/antibiotic-use/stewardship-report/pdf/steward ship-report.pdf).

b) **Pressuring doctors and veterinarians on antibiotics prescription**, regardless they are necessary. This is one of the unintended side effects of the diffusion of antibiotics. The "just in case" medication is often required by the patient, or in case of children or animals, by parents and owners;

c) **Not following recommendations**. The goal of antibiotic treatment includes to treat the right patients with the right antibiotics, right dose, via the right route, and for the right amount of time, avoiding self-medication and shared prescriptions. Correct choice of treatment antibiotic, when indicated, is critical not only to prevent development of resistance but also to ensure that patients with significant infections are treated appropriately. This concept is included in the "antimicrobial stewardship" *modus operandi,* and it is aided by practice

guidelines and local antibiograms, both of which may change frequently as antibiotic resistance grows (12). To ensure that antimicrobial stewardship works, both sides (the patients and the human or veterinary doctor) must be aware of its importance.

d) **The absence of a regulation on antibiotics sells or on AMR policies**. At a national level, this is specifically important in low- and middle-income countries, where often the medical prescription is not required to get an antibiotic drug, or it is obtained from an illegal source at lower costs. These countries have often to face more difficulties in the AMR fight, related with limited diagnostics and laboratory tools, poor literacy, lack of access to appropriately trained medical professionals, and competing governmental priorities (10). A recent study by Allel et al. (13) showed that AMR at a country-level in both humans and animals is related not only with direct antibiotics consumption, but also with socioeconomic factors, such as governance, GDP and legislation.

e) **Using antibiotics as growth promoters in animal farming**: although this practice has played a crucial role in industrial-scale animal agriculture for over half a century, it represents an abundant source of AMR. Over 70% of antimicrobials produced globally are used in animal agriculture (14), and the magnitude of the impact of growth promoters is still hard to quantify (15). For these reasons, antibiotics as growth-promoters have been banned in many countries, including the European Union in 2006 and the United States in 2017. Also, many low and middle-income countries are progressively following Europe in limiting and banning the use of antibiotics as growth promoters. For example, Nigeria, Vietnam, China, and Brazil (the latter two are countries with the largest antibiotic consumption in livestock). Nevertheless, this practice is still legal in some countries such as India (16).

f) **Not correctly disposing antibiotics after use:** this will enhance the presence of antibiotics in the environment, with a subsequent increase of AMR levels. This is particularly important in countries where wastewater treatment systems are not still commonly used.

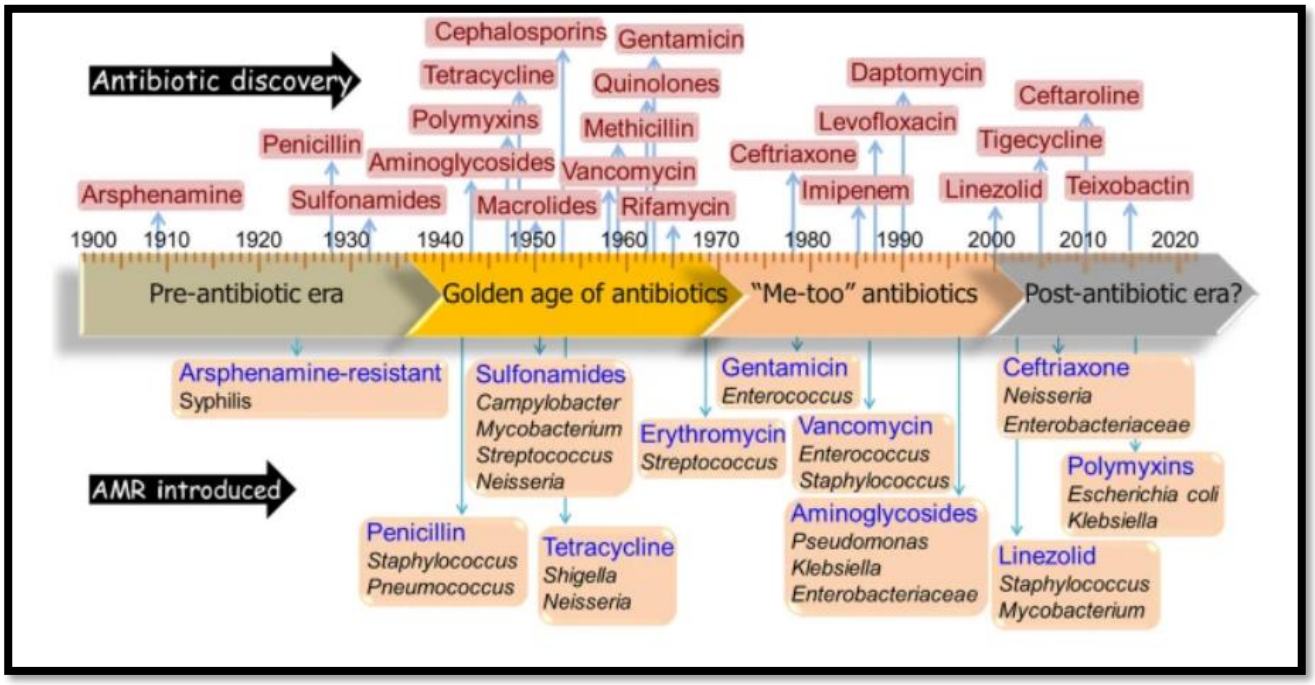
To reduce antibiotic misuse or overuse, the so-called “antibiotic optimization” includes, in addition to avoiding the mentioned examples, the development of vaccination policies, the implementation of common hygiene practices, such as hand washing, the promotion of antibiotic-free animal products and the education of people, including veterinarians and physicians, on the topic.

3) **Nothing new in the West.**

A logic possible solution for the AMR burden could be found in the invention and production of more antibiotics by pharmaceutical companies. But it comes out that this is easy only in theory, as Experts estimate that research and development into antibiotics has deeply fallen in the past decades. (17). Comparing with the early days of the antibiotic era, where new antibiotic classes where discovered every few years, now the rhythm has worryingly slowed down (see Figure 3). This can be explained with the fact that new

antibiotic classes are inevitably more difficult to find, but also the development of a new drug and its commercialization is quite expensive in terms of time and money (around 1.5 billion dollars and 12 years, according to Tawse et al. (18)) and the investment needed to research and produce such drug cannot be efficient in terms of profitability. Furthermore, the current AMR situation is forcing countries to limit the selling of recently discovered drugs, in order preserve their efficacy in a medium-long period. This is an additional deterrent for pharma companies, that have to consider this economic limitation when deciding to invest on a new antibiotic development. In the last years, European Union is trying to take the lead on this issue by incentivizing drug companies to research (19).

Figure 3. Timeline depicting the discovery of major antibiotics and subsequent emergence of resistance against them in various bacterial strains. from Chakraborty et al. *(20)*.



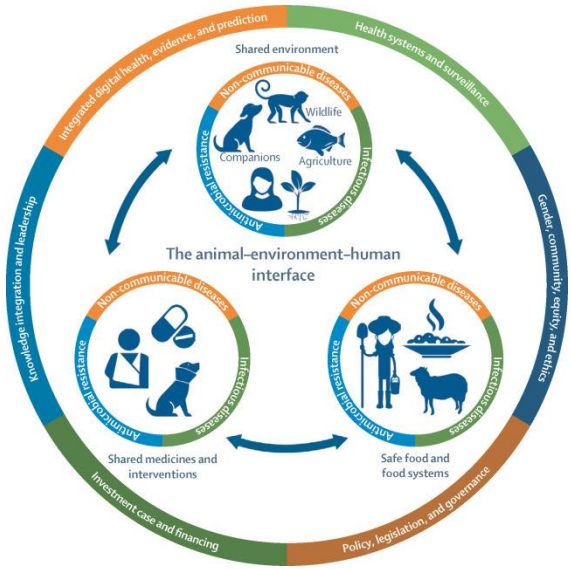
4) Globalization

As COVID-19 taught us, in a globalized world also infectious agents (and in this case, their resistance genes) can travel through continents. This inevitably leads to an exacerbation of the AMR phenomenon at a macroscopic scale. This model of spread occurs mainly through general travel (of people, animals, and food), but also medical tourism, emigration, or military service (12). This risk of acquiring resistant organisms with travel is significantly increased if there is contact with the healthcare system while abroad (21). This organism transport can act bidirectionally between high and low-and middle-income countries. One example of this resistance spread is the history of New Delhi metallo-beta-lactamases (NDM) gene, first isolated in 2008 in Sweden from a patient of Indian descent. The patient travelled to India, where he was treated with multiple antibiotics for an abscess, then came back to Sweden where he was found to be infected with a bacterial species, *Klebsiella pneumoniae,* with such resistance gene, able to confer resistance to carbapenems. Medical tourism, considered a rising niche of global medical care, has been reported as a risk for acquisition (21); military conflict and soldier repatriation have been also implicated in the spread of resistance (22).

Furthermore, the massive global movement of people trough emigration and immigration offers a continuing opportunity for AMR to be transferred bidirectionally (23).

All these key points highlight how AMR, in its development and spread, must be considered as a multifactorial problem, that raises both difficulties and opportunities, and necessarily need an integrate approach that includes a deep vision, resumed in the concept of One Health, or Global Health (24) (see Figure 4). In this approach, that is being promoted by several governments and organizations (including the WHO), understanding the role of animals becomes crucial.

Figure 4. The One Health concept summarized. Adapted from *(25)*.



1.B. More than a companionship?

1. Companion animals in the global AMR epidemiology

The animal kingdom is undoubtedly the major reservoir for infectious organisms, causing 60-70% of total infectious diseases in humans (26). That is also the case of AMR, for which animals contribute to select for antimicrobial resistance genes (ARGs) (27). Considering only domestic animals, several efforts have been made to better understand and control the spread of AMR in livestock animals, because the major proportion of veterinary antibiotics (in Europe, around 90-95% according to the European Medicines Agency(28)) is used on them. The efforts to reduce antibiotics consumption in animal agriculture were prioritized, with encouraging results in Europe, as demonstrated by the annual reports (28). In Europe, this sectoral approach reflects in legislation, referring to European Commission Decision 2013/652/EU dedicated to assessing the risk of transmission of AMR from livestock animals to humans and not considering other animals such as wildlife and companion animals (29,30), that have been neglected and overlooked from health reports for a long time, also due to the inaccessibility to data (31). But in the last years, following to the mentioned One Health approach, they are

receiving more and more attention in the global overview of AMR. Specifically, companion animals such as dogs and cats are a matter of debate. The evidence of bidirectional transmission of AMR bacteria, genes and genetic mobile elements have been described by multiple studies (6–8) and their role in the AMR epidemiology is obtaining an increasing importance. The reasons can be resumed in four factors:

a. **Increasing presence:** dogs and cats are increasingly popular in the family households, especially in high income countries. In Europe, there are over 200 million owned-pets (https://www.statista.com/statistics/515010/pet-population-european-union-eu-by-animal/), and the number is increasing especially after COVID-19 pandemic (32,33).This estimation does not include stray dogs and cats, that also represent a potential reservoir of AMR (34,35). Approximately, 46% of European households own at least one pet (https://europeanpetfood.org/_/news/new-fediaf-facts-figures-highlights-the-growth-of-european-pet-ownership/). Owning a pet gives physical and mental health benefits, including decreased loneliness, alienation or depression, and increased mobility, exercise and social development (36,37). Furthermore, pets (particularly dogs) can be also used in Animal Assisted Interventions (AAIs) in hospitals and other healthcare facilities, or can be trained for specific tasks to help people with disabilities. But if from one side their presence represents a clear benefit, on the other hand it poses a further question: is there a risk for both people and companion animals to share and spread AMR?

b. **Direct contact:** Comparing with livestock or wildlife animals, companion animals are generally in closer direct contact with humans. For instance, dogs and cats may share housing, food, sofas, and even beds with their owners. A 2023 study by Menezes et al. (38) showed that the majority of the owners interviewed declared to have close and direct contact with their pets. Hygienic measures like handwash after direct and indirect contact are not always performed. Hence, the risk of potential direct transmission of AMR bacteria or ARGs is more concerning than for livestock. But the knowledge gap about the risk of sharing between pets and owners does not provide an evidence-based risk-assessment of the importance of direct contact. Indeed, studies that reported sharing rates of ARGs between humans and companion animals are present also in countries where generally the direct contact with pets is less frequent for cultural and religious reasons such as Islamic countries (39,40), in which some behaviors such as allowing dogs to lick the owner's face are not common, while washing hands after petting animals is a normal practice. This suggests that the shared environment, such as the household, could be a more impactful factor rather than the direct contact. But once again, little evidence is provided, and more studies on the topic are needed.

c. **Same antibiotics.** Antibiotics are very often used in the small animal clinical practice, considering that owners are more and more often asking for the same level of care and cure expected for a family member (41). Pets and owners share similar pathologies and therapeutic protocols, including antibiotics. Comparing with livestock animals, the decision to use antibiotics is more linked with an infection in a single patient, such as it happens in human medicine, rather than with prophylactic purposes (42). This similarity enhances the opportunity for resistant pathogen

spillover (43). Studies from the United Kingdom estimated that over a two-year period, one in four dogs and one in five cats received antibiotics (44). Although the general antibiotic consumption in pets is gradually declining in most European countries (28,45,46), the main issue regards the use of antibiotics shared with human medicine, with a logic, subsequential risk to develop the same resistance mechanisms. A study by Ferreira Raro et al. (47) demonstrated that sub-inhibitory concentrations of some antibiotics used in veterinary medicine are able to increase plasmid conjugation frequencies in some *E.coli* strains. Most antibiotics commonly used in small animal practice are categorized as of critically importance (CIAs) by the WHO (48); for example, fluoroquinolones and 3rd generation cephalosporins. Regulatory agencies, such as the European Medicines Agency (EMA) did classify antibiotics according to their recommended use in veterinary medicine, putting some of the most recent molecules (such as carbapenems and glycopeptides) in the "A" ("avoid") category, and others (fluoroquinolones, 3rd and 4th generation cephalosporins) in the B ("restrict") category (see Figure 5). That was followed by the 2022/1255 UE regulation that officially banned most category "A" antimicrobial classes from veterinary use. In cats, studies show that the most commonly used drug id cefovecin, due to its broad-spectrum and its long-acting effect after a single injection, while in dogs is amoxicillin-clavulanic acid, even though it should not be used as first choice drug according to the categorization redacted by the EMA (41,49,50). A list of the most commonly used antibiotics (including the proportion of CIAs) redacted by De Bryine et al. (51) in companion animals in Europe is present in Table 3 and 4. Furthermore, small animal practice routine sometimes needs to start an empiric therapy before a complete bacteriological diagnosis. However, this should be limited as much as possible, and when inevitable, in accordance with the guidelines that assist the veterinarian in choosing the most appropriate antibiotic to use. But a complete uniformity in protocols and in guidelines are not defined in most countries (41,52,53). Another factor that should be considered is the so-called extra-label administration of antibiotics, according to the cascade rule, that may increase the risk of emergence of AMR infections in animals resistant to priority antibiotics for humans, such as carbapenems or 4th generation cephalosporins. This practice is still legal in some countries such as the United States (52).

Figure 5. Infographics for the categorization of antibiotic for use in animals redacted by EMA. Adapted from *(50)*.

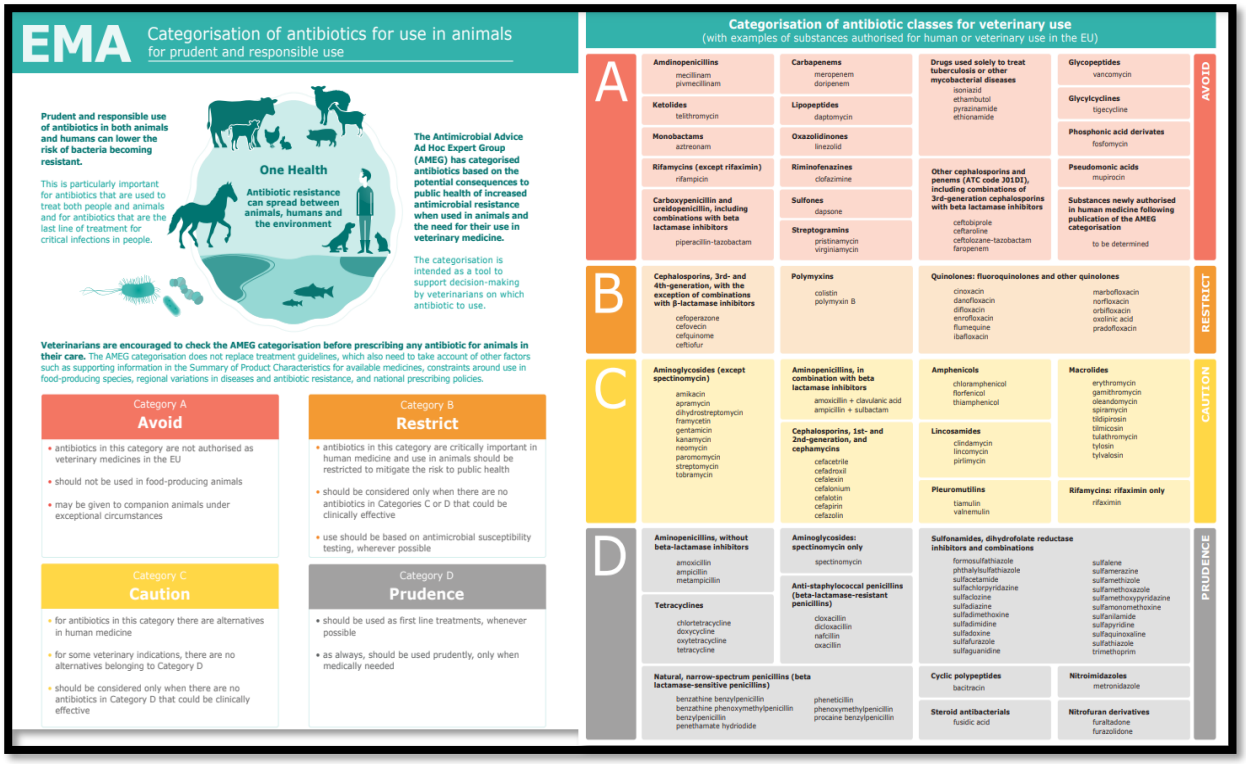


Table 3. List of antibiotics used (including CIAs) for every therapeutic area in dogs in Europe, in a study from De Bryne et al. *(51)*.

Therapeutic area	Percentage mentioned*	Percentage critically important antibiotics (CIAs) v percentage other antibiotics used for treatment	Frequency of use of the different classes of antibiotics (top 5)
Skin disease including:	52%	**CIAs: 10%** Non-CIAs:90%	1st and 2nd generation cephalosporins 35%, Penicillins 31%, **(Fluoro)quinolone 9%,** Potentiated sulphonamides 8%, Lincosamides 8%
(pyo)derma	*(36%)*		
otitis	*(9%)*		
wounds	*(7%)*		
Urogenital infection	11%	**CIAs: 27%** Non-CIAs: 73%	Penicillins 55%, **(Fluoro)quinolone 26%,** 1st and 2nd generation cephalosporins 9%, Potentiated sulphonamides 6%
Respiratory disease	10%	**CIAs: 16%** Non-CIAs: 84%	Penicillins 45%, Tetracyclines 26% **(Fluoro)quinolones 13%,** 1st and 2nd generation cephalosporins 8%
Gastrointestinal disease	10%	**CIAs: 7%** Non-CIAs: 93%	Metronidazole 37%, Penicillins 31%, (Potentiated) sulphonamides 14%, Aminoglycosides 7%, **(Fluoro)quinolones 5%**
Dental disease	7%	**CIAs: 36%** Non-CIAs: 64%	**Macrolides 34%,** Metronidazole 31%, Lincosamides 20%, Penicillins 10%
Others	10%	**CIAs: 18%** Non-CIAs: 82%	Penicillins 35%, Tetracyclines 19%, **(Fluoro)quinolones 17%,** 1st and 2nd generation cephalosporins 11%, Lincosamides 4%

*Percentages in brackets are part of the percentage mentioned of each therapeutic area.

Table 4. List of antibiotics used (including CIAs) for every therapeutic area in cats in Europe, in a study from De Bryne et al. *(51)*.

Therapeutic Area	Percentage mentioned*	Percentage critically important antibiotics (CIAs) v percentage other antibiotics used for treatment	Frequency of use of the different classes of antibiotics (top 5)
Skin diseases including: Wounds and abscesses (Pyo)dermatitis	42% *(39%)* *(3%)*	**CIAs**: 24% Non-CIAs: 76%	Penicillins 51%, **3rd and 4th generation cephalosporins 16%,** Lincosamides 11%, **(Fluoro)quinolones 8%,** 1st and 2nd generation cephalosporins 8%
Respiratory disease	24%	**CIAs**: 16% Non-CIAs: 84%	Tetracyclines 50%, Penicillins 28%, **(Fluoro)quinolones 11%,** 1st and 2nd generation cephalosporins 4%, **3rd and 4th generation cephalosporins 3%**
Urinary tract infection	16%	**CIAs**: 62% Non-CIAs: 38%	**(Fluoro)quinolones 49%,** Penicillins 25%, **3rd and 4th generation cephalosporins 13%,** 1st and 2nd generation cephalosporins 9% (*Remaining classes 1% or less*)
Periodontal disease	14%	**CIAs**: 38% Non-CIAs: 62%	Penicillins 21%, **3rd and 4th generation cephalosporins 21%,** Lincosamides 19%, **Macrolides 14%,** Metronidazole 12%
Perioperative	1%	**CIAs**: 19% Non-CIAs: 81%	Penicillins 75%, **3rd and 4th generation cephalosporins 9%,** **(Fluoro)quinolones 9%,** Aminoglycosides 6% *(no other classes cited)*
Other	3%	**CIAs**: 25% Non-CIAs: 75%	Penicillins 41%, **(Fluoro)quinolones 15%,** Metronidazole 10%, **3rd and 4th generation cephalosporins 8%,** 1st and 2nd generation cephalosporins 7%

*Percentages in brackets are part of the percentage mentioned of each therapeutic area.

d. **Direction of the transmission:** Given that AMR transmission between companion animals and people occurs (38,54), the strength of the direction in both sides remains unclear. Such transmission – including direct transmission of bacteria or ARGs - is bidirectional, with an important involvement of the environment (water, soil, air) in the preservation and the spread of AMR. Regarding companion animals, we have to consider not only the classic anthropocentric view of the animal-to-human risk of transmission, but also the human-to-animal, in the so-called reverse zoonosis. This is particularly important when we consider resistances to antibiotics whose use is limited but still conceded in pets (such as fluoroquinolones or cephalosporins), because the acquisition of such resistances by animals implicates a major risk of developing infections not treatable with drugs licensed for companion animals, with a subsequent therapeutic failure. This consideration could be particularly important for pets living with people working in healthcare or who have been recently hospitalized(55) Furthermore, this bidirectional way of transmission must be matter of concern also for the potential role of maintenance reservoir companion animals might have. This topic has been highlighted by various studies (37,56–59), that highlighted as pets can accumulate different types of ARGs and AMR bacteria, even towards drugs that are not allowed for veterinary use, such as carbapenems. This represents a threat from a public health point of view because it potentially allows the persistence of AMR even in absence of infections in both people and animals, with a consequent risk of overlooking or underestimation of the real problem in the future.

2. **Most concerning Antimicrobial Resistant Organisms (AMROs) in small animal practice.** Between the different AMROs listed by the WHO in 2017 (60), some of them belonging to the "High" and "Critical" priority group are considered specifically important also for small animal practice.

GRAM-POSITIVES BACTERIA

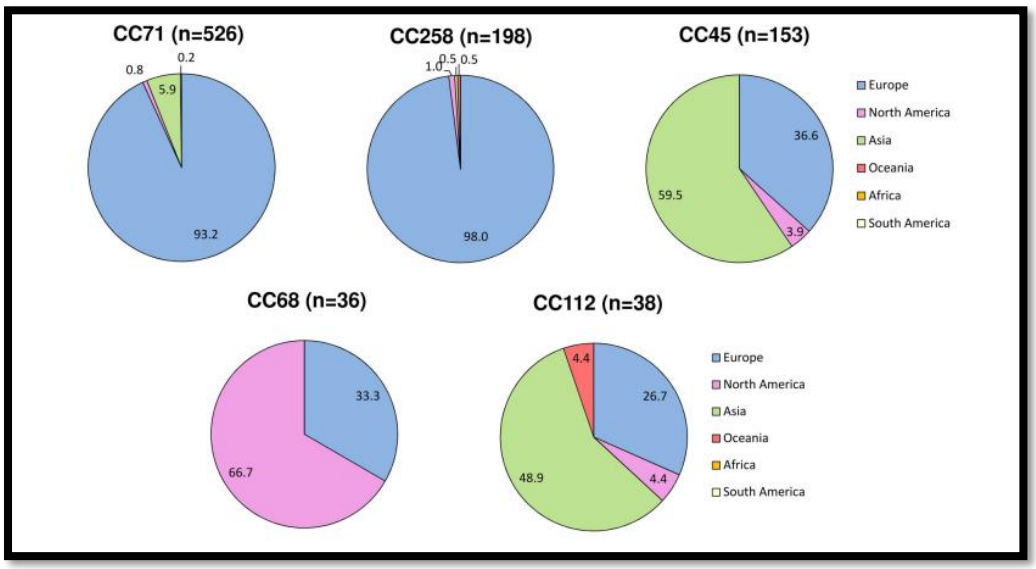
a. **Methicillin -Resistant Staphylococci (MRS).** MRS represent a serious and emerging health problem in both human and veterinary medicine. The genus *Staphylococcus* spp. a variety of species (over 40) of gram-positives, non-spore forming and non-mobile bacteria. They are normally present in the skin and mucosa flora of birds and mammals, including humans, and can survive in the environment even in extreme conditions, including after disinfection (61). The acquisition of resistance towards methicillin gives them the ability to alter the affinity to all the beta-lactams antibiotics, such as cephalosporins and penicillins, and often to drugs from other classes, such as fluoroquinolones, tetracyclines and sulfonamides (62), in the so-called multi-drug resistance (MDR). Methicillin resistance is mainly mediated by the *mecA* gene, or its homologue *mecC,* that codify for a modified Penicillin-Binding-Protein (PBPa). PBPa is involved in the cell wall synthesis: by altering the target, it confers resistance to beta-lactams and other antibiotic classes (63), that are no more able to block wall cell synthesis. These genes are located in a mobile genetic element, called Staphylococcal chromosome cassette mec (SCC*mec*) (64). Specifically, methicillin resistance in coagulase-positive Staphylococci (CoPS) is considered more important due to their higher pathogenicity rates compared with coagulase-negatives (CoNS). The importance of CoNS is related with the maintenance and diffusion of AMR genes more than their ability to cause infections (65). Some CoNS species frequently found in pets are *Staphylococcus felis, Staphylococcus epidermidis, Staphylococcus sciuri, Staphylococcus haemolyticus*, *Staphylococcus warnerii* and *Staphylococcus saprophyticus.* On the other hand, the most concerning MR CoPS in small animal practice are MR *Staphylococcus aureus* (MRSA) and MR *Staphylococcus pseudintermedius* (MRSP).

i. **MRSA.** The emergence of MRSA is a global health threat. In human medicine, MRSA started to emerge as a pathogen in the '70s in the United Stares (66), to subsequently spread pandemically in all the continents in the '90s (67). In companion animals, MRSA infections were gradually reported since 2000s (68). It is considered a primary human pathogen, with prevalence in healthy cats and dogs reported to be between 0 and 5.7%, with an increasing trend (69–71). Infections sustained by MRSA in pets are mostly skin infections (pyoderma), surgical site infections (SSIs), urinary tract infections (UTIs), otitis and wounds (72). They have been associated with hospital-acquired infections in veterinary hospitals (69,73,74). Several studies have highlighted that MRSA strains isolated from pets are indistinguishable from MRSA strains isolated in human infections, although the interspecies transmission is considered a rare event (75,76). This suggests the potential role of reservoir of companion animals, specifically dogs (77), that may act as "transient carriers" of MRSA from an infected human or a human carrier living in the same

household. Indeed, MRSA infections in pets are considered primarily of human origin (78), and contact with kids and tendency to lick people have been reported as a risk factors for cats and dogs to be colonized by MRSA (79). At the same time, it is important to remember only a minority of human MRSA infections are directly associated with animals. The presence of MRSA in a pet does not necessarily indicate that the animal is the primary source for zoonotic infection, but more probably that was colonized from a human origin (80). However, a general lack of host specificity is likely, since MRSA genotypic profiles reflect more the geographic area rather than the host origin (81).

ii. **MRSP**. *S. pseudintermedius* is a normal commensal of the cutaneous and mucosal flora of pets (82), that can become an opportunistic pathogen causing pyoderma, otitis, SSIs, conjuntivitis, dermatitis, UTIs and respiratory tract infections (83,84). Skin or soft tissue infections associated with *S.pseudintermedius* that can be managed successfully by topical treatment (85). It belongs to the Staphylococcus Intermedius Group (SIG), together with *Staphylococcus intermedius* and *Staphylococcus delphini* (86), due to their genetic affinity. It is generally more frequent in dogs than in cats (87), and this could be explained by a study by Lu & McEvans (88) that demonstrated that *S.pseudintermedius in vitro* is less capable to adhere to feline's skin corneocytes compared with canine's. MRSP is considered a primary animal opportunistic pathogen, with a high-variable prevalence in both diseased and healthy pets; virtually, any dog or cat could be colonized by MRSP, transiently or permanently (56,57). Healthy carriers are a source for transfer of the bacteria (not the disease) to other animals (90), through direct contact or contaminated environment, as described in a 2021 study by Papic et al. (91). A 2018 study report a prevalence of 63% of MRSP between all the *S.pseudintermedius* strains isolated from infected dogs: of these, 78% was multi-drug resistant (92). The variability of MRSP isolation in infected animals varies in relation with infection site and geographic area, as emerged in a multicentric study on UTIs in European dogs (93), that highlighted differences at a national level: for example, in Italy *S.pseudintermedius* represented the causative agent of 94.7% of total Staphylococcal infection, of which 50% was MRSP. Similar results were obtained by other Italian studies (94,95). MR transmission is mainly due to the global diffusion of few epidemic clonal complexes, responsible for the vast majority of MRSP infections worldwide (CC71, CC68, CC258, CC45 and CC112) of which the most important in Europe is the CC71 (96) (Figure 6).

Figure 6. Geographic variation in the reported frequency of major MRSP clonal complexes (CC). Adapted from *(96)*.



The zoonotic risk of transmission of MRSP to humans has been described firstly in 1999 in South Korea (97). Owners of dogs suffering from *S.pseudintermedius* infections and veterinarians appear to be more likely to be nasally colonized with *S.pseudintermedius* than other individuals (98,99), There is no evidence that MRSP can be transmitted via contaminated food, neither that MRSP is more likely to be transmitted to humans than susceptible variants of *S.pseudintermedius*. People exposed to MRSP tend to eliminate it without developing infections; however, in rare cases (e.g. immunocompromised persons) can lead to disease, normally mild or moderate skin and soft tissue infections (100–102). Differently from MRSA, MRSP infections in humans are mainly caused by an animal source (103).

b. *Enterococcus* spp. Enterococci are gram-positives, opportunistic, non-motile bacteria often found in the gastrointestinal tract of animals and humans. In small animal practice, the most important species from a pathogenic point of view are *Enterococcus faecium* and *Enterococcus faecalis*. In contrast with human medicine, *E.faecalis* seem to be more predominant than *E.faecium* in companion animals (104). Enterococci are intrinsically resistant to several antibiotics, such as cephalosporins, clindamycin, erythromycin and trimethoprim(105), but are also able to acquire resistances to other antibiotic classes. But despite of their AMR rates, in some cases the isolation of *Enterococcus* spp. alone does not require specific treatment. Indeed, they tend to be of limited virulence (106), and in a patient with no clinical signs and an immune-competent system, antibiotic treatment may be avoided. Despite this, they are difficult to eradicate when they cause disease (e.g. in compromised hosts). The high resistance rates, summed with their ability to persist in the environment and in the gut microbiota make Enterococci particularly challenging in the global AMR overview. The spread of Vancomycin-resistant Enterococci (VRE), considered an increasing concern in human medicine, still appears to be rare in small animal practice (107). However, MDR Enterococci are regularly recognized also in pets (104,108).

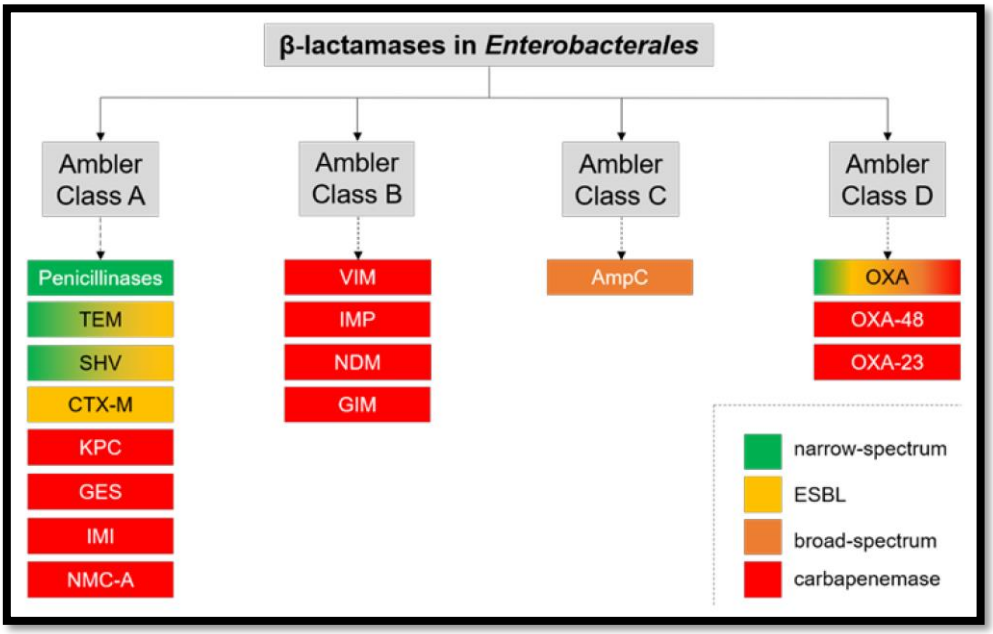
GRAM-NEGATIVES BACTERIA

c. **Beta-lactams resistant Enterobacterales.** The Enterobacterales order includes several species of gram-negatives bacteria such as *Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp., *Serratia marcescens* and *Salmonella* spp. These bacteria are opportunistic, motile and non-spore forming rods present in the gut of mammals as commensals or pathogens. They have been associated with several intestinal and extra-intestinal infections in both humans and animals (109,110). In pets, the two most common species are *E.coli* and *K.pneumoniae,* found in abscesses, SSIs, otitis, septicemia, gastroenteritis, cholangiohepatitis and UTIs (111–113). On the other hand, *Salmonella* spp. seem to be most frequently isolated in equine medicine(106), but it still must be considered an important pathogen also in companion animals, especially in relation with the occurrence of zoonotic transmission and the high proportion of subclinical infections in pets. *E.coli, K.pneumoniae* and *Enterobacter* spp. can be frequently found as a normal part of the commensal gut flora of healthy and sick animals, sometimes harboring several ARGs. Indeed, these organisms present a high capacity to acquire resistance genes (112,114), as well as genes related with hypervirulence (especially *K.pneumoniae* (115)) normally through a rapid, horizontal transmission mediated by plasmids (conjugation). These plasmids possess the ability to accumulate resistance genes, increasing their pathogenicity (116,117). Specifically, two typologies of resistance are considered a matter of concern in Enterobacterales, for which they have been included in the list 1 "Priority pathogens" redacted by the WHO (60):

- **Third generation cephalosporins resistance (3GCR):** mediated by genes encoding enzymes able to hydrolyze such antibiotics. The most epidemiologically important group of such enzymes are the Extended-spectrum beta-lactamases (ESBL), which have become endemic worldwide. ESBLs are serine beta-lactamases, belonging to Class A Ambler (with the exception of OXA-type enzymes, that belong to the class D) (see Figure 7). Biochemically, they are able to hydrolyze expanded spectrum beta-lactam antibiotics such as 3rd generation cephalosporins, while are inhibited by b-lactamase inhibitors such as clavulanate, sulbactam, tazobactam and avibactam (118). Due to genetic linkages, ESBLs are often associated with MDR (38,106,119,120). The first report on the production of ESBL was at the end of the '80s (121), while the first clinical report in small animal practice was in 1998 (122). As previously mentioned, 3GCs are commonly used in small animal practice, so consequently resistance genes are rapidly disseminating. Between the ESBL enzymes of pandemic relevance, the most described in small animal practice are the CTX-M, SHV, TEM and OXA type (123,124) (see Table 5). A study by van den Bunt et al. in 2020 shows that 12% of the people included with a dog carrier of ESBL-producing Enterobacterales were carriers as well of the same resistances (125). The carriage of ESBLs in asymptomatic patients is considered the

main reservoir for ESBLs infections in human medicine (126), increasing the chances of a severe infection in case of immunocompromised patients. In small animal practice, data are scarcer, but there are indications that colonized pets could act as reservoirs (37,127). A recent phylogenetic study by Perestrelo et al. () on ESBL-producing *E.coli* from human cases and different sources suggests as dogs may considerably contribute to the human cases as reservoir (128). The most relevant risk factors for the ESBL-producing Enterobacterales colonization identified by scientific literature are previous hospitalization, raw meat diet, age and previous antibiotic therapy (125,129). Having a frequent contact with pets, such as veterinary workers and owners have, seem to be related to a higher risk to carry ESBLs compared with the general population, as reported by studies in Germany and the Netherlands (130,131). Another 3GCR resistance pattern is mediated by AmpC enzymes. AmpCs belong to Amber class C cephalosporinases (see Figure 7) and beyond 3GCR, confer resistance to penicillins and old beta-lactamases inhibitors such as clavulanate, sulbactam and tazobactam, but not more recent such as avibactam and relebactam.

Figure 7. Ambler's classification with examples of main β-lactamases in Enterobacterales. Adapted from (132).



The differences a between ESBLs and AmpCs are related with the degradation modality (133). Generally speaking, AmpC prevalence is less prominent than ESBLs, and the most important AmpC type in small animal practice is plasmid-mediated and human-shared CMY-2. Reported prevalences of 3GCR Enterobacterales in healthy dogs and cats are increasing worldwide, with % up to 45% (134–138). In diseased animals, 3GCR prevalence is widely variable, with no evident difference between dogs and cats (123), and with frequent

association with MDR (139). Furthermore, several studies have demonstrated that 3GCR Enterobacterales isolated in pets and humans that shared the same household were indistinguishable (38,93,125,140,141). Despite 3GCR in Enterobacterales is considered a public health problem (142), the extension of the in companion animals has not still received considerable attention, with only few national programs present in high-income countries (123).

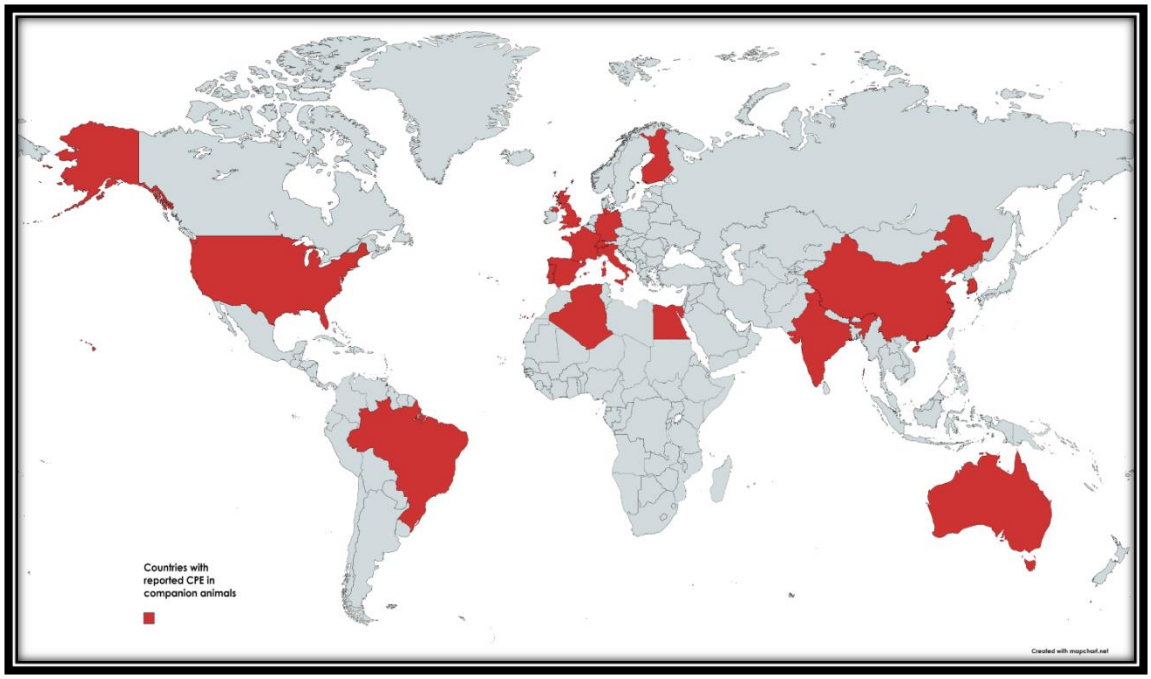
Table 5. Types and characteristics of ESBL enzymes. The most prevalent in small animal practice are TEM, SHV, CTX-M, GES. Adapted from *(118)*.

Family	Nomenclature	Characteristics
TEM	Temoneira, the patient infected with the first isolate expressing TEM-1	Point mutation variants of TEM-1 or TEM-2
SHV	Sulfhydryl reagent variable	Point mutation variants of SHV-1
IRT	Inhibitor-resistant TEM	TEM variants that are resistant to inhibition by clavulanate and sulbactam, but do not have ESBL phenotype
CMT	Complex mutant derived from TEM-1	TEM variants that are resistant to inhibition by clavulanate and sulbactam and also have ESBL phenotype
CTX-M	Cefotaxime-hydrolysing β-lactamase isolated in Munich	Derived from the chromosomal β-lactamase from *Kluyvera* spp. Preferentially hydrolyses cefotaxime
GES	Guiana-extended spectrum	More prevalent in *P. aeruginosa* than Enterobacterales Some variants also hydrolyse carbapenems
PER	*Pseudomonas* extended resistant	More prevalent in *P. aeruginosa* and *A. baumannii* than Enterobacterales Inhibition by newer β-lactamase inhibitors is variable
VEB	Vietnam extended-spectrum β-lactamase	Preferentially hydrolyses ceftazidime and aztreonam compared with cefotaxime Inhibition by newer β-lactamase inhibitors is variable
BEL	Belgium extended β-lactamase	Preferentially hydrolyses ceftazidime and aztreonam compared with cefotaxime
TLA	Named after the Tlahuica Indians (Mexico), from whom the first isolate was obtained	Preferentially hydrolyses ceftazidime and aztreonam compared with cefotaxime
SFO	From *Serratia fonticola*	Inducible
OXY	From *Klebsiella oxytoca*	Chromosomally encoded

- **Carbapenems Resistant Enterobacterales (CR-E)**. Carbapenems are recently discovered β-lactam antibiotics with broad antimicrobial spectrum. With the emergence of 3GCR and other resistances, carbapenems became the antibiotics of last resort in hospitals, and their use is typically preserved to treat critical infections (143). But once again, their efficacy is threatened by the emergence of carbapenem-hydrolysing enzymes, the carbapenemases (57,143). Carbapenemases are classified in three different Amber classes (144): (i) class A, including the KPC, IMI/NMC, SFC, GES type enzymes; (ii) class B, including VIM, IMP, and NDM metallo-β-lactamases (MBL); and (iii) class D, including OXA-48-like type enzymes. In veterinary medicine, regulation on the use of carbapenems varies worldwide, but they are normally preserved for human medicine. In the EU, they belong to the category A ("avoid"), meaning that they are not authorized (See Figure 1). But nevertheless, reports of CR Enterobacterales detection among companion animals are emerging worldwide (See Figure 8), rising a public health concern in relation to the dissemination of CR genes and the potential role of pets as reservoirs (57). The reasons behind the diffusion of CR remain quite unclear, as no global surveillance protocol is currently in place for companion animals. Resistance to carbapenems is frequently

associated not only with resistance to all beta-lactams, but also with MDR (57).

Figure 8. Global overview of countries that reported carbapenemases production (CP) by 2022 in companion animals. Adapted from *(57)*.



d. ***Pseudomonas aeruginosa.*** *P.aeruginosa* is an ubiquitarious, motile, gram-negative rod that belongs to the family Pseudomonadaceae. Its nutritional versatility and wide distribution made *P.aeruginosa* one of the most problematic opportunistic pathogens in both human and veterinary medicine (145,146). It has been isolated from various biological samples and different inorganic surfaces (145,147). In pets, it has been associated with chronic and recurrent disease such as otitis, SSIs, UTIs and pyoderma (148,149). Due to the presence of several drug efflux systems and porins, *P.aeruginosa* is intrinsically resistant to a wide range of antimicrobials including benzylpenicillins, aminobenzylpenicillins, carboxy-penicillins, first and second generation cephalosporins, chloramphenicol and potentiated sulfonamides (146); however, acquired mechanisms of antimicrobial resistance, such as efflux pumps, but also the aforementioned hydrolyzing enzymes such as ESBL and carbapenemases have been described in small animal practice (57). It also often forms biofilms, which are a major challenge from a therapeutic point of view (146).

e. ***Acinetobacter* spp**. Member of the Moraxellaceae family, the genus *Acinetobacter* comprehends Gram-negative, strictly aerobic, motile bacteria (150), often associated with nosocomial infections in humans (151). The majority of clinical infections caused by *Acinetobacter* spp. are attributed to a specific group belonging to the *A.calcoaceticus* complex (*A.baumannii*, *A. pittii* and *A. calcoaceticus*). *A.baumannii* is unsurprisingly included in the ESKAPE bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp.), listed as one of the most challenging pathogens for health care professionals (152). Apart of the ability to persist in the environment for extended periods, the main characteristic of *Acinetobacter* spp. is

the intrinsic resistance to a variety of antibiotic classes, such as aminopenicillins (even when potentiated with the beta-lactamase inhibitors, like clavulanic acid), 1st and 2nd generation cephalosporins, chloramphenicol and fosfomycin (151,153). Furthermore, *Acinetobacter* spp. is also frequently associated with acquired resistance against other classes of antibiotics, such as 3rd generation cephalosporins and carbapenems. The worldwide emergence of carbapenem-resistant strains in *A.baumannii* is regularly associated with MDR, possibly due to the multiple acquisition of ARGs (150,151). In small animal practice, epidemiologic data about *Acinetobacter* are scarce (154). However, recent studies have provided the evidence of disease also in pets, especially in relation with UTIs and SSIs (155–157).

1.C. The paradox of healthcare

If we consider the AMR phenomenon as an iceberg, the bigger, submerged part would be represented by the AMROs and ARGs spread in the environment, humans, and animals; on the other hand, the emerged part (what we really see and suffer for) is represented by AMR infections. Indeed, infections caused by AMROs are the concrete and visible problem, and the final aim of the fight against AMR is to avoid the post-antibiotic era, in which bacterial infections are no more treatable. Bacterial infections are historically categorized into Community-Acquired Infections (CAIs) or Healthcare-Associated Infections (HAIs). By definition, CAIs are infection contracted outside of an healthcare facility, or within 48 hours after the admission to such facility, while HAIs are infections acquired while receiving healthcare, detected after 48 hours from admission or within 30 days after discharge (90 days in case of surgical implants (158)). Initially, the term HAIs was referred to infection linked with admission to acute-care hospitals (earlier called nosocomial infections), but now the term has been extended to infections developed in settings where patients obtain healthcare. HAIs be considered either endemic (most common) or epidemic. Epidemic infections occur during outbreaks, defined as an unusual increase above the baseline of a specific infection or infecting organism (159).HAIs are considered a worldwide major public health problem (WHO). for some reasons:

- **High mortality and morbidity rates:** HAIs are often sustained by hypervirulent organisms, and/or contracted by patients with comorbidities or immunocompromised systems, that are less able to contrast infections adequately. This is reflected to the increasingly high morbidity and mortality rates. Overall, HAIs are considered the second most prevalent death cause in the world, with rising trends (160). The ECDC estimates that more than **8.8 million cases of HAIs** occur in the European Union yearly (see Figure 9), leading to more than **90 000 deaths** and corresponding to approximately **2.5 million disability adjusted life years** (DALYs) (161). In low- and middle-income countries, the escalation is even more alarming, with rates 3-20 times higher than high-income countries and prevalences between 5.7% and 19.1% (162). According to the WHO, out of every 100 patients in acute-care hospitals, seven patients in high-income countries and 15 patients in low- and middle-income countries will acquire at least one HAI and on average, 1 in every 10 affected patients will die (162).

- **Economic and reputational burden:** HAIs are related also with substantial economic and reputational losses for healthcare facilities, related with longer hospital stays and the use of more expensive drugs. In Europe, the ECDC estimated that they are responsible of 16 million extra days of hospitalization, and an approximate economic cost around 7 billion euros (163). In Italy alone, the Ministry of Health reports that between 450,000

and 700,000 HAIs are recorded annually, with a rate of 4-7% considering total hospitalizations. A single HAI is equivalent to spending between 5,000 and 10,500 euros(164). Furthermore, there are indirect, costs related with the onset of HAIs that are more difficult to measure but that can have a massive impact, such as community care costs, lost wages, productivity by the patient and caregivers, and reputational costs. Indeed, the paradoxical situation in which a patient is admitted to a healthcare facility to get medical assistance but contract an infection (often severe) inside the same facility, can mine the credibility not only of the facility, but of the entire healthcare system in the long term. Additionally, the fear of a negative reputation for both the healthcare facility and the physician could lead to the under-reporting of HAIs rate, with a subsequent underestimation of the real size of the problem (165).

- **High AMR rates**: HAIs are contracted by definition in healthcare facilities, where antibiotics are commonly used for treatment. The selective pressure put on such facilities represent the perfect *pabulum* for bacteria to develop AMR, far more frequently if compared with CAIs. It is estimated that in Europe HAIs constitute **71% of all cases of infections with AMR bacteria** (166), including MDR bacteria resistant to last-resort antibiotics, such as CR Enterobacterales or MRS (the so-called "superbugs"), associated with severe infections such as sepsis (see Figure 10). The association between AMR and HAIs represent the real issue and is directly related with the high mortality rates aforementioned. HAIs constitute the real, concrete demonstration of the AMR burden.

Figure 9. Infograhpics redacted by the ECDC on Healthcare-associated infections in Europe. From https://www.ecdc.europa.eu/en/publications-data/infographic-healthcare-associated-infections-threat-patient-safety-europe

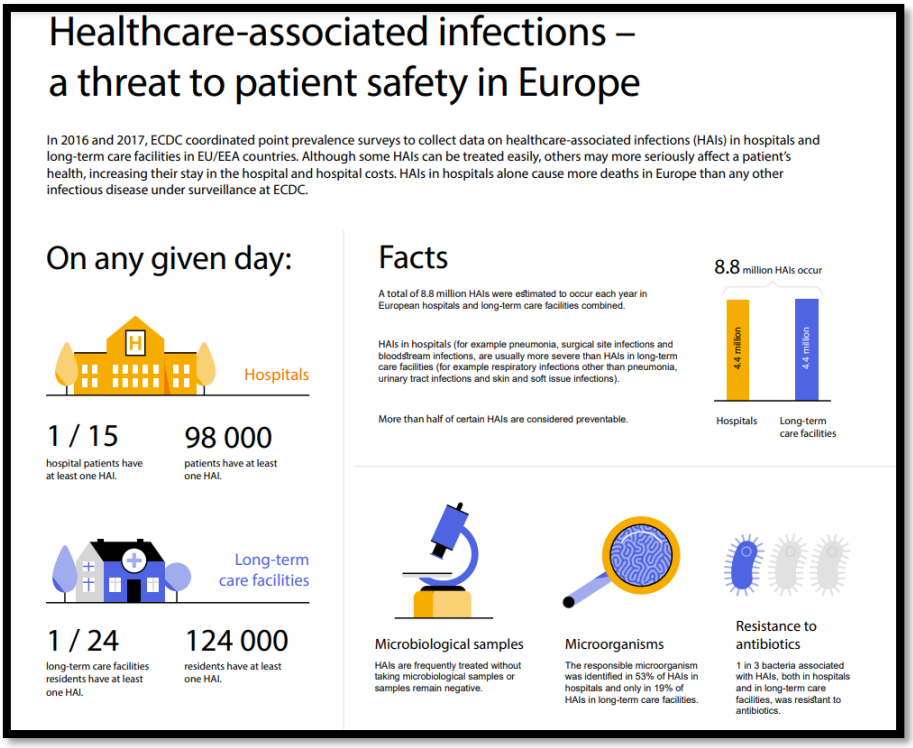
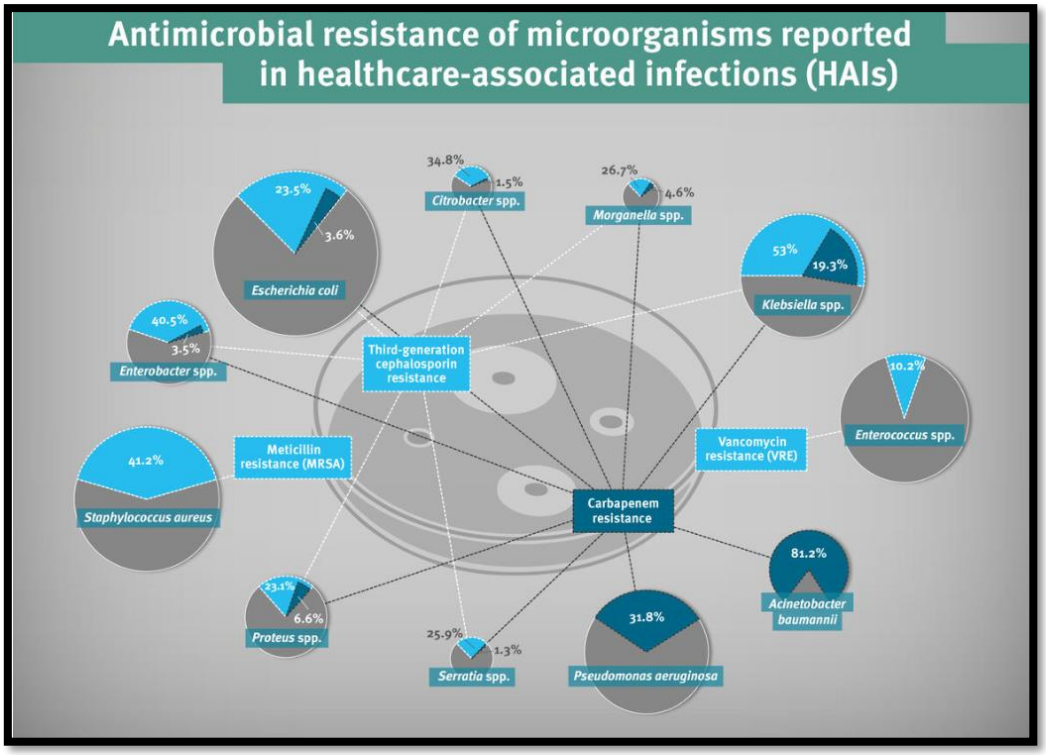


Figure 10. Infographics redacted by the ECDC on the most common bacterial agents responsible of HAIs in Europe. From *https://www.ecdc.europa.eu/en/publications-data/antimicrobial-resistance-microorganisms-reported-healthcare-associated-infections*



What about companion animals?

Veterinary medicine, and specifically small animal practice, is also experiencing the paradox of healthcare (106,167). Compared with human health, the "delay" on the topic could be estimated on 10-15 years; on one hand, this means that the scientific knowledge is less abundant, on the other hand, we have the chance to learn from the experiences of human medicine and take steps to prevent the escalation of this emerging problem (168). Data available for the veterinary field showed similar HAIs rates (or even higher), such as 16% in Intensive Care Unit in one study (169). In a recent review on HAIs in veterinary medicine (170), 63% out of 27 included papers were from companion animal clinics/hospitals. Similarly with human medicine, the HAIs burden affect the owners with increased anxiety and expenditure, and veterinary hospitals through potential legal claims. Additionally, there are some aspects that make HAIs an emerging threat specifically in small animal practice:

- **The growing quality of the healthcare assistance:** in the last years, small animal practice is experiencing a substantial improvement in terms of length and quality of animal patients' lives. The use of more invasive surgical procedures, more complex antimicrobial and immunosuppressive therapies, the greater intensity of critical care management, long-term hospitalizations and geriatric assistance are examples of how medical progress is enhancing the quality of the healthcare (171). This implies not only a greater attention to HAIs compared with years ago, when they were often neglected; but also a greater chance for HAIs to develop in patients that probably would have not survived their underlying disease in the past.

- **Less therapeutic options:** as previously mentioned, veterinary medicine has some limitations on antibiotic use in order to preserve their effectiveness for human medicine. In Europe, antibiotics such as carbapenems, glycopeptides or ureidopenicillins, have been banned with the 2022/1255 Regulation. From a strict clinical point of view, this implicates that less therapeutic options are available to treat a pet with an HAI, with a subsequent higher risk of treatment failure, euthanasia or death;

- **Harder to recognize and manage:** in animals, to differentiate between HAIs and CAIs could be challenging because of the lack of common, standardized definitions; furthermore, the majority of HAIs usually appear when the patient is already at home, because the duration of hospitalization is usually short and many elective procedures are performed as outpatient surgery (172). The lower presence of long-term patients, together with a relatively lower proportion of profoundly immunocompromised patients and highly invasive procedures, could suggest a lower impact of HAIs in small animal practice; but other factors must be considered. For example, the considerably greater difficult to achieve patient compliance (e.g. licking wounds), and to ensure hygienic standards (106).

- **Public health concern:** it is well recognized that veterinary settings contribute to the selection of AMROs and ARGs with zoonotic potential (81,168). Small animal hospital environment is a human-animal interface, where the inter-species transmission is more likely with a subsequent epidemiologic risk for disease transmission. Several studies demonstrated that humans, animals and the environment can act as reservoir for AMROs

capable to cause HAIs, even for years (173–175)**.** The patient microbiota, healthcare workers, fomites and the hospital environment have been identified as possible sources of organisms associated with HAIs (170). This deep interconnection makes that veterinary facilities must be considered under a One Health lens in terms of infection control policies. Veterinary healthcare workers and owners are considered at a higher risk: for example, a research on ESBL-producing *E.coli* revealed that contact with pets increases the chance to be colonized more than six-fold (131). Additionally, studies on dogs of veterinary students revealed that they were associated with a higher risk of MRSA colonization, highlighting the impact of veterinary settings in the dissemination of AMROs in the community also through their owned pet (176). Similarly, a study on the occurrence of ESBL-producing *E.coli* from fecal samples in infected dogs collected in the nearby of the veterinary hospital showed a considerable environmental contamination (134).

Characteristics of HAIs in Small Animal Practice

1. **Localization**. The most reported HAIs in pets include surgical site infections (SSIs) and implants, catheter-associated urinary tract infections (UTIs) and bloodstream infections (106)**.** Other HAIs include gastrointestinal disease (especially salmonellosis, although the determination of the cause is often difficult), upper respiratory tract infection or dermatophyte infections. **Ventilator-associated infections, common in human medicine, are comparably rarer, since long term** artificial respiration is not a common procedure in companion animals (177).

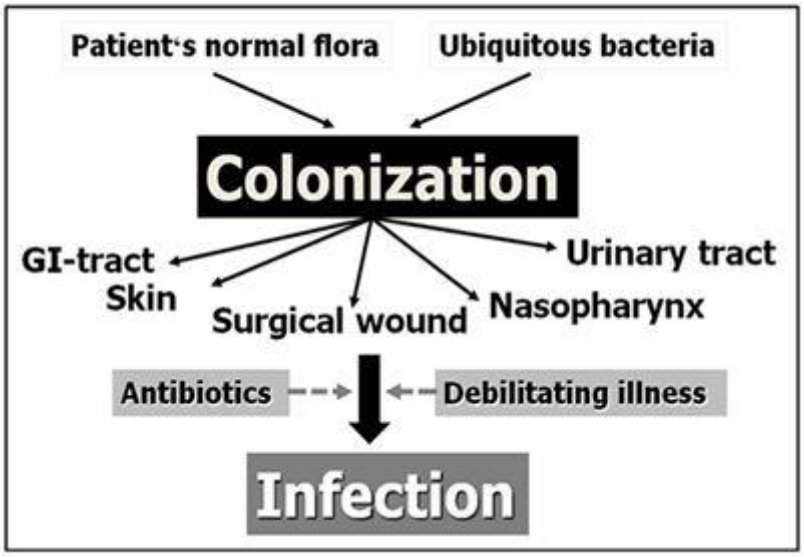
    - **HA UTIs.** UTIs are one of the most common HAIs in pets (106)**,** often associated with a prolonged catheter use, that interferes with the normal defense mechanisms such as the mucosa-secreted adhesion inhibitors. The presence of comorbidities and an inadequate catheter handling are other factors that facilitate bacterial colonization of the catheter and the subsequent ascension of the organism to the bladder. AMROs involved in HA UTIs can be endogenous (arising from the rectum or perineum) or exogenous from the hospital environment or people (through direct contamination). AMROs can ascend from the collection system into the bladder if there is retrograde flow of urine (e.g. if the collection system is elevated above the level of the patient, or if there is an obstruction). In addition, AMROs capable to produce biofilm on the surface of the catheter are less likely to be killed by antibiotics(106).

    - **Bloodstream infections.** These infections are mostly associated with intravascular devices. In human medicine, duration of catheterization has been recognized as the most important risk factor for the development of catheter-related bloodstream infections (most developing 4–5 days after placement) (178). But despite this increased risk, studies have not documented a benefit with prophylactic catheter changes (eg, every 3 days). the current recommendation is for catheters to be removed as soon as medically indicated, but for routine changes to be avoided. Veterinary studies have revealed that jugular and intravenous catheters are frequently contaminated with enteric or environmental pathogens (179). Several factors have been positively associated with intravenous catheter contamination in dogs and cats, including receipt of dextrose infusion, longer duration of catheter placement, and patient immunosuppression(180). Contamination may occur from the hands of people placing or handling the catheter, the

patient's own flora, or the hospital environment. However, there is little evidence indicating that contaminated but not infected catheters (ie, catheters from which bacteria can be isolated but where the catheter insertion site and vein are clinically normal) pose a risk for subsequent bloodstream infection. Routine culture of catheters at time of removal or culture of catheter insertion sites is not recommended because skin bacteria are expected to be present. Veterinary outbreaks of bloodstream infections have been associated with inadequate skin preparation or contaminated materials used in skin preparation (106), often linked with the preparation of antiseptic solutions by refilling bottles or containers, which can become contaminated with biocide-resistant bacteria over time.

- **Surgical Site Infections (SSIs).** SSIs are typically a burden of surgery, being the most common cause of infections in human surgical patients, and also the most preventable of all the HAIs (181). SSIs are infections associated with a particular operative procedure and the facility in which the procedure was performed, including infections occurring in wounds not closed by primary intention (182). They can be classified according to superficial, deep, or organ/space infections. Despite the array of preventive measures, several veterinary studies have reported high SSI rates, with incidences up to 21.3% (181,183). AMROs such as MRSP and Enterococci have been frequently associated with SSIs (184). As well as with UTIs and bloodstream infections, the source of bacterial contamination can be endogenous (skin, oropharynx, gut) or exogenous (surgical team, environment, materials, and instruments used) (see Figure 11). While endogenous sources may be limited with a careful patient preparation, exogenous sources are more challenging, and often related with a poor compliance by the personnel(185) . The implementation of surgical asepsis, preoperative bathing, trichotomy, hand hygiene procedures, appropriate antimicrobial prophylaxis (timing and selection) and intraoperative skin preparation are all milestones of the current many guidelines accessible (181), but their execution is not always correctly complied by the surgical team. Biofilm formation, such as in MRSP, can often complicate the treatment of SSIs (186).

Figure 11. Development of a healthcare-associated infection. Adapted from: https://www.vin.com/apputil/content/defaultadv1.aspx?pId=11268&id=3866711&print=1



2. **Etiological agents.** In small animal bacterial HAIs, pathogens often have one or more of the following characteristics: opportunistic pathogen normally present in healthy animals, environmentally stable and multidrug-resistant. The most frequently isolated are the previously mentioned **MRS, *Enterococcus* spp., 3GCR and CR Enterobacterales, *Pseudomonas* spp. and *A.baumannii*** (106,168,170). They are not inherently more virulent than susceptible organisms, but treatment options are limited, worsening the prognosis. The frequency of each pathogen varies for each veterinary practice, and it is influenced by antibiotic pressure, geography, animal species, vaccine coverage of animals in "catchment" area and level of care provided (106). These organisms are able to accumulate ARGs over time, suggesting an adaptation (187). They can survive in the patient microbiota, or in the hospital environment for long periods in absence of evident cases, and even in presence of high temperatures or disinfectants (188). For example, *A.baumannii* survives on dry surfaces (189,190); therefore, commonly used fomites, bed rails, cages, and examination tables could serve as reservoirs. Furthermore, hospital personnel can act both as mechanic vector and source of infection. *E.coli* infections have been linked with fecal contaminated hands of veterinary workers and shared equipment, showing the ability to cause outbreaks (191).

3. **Risk factors**. Such as in human medicine, infectious agents need normally an additional predisposition to cause an HAI. Such risk factors can be related with the patient (intrinsic risk factors) or with the environment (extrinsic risk factors), or with both. Intrinsic risk factors to develop an HAI include age, obesity, or the presence of comorbidities such as immunosuppression, endocrinopathies, other infectious disease or neoplasia (106), but also factors related with the history of the patient, such as previous hospitalization, antibiotic treatment or prolonged perioperative prophylaxis (192); furthermore, becoming carrier of AMROs directly enhance the chance to develop an HAI:; when an outbreak happens, more patients are colonized than infected (172). Extrinsic risk factors are related with a poor staff' hygienic compliance (including handwashing), the absence of infection control policies and high environmental contamination, overcrowding (181), long and invasive surgeries, prolonged use of invasive devices, and the length of the hospital stay (73,181,193). Long term patients are not only at higher risk to develop an HAI, but they also represent a potential source for AMRO dissemination (187). Additionally, other factors, such as the size of the hospital, the location, the variety of medical services offered and the patient groups admitted, can play a role in the onset of HAIs (194). Generally speaking, a clinic with more referral patients is at higher risk to introduce AMRO via colonized or infected patients than first-line practitioners. This is confirmed by a survey by Benedikt et al. (195), that underlined that Veterinary Teaching Hospital (VTHs) should be considered high-risk places, with 82% of such facilities reporting at least one HAI outbreak in the last five years. In addition to the high number of referred patients that are more likely to have received previous antimicrobic treatment, VTHs possess other potential risks, such as the co-presence of different animal species and the overcrowding due to the presence of students (196).

1.D. You can't manage what you don't measure

HAIs are considered an undesirable outcome, and their rate is considered an indicator of the quality of patient care. There are several strategies to reduce the impact of HAIs in healthcare facilities. These methods will also reduce the chance of patient in-hospital colonization with an AMRO, which can become part of the patient's commensal microbiota, potentially increasing disease risk

and posing a risk to other animals and humans. According with human medicine (160), such infection control strategies can be divided into the following main categories:

- **Surveillance** (data collection on AMR and HAIs);
- **Prevention,** that includes:
  - Hand hygiene and use of personal protective equipment (e.g., clothing and/or gloves);
  - Environmental hygiene (environmental surfaces and patient equipment)
  - Patient management (e.g., patients cohorting based on risk, isolating high-risk patients, discontinuing the use of invasive devices when indicated);
  - Education, training and compliance with guidelines (owners, staff).
- **Treatment:** Antimicrobial stewardship policies (ASPs) (prudent antimicrobial use).

These methods should be combined in order to obtain the maximum effect, and organized in a written, well-defined infection control plan (ICP). In human medicine, it is estimated that 30-70% of HAIs is preventable (197), and that the costs related with such plan would equal the amount saved when approximately 6% of the HAIs are prevented (198). Structuring an effective system not only maximizes the quality of healthcare but also enhances the facility's reputation, minimizes the costs related with AMR, also reduces zoonotic risk for clinical staff and protects against possible legal actions by owners(69). Unfortunately, studies on the area indicate only a minority of small animal veterinary hospitals have such programs (0%–31%) (199,200), and shared guidelines are still scarce (201). However, the first step to start (the "basis of the pyramid" in Figure 12) is represented by surveillance. Indeed, although it is well recognized that the old claim that "you can't manage what you don't measure" is not always necessarily true, in the field of healthcare it is certainly much easier to manage what you can measure.

Figure 22. The pyramid of infection control. Adapted from *(202)*

The importance of surveillance

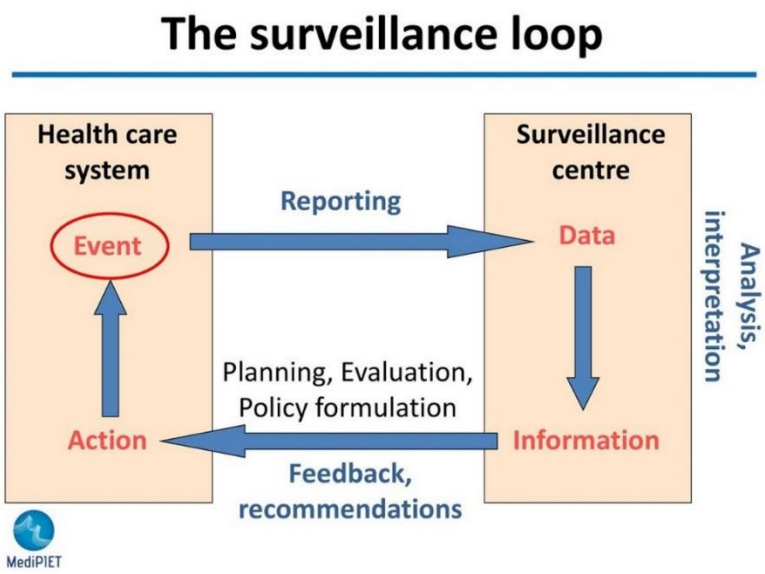
"*Good surveillance does not necessarily ensure the making of the right decisions, but it reduces the chances of wrong ones***."**

Alexander D. Langmuir (1963)(203)

Surveillance is defined as "systematic collection, analysis, interpretation, and dissemination of data regarding a health-related event for use in public health action to reduce morbidity and mortality and to improve health" (160). Collecting, analyzing, and interpreting data is indeed the initial step to identify critical issues and priorities. Estimating the endemic (expected) AMR and HAI rates, the spread of specific pathogen and the degree of environmental contamination is the basis for implementing effective and tailored strategies such as ICPs and ASPs, as much specific as possible for the identified priorities and based on the evidence of data, rather than theoretic threat. Understanding of endemic rates can be useful not only because they actually accounts for the majority of HAIs (204), but also to allow the comparison with other facilities, to establish benchmarks, to serve as a baseline for interventions, to allow for more accurate risk analysis (e.g., SSI rates), to indicate where to allocate the economic efforts, and to provide a greater overall awareness of the importance of HAIs and AMR. Furthermore, surveillance allows the early identification of potential HAI cases, avoiding that they pass below the radar for extended periods because of the lack of centralized data reporting or communication, resulting in outbreaks, with substantial environmental contamination, patient morbidity (and potentially mortality), and even increased zoonotic disease risk for staff and clients. Indeed, the detection and isolation of patients infected with MDR bacteria is a top priority in the prevention and control of HAI outbreaks (205). One Scottish study from human medicine suggested that the lack of a well-organized surveillance system brought to a longer time taken to first recognize HAIs (206); one recent Indian study showed that a low incidence of HAIs was related with the strict practice of surveillance in a neurosurgery unit (207). Additionally, performing surveillance over time allows to evaluate the real effectiveness of ICPs and ASPs in the specific setting (208) (see Figure 13).

In small animal practice, surveillance systems are still rare in hospitals and big clinics (170,208); at a National level, in Europe, only few countries (Denmark, Finland, France, Norway, Sweden, Switzerland) have a National Surveillance Program specific for AMR in companion animals (31). Veterinary medicine has few mechanisms to incentivize generating or sharing AMR data (209); nevertheless, the ability to aggregate, analyze, and share this type of information in a manner that is region specific and timely would allow for (i) developing cumulative susceptibility information to guide prescribing practices, (ii) monitoring resistance trends, (iii) detecting emerging diseases, and (iv) potentially improving clinical decision making. In this context, the EU is planning to launch the European Antimicrobial Resistance Surveillance Network in Veterinary Medicine (EARS-Vet), that will include both livestock and companion animals, to get an overview of the current situation and establish European AMR monitoring systems (210).

Figure 13. Example of the surveillance workflow. Adapted from https://slideplayer.com/slide/12483038/



Types of surveillance

Currently, there is no standardization in defining surveillance programs in small animal veterinary hospitals. Given that every facility has unique structural, organizational and management characteristics, infection control strategies (including surveillance efforts) should be tailored for each facility; there is no "one size fits all." Nevertheless, there are key characteristics that each surveillance system should take into consideration when developing the program (208):

- Delineate specific objectives and identify methods to address each;
- Select and define outcome and processes to be measured;
- Define the population to be monitored;
- Outline the data collection process, time period, and analytical method;
- Establish critical limit that will activate action;
- Develop a feedback mechanism;
- Document the plan in writing.

An approach that could be useful for this purpose is the use of electronic medical recording systems (EMRS), which can also automate data collection. In this regard, a working group from the University of Berlin has presented a software, following the model of what already happens in human medicine, called VISKIT (Veterinary Infection Surveillance Kit), which aims to harmonize the recording, surveillance, and evaluation of HAIs in veterinary medicine (211). Nevertheless, although this automatic data collection may improve efficiency and offer a unique opportunity to create specific data entry/collection systems, it does not necessarily improve accuracy (208). There are different types of surveillance, each with different characteristics, which can also be combined to achieve more effective results (see Table 5).

1109 **Table 5. Example of different surveillance systems that can be implemented. Adapted from** *(208)*

Type of surveillance	Advantages	Disadvantages	Examples
Passive surveillance	Easy; relatively inexpensive	Limitations in data quality; Data not specifically collected for surveillance purposes; Potentially very time-consuming.	All diagnostic samples culture positives from hospitalized patients are reported; Retrospective analysis of laboratory and pharmacy databases.
Active surveillance	Primary data source; Better quality and more complete data; Can be representative of specific patient groups of interest and the overall hospital population; Allows collection of data customized to address specific questions of interest	Greater investments required (time and money)	Fecal sample culture of all hospitalized patients on admission and twice weekly for the duration of hospitalization; Regular environmental cultures throughout the hospital; Collection of information about risk factors and patient movements during hospitalization
Syndromic surveillance	Easy; Inexpensive.	*No laboratory diagnosis;* *Nonspecific indicators of disease*	3–5 fecal cultures from all patients (small and large animal) developing specific disease (e.g. diarrhea of unknown origin) during hospitalization
Targeted surveillance	Generally accurate (both if active and passive); Focuses on patient population/agents/infections of concern; Less difficult and less expensive than larger surveillance programs.	Needs a previous, larger surveillance program that highlights risks and endemic baselines.	Sample culture of all patients with pre-determined risk factors highlighted; Environmental culture of selected locations when an increase (above baseline) in positive patients or positive environmental samples is detected

1110

Microbiological Surveillance. Microbiological surveillance, which uses laboratory methods for the identification and possible typing of bacterial strains, is normally divided into two types:

- **Passive Surveillance**: Involves using data from samples collected for diagnostic purposes or other purposes. By collecting and organizing data already available, the epidemiological situation can be monitored. It can be applied to all hospitalized patients or directed categories at risk (targeted passive surveillance), such as patients in intensive care, for example. This type of microbiological surveillance has the advantage of being practical and inexpensive, as it does not require the use of additional diagnostic investigations (212). Passive surveillance leads to the creation of a centralized database that allows the estimation of the endemic rates and the comparison of results over time. Data collection can be both retrospective (considering the patients' history) and prospective (performed on new patients). An example of retrospective surveillance is represented by an Australian study from 2017 (213), which collected and analyzed coagulase-positive staphylococci strains from 22 veterinary laboratories isolated for diagnostic purposes from dogs and cats. Another example is an Italian study conducted on 590 clinical samples taken from dogs for diagnostic purposes, showing a 2% prevalence of MRSP of the total (214). An example of prospective passive surveillance is represented by a Canadian study from 2014 on surgical site infections (SSI) in dogs: through telephone follow-ups up to 30 days (1 year for surgical implants) after surgery, an estimated prevalence of 3% SSI was determined (184). Furthermore, passive surveillance can be analyzed in relation to antimicrobial use (AMU) over time, in order to monitor the relation between AMR and AMU (160).

- **Active Surveillance**: Involves actively sampling patients and/or other matrices (environment or hospital staff) to search for agents potentially responsible for HAIs. This is a more expensive method that requires more time but provides higher quality and sensitivity information(69). It is usually performed in large facilities such as Veterinary Teaching Hospitals, which have dedicated staff. As for passive surveillance, active surveillance can be targeted only at certain types of patients or departments: an example is the MRSP surveillance program at the Colorado State University Veterinary Teaching Hospital (CSU-VTH) for dogs with dermatological and oncological pathologies (215,216). Active surveillance can also be aimed at searching for a specific etiological agent, as in a Spanish study from 2016 that describes a prevalence of 0.6% carbapenemase-producing *Enterobacter*iaceae among 160 dogs arriving at the Fuenlabrada hospital undergoing rectal swab(217). Or targeted only in the case of a suspected outbreak, as described by a Canadian study from 2007, in which, after isolating a strain of methicillin-resistant Staphylococcus aureus (MRSA) from the tracheostomy tube of a dog hospitalized in intensive care, active sampling was performed in the following days, showing a 23% MRSA prevalence from nasal swabs of other animals and an apparent acquisition incidence of 20% (218). Another example is the description of an MRSP outbreak among dogs and cats hospitalized at the Helsinki Veterinary Hospital (219). An alternative option is to carry out active surveillance, whether targeted or not, in a pulsed manner, i.e., at programmed time intervals (for example, monthly, quarterly, or on a specific day of the week)(220). In a study on veterinary hospitals accredited by the American

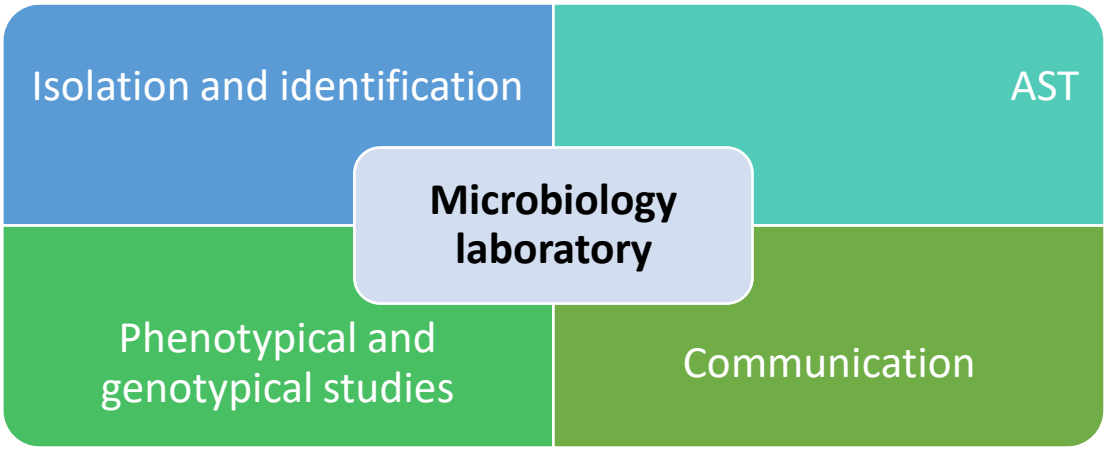
Veterinary Medical Association, it emerged that many had surveillance systems but were not performed at defined intervals, making estimates of endemic rates imprecise and not systematic (195). Active surveillance can be applied, for example, for the study of commensal bacteria resistance profiles to identify patients carrying bacteria with MDR patterns and treat them accordingly (e.g., isolating them), as described in the study conducted by Dazio et al. for the research of multi-resistant bacteria in small animals hospitalized at different veterinary facilities in Switzerland (221). Active surveillance can be performed on patients or on the environment and clinical staff (environmental surveillance). In fact, the environmental contamination of veterinary hospitals has been associated with various HAI outbreaks (219,222,223). For example, a report from the Ohio State University College of Veterinary Medicine (224) describes the use of environmental active surveillance within a broader infection control program. Devices can also be potential reservoirs of HAIs, such as cellphones in a Canadian study from 2012, where a prevalence of 1.6% MRSP and 0.8% MRSA was detected, respectively (225). The same goes for the hands and clothing of healthcare personnel, which can often act as vectors for HAI transmission: as in the case of a veterinarian carrying asymptomatic MRSA associated with post-operative MRSA infections in five dogs (226). Another example is a Canadian study from 2013 on the laboratory coats of veterinary medical staff at the Ontario Veterinary College Health Sciences Centre for MRS research through electrostatic cloths, with a prevalence of 17.5% (227). The air circulation system can also host potentially HAI-causing bacteria: for example, a study from 2013 revealed a 12.5% prevalence of MRS in the air of various departments of the University of Arizona Veterinary Hospital (173). Active environmental surveillance can be combined with patient surveillance, as reported by a study from Ohio State University, where annual surveillance for MRSA was conducted on both the environment and visiting dogs, with a prevalence of 13.5% and 5.7%, respectively (174).

- **Syndromic Surveillance**. Syndromic surveillance is based on the observation of clinical events/signs that may be indicative of infection before confirmation from the laboratory arrives (228). It has the advantage of being easy to apply and immediate. Some European countries, such as Switzerland, the United Kingdom, or the Netherlands, have developed veterinary syndromic surveillance programs for the prevention and containment of outbreaks (229). By monitoring clinical conditions such as gastrointestinal infections or fever of unknown origin, veterinarians can implement control measures to reduce the risk of transmission. In this case, syndromic surveillance can also be targeted towards a single event, such as in a study by Jones et al. on diarrhea (230). It can be used to collect data on prevalence, incidence, and risk factors within the patient population (231). A study from 2013 showed that 16.3% of dogs and 12% of cats admitted to intensive care had recorded 1 or more syndromic events during hospitalization (169). It is a method that has low specificity in case of outbreaks but high sensitivity guaranteed by specific clinical markers (232), such as fever of unknown origin, acute diarrhea, localized inflammation or surgical site infections.

The role of the microbiology laboratory

Success in surveillance programs relies on active involvement of the microbiology laboratory. The laboratory personnel must be sensitized and involved with the clinical staff, suggesting necessary approaches, and actively supporting and collaborating within the program (208). The microbiology laboratory plays a crucial role in producing information to support the control and management of AMR and HAIs within hospital facilities, primarily through: i) isolation and identification of bacterial agents involved; ii) assessment of antimicrobial sensibility; iii) phenotypic and genotypic typing studies to evaluate epidemiological aspects (233); iv) communication with clinical staff (see Figure 14).

Figure 14. Schematic representation of the different tasks of the laboratory of microbiology in the surveillance program.



i) **Isolation and identification of bacterial agents.** Bacterial isolation and correct identification must be carried out with extreme precision. The first step involves sample collection and transport: improperly collected or transported samples can lead to inaccurate results even in the best laboratories, and may result in improper clinical and therapeutic management of the patient. Bacterial identification is generally performed using phenotypic methods, including the evaluation of growth conditions, macroscopic and microscopic colony morphological aspects, and biochemical tests 17,18. Complete identification ideally takes a couple of days, delaying proper patient and/or outbreak management. An alternative method emerging in clinical microbiology for bacterial identification is the use of mass spectrometry through the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) instrument, which is a rapid, economical, and reliable bacterial identification method described in both human and veterinary microbiology, providing correct identification from isolated colonies within minutes (234,235). During an epidemiological investigation, targeted analyses may be necessary on patient samples, hospital staff, medical devices, hospital environment, infusion-related products and devices, disinfectants, and others 23. Special culture media, such as selective (inhibiting the growth of species other than the one of interest), differential (differentiating the species of interest from others), or enrichment media may be used for this purpose, depending on the suspected bacterial load.

ii) **Evaluation of antimicrobial sensibility.** This must be carried out using standardized methods. The antibiogram, generally performed using the Kirby-Bauer method, involves the in vitro assessment of the efficacy of different classes of antibiotic molecules against a specific bacterial agent. Antimicrobial sensibility tests (ASTs) can be performed using phenotypic methods (such as the Kirby-Bauer technique or measurement of the minimum inhibitory concentration) or genotypic methods, to highlight resistance genes or products of resistance mechanisms Regardless of the method used, it is important that it be carried out using procedures defined by accredited scientific entities such as the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST), both of which have specific areas for veterinary medicine. Laboratory personnel must immediately inform clinical staff when resistant organisms are identified and when new or unusual phenotypic resistance patterns are detected, so that appropriate precautions can be instituted(236).

iii) **Phenotypic and genotypic typing studies to evaluate epidemiological aspects.** Typing nosocomial pathogens is extremely useful for epidemiological investigations, in order to determine if different isolates produce identical or different results in one or more tests. If isolates from different patients give the same result, it means they may have the same origin, and were therefore likely transmitted from patient to patient or from a common source (236).

iv) **Communication with clinical staff.** The laboratory should report all results as quickly as possible. In most situations, routine reporting on the hospital's patient management system is adequate. However, some results take precedence over others because they will affect patient care or require an immediate response from staff. The microbiologist should record all data so that they can be analyzed comprehensively to establish the frequency with which specific microorganisms cause infections and to assess any trends that may suggest the existence of an HAI outbreak. It is important to establish a routine relationship between the microbiologist and the clinician for the review of microbiological results, which must necessarily be correlated with clinical and epidemiological results. These moments allow clarifying whether patients are colonized or infected, guiding clinicians in the choice of antimicrobials to use following the evaluation of sensitivity profiles, ensuring that samples from epidemiological investigations are properly evaluated, and focusing laboratory efforts optimally (237).

Chapter 2

Objectives and Project Design

This PhD project was conducted in the Operative Unit of the Veterinary Laboratory of Bacteriology (VeLaBac), part of the Avian Pathology Service (SEPAV) of the Department of Veterinary Medical Sciences, University of Bologna. It had different aims:

1) To obtain, analyze and compare local, epidemiological data about HAIs and AMR in pets through the development of a pilot surveillance program at the Small Animal Veterinary Teaching Hospital of Bologna;

2) Starting from the results, to highlight the key points for the elaboration of tailored guidelines for an internal Infection Control Program, that includes:
   - Long-term surveillance;
   - Prevention policies;
   - Outbreak management,
   - Antimicrobial Stewardship;
   - Communication and dissemination.
3) To expand the knowledge about the role of small animal practice (specifically Veterinary University Hospitals) in a global overview of AMR, with a focus on specific pathogens in the human-animal interface.

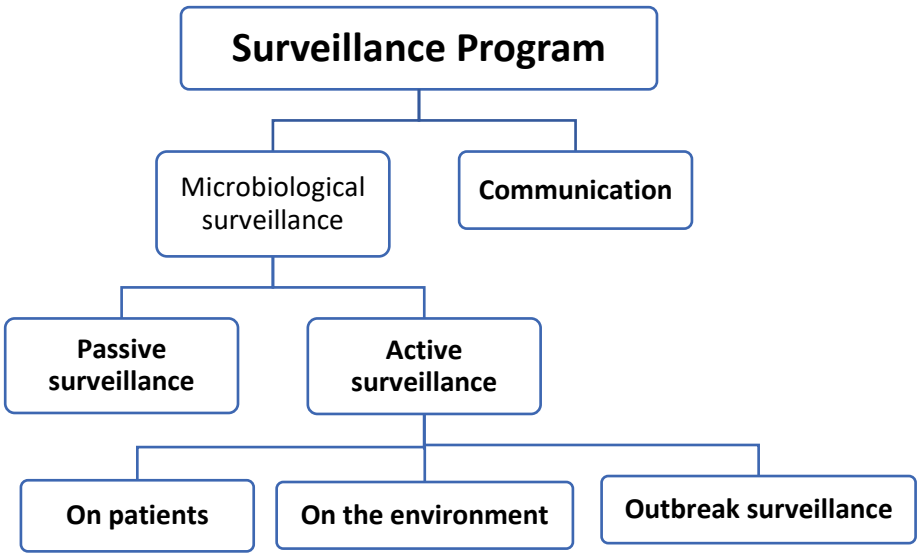
Project design

This PhD thesis will be organized following the project design. The surveillance program started in November 23th, 2020, and was concluded on May 30st, 2023, and was composed by five parts:

- Passive surveillance;
- Active surveillance on patients;
- Active surveillance on the environment;
- Outbreak surveillance;
- Education and communication.

Each part will be described in separate chapters, with a detailed description of the specific aims, the methodology and the discussion of the results. The last chapter ("Conclusions") will organize all the results in order to address the aims 2) and 3).

Figure 3. Schematic overview of the Pilot Surveillance Program organization.



Chapter 3

Passive surveillance

3.A. Specific aims

Passive surveillance aimed at collecting and analyzing data of clinical samples from the Bologna VTH submitted to the Veterinary Laboratory of Bacteriology for diagnostics purposes. Information about specimen type, bacterial species, AMR/MDR percentages and associated risk factors were recorded, with a specific focus on potential HAIs and ESKAPE pathogens.

3.B. Materials and methods

Passive surveillance was executed through the perspective collection and analysis of data originated from routine diagnostic bacteriological cases received by the Veterinary Laboratory of Bacteriology (VeLaBac) at the Department of Veterinary Medical Sciences, from the internal Small Animals VTH. We collected data from November 23rd 2020 to May 31st 2023 on isolates from biological specimens from dogs and cats referred to the Bologna VTH, submitted for culture, bacterial isolation, identification, and antimicrobial susceptibility testing (AST). Only isolates with a reported AST result were included in the study.

3.1. Culture and Identification of Bacterial Isolates. Depending on the specimen type, standard microbiological procedures were used and are listed in Table S1. The different specimen types, sampled by the submitting veterinarian, were classified as follows: abdominal/pleural/peritoneal effusion, abscess, bile, biopsy, bone fragment, bladder stone, blood culture, bronchoalveolar lavage, ear swab, exudate, nasal swab, synovial liquid, SSI and surgical implants, transtracheal lavage/bronchial brush, urine (both by cystocentesis and from catheterization), vaginal/uterine swab, wound and "others." The "others" category included specimens with very few frequencies or without specific indications.

After the incubation of 24–48 hours at 37+−1°C, plates that presented adequate growth were considered positive according to guidelines (238). Colonies were evaluated morphologically and the identification at species level of each isolate was assessed through the matrix-assisted laser desorption–ionization time-of-flight mass spectrometry method (MALDI-TOF MS) using a Bruker Daltonics MBT SMART equipment, and the Biotyper Real Time Classification software v3.1 (Bruker Daltonics, Germany), following manufacturer's instructions and considering a species-level identification when the ID score was >2 (green—high accuracy), or >1.8 (for *Staphylococcus* spp. isolates). If isolates identified as the same bacterial species were isolated from different specimens in the same patient at the same time, they were considered separately only in the specimen analysis.

3.2. **Data Collection**. For every isolate, the following variables were collected from the patient: species (dog/cat), age (years, rounded up), type of specimen (see above), previous hospitalization/surgery in the past 30 days (yes/no), hospitalization at the time of the sampling (yes/no), hospitalization in intensive care unit (yes/no), surgery at the time of the sampling (yes/no), days of hospitalization at the time of the sampling (number of days), invasive device use in the previous 90 days (yes/no), antimicrobial use in the past 90 days (yes/no), and current antimicrobial use (yes/no).

3.3. Antimicrobial Susceptibility Testing. AST of all the isolates was performed using the Kirby–Bauer disc diffusion method, according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (239). For every tested drug, each isolate was classified as susceptible (S), intermediate

(I), or resistant (R) based on the CLSI(239) veterinary breakpoints. Overall, 12 antimicrobials from 8 antimicrobial classes were included in the final analysis (Table S2). All the discs were purchased from Oxoid (Oxoid, Milan, Italy). For Gram-negative bacteria, clindamycin and erythromycin were not tested due to their low-activity rates (240). For certain species, antimicrobial agents known to exhibit expected resistance phenotypes according to the National Reference Laboratory for AMR (241) were not tested. Isolates identified as the same bacterial species found in the same patient at different time points were considered separately only when they showed different AST results. For AST interpretation, the strains were divided into "susceptible" and "non-susceptible," as suggested by Sweeney et al. (68), where the "non-susceptible" category included resistant and intermediate isolates. Isolates that were non-susceptible to at least one antimicrobial drug were considered as AMR isolates, and isolates that were not susceptible to at least one antimicrobial drug in three or more antimicrobial classes were considered as MDR(242).

3.4. Phenotypical resistance assessment and whole genome sequencing of Enterobacterales resistant to piperacillin-tazobactam. A further analysis on some selected Enterobacterales isolates from different periods that expressed phenotypic resistance towards piperacillin-tazobactam at the AST was performed in collaboration with the Antimicrobial Resistance Unit of the Veterinary Medicine Department at the Complutense University, Madrid, Spain. This analysis included a deeper assessment of piperacillin-tazobactam resistance through a Minimum Inhibitory Concentration by broth microdilution, and a complete last-resort antibiotic resistance profile through the Sensititre GN7F AST Plates (ThermoFisher Scientific, Lenexa, US). Additionally, some of these isolates were submitted for Whole Genome Sequencing through Illumina and Nanopore technology.

3.5 Statistical Analysis. Descriptive statistics was performed considering type of specimen, bacterial species, Gram stain, single-drug non-susceptibility percentages and AMR/MDR proportions, that were evaluated by dividing the number of AMR/MDR strains by the number of total tested strains. A temporal analysis was performed for AMR/MDR percentages based on monthly trends. Besides this general overview, descriptive statistics on isolates belonging to species part of the ESKAPE group (*E.faecium, S.aureus, K.pneumoniae, A.baumannii, P.aeruginosa, Enterobacter* spp.(152)) and from suspected HAIs (following the definition by Haque et al. (158), was performed, considering AMR/MDR proportion, single nonsusceptibility percentages, and bacterial species/specimen relation (for isolates from suspected HAIs). Furthermore, a temporal analysis of suspected HAIs was included in the study, considering only monthly new suspected HAI cases. A new case was defined as a new patient with one or more bacterial isolates from a specimen considered a suspected HAI.

The association between Gram stain and AMR/MDR was tested with the χ 2 test. The alpha risk was set to 0.05. The association between AMR/MDR isolates and the independent variables was calculated using univariable logistic regression analyses. The same analysis was used to assess the association between isolates from suspected HAIs and the independent variables, excluding "previous hospitalization/surgery" and "hospitalization at the moment of the sampling", and considering only patients with 2 or more days of hospitalization. Normality and heteroskedasticity of data were assessed with the Shapiro–Wilk test and the Levene's test. Only variables with a $p < 0.05$ in the initial assessment were included in the multivariable logistic model built using forward stepwise selection at $p < 0.05$. Data were checked for multicollinearity with the Belsley–Kuh–Welsch technique. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. Statistical analysis was performed with MedCalc (version v22.009).

3.C. Results

NB: the partial results of this analysis has been published on the Journal "Transboundary and Emerging Disease" in a paper titled "Monitoring the Prevalence of Antimicrobial Resistance in Companion Animals: Results from Clinical Isolates in an Italian University Veterinary Hospital (243)**.**

From a total of 3727 specimens (average 124 specimens/moths) collected from 2062 patients (1493 dogs and 569 cats), 1204 (32.3%, 970 from dogs and 179 from cats) were positive for bacterial growth (average 40 positive specimens/month). One thousand and forty-eight (87%) positive specimens grown in monoculture (with a single bacterial species isolated), while 156 (13%) were mixed infections (with two or more different bacterial species). A total of 1342 isolates (average 45 isolates/month) with a reported AST was recorded.

Specimen analysis. The specimen type distribution is listed in Table 1. Urine (n = 680, 56.5%) was the most frequent specimen in both cats and dogs, followed by ear swab in dogs (n = 81, 8.4%) and nasal swab (n = 17, 7.3%) in cats.

Table 1. Distribution of the 1204 specimens (total number and percentages) considering specimen type and species of origin (dogs/cats).

Type of specimen	total	%	cats	%	dogs	%
	1204		234		970	
Abdominal/pleural/peritoneal effusion	32	2.7%	9	3.9%	23	2.4%
Abcess	22	1.8%	6	2.6%	16	1.7%
Bile	13	1.1%	6	2.6%	7	0.7%
Biopsy	30	2.5%	5	2.1%	25	2.6%
Bone fragment	1	0.1%	1	0.4%	0	0.0%
Bladder stone	2	0.2%	0	0.0%	2	0.2%
Blood culture	60	5.0%	13	5.6%	47	4.9%
Bronchoalveolar lavage	10	0.8%	0	0.0%	10	1.0%
Ear swab	95	7.9%	14	6.0%	81	8.4%
Exudate	19	1.6%	5	2.1%	14	1.4%
Nasal swab	31	2.6%	17	7.3%	14	1.4%
Synovial liquid	2	0.2%	0	0.0%	2	0.2%
SSI and surgical implants	60	5.0%	10	4.3%	50	5.2%
Transtracheal lavage/bronchial brush	3	0.3%	1	0.4%	2	0.2%
Urine	680	56.5%	117	50.0%	563	58.0%
Vaginal/uterine swab	55	4.6%	12	5.1%	43	4.4%
Wound	58	4.8%	14	6.0%	44	4.5%
Others	31	2.6%	4	1.7%	27	2.8%

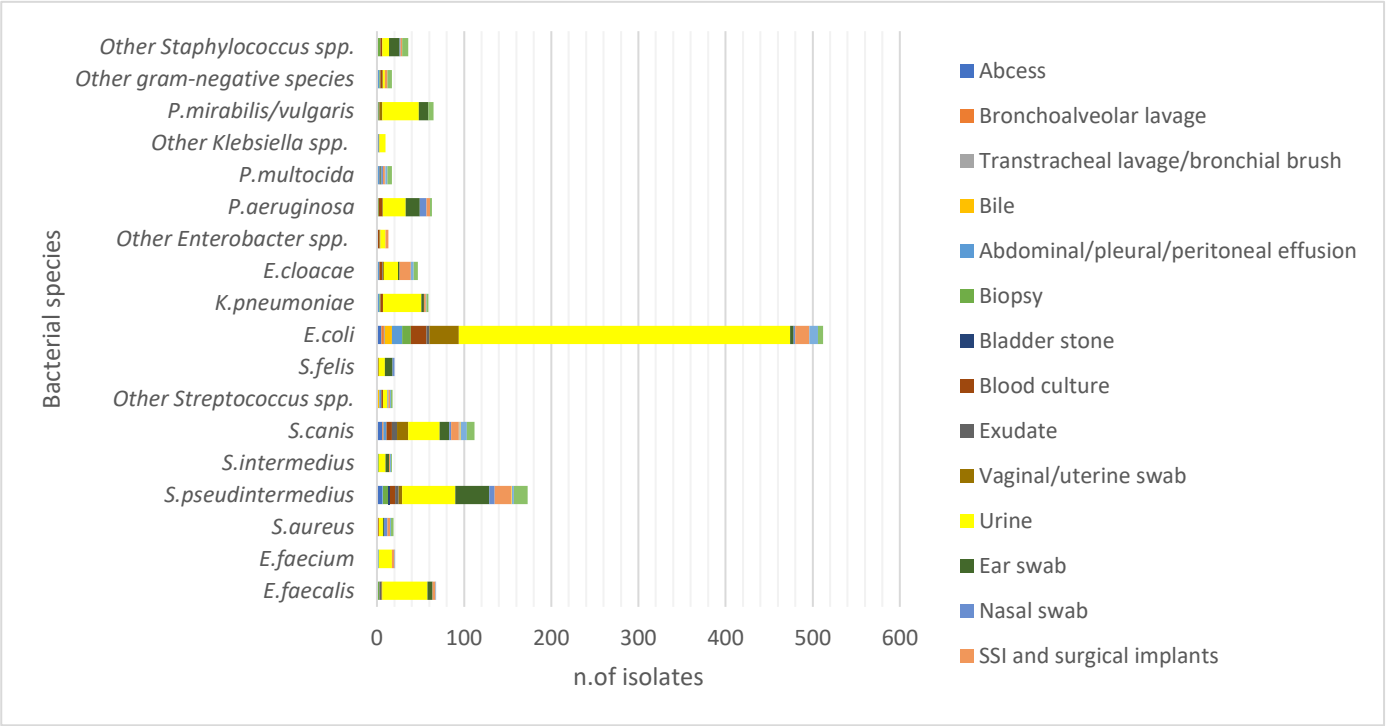
Isolates analysis. Bacterial species. Isolates from cats were 19.7 % (n = 264) of the total, while 80.3% (n = 1078) were isolated from dogs. Eight-hundred thirty-two (62 %) strains were classified as Gram-negative, while 510 (38%) as Gram-positive. Distribution of bacterial species is shown in Table 2. *E.coli* (37.1%) was the most commonly isolated species, followed by *S. pseudintermedius* (12.5*%), S. canis* (8.1%), and *E. faecalis (*5.1%). Notably, *S. pseudintermedius* was considerably less frequent in cats than in dogs (5.4% vs. 14.4%), while *E. faecalis* was more frequent in cats

(10.42 % vs. 3.99%). The species distribution according to specimen of origin is shown in Figure 1. Considering the five most common specimen types, *E.coli* was the most isolated species in urines and vaginal/uterine swabs, while *S.pseudintermedius* in ear swabs, SSIs and wounds.

Table 2. **Distribution of the 1342 analyzed isolates (number of isolates and %) based on bacterial species identification, considering species of origin (cats/dogs).**

	n.of isolates	%	dogs	%	cats	%
A.baumannii	3	0.2%	3	0.3%	0	0.0%
B.bronchiseptica	4	0.3%	3	0.3%	1	0.4%
B.pyogenes	8	0.6%	4	0.4%	4	1.7%
C.auriscanis	8	0.6%	7	0.7%	1	0.4%
C.perfringens	6	0.5%	6	0.6%	0	0.0%
C.urealyticum	5	0.4%	2	0.2%	3	1.3%
Citrobacter spp.	8	0.6%	8	0.7%	0	0.0%
E.cloacae	47	3.5%	39	3.6%	8	3.3%
E.coli	498	37.1%	413	38.3%	85	35.4%
E.faecalis	68	5.1%	43	4.0%	25	10.4%
E.faecium	20	1.5%	15	1.4%	5	2.1%
H.haemoglobinophilus	4	0.3%	4	0.4%	0	0.0%
K.pneumoniae	58	4.3%	48	4.5%	10	4.2%
Neisseria spp.	4	0.3%	3	0.3%	1	0.4%
Other gram positive species	7	0.5%	5	0.5%	2	0.8%
Other gram negative species	16	1.2%	14	1.3%	2	0.8%
Other *Corynebacterium* spp.	5	0.4%	5	0.5%	0	0.0%
Other *Enterobacter* spp.	13	1.0%	10	0.9%	3	1.3%
Other *Enterococcus* spp.	6	0.5%	5	0.5%	1	0.4%
Other *Klebsiella* spp.	10	0.8%	10	0.9%	0	0.0%
Other *Staphylococcus* spp.	36	2.7%	27	2.5%	9	3.8%
Other *Streptocuccus* spp.	17	1.3%	16	1.5%	1	0.4%
P.aeruginosa	61	4.6%	50	4.6%	11	4.6%
P.canis	8	0.6%	7	0.7%	1	0.4%
P.mirabilis/vulgaris	64	4.8%	52	4.8%	12	5.0%
P.multocida	17	1.3%	2	0.2%	15	6.3%
S.aureus	19	1.4%	11	1.0%	8	3.3%
S.canis	108	8.1%	88	8.2%	20	8.3%
S.felis	20	1.5%	0	0.0%	20	8.3%
S.intermedius	17	1.3%	15	1.4%	2	0.8%
S.maltophilia	3	0.2%	3	0.3%	0	0.0%
S.marcescens	4	0.3%	3	0.3%	1	0.4%
S.pseudintermedius	168	12.5%	155	14.4%	13	5.4%
*Salmonell*a spp.	2	0.2%	2	0.2%	0	0.0%

Figure 1. Distribution of the 1342 isolates according to bacterial species and specimen of origin. Only species with >10 isolates recorded were included.



AMR and MDR percentages. The overall AMR percentage was 77% (n = 1033), while MDR percentage was 41.6 % (n = 559). Considering only AMR strains, 54.1% had an MDR pattern. The AMR percentages were, respectively, 70.3% and 87.8 % in Gram-negative and Gram-positive isolates (OR = 3.05; CI 95%2.25–4.14; p<0.01), while the MDR percentages were, respectively, 33.1 % and 55.7% (OR = 2.54; CI 95% 2.02–3.19; p<0.01). Table 3 shows the single non-susceptibility percentages for every tested drug. The highest nonsusceptibility percentages were recorded for clindamycin, erythromycin, ampicillin, and enrofloxacin (58.4%, 54.6%, 50%, and 45.8%, respectively). The lowest non-susceptibility percentages were recorded for amikacin and piperacillin–tazobactam (9.4 %and 15%, respectively). Non-susceptibility and AMR and MDR percentages considering bacterial species are shown in Table 4. The highest AMR percentages were recorded for *E.faecium, P.aeruginosa* (both 100%) and *Staphylococcus* spp. other than *S.pseudintermedius, S.intermedius* and *S.aureus* (94.1%), while the highest MDR percentages were recorded for *E.faecium* (90%), *Enterobacter* spp. other than *E.cloacae* (76.9%) and *S.pseudintermedius* (73.2%).

Table 3. Nonsusceptibility percentages for every single tested antimicrobial of the 1342 analyzed isolates.

	n.isolates tested (%of total isolates)	% NS	GRAM + tested	% NS	GRAM - tested	% NS
Amikacin	1122 (83.6%)	9.4%	290	5.9%	832	10.7%
Gentamicin	1215 (90.5%)	20.3%	383	30.3%	832	15.7%
Ampicillin	1141 (85%)	50%	510	46.5%	631	52.8%
Amoxicillin-clavulanate	1214 (90.5%)	20.3%	510	17.8%	704	22.2%
Piperacillin-tazobactam	1342 (100%)	15%	510	14.5%	832	15.3%
Cefazolin/cephalotin	1123 (83.7%	27.8%	420	18.6%	703	33.3%
Ceftiofur	1188 (88.5%)	24.2%	420	24.5%	768	24%
Tetracycline	1274 (94.9%)	43.6%	510	59.8%	764	32.8%
Clindamycin	421 (31.4%)	58.4%	421	58.4%	NT	NT

Erithromycin	421 (31.4%)	54.6%	421	54.6%	NT	NT
Enrofloxacin	1342 (100%)	45.8%	510	58.8%	832	37.9%
Trimethoprim–sulfamethoxazole	1281 (95.4%)	24.8%	510	23.1%	701	28.5%

FOOTNOTE: in the first column, the number of isolates tested (excluding intrinsic resistances) and its percentage in relation with the total number of isolates is reported. In the second column, the percentage and the number of nonsusceptible isolates for every tested drug is described. In the third and fourth column, the nonsusceptibility percentages and number (number of nonsusceptible isolates/number of total isolates tested) of isolates identified as Gram positive and Gram negative is reported. NT=Not tested (due to intrinsic resistance/low activity rates.

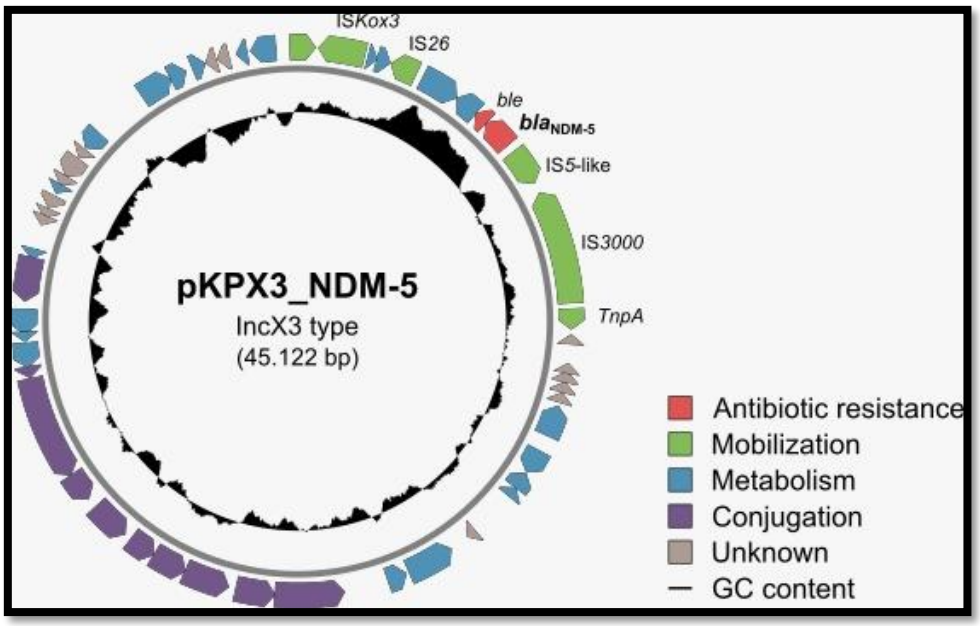
Table 4. Non-susceptibility, AMR and MDR percentages considering bacterial species (only for species with >10 isolates). In bold the highest percentages for every tested drug.

Species	AK	CN	AMP	AMC	TZP	KF/KZ	EFT	TE	CLI	ERI	ENR	SXT	% AMR	% MDR
E.cloacae	**14.9%**	19.2%	NT	NT	40.4%	NT	48.9%	25.5%	NT	NT	59.6%	51.1%	70.2%	46.8%
E.coli	10.6%	13.9%	61.5%	23.5%	12.7%	32.3%	20.1%	28.3%	NT	NT	30.1%	21.1%	71.3%	33.5%
E.faecalis	NT	16.2%	8.8%	7.4%	5.9%	NT	NT	55.9%	NT	NT	80.9%	25.0%	91.2%	27.9%
E.faecium	NT	35.0%	85.0%	**85.0%**	**80.0%**	NT	NT	85.0%	NT	NT	**100.0 %**	40.0%	**100.0 %**	**90.0%**
K.pneumoniae	8.6%	24.1%	NT	48.3%	44.8%	65.5%	60.3%	46.6%	NT	NT	70.7%	41.4%	84.5%	55.2%
Other *Enterobacter* spp.	0.0%	15.4%	NT	NT	61.5%	NT	**76.9%**	46.2%	NT	NT	69.2%	**53.9%**	84.6%	76.9%
Other *Klebsiella* spp.	10.0%	20.0%	NT	60.0%	40.0%	**80.0%**	50.0%	20.0%	NT	NT	30.0%	0.0%	90.0%	40.0%
Other gram-negative species	18.8%	31.3%	25.0%	12.0%	6.2%	18.7%	12.0%	18.7%	NT	NT	25.0%	31.2%	56.3%	18.8%
Other *Staphylococcus* spp.	5.9%	35.3%	76.5%	17.7%	5.9%	17.7%	17.7%	47.1%	**70.6%**	**76.5%**	94.1%	35.3%	83.3%	27.8%
Other *Streptococcus* spp.	NT	NT	11.8%	0.0%	0.0%	5.9%	5.9%	58.9%	52.9%	64.7%	70.6%	17.6%	94.1%	52.9%
P.aeruginosa	11.5%	14.8%	NT	NT	3.3%	NT	NT	**91.8%**	NT	NT	85.3%	NT	**100.0 %**	26.2%
P.mirabilis/vulgaris	12.5%	20.3%	18.8%	1.6%	0.0%	18.8%	3.1%	NT	NT	NT	28.1%	31.3%	51.6%	20.3%
P.multocida	11.8%	17.7%	5.9%	0.0%	5.9%	17.7%	5.9%	0.0%	NT	NT	5.9%	0.0%	29.4%	5.9%
S.pseudintermedius	3.6%	41.1%	**85.7%**	26.2%	20.2%	27.4%	38.7%	67.9%	66.1%	67.3%	50.0%	39.3%	89.3%	73.2%
S.aureus	5.3%	15.8%	79.0%	21.1%	15.8%	26.3%	31.6%	42.1%	21.1%	26.3%	47.4%	5.3%	84.2%	52.6%
S.canis	NT	NT	5.6%	0.9%	0.0%	2.8%	2.8%	79.6%	65.7%	50.0%	75.0%	3.7%	90.7%	58.3%
S.intermedius	5.9%	**47.1%**	82.4%	29.4%	17.7%	29.4%	41.2%	58.8%	58.8%	58.8%	41.2%	35.3%	82.4%	58.8%
S.felis	0.0%	5.0%	25.0%	0.0%	0.0%	0.0%	10.0%	5.0%	20.0%	20.0%	10.0%	0.0%	40.0%	10.0%

FOOTNOTE: AK=Amikacin; CN=Gentamicin; AMP=Ampicillin; AMC=Amoxicillin-clavulanate; TZP=Piperacillin-tazobactam; KF/KZ=Cephalotin/Cefazolin; EFT=Ceftiofur; TE=Tetracycline; CLI=Clindamycin, ERI=Erithromycin; ENR=Enrofloxacin; SXT= Trimethoprim–sulfamethoxazole; NT: Not tested due to intrinsic resistance/low activity rates.

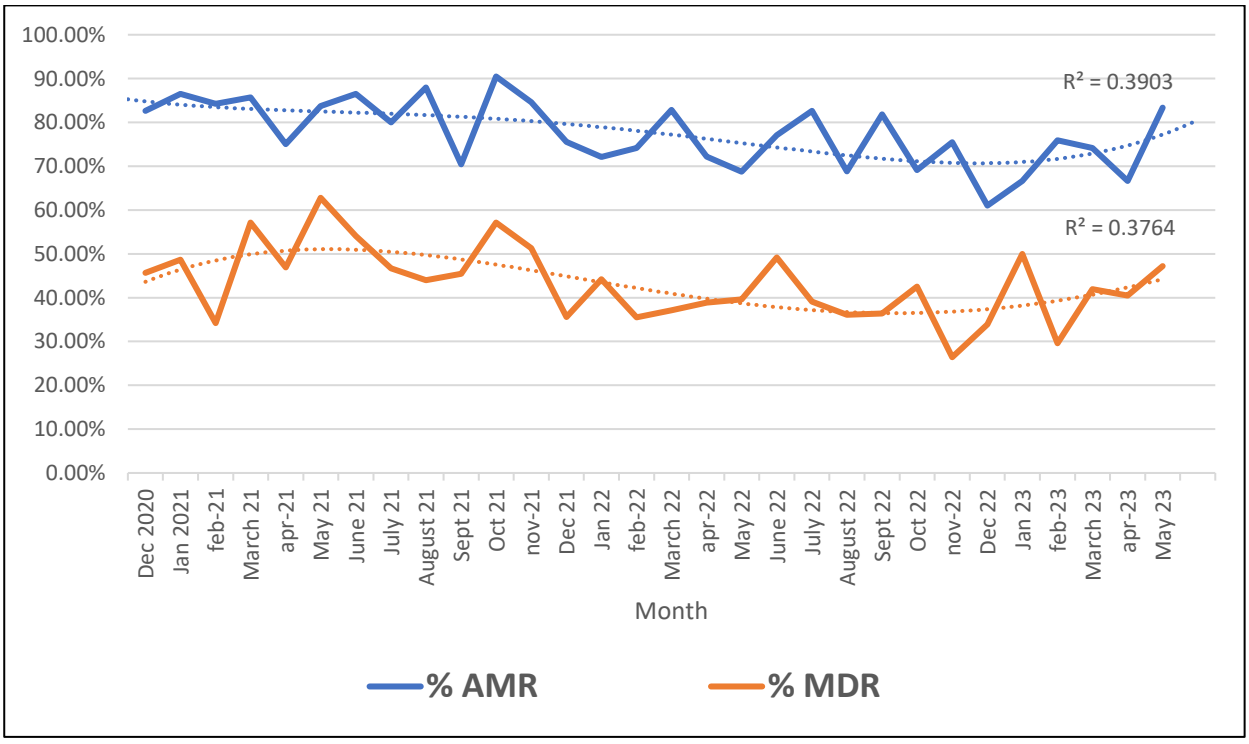
Thirty-two Enterobacterales isolates that expressed resistance to piperacillin-tazobactam were selected to be further analyzed. Twenty-two (68.8%) exhibited resistance levels higher than 64/4 mg/l. All of them also exhibited resistance to a wide variety of critical antibiotics, including human-reserved potentiated penicillins such as ceftolozane-tazobactam (17/32, 53.1%), ceftazidime-avibactam (11/32, 34.4%), and carbapenems such as imipenem (9/32, 28.2%) and ertapenem (15/32, 46.9%) (see Table S4). Ten isolates (4 *E.coli*, 4 *K.pneumoniae*, 1 *E.cloacae* and 1 *K.oxytoca*) were submitted to WGS, that revealed the presence of a wide number of various 3GCR genes (see Table S5), including $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{SHV-1}}$ *and* $bla_{\text{CMY-2}}$. Additionally, 3 isolates (two *K.pneumoniae* and one *E.coli*) with resistance levels to piperacillin-tazobactam >1024 mg/l and resistant to all carbapenems were found to possess the $bla_{\text{NDM-5}}$ gene on the IncX3 plasmid (see Figure 2). Notably, these isolates were all from suspected HAIs (two urines and one abdominal effusion).

Figure 2. Visual representation of the bla_{NDM-5} carrying IncX3 type plasmid found on 3/10 isolates (one *E.coli* and two *K.pneumoniae*) submitted for whole genome sequencing.



AMR/MDR temporal trend. The temporal trend of the percentage of AMR and MDR isolates is shown in Figure 3. Temporal trends of every tested drug are also shown separately (Table S6).

Figure 3. Temporal trend of AMR and MDR rates based on monthly records. The dotted line represents the polynomial trendline of the values. Also the value of R^2 is reported.



Risk factors analysis. Univariable analysis results for AMR/MDR and suspected HAI are shown in Table 5. In the multivariable analysis for AMR (Table 6) , species cat (OR 0.63, CI 95% 0.46–0.87; p <0.01), was associated with a lower proportion of AMR, while invasive device use in the previous 90 days (OR 1.59,CI 95% 1.03-2.47, p=0.02), antimicrobial use in the past 90 days (OR

2.58, CI 95% 1.94–3.43, p<0.01) and previous hospitalization/surgery in the past 30 days (OR 2.12, CI 95% 1.22-3.69, p=0.03) were associated with a higher proportion of AMR. In the multivariable analysis for MDR, invasive device use in the previous 90 days (OR 1.92, CI 95% 1.48-2.49, p<0.01), antimicrobial use in the past 90 days (OR 2.36, CI 95% 1.94–2.88, p<0.01), and current antimicrobial use (OR 1.48, CI 95% 1.18–1.86, p<0.01) were associated with higher percentages of MDR. In the multivariable analysis for suspected HAIs, antimicrobial use in the past 90 days (OR 1.84, CI 95% 1.39–2.42, p<0.01), current antimicrobial use (OR 2.39, CI 95% 1.73-3.31, p<0.01), invasive device use in the previous 90 days (OR 10.43, CI 95% 6.83-15.94, p<0.01) and days of hospitalization at the time of the sampling (OR 1.23, CI 95% 1.14-1.33, p<0.01) were associated with a higher proportion of isolates from suspected HAIs.

Table 5. Univariable logistic regression results from a study associating risk factors to AMR, MDR or suspected HAI in the 1342 analyzed isolates. Results considered significant (p<0.05, marked with *) were included in the multivariable analysis model. Odds ratios (OR) are reported with 95% Confidence Interval (CI).

Variables	**AMR**		**MDR**		**Suspected HAI**	
	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)
Species (dog)	**0.001***	0.62 (0.46 to 0.84)	0.16	0.82 (0.0.62 to 1.08)	0.52	1.13 (0.77 to 1.65)
Age (years)	0,37	0.99 (0.95 to 1.03)	0.25	0.98 (0.96 to 1.01)	**0.03***	0.96 (0.93 to 0.99)
Previous hospitalization/surgery in the past 30 days (yes)	**<0.0001***	4.18 (2.67 to 6.53)	**<0.0001***	2.95 (2.24 to 3.87)	NT	
Hospitalization at the time of the sampling (yes)	0.39	1.13 (0.85 to 1.50)	**0.0002***	1.57 (1.24 to 1.98)	NT	
Hospitalization in intensive care unit (yes)	0.41	0.82 (0.51 to 1.32)	0.73	1.07 (1.24 to 1.98)	0.28	0.78 (0.50 to 1.22)
Surgery at the time of the sampling (yes)	0.35	1.17 (0.84 to 1.65)	0.56	1.09 (0.82 to 1.44)	**0.0002***	1.98 (1.39 to 2.82)
Days of hospitalization at the time of the sampling (number of days)	**0.02***	1.14 (1.02 to 1.27)	**0.01***	1.08 (1.02 to 1.15)	**0.0002***	1.24 (1.11 to 1.38)
Invasive device use in the previous 90 days (yes)	**<0.0001***	3.35 (2.37 to 4.74)	**<0.0001***	2.89 (2.27 to 3.67)	**<0.0001***	15.36 (10.39 to 22.74)
Antimicrobial use in the past 90 days (yes)	**<0.0001***	3.29 (2.49 to 4.34)	**<0.01***	2.85 (2.36 to 3.45)	**<0.0001***	2.98 (2.39 to 3.71)
Current antimicrobial use (yes)	**0.01***	1.41 (1.08 to 1.83)	**<0.0001***	1.86 (1.51 to 2.30)	**<0.0001***	3.88 (2.99 to 5.05)

FOOTNOTE: *Included in the multivariable analysis.

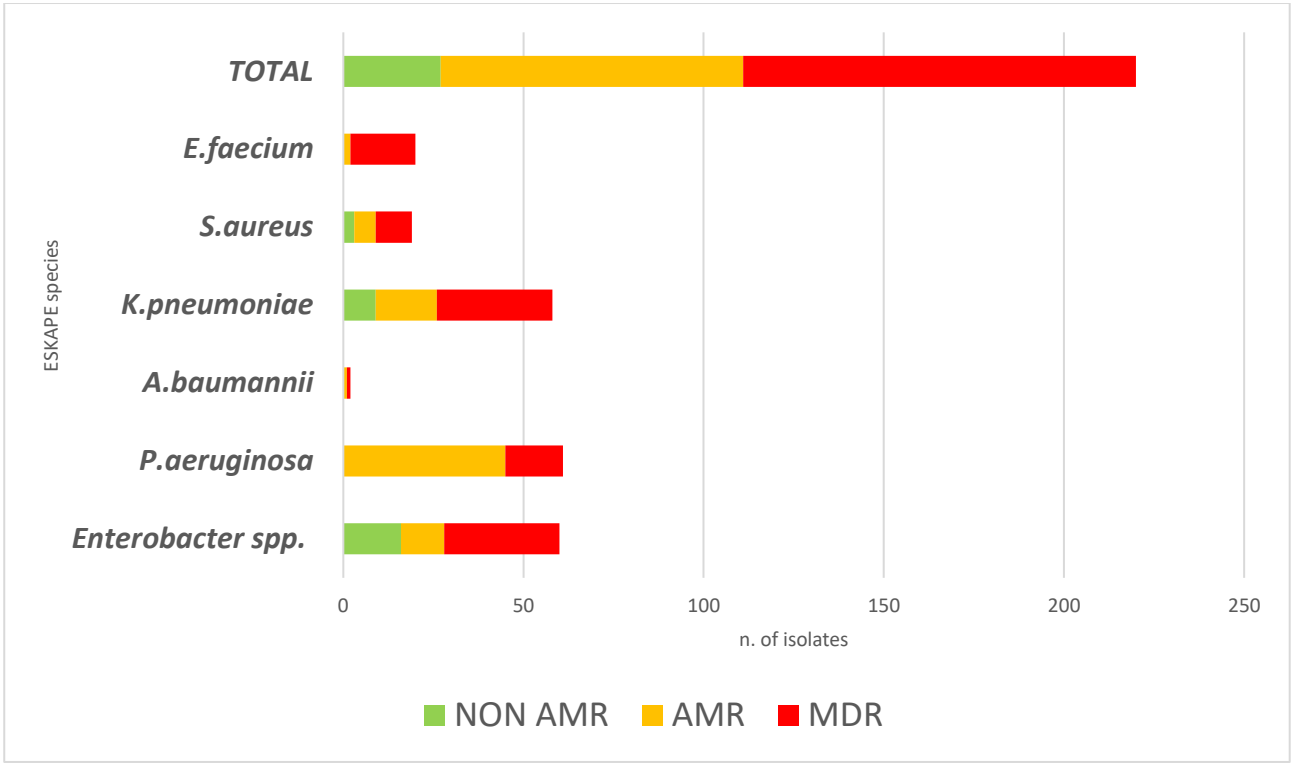
Table 6. Results of the multivariable analysis results from a study to assess the association of risk factors included in the model to antimicrobial resistance (AMR) or multi-drug resistance (MDR) in the 1342 analyzed isolates. Results were considered significant with a p <0.05. Only results with a significant p-value (marked with *) are shown.

Variables	AMR p-value	MDR p-value	Suspected HAI p-value
Species (dog)	**<0.01***		
Age (years)			
Previous hospitalization/surgery in the past 30 days (yes)	**0.03***		
Surgery at the time of the sampling (yes)			
Days of hospitalization at the time of the sampling (number of days)			**<0.01***
Invasive device use in the previous 90 days (yes)	**0.02***	**<0.01***	**<0.01***
Antimicrobial use in the past 90 days (yes)	**<0.01***	**<0.01***	**<0.01***
Current antimicrobial use (yes)		**<0.01***	**<0.01***

FOOTNOTE: *Significant according to the p-value (<0.05).

ESKAPE group. Considering only the identified isolates belonging to the ESKAPE group (n = 221, 16.5 % of the total), AMR and MDR percentages were 87.3% (N=193) and 49.3% (N=109), respectively (see Figure 4). *Pseudomonas aeruginosa* was the most commonly isolated species (n = 61), followed by *Enterobacter* spp. (n = 60) and *K.pneumoniae* (n = 58). *P.aeruginosa, A.baumannii*. *and E. faecium* strains showed the highest AMR percentages (100%, see Table 4), while *E. faecium* showed the highest MDR percentage (90%), followed by *K.pneumoniae* (55.2%).

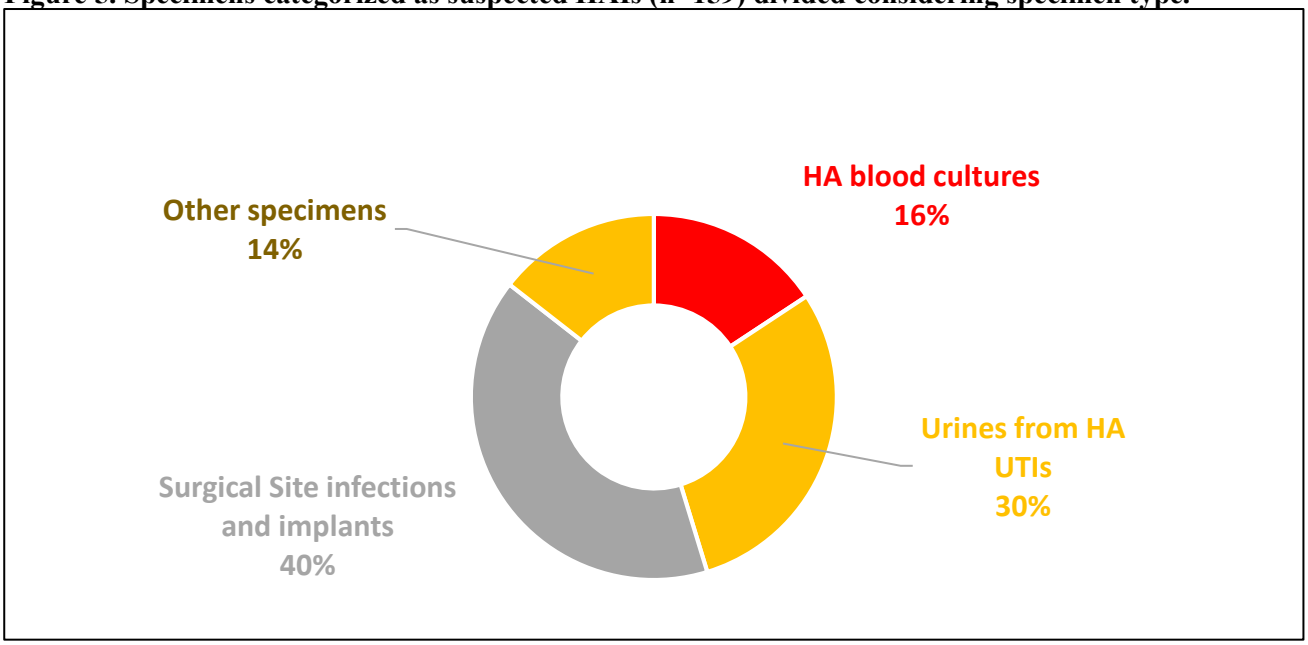
Figure 4. Distribution and AMR/MDR profiles of bacterial species belonging to the ESKAPE group.



FOOTNOTE: the term "NON AMR" include isolates susceptible to every tested drug; "AMR" refers to isolates non-susceptible to drugs belonging to <3 different classes; while "MDR" refers to isolates non-susceptible to drugs from 3 or more antimicrobial classes.

Suspected HAIs. Specimens from suspected HAIs accounted for 13.2% of the total (n=159, see Figure 5). Twenty-five were classified as blood cultures from suspected HA bloodstream infections, 47 urines from suspected HA UTIs, 64 SSIs and surgical implants, while 23 were samples taken from other infections. Of these 159 specimens, 123 (77.4%) grown in monoculture, while 36 (22.6%) were mixed infections.

Figure 5. Specimens categorized as suspected HAIs (n=159) divided considering specimen type.

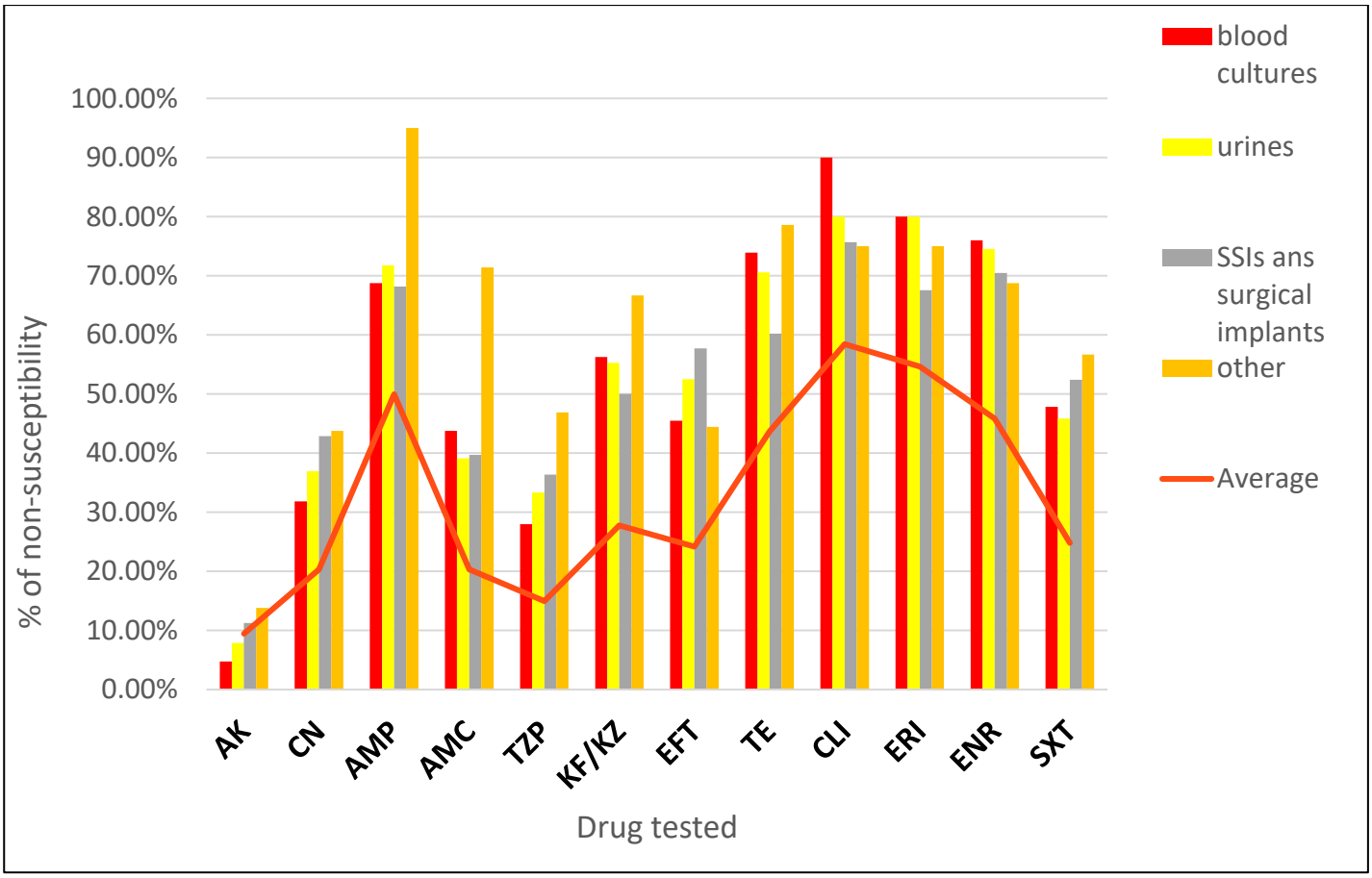


FOOTNOTE: the term "Other specimens" include abdominal/pleural/peritoneal effusion (n=9), non-surgical wound (n=6), abscess (n=1), bronchoalveolar lavage (n=1), biopsy (n=1), bile (n=1), exudate (n=1), ear swab (n=1), and specimen classified as "others" (n=2).

A total of 187 isolates (13.9% of the total number) from suspected HAIs was recorded. In 8 cases, the same isolate was found in more than one specimen in the same patient at the same time. AMR and MDR percentages of suspected HA pathogens (90.9% and 71.7 %) were considerably higher than the average ones observed in all the isolates. Considering single analysis for each tested drug (Figure 6), the nonsusceptibility percentage toward clindamycin was the one with the largest difference compared with the average percentage in blood cultures (90% vs. 58.4%), while it was enrofloxacin in urines from potential HAIs (74.5% vs 45.8%), ceftiofur in SSIs and surgical implants (57.7% vs 24.2%), and amoxicillin-clavulanate for other specimens (71.4% vs 20.3%). Considering drugs with the lowest nonsusceptibility percentages between the 12 tested, a considerable difference was observed for piperacillin–tazobactam, with nonsusceptibility percentages from 28% to 46.87% in specimens from potential HAIs, compared with the average nonsusceptibility percentage of 14.98%. On the other hand, no significant differences in nonsusceptibility percentages compared with the average were recorded for amikacin. Figure 7 shows the most commonly isolated species considering specimen type: *E.coli* was frequent in every specimen, while *S.pseudintermedius* and *Enterobacter* spp. were more common in SSIs/surgical implants, and *K.pneumoniae* and *E.faecium* in suspected HA UTIs. Notably, 64 isolates belonged to the ESKAPE group (28.9% of total ESKAPE isolates). Considering the bacterial species involved in suspected HAIs compared with the average, *Enterobacter* spp., *E.faecium* and *S.marcescens* were

isolated in a high proportion in suspected HAIs (table 7). The single nonsusceptibility percentages of the major bacterial species isolated in suspected HAIs are shown in Figure 8, including a visual comparison with the average percentages registered in the general analysis.

Figure 6. Nonsusceptibility percentages for every single tested drug considering the 187 isolates from suspected HAIs (divided into blood cultures, urines, SSIs and surgical implants and other), compared with the average percentages (orange line).



FOOTNOTE: AK=Amikacin; CN=Gentamicin; AMP=Ampicillin; AMC=Amoxicillin-clavulanate; TZP=Piperacillin-tazobactam; KF/KZ=Cephalotin/Cefazolin; EFT=Ceftiofur; TE=Tetracycline; CLI=Clindamycin, ERI=Erithromycin; ENR=Enrofloxacin; SXT= Trimethoprim–sulfamethoxazole.

Figure 7. Distribution of isolates from suspected HAIs according to bacterial species and specimen type. Only species with >2 isolates are shown.

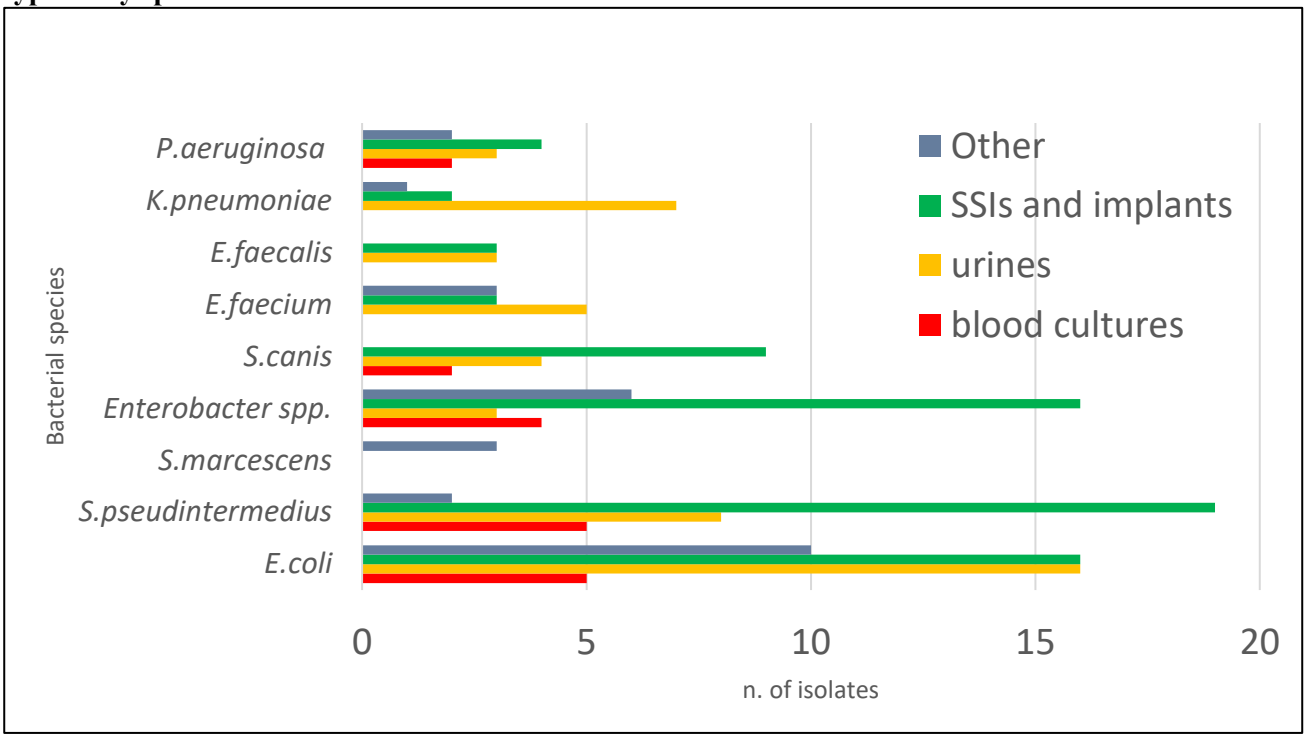
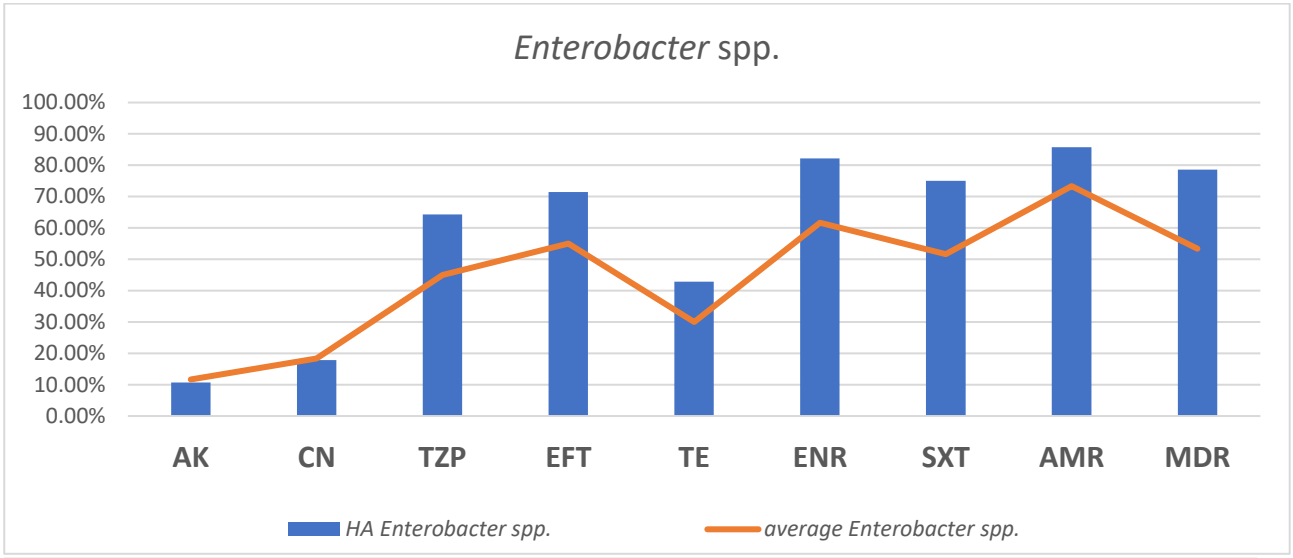
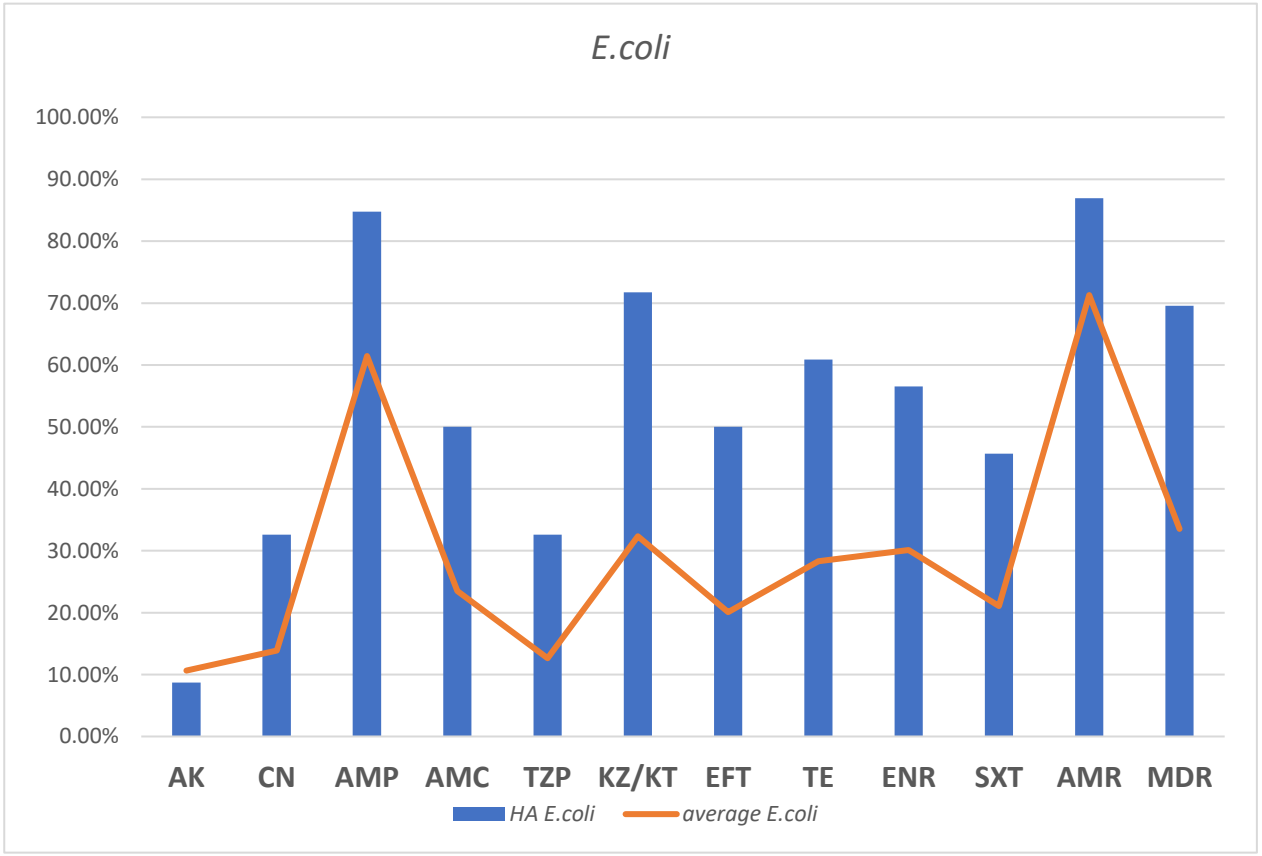


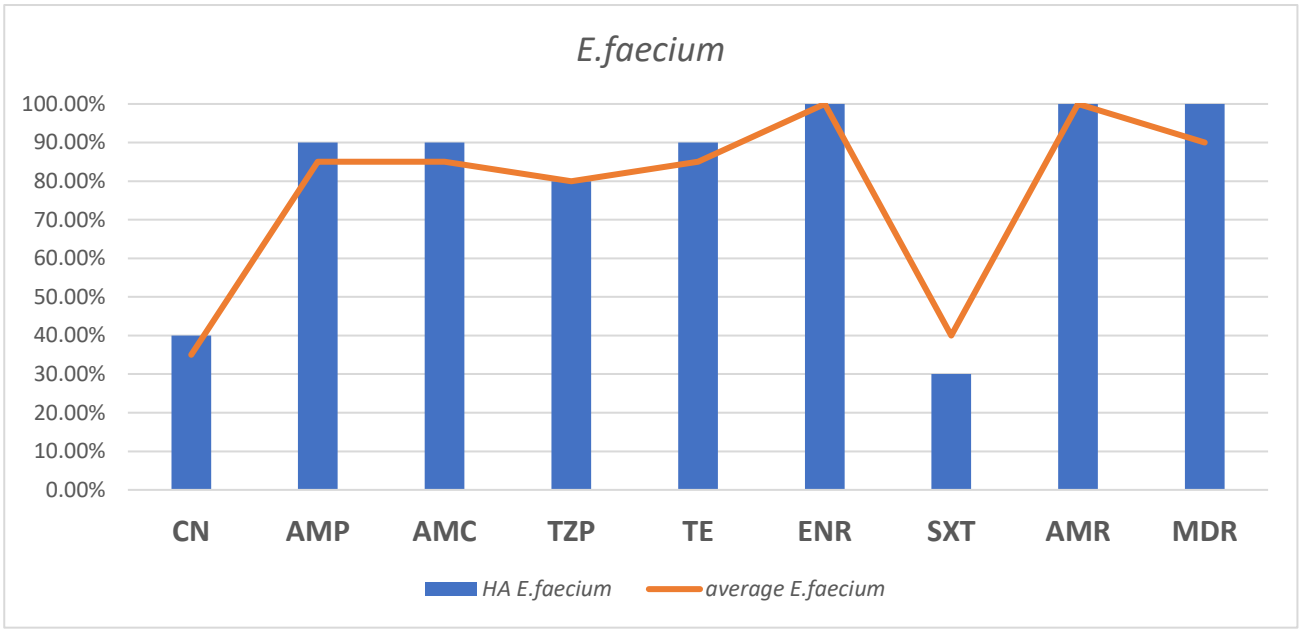
Table 7. Distribution of the 187 bacterial isolates associated with suspected HAIs according to bacterial species. Only species with >2 isolates were included.

Bacterial species	n. of suspected HA isolates	% of total isolates from suspected HAIs	**% of total isolates of the same species**
E.coli	46	24.6%	9.2%
Enterobacter spp.	28	15%	46.7%
K.pneumoniae	10	5.3%	17.2%
P.aeruginosa	11	5.9%	18%
S.pseudintermedius	33	17.6%	19.6%
S.aureus	4	2.1%	21%
S.canis	14	7.5%	13%
E.faecium	10	5.3%	50%
E.faecalis	6	3.2%	8.8%
S.marcescens	3	1.6%	75%)

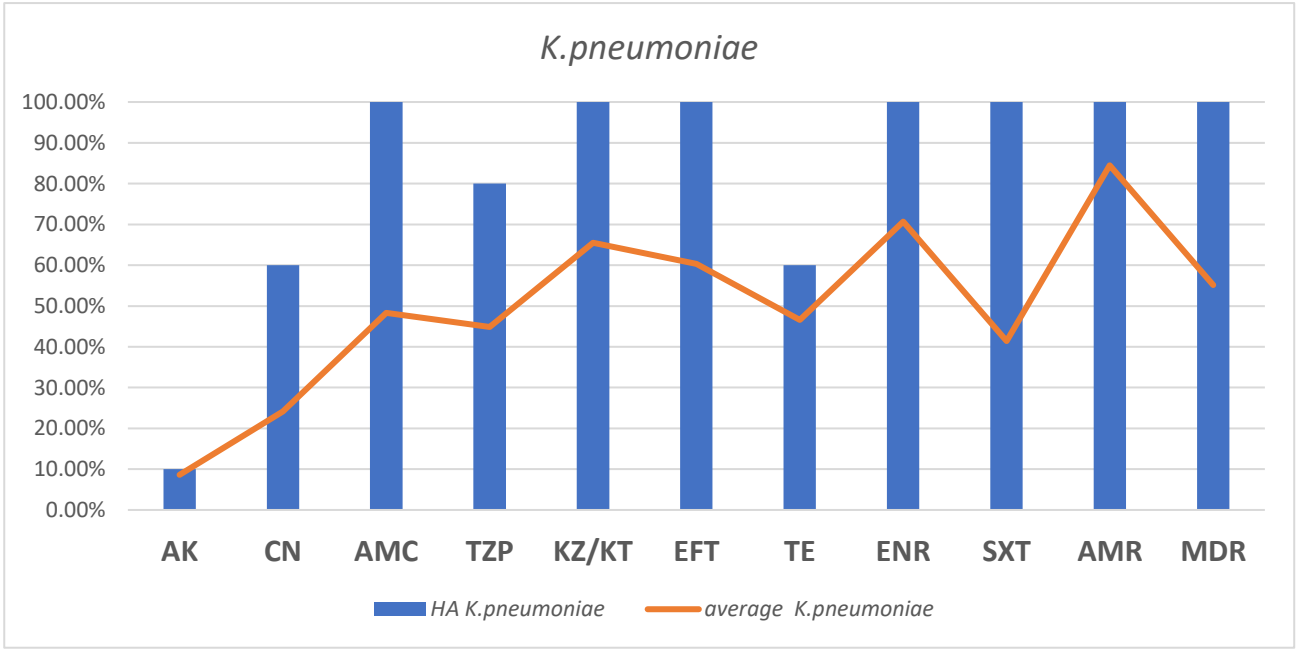
Figure 8. Non-susceptibility percentages of the most common pathogens isolated from suspected HAIs, divided considering bacterial species. The orange line refers to the percentages registered in the general analysis for each species species considered.



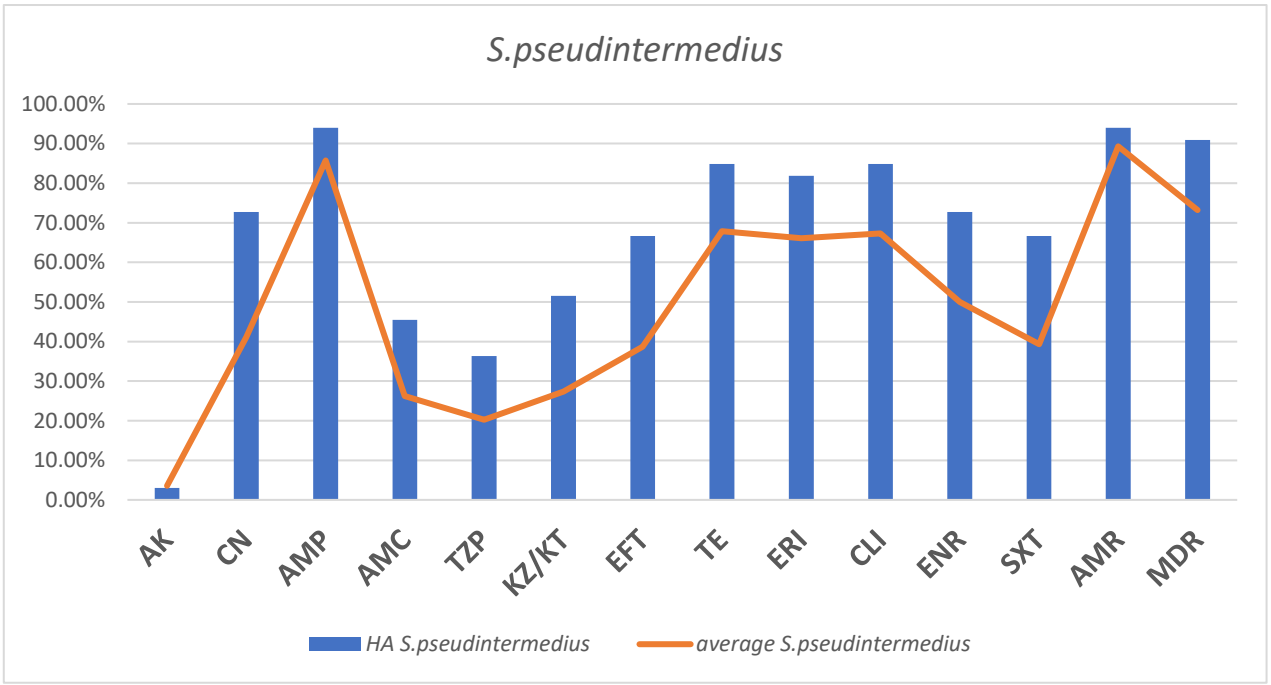



E.faecium
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
CN
AMP
AMC
TZP
TE
ENR
SXT
AMR
MDR
HA E.faecium
average E.faecium

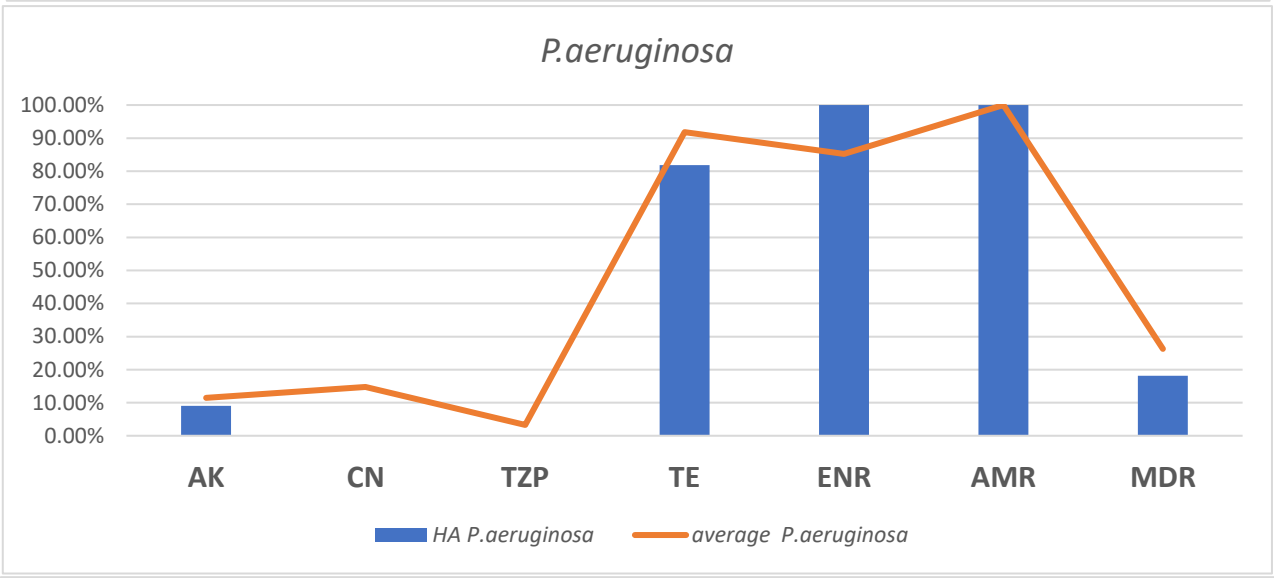
1564


K.pneumoniae
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
AK
CN
AMC
TZP
KZ/KT
EFT
TE
ENR
SXT
AMR
MDR
HA K.pneumoniae
average K.pneumoniae

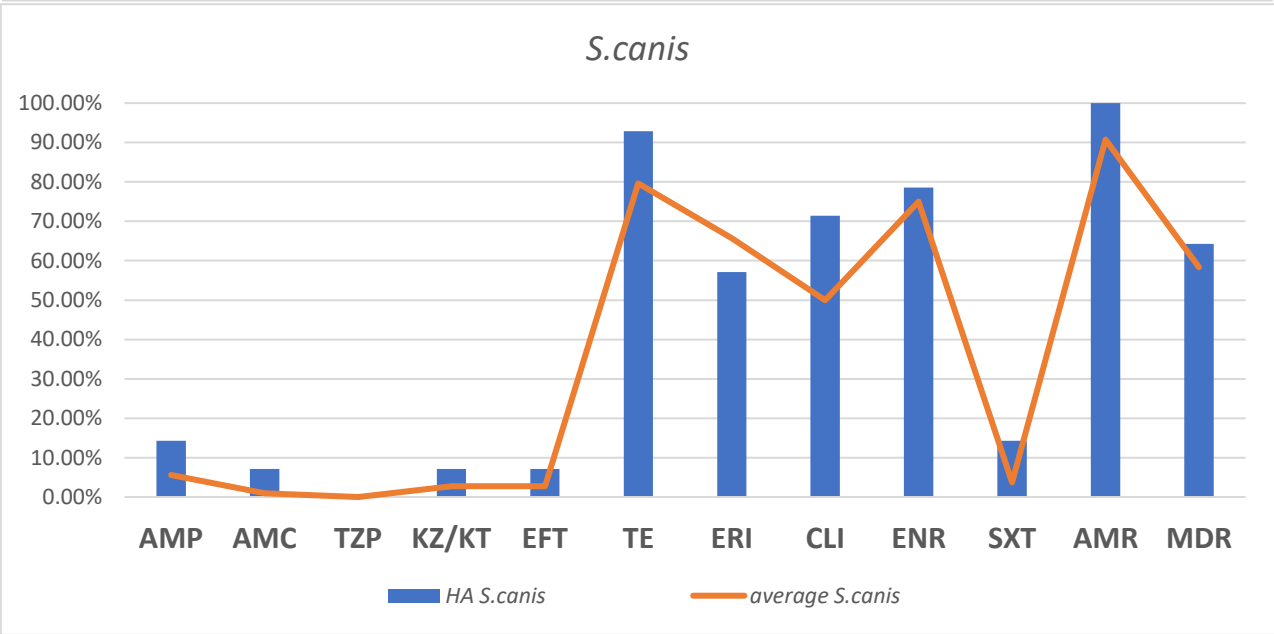
1565



1566



1567

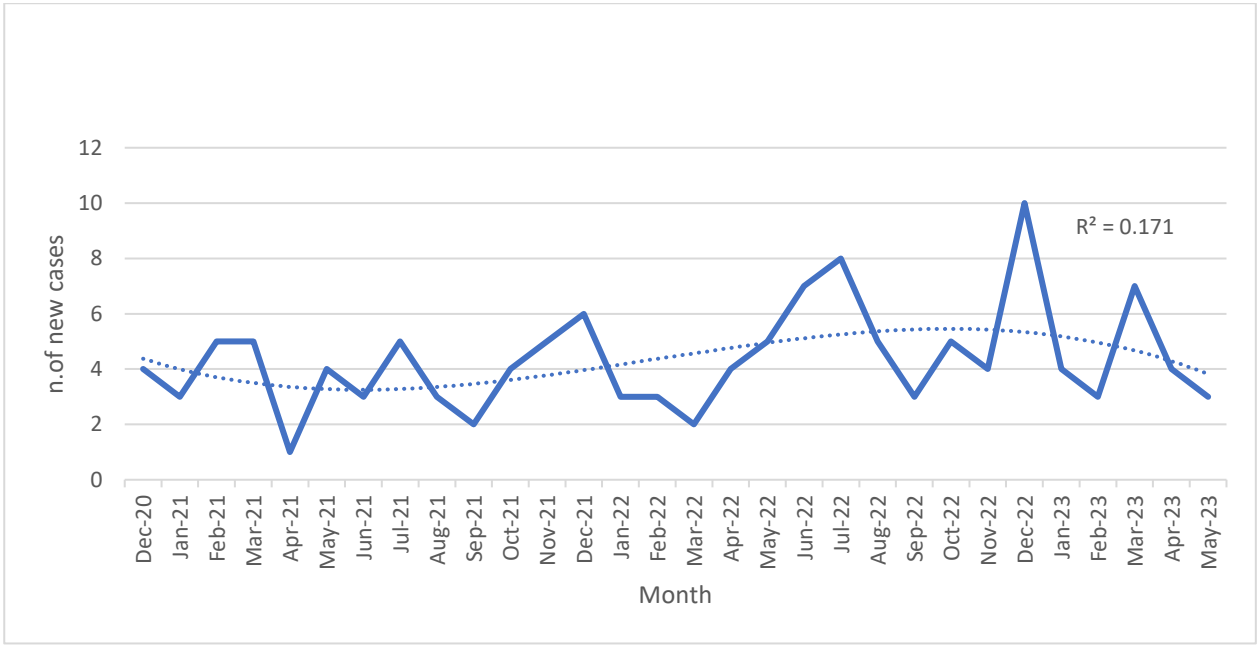


1568

FOOTNOTE: AK=Amikacin; CN=Gentamicin; AMP=Ampicillin; AMC=Amoxicillin-clavulanate; TZP=Piperacillin-tazobactam; KF/KZ=Cephalotin/Cefazolin; EFT=Ceftiofur; TE=Tetracycline; CLI=Clindamycin, ERI=Erithromycin; ENR=Enrofloxacin; SXT= Trimethoprim–sulfamethoxazole. The "average" line (in orange) refers to the non-susceptibility percentages recorded for each species in the general analysis. HA= Healthcare-associated.

Considering suspected HAIs cases (n=130, average = 4.3 new cases per month), they represented 5.03% of total patients that were hospitalized during the period considered. Temporal trend of new suspected HAI cases is shown in Figure 9.

Figure 9. Temporal trend of new suspected cases of HAIs based on monthly records. The dotted line represents the polynomial trendline of the values. Also R^2 is reported.



3.D. Discussion

The execution of this internal passive surveillance system allowed to obtain an overview about the bacterial species isolated from cat and dog infections in the Bologna Small Animal VTH, the most submitted specimens, the AMR/MDR profiles and associated risk factors, the single drug non-susceptibility percentages with a focus on specific bacterial species (ESKAPE group) and specimens (suspected HAIs). In both cats and dogs, the most frequently submitted sample was urine (56.48% of the total). Although this result is in contrast with other similar studies worldwide in which it was found to be wounds (244–246), or ear swabs in dogs (84,247,248), its high prevalence was expected in Italy because it is considered to be one of the most frequently cultured biological materials (249). Nasal swabs were considerably more frequent in cats than in dogs (7.26% vs. 2.57%). Those results are comparable with a Spanish study (246) over 5,875 clinical samples from dogs and cats, in which otitis was shown to be more prevalent in dogs and respiratory tract disease in cats. Considering bacterial species analysis, *E.coli* was found to be the most common in both cats and dogs. Its high prevalence is probably related with the high prevalence of urine specimens. Indeed, other studies worldwide have highlighted that *E.coli* is the most common agent in urinary tract infections in pets (250–255). *S.pseudintermedius* was not surprisingly found to be the second most common species in dog infections; on the other hand, the most present staphylococcal species in cats was *S.felis*. Notably, *S.pseudintermedius* prevalence was far higher than *S. aureus*, as expected and highlighted by other works (84,96,256–258). In this study, *E.faecalis* was reported to be the second most isolated species

in cats, with a considerably higher prevalence (10.4% vs 4%) compared with dogs. Substantial differences among *E.faecalis* prevalence between dogs and cats were not reported in other studies on enterococcal isolations in pets (259,260), so this finding could be due to the difference in the population size of the two species. Anyway, the higher prevalence registered for *E.faecalis* compared with *E.faecium* (5.1% versus 1.5%) confirms that, in contrast with human medicine, *E.faecalis* seem to be more present in pets, but with lower MDR percentages (90% vs 27.9%, in our case), as reported in other studies (261).

In contrast with other studies on AMR in companion animals that have been conducted on specific bacterial species or specimens, this study analyzed all the isolates in a determined timeframe, from both dogs and cats. A recent Colombian study over a 4-year period on 1,413 isolates from dogs and cats showed MDR percentages of 18.7% and 22%, respectively (244), whereas a Malaysian study on 780 isolates from 2015 to 2017 reported MDR percentages of 75.8% in cats and 71.6% in dogs (245). A longer Canadian study from 1994 to 2013 on more than 15,000 specimens showed MDR rates of 9% and 12% in cats and dogs isolates, respectively (247). In Italy, Smoglica et al.(262) described very similar results to our report in isolates from urines in companion animals, with AMR and MDR rates of 74.3% and 44.8%, respectively. Since the reasons that could explain the difference between the results are primarily geographical, comparisons, and data extrapolation should be done with prudence. The high nonsusceptibility percentages have to be considered as a partial reflection of the Italian epidemiological situation about AMR, which is currently one of the most concerning in Europe (263). A multicentric study on uropathogenic bacteria isolated from dogs and cats (93) showed that Southern European countries generally presented higher levels of antimicrobial resistance when compared with Northern European ones. AMR and MDR occurrence in companion animals is influenced by policies in antimicrobial use in the local community, not only in small animal settings but also in livestock production and human medicine (264). Notably, the present study was conducted in a VTH, which is considered a higher risk place due to the large number of personnel and referred patients (196). This could mean that the percentages reported in this study could be higher if compared with the other veterinary settings from the same geographical area. Moreover, the study describes only bacterial strains from diagnostic cases, so it does not provide an overall picture about AMR in the general dogs and cats population.

AMR and MDR percentages were found to be statistically more relevant in gram-positive bacteria. This result, in contrast with the classic consideration that gram-negatives tend to be more resistant than gram-positives (265), could be due to the intrinsic resistances considered and the limited number of drug tested, that could have underestimated the AMR in gram-negatives. Considering the non-susceptibility for the single drug, this study highlights that clindamycin, amoxicillin–clavulanate, ceftiofur, piperacillin–tazobactam, and enrofloxacin percentages represent a serious threat from a One Health perspective. In Europe, reported clindamycin nonsusceptibility percentages in pets reached a maximum of 100% in 39 *S.aureus* isolates from canine dermatitis in an Italian study (266). Amoxicillin–clavulanate nonsusceptibility percentages, reported to be 20.35% in our study, ranged from 10.9% to 100% in other European studies on *E.coli* (267–269). They are also comparable with the ones reported both by the French and the German national surveillance systems for AMR in companion animals, with percentages of nonsusceptibility of 35.5%–40% for *E.coli* in France (270), and of between 11% and 22% in *E.coli* isolated from dogs and cats with UTIs in Germany (271). An Italian study on 334 isolates from urines in pets empirically treated within one month from urine sample collection described non-susceptibility rates of 76.5–91.7% for amoxicillin-clavulanate and of 71.4-91.7% for ceftriaxone in *E.coli* and *K.pneumoniae* strains, respectively (249). The rates reported are possibly proportional to the high number of prescriptions of amoxicillin-clavulanate in

pets, often as first choice treatment (272). Notably, the non-susceptibility percentage for ceftiofur in *E.coli* isolates (20.1%) are very similar to the percentage of 3GCR *E.coli* in the Emilia–Romagna region (21.4%) reported by the Italian surveillance system for AMR in humans (273), suggesting a similar spread of 3GCR-Enterobacterales. In pets, resistance rates to at least one of the third-generation cephalosporins tested reached 36% among 125 *Enterobacter*iaceae isolates from diseased cats (274). Reports on piperacillin–tazobactam rates are scarcely present in veterinary literature, but the report from the Italian surveillance system in humans described nonsusceptibility percentages from 8.5% for *E.coli* to 46.3% for *K.pneumoniae* (273). Considering enrofloxacin, the high nonsusceptibility percentages are not surprising since fluoroquinolones are one of the most commonly prescribed antimicrobial classes in small animal practice (51). In Italy, a study on 70 *S. pseudintermedius* isolates from canine pyoderma (275) described a resistance rate of 94%, while others Italian works on *P.aeruginosa* and *S.aureus* reported non-susceptibility rated of 43.5% and 51.3%, respectively (266,276). Overall, these results reflect the widespread consumption of antibiotics such as clindamycin, amoxicillin–clavulanate, ceftiofur, piperacillin–tazobactam, and enrofloxacin in the Italian small animal practice. Although the major limitation of the study is the absence of data on antibiotics prescriptions and consumption, from our results it can be assumed that nonsusceptibility percentages are proportional to them. If we consider the two most isolated species, *E.coli* and *S.pseudintermedius,* the latter presented more alarming AMR/MDR profiles (89.3% versus 71.3% and 73.2% versus 33.5%, respectively). *E.coli* MDR percentage is in line with the rates reported by studies from Argentina (248), but higher than the ones reported from USA, Australia and Egypt (213,277,278). *S.pseudintermedius* MDR percentages (73.2%) were similar to the rates reported by a Taiwanese study on isolates from superficial pyoderma in dogs (70.21%) (279), but considerably higher than the others recorded in other studies over the world (248,255,280,281), indicating the potential high prevalence of MRSP. Unfortunately, methicillin was not included in the drugs routinely tested for diagnostic purposes, therefore an evaluation of the percentage of phenotypically evident MRS (including MRSP) was not performed. Nevertheless, it can be assumed that such percentage is proportional to the high rate of MDR isolates between the genus *Staphylococcus* spp.

The analysis of the selected Enterobacterales isolates showed that resistance to piperacillin-tazobactam was associated with resistance towards several other critically important antibiotics, such as potentiated penicillins, carbapenems and fluoroquinolones. Such findings are alarming from a One Health perspective and confirm the role of pets in the acquisition and dissemination of AMR, including for antibiotics that are not allowed in small animal practice in Europe. Piperacillin-tazobactam selects for changes in membrane permeability and maintenance associated with multidrug-resistance (282). Although a small proportion of the patients considered (5 out of 28) were directly treated with piperacillin-tazobactam in the previous 90 days, its use (until the ban from the EU with the 2022/1255 regulation) could have helped in the selection and maintenance of multiple resistance genes within the VTH for long periods. Indeed, 17 out of 32 (53.1%) of the isolates were from suspected HAIs, confirming the fact that an intra-hospital spread of ARGs selected by piperacillin-tazobactam use was likely. The WGS analysis confirmed this suspect, showing that several ARGs, including towards other antibiotic classes, were found in all the selected isolates. In most of them, piperacillin-tazobactam resistance was due to the expression of *bla*$_{\text{OXA-1}}$, or through a combination/overexpression of other 3GCR genes such as *bla*$_{\text{TEM-1B}}$, bla $_{\text{CTX-M-15}}$, *bla*$_{\text{OXY}}$ and *bla*$_{\text{CMY2}}$, found both on the chromosome and plasmids. The ability of such genes to confer resistance towards piperacillin-tazobactam was already confirmed by other studies from human medicine (283–286). In small animal practice, reports on piperacillin-tazobactam resistance are scarce; recently, Woerde et al. (139) described a non-susceptibility rate of 23% between 30

ESBL-producing Enterobacterales from clinical isolates, but without a direct association with the involved resistance genes. The identification of three isolates carrying the carbapenem-resistance *bla*$_{NDM-5}$ gene on the IncX3 plasmid represent an additional worrisome finding from a public health point of view. As previously mentioned, NDM-5 is one of the most concerning carbapenemases, and its emergence in small animal practice is being increasingly described worldwide (40,54,56,287–291), including Italy, where it was firstly described in 2021 by Alba et al. in a urine from an infected dog in Northern Italy (292). In that case, the *bla*$_{NDM-5}$ gene was encoded by the IncF mosaic plasmid, while in our study the gene was found on the IncX3 plasmid. Notably, all the three isolates where associated with suspected HAIs, and the two *K.pnemoniae* isolates were the same Sequence Type (ST307), considered a high-risk human pandemic clone (293) and already found in dogs (294), so the possibility of a primary interspecies transmission from humans (reverse zoonosis) should be considered. Once again, given that carbapenems were not used in the VTH in the study period, such results reinforce the possibility of a cross-selection of resistance due to the use of piperacillin-tazobactam, with a subsequent clonal and plasmidic spread over time. The conjugative plasmid harbored other resistance genes and have the ability to spread between different bacterial species, making it even more dangerous. Potentially, it could be further co-selected and amplified by the use of other classes of antibiotics before spreading back to humans.

If we look at the temporal evaluation of AMR and MDR over time (Figure 2), the trend seems not particularly increasing, although monthly variations are present, suggesting that the overall situation is stable. However, the low value of R^2 and the relatively short timeframe (30 months) considered implicate that such evaluation should be done with prudence.

Risk factor analysis for AMR and MDR confirms the extremely strict relationship between antimicrobial use and the onset of resistance, as reported in other studies in both human (295) and veterinary medicine (213,296,297). Also, the use of invasive devices, related with the occurrence of HAIs in other studies (298), was linked with a higher chance of AMR/MDR and HAIs. Compared with dogs, cats seems to have a lower predisposition to develop an infection caused by AMR bacteria; this result could be due to the differences in population size between the two species, but it may also reflect less frequent use of antimicrobials in cats, as described by Hur et al. (49) in Australia and by Joosten (299) in Italy in a multicentric study. Another study by Choi et al (300) describe higher resistance rates towards cefovecin and enrofloxacin in clinical isolates from dogs than in cats. In contrast, other studies by Escher et al. (301) and Singleton et al. (41), described a higher systemic antimicrobial prescription and AMR rates in cats than in dogs. Additionally, the length of hospitalization was statistically related with isolates from suspected HAIs, confirming the findings from other studies from both human and veterinary medicine (172,302,303) that individuated in the prolonged hospital stay one of the most important factors linked with HAIs.

Isolates belonging to the ESKAPE group (n=220) represented 16.4% of the total strains. AMR and MDR percentages were alarmingly high in every species considered. Particular attention should be paid to the species that were isolated more frequently, such as *K.pneumoniae*, *P.aeruginosa*, and *Enterobacter* spp. The isolates of *K.pneumoniae* had high nonsusceptibility percentages towards potentiated penicillins, very similar to the ones described by the Italian national surveillance system in humans (273), with percentages of 54.4% for amoxicillin–clavulanate and 46.3% for piperacillin–tazobactam. The high nonsusceptibility percentage towards enrofloxacin described in this study (70.7%) should also be considered, since it is very similar to a Portuguese study by Marques et al. (304), that reported resistance rates of 72% for enrofloxacin and ciprofloxacin in 25 *K.pneumoniae* isolates from pets with UTI. Furthermore, of particularly interest is the alarming

MDR percentage (100%, with 80% of non-susceptibility towards piperacillin-tazobactam) of *K.pneumoniae* isolates from suspected HAIs (Figure 8), that highlights once again as this pathogen, when of nosocomial origin, can be extremely dangerous also in small animal practice, with the highest resistance rates between the Enterobacterales (248,277). Notably, *P.aeruginosa* strains showed a lower MDR percentage, in line with other studies (248). This could be due to the high number of intrinsic resistances (7 out of 12) toward the drugs considered. The relatively low nonsusceptibility percentages toward amikacin and gentamicin, similar to the ones reported by the Italian national surveillance system in humans (2.7%–12.6%)(273) suggest that aminoglycosides should be considered when facing a pseudomonal infection, as confirmed by previous studies (246,247). On the other hand, enrofloxacin high non-susceptibility percentage (85.3%) must be highlighted, and is similar to other European studies, in which it ranged from 43.5% to 99% (149,256,276). *P.aeruginosa* isolates from suspected HAIs do not seem to be specifically more resistant than the average (Figure 8): this could be related to the fact that *P.aeruginosa* resistance genes are not primarily related with plasmidic conjugation (146), with a lower chance to acquire them through the transmission from other gram-negative bacteria stimulated by selective pressure exerted within the VTH. For *Enterobacter* spp. isolates, their importance must be considered in relation to their high nonsusceptibility proportion even in the presence of several intrinsic resistances (5 out of 12). A Japanese study (305) on 60 *Enterobacter* spp. isolates from cats and dogs revealed general lower resistance percentages, except for tetracycline (40% versus 25.5% of our study). In particular, nonsusceptibility percentages toward 3rdgeneration cephalosporins such as ceftiofur (55%) recorded for *Enterobacter* spp. are far higher than the results from a French study by Haenni et al. (306), in which it ranged from 3.5% to 10.2%. Furthermore, a considerable proportion of *Enterobacter* spp. isolates (46.7%, with higher nonsusceptibility percentages) was from suspected HAIs; this finding could indicate that a specific attention should be paid to this bacterial genus, as it could be relevant as an endemic hospital pathogen. The same consideration could be done towards *E. faecium* isolates: despite a low number of isolates (n=20), 50% of them (n=10) was associated with suspected HAIs. Additionally, *E.faecium* isolates showed a high MDR proportion (90%), with high nonsusceptibility percentages for penicillins +/− beta-lactamases inhibitors (83.33%–77.78%). Such findings are comparable with the ones for ampicillin (89.3%) described by the Italian surveillance system in humans (273) and suggest that, in contrast to other studies (247), the empiric treatment with this antimicrobial class in case of enterococcal infections should be avoided. Data about the ESKAPE group should address veterinary hospitals and clinics to enforce surveillance for these specific pathogens, but some differences with human medicine are present. *E.faecium, S.aureus* and *A.baumannii* seem to have a lower impact in terms of number of isolates; therefore, a "veterinary customized" approach for the ESKAPE pathogens, in which the first "E" include also *E.faecalis*, the "S" include *S.pseudintermedius* and *S.intermedius* is suggested in order to reach a better overview. For example, *S.pseudintermedius* isolates, far more prevalent than *S.aureus* (n=168 versus n=19) are also frequently involved in suspected HAIs (19.6% of total isolates) and with high MDR rates (90.9%), underlying its major importance as HAI agent in small animal practice.

Considering only isolates from suspected HAIs (n=187), they represented 13.9% of the total. This percentage is probably an overestimation, because a precise evaluation of every single case was not possible, but it can be considered as a good marker for HAIs impact in the VTH. As expected, they were mainly bloodstream infections, UTIs and SSIs/surgical implants (86% of the total). The higher AMR and MDR percentages (90.9 % and 71.7 %, respectively) compared with the average were expected, with relevant increases registered for clindamycin, enrofloxacin, ceftiofur, amoxicillin-clavulanate and piperacillin–tazobactam, the same antibiotics which non-susceptibility percentages

were highlighted as critical in the general overview. Such increases could be proportional with the common, higher use of such antibiotics in those infections; unfortunately, the absence of data about antibiotics consumption does not allow to go deeper in this speculation. Data about HAIs are scarce in veterinary medicine; nevertheless, a Swedish study (307) on SSIs in dogs confirmed that *S.pseudintermedius* was the most common species (46%) with resistance rates of 80% for ampicillin, 24.4% for trimethoprim-sulfamethoxazole, 5.6% for enrofloxacin and 6.7% for gentamicin. Another French study by Haenni et al. (308) on MRSP isolates from SSIs showed as 13/15 had probably a nosocomial origin, all with linked genetic profiles. The results, especially the high nonsusceptibility increases, should remark the importance of a correct antimicrobial stewardship policy to avoid therapeutic failure in severe infections such as HAIs, in which antimicrobial use is required to save the patient's life. Although referred to *in vitro* results, these data should also highlight that passive microbiological surveillance can serve as a guide for clinicians to make rational therapeutic decisions and to limit the selection and spread of AMR in the local community.

Considering the temporal evaluation of suspected HAIs cases, the trend looks very swinging (Figure 9), with no evidence of increase or decrease over time. Again, this could be due to the relatively short timeframe considered. Additionally, the VTH studied is a reference clinic, so many cases of suspected HAIs (around 30% of the total, from an anecdotical evaluation) were referred from other facilities and did not primarily arise within the VTH. This explains also that the monthly peaks of cases registered (e.g. in December 2022) did not necessarily correspond to HAIs outbreaks in the hospital (see Chapter 6), and means that this temporal evaluation cannot be considered a pure indicator of the healthcare quality in the VTH.

In conclusion, the passive surveillance system implemented over 30 months in the Bologna VTH furnished some useful data on AMR and HAIs in companion animals, highlighting the priorities that should be faced with more urgence. By knowing the most concerning bacterial species and infections, AMR/MDR percentages and risk factors, it is possible to obtain an overview of the problem and consequently decide where and how concentrate the attention, creating a tailored surveillance plan and posing the basis for a tailored antimicrobial stewardship program. Furthermore, considering the absence of shared control programs in the EU for AMR in companion animals, facilities such as VTHs should be encouraged to develop internal surveillance plans to obtain and to share such data.

Chapter 4

Active surveillance on patients

4.A. Specific aims

Active surveillance on patient aimed at collecting and analyzing data about AMR in the commensal flora of patients hospitalized at the Bologna VTH. Three AMROs, MRS, 3GCR and CR gram-negative bacteria (GNB) were the focus of the study. Information about AMRO carriage at admission, in-hospital acquisition and the associated risk factors were recorded over time.

4.B. Materials and methods.

1. Study design. A perspective, cross-sectional and observational study was conducted into the Bologna VTH as a part of the pilot surveillance program from May 2021 to May 2023. The surveillance was executed in sessions performed quarterly (pulsed surveillance). According with

clinical staff's indications, every patient expected to be hospitalized for more than 48 hours was sampled in each session, until reaching twenty-five patients per session.

2. Data collection. For every patient, anamnestic data (species, sex, age, previous antibiotic use in the past 90 days, previous hospitalization/surgery in the past 90 days) and hospitalization data (length of hospitalization, antibiotic treatment, use of corticosteroids and analgesics, hospitalization in intensive care unit, surgery, anesthesia, use of invasive devices, onset of a suspected HAI according with passive surveillance) were recorded.

3. **Sampling.** Oral and perirectal swabs were collected from patient at the admission (within 12 hours) and on the day of discharge using sterile swabs with Amies transport medium. Oral sampling was performed by gentling inserting the swab for 10-15 seconds into the oral cavity, lateral to the tongue (Figure 1). Perirectal samplings were performed by gently putting the swab in the perirectal area and rotating it for 10-15 seconds. Samples were stored at 4°C for a maximum of 24 hours before being processed.

Figure 4. Example of oral sampling executed in a dog.

3. **Isolation and identification**. Every sample was then processed by rubbing the swab into selective chromogenic media, over approximately one third of the surface of each plate, and subsequently streaking using a sterile loop. Perirectal swabs were cultured on CHROMAGAR TM KPC (Chromagar ^{TM}, Paris, France) for CR-GNB, and on CHROMAGAR TM ESBL (Chromagar ^{TM}, Paris, France) for 3GCR-GNB. Oral swabs were cultured on Oxacillin Resistance Screening Agar Base (ORSAB, Oxoid, Wesel, Germany) for MRS. After 24-48 hours of incubation at 37+/-1 °C in aerobic conditions, colonies chromogenically distinguishable isolated from positive cultures were subcultured on tryptone-soy agar (Oxoid, Wesel, Germany) and subsequently identified through the matrix-assisted laser desorption–ionization time-of-flight mass spectrometry method (MALDI-TOF MS) using a Bruker Daltonics MBT SMART equipment, and the Biotyper Real Time Classification software v3.1 (Bruker Daltonics, Germany), following manufacturer's instructions. Colonies identified with a score between 1.5 and 2 were considered only at the genus level.

4. PCR. Single and multiplex PCR were performed to assess the presence of resistance genes. Specifically, isolates grown on selective media for MRS were tested for the presence of *mecA* and *mecC* and following the protocol described by Stegger et al. (309); isolates grown on selective media for 3GCR-GNB were tested for the presence of $bla_{\text{CTX-M}}$ group 1, bla_{SHV}, $bla_{\text{OXA-1-like}}$ and bla_{TEM} following the protocols described by Dallenne et al. (310) In addition, CR-GNB isolates were tested were tested for bla_{NDM}, $bla_{\text{KPC 1-5}}$, bla_{VIM}, bla_{IMP}, and various AmpC genes ($bla_{\text{CMY 2-7, 12-18, 21-23}}$, $bla_{\text{LAT 1-3}}$, $bla_{\text{BIL-1}}$), following the same procotol (310) and, for bla_{NDM}, the one described by Poirel et al. (311)

5. Antimicrobial susceptibility testing. GNB isolates that tested negative for all the genes on the PCRs were tested for confirmation of the phenotypical resistances (3GCR, CR) through the disc diffusion test. Following CLSI breakpoints (4), 3GCR was defined as non-susceptibility (intermediate or resistant isolate) to cefotaxime (5 μg) and ceftazidime (10 μg), while CR was defined as nonsusceptibility to ertapenem and/or imipenem (10 μg), in accordance with reports from previous studies (103,312,313).

5. Data Analysis. Descriptive statistics was performed. In patients, frequency of detection (FD) of carriers at admission was determined in patients with a positive sample at admission. In-hospital acquisition was determined in animals with that were negative at the admission sampling and positive at discharge. If all the three investigated AMROs was detected in a patient at admission, it was excluded from the in-hospital acquisition evaluation. A temporal trend description of the % of in-hospital acquisition in the different sessions was also performed. Furthermore, patients with reported infections suspected to be HAIs (collected through passive surveillance) were registered. Risk factors analysis was executed. The association between carriage at admission (general and for each AMRO) and the anamnestic data was assessed using univariable logistic regression (enter), and variables with a $p<0.01$ were included in the multivariable logistic model built using forward stepwise selection at $p<0.05$. Normality and heteroskedasticity of data were corrected with the Shapiro–Wilk test and the Levene's test. The same analysis was used to assess the association between the anamnestic/hospitalization data and both the in-hospital acquisition (general and for each AMRO) and the onset of potential HAIs (according with passive surveillance data). Data were checked for multicollinearity with the Belsley–Kuh–Welsch technique. Statistical analysis was performed with MedCalc (version v22.009).

4.C. Results

A total of six sampling sessions (150 patients) was performed. One-hundred and five (70%, 56 males and 49 females) were dogs, while 45 (36 males, 9 females) were cats. Average age was 9.1 years (95% CI, 8.5 to 9.9). Forty-four patients (29.3%) received antimicrobial treatment in the previous 90 days, of which 9/150 (6%) were treated with more than one drug, and 35 (23.3%) were hospitalized or underwent surgery in the previous 90 days. The average length of hospitalization was 5.3 days (95% CI 4.6 to 6.1). One-hundred and six (70.7%) received antimicrobial treatment during the hospitalization, of which 22 (22/150, 14.7%) were treated with more than one drug (see Table 1), while 93 (62%) received opioid analgesics and 30 (20%) corticosteroids. One-hundred patients (66.7%) stayed in the Intensive Care Unit, 59 (39.3%) underwent surgery, and 73 (48.7%) stayed in the anesthesia room. Urinary catheter was used in 20 patients (13.3%), nasogastric tube in 34 (22.7%), while other invasive devices (central venous catheter, surgical drainage tube, ureteral stent) in 25 patients (16.7%).

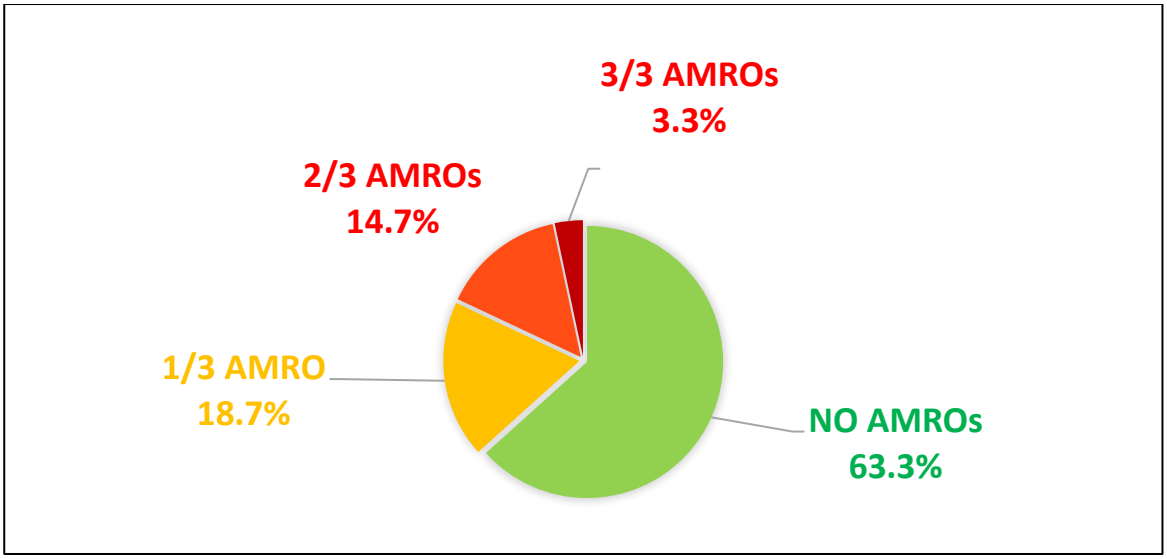
Table 1. Antibiotics use (prior and during hospitalization) in the 150 patients (dogs and cats) included in the study during six different sessions, from May 2021 to May 2023.

	number of patients treated up to 90 days prior to hospitalization	%	Number of patients treated during hospitalization	%
AMC	16	10.7%	10	6.7%
AMS	6	4.0%	57	38.0%
PTZ	1	0.7%	15	10.0%
MRB	6	4.0%	23	15.3%
DOXI	4	2.7%	7	4.7%
First generation cephalosporins	5	3.3%	17	11.3%
3rd generation cephalosporins	2	1.3%	0	0.0%
AK	0	0.0%	3	2.0%
ENR	6	4.0%	0	0.0%
Others/unknown	8	5.3%	3	2.0%

FOOTNOTE: AK=Amikacin; AMC= Amoxicillin-clavulanate; AMS= Ampicillin-sulbactam; DOXI=Doxycycline; ENR=Enrofloxacin; MRB=Marbofloxacin; PTZ=Piperazillin-tazobactam.

Carriage at admission. Fifty-five patients (36.7%, 44 dogs and 11 cats) were detected as carrier at admission of at least one of the three investigated AMROs. Specifically, 28 patients (18.7%) were carriers of one AMRO, 22 (14.7%) were carriers of two and five (3.3%) were carriers of three (see Figure 2). Considering AMRO typology, MRS were detected in 33 patients (22%), 3GR-GNB in 37 (24.7%) and CR-GNB in 17 (11.3%) (see Figure 4).

Figure 2. Distribution of AMRO carriage at admission of the 150 patients sampled.



FOOTNOTE: AMROs=Antimicrobial-resistant organisms.

In-hospital acquisition. Fifty-nine patients out of 145 (40.6%) acquired at least one of the investigated AMROs: 27 (18.6%) acquired one, 24 (16.5%) acquired two and 8 (5.5%) acquired all the three AMROs (Figure 3). Considering AMRO typology, 23/117 patients (19.7%) acquired MRS, 41/113 acquired 3GCR-GNB (36.3%), and 34/133 (25.6%) acquired CR-GNB (see Figure 4). The temporal trend of the percentages of in-hospital acquisition in the different session is shown in Figure 5.

Figure 3. Distribution of AMROs in-hospital acquisition in the 150 investigated patients, according with the number of acquisitions.

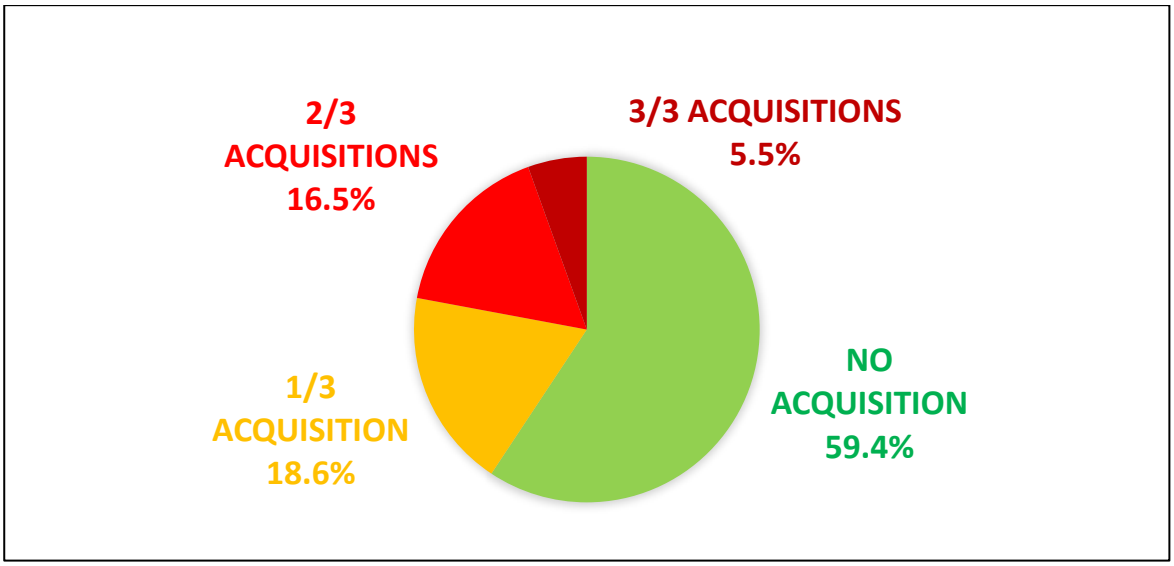
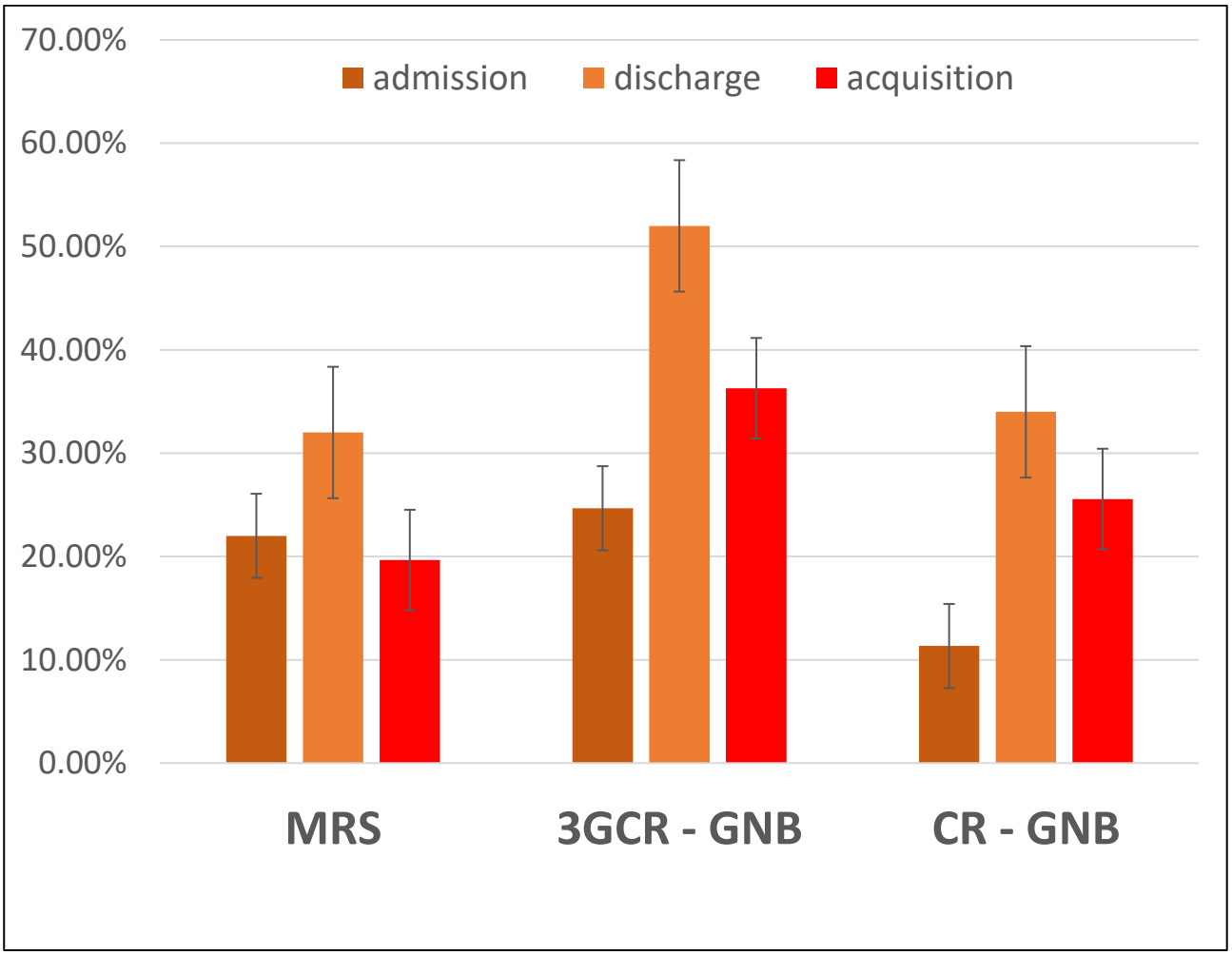
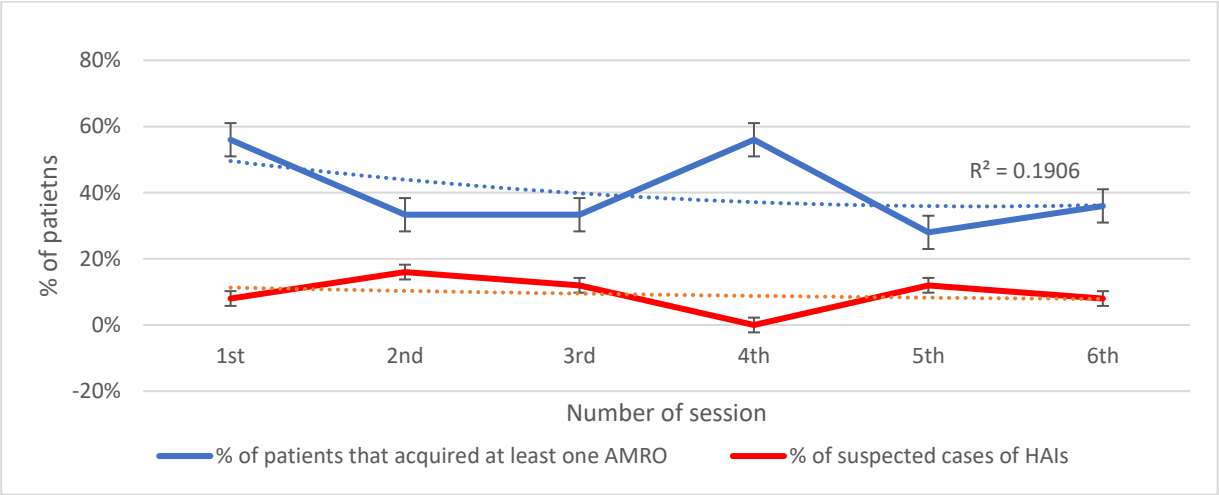


Figure 4. Distribution of the frequency of AMROs detection in admission, discharge (and acquisition), according with AMRO typology (MRS, 3CGR-GNB and CR-GNB) in the 150 investigated patients.



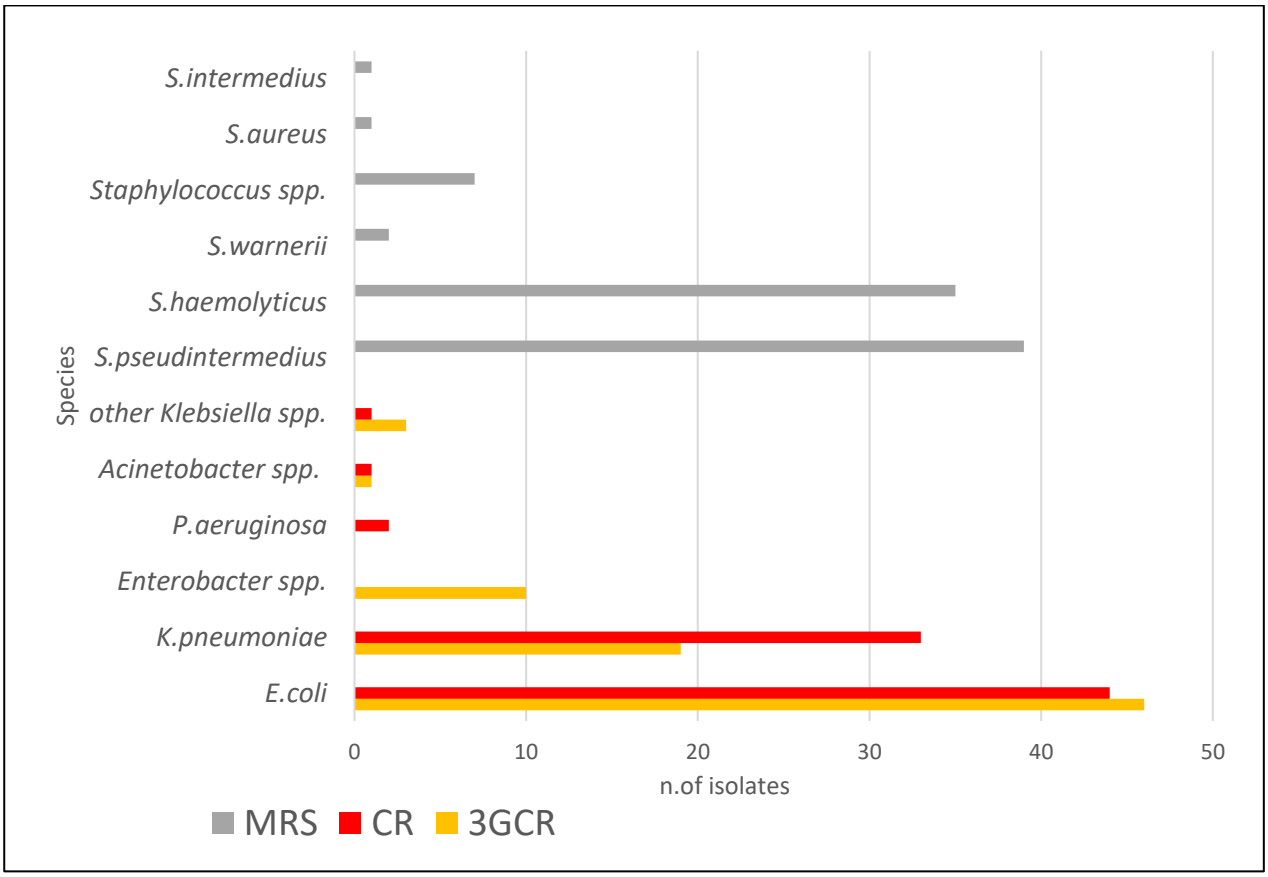
FOOTNOTE: CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. MRS: Methicillin-Resistant Staphylococci

Figure 5. Temporal trend of the percentage of patients that acquired at least one of three investigated AMROs in the six sessions, and of patients with a reported suspected case of healthcare-associated infection (HAI). A polynomial tendency line with the R^2 value is also shown.



Bacterial identification and phenotypical/genotypical analysis. A total of 247 isolates (85 MRS, and 162 GCR-GNB, including 81 CR-GNB) was identified (see figure 6). Between the MRS, the most common species were MRSP (n=39) and MR *S.haemolyticus* (n=35), while the most common 3GCR and CR GNB were *E.coli* (n=91, 46 3GCR and 45 CR) and *K.pneumoniae* (n=53, 19 3GCR and 34 CR). Genotypical analysis (see Table 1) on MRS confirmed all the isolates possessed the *mecA* gene. Additionally, 3 isolates (3.5%) the presence of the *mecC* gene was detected. Considering 3GCR resistance genes detection, $bla_{OXA\text{-}1\text{-}like}$ was the most common resistance gene found (66.7%) followed by $bla_{\text{CTX-M}}$ and bla_{TEM} (66.1%), and bla_{SHV} (34.6%). The co-presence of one or more of the investigated genes was confirmed in 102 isolates (63%), including 48 isolates (29.6%) in which all the four genes were found. Considering CR resistance, bla_{NDM} was found in 78 out of the 81 (96.3%) tested isolates, while the other genes (bla_{VIM}, bla_{IMP}, $bla_{\text{OXA-48-like}}$ and $bla_{\text{KPC}\ 1\text{-}5}$) were not found. AmpC genes were found in 21 (25.9%) of tested isolates. Co-presence of bla_{NDM} and at least one of the 3GCR genes investigated was confirmed in 67 isolates (85.9%). Ten 3GCR isolates (including 3 CR) tested negatives for all the genes searched, but their phenotypic resistance was confirmed in all the cases.

Figure 6. Species distribution of the 247 AMROs isolates identified from the 150 investigated patients.



FOOTNOTE: CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. MRS: Methicillin-Resistant Staphylococci

Table 2. Distribution and description of the prevalence of the AMR genes investigated on the 249 isolates considering AMRO typology (MRS, 3GCR-GNB, CR-GNB).

AMRO typology	Resistance gene tested	total isolates tested	n.of positive isolates	Prevalence
MRS	*mecA*	85	85	100%
	mecC	85	85	3.3%
3GCR-GNB	*bla*$_{TEM}$	162	107	66.1%
	bla$_{SHV}$	162	56	34.6%
	bla$_{OXA-1,-4,-30}$	162	108	66.7%
	bla$_{CTX-M}$ group 1	162	107	66.1%
CR-GNB	*bla*$_{VIM}$	81		0%

		0	
*bla*IMP	81	0	0%
*bla*OXA-48-like	81	0	0%
*bla*KPC 1-5	81	0	0%
*bla*NDM	81	81	96.3%
AmpC (*bla*CMY 2-7, 12-18, 21-23, *bla*LAT 1-3, *bla*BIL-1)	81	21	25.9%

FOOTNOTE: CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. MRS: Methicillin-Resistant Staphylococci

Risk factor analysis. In the multivariate analysis for AMRO carriage at admission (Table 2), hospitalization in the previous 90 days was associated with general AMRO carriage (p=0.0005), 3GCR-GNB carriage (p=0.0079), and MRS carriage in dogs (p=0.0166). Quinolones use in the previous 90 days was associated with general carriage (p=0.0272) and 3GCR-GNB carriage (p=0.0488). Species dog was associated with MRS carriage at admission (p=0.0085). In dogs, antibiotic use in the previous 90 days was associated with general AMRO carriage (p=0.0047) and 3GCR-GNB carriage (p=0.0049). Furthermore, the carriage of 3GCR-GNB was associated with the onset of a suspected HAI during the hospitalization. (p=0.0161). In the multivariate analysis for AMRO in-hospital acquisition, the length of hospitalization was associated with general AMRO acquisition (p=0.0006) and CR-GNB acquisition (p=0.0427). In dogs, it was also associated with MRS (p=0.0009), and CR-GNB acquisition (p=0.0016). The use of nasogastric tube (p=0.0002) was associated with 3GCR-GNB in-hospital acquisition. The treatment with piperacillin-tazobactam during the hospitalization was associated with CR-GNB acquisition (p=0.0380).

Table 3. Results of the multivariate analysis for AMRO carriage at admission and AMRO in-hospital acquisition. Only variables retained by the univariate regression results were included. Only significant values (p<0.05) are shown.

	General			**MRS**			**3GCR-GNB**			**CR-GNB**		
Risk factors for AMRO carriage	all patients	dogs	cats	all patients	dogs	cats	all patients	dogs	cats	all patients	dogs	cats
Hospitalization/ surgery in the previous 90 days	0.0005	0.0418			0.0166		0.0079	0.0489				
Antibiotic treatment in the previous 90 days		0.0047						0.0049				
Quinolones treatment in the	0.0272						0.0488					

previous 90 days							
Species dogs			0.0085				
Risk factors for AMRO acquisition							
Length of hospitalization	0.0006	0.003	0.0009	0.0427	<0.0001	0.0027	0.0016
Use of nasogastric tube during hospitalization				0.0427			
Treatment with piperacillin-tazobactam during hospitalization						0.0380	

FOOTNOTE: CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. MRS: Methicillin-Resistant Staphylococci.

4.D. Discussion

The active surveillance on patients was developed to obtain information about specific AMROs considered of particular interest for small animal practice (MRS, 3GCR and CR-GNB) carried by patients hospitalized for more than 48 hours at the Bologna VTH. Indeed, it is well established that patients become colonized by AMROs before that an infection becomes evident (314), so monitoring AMROs presence in the patients' microbiota (the "submerged" part of the AMR iceberg) can be a useful indicator for a general evaluation, possibly allowing to anticipate and to limit the onset of AMR infection. The choice to perform this type of surveillance in pulsed sessions (every four month, until reaching 25 patients in each session), was in line with guidelines for Infection Control by the Anderson et al. (220) . In a real-life scenario, few veterinary healthcare settings can afford the costs, both in terms of time and money, related with an extensive and continuous surveillance system. Pulsed surveillance is able to optimize such costs, still furnishing a detailed evaluation in determined timeframes (in our case, each session lasted approximately 30 days), which sum can be used to obtain a general overview and to individuate priorities.

The hospital environment exposes the bacterial commensal flora to a high selective pressure, mainly due to the massive use of antibiotics and to the presence of other patients treated or potentially carriers of AMROs. Considering MRS, the results are in line compared with other studies in hospitalized pets, in which MRS prevalence at admission ranged from 1.3% in the US to 51.1% in Nigeria (129,174,220,221,315–320), while MRS in-hospital acquisition varied from 1.4% to 26.4% (221,315,316,321). The same consideration could be done for 3GCR-GNB, for which studies in small animal healthcare facilities were mainly focused on *E.coli,* reporting prevalences at admission from 1.4% to 45% (112,125,137,221,322–325), with in-hospital acquisition frequencies up to 40% (221,322,325,326). The differences could be due to the different methodologies, patients or targets

used, but once again, differences (especially about the FD at admission) are primarily attributable to geographical reasons, so comparisons should be done with prudence.

Considering CR-GNB, the high detection frequency found is concerning from a One Health perspective, and highlights as pets could serve as reservoirs for carbapenems resistance. Even though at the time of the samplings carbapenems use for pets in Italy was first restricted, and then (from 2023) banned, 11.3% of hospitalized patients had CR-GNB in their gut flora at admission and almost one fourth acquired them. Such rates are considerably higher compared with the few other studies in Europe (38,221,322,327). The high CR-GNB detection frequency at admission (11.3%) could be partially explained by the fact that more than 50% of positive patients (9/17) were hospitalized at the same VTH in the previous 90 days, where they possibly acquired CR, subsequently detected when they were back to the hospital. Furthermore, considering the turnover time from the admission to the first sampling (< 12 hours), the chance that a patient became colonized by AMROs (including CR) in such timeframe, with a consequent overestimation of the detection frequency at admission and an underestimation of the in-hospital acquisition rate, must be evaluated. In a similar study from Switzerland by Nigg et al. (328), dogs positive for CR Enterobacterales at discharge were still carriers after 4 months, but were negative after 6 months. In our case, we did not conduct follow-up studies, but we can assume a similar duration of AMR persistence, although studies from human medicine describe durations of CR colonization over 12 months (329). The results indicate that pets can maintain and disseminate CR after hospitalization, representing a concerning risk for infection and for transmission to humans. A study from human medicine on CR-GNB infections reports an average time of 21 days from colonization to infection (330). Generally speaking, CR-GNB prevalence seems to be increasing in small animal practice and it has been growingly described (57). Considering that resistance to carbapenems is one of the most concerning public health problems, surveillance of CR-GNB should be enhanced also in veterinary settings to avoid that colonized animals could silently contribute in the dissemination to the community. Furthermore, the role of piperacillin-tazobactam in the selection of CR-GNB should be further investigated also in small animal hospitals.

The temporal trend of in-hospital acquisition during time indicates that the rate ranged from 28 to 56%. After a significant decrease observed in the second and third session from a starting rate of 56%, the fourth session was characterized by a "rebound" effect and the acquisition was back to 56%, but then the fifth and sixth session restored levels of approximately 30%, that could be considered endemic. Notably, there is no apparent correspondence between the rate of suspected HAIs and the percentage of patients that acquired AMROs in the hospital, confirming the fact that AMRO colonization could silently spread within a healthcare setting, in absence of clinical evidence.

The species analysis confirms that *S.pseudintermedius* is the most common MRS in small animal practice, not only as infectious agent but also as colonizer. Particular attention should be given to MR *S.haemolyticus,* a CoNS that is not frequently involved in infections, but can play an important role in the maintenance and dissemination of MR. Considering GNB, the high detection frequency of *E.coli* and *K.pneumoniae*, both 3GCR and CR, is not surprising, given that those two species are typically found in the gut microbiota and frequently associated with such resistances. A study from an Israeli VTH shows how these two species represented more than the 85% of all the ESBL-producing Enterobacterales isolated from cats and dogs (129). On the other hand, other AMROs such as *P.aeruginosa* and *Acinetobacter* spp., that typically are characterized by high MDR rates, seem to be less frequent in the patients commensal gut flora.

Considering the genotypical analysis, the 100% prevalence of the *mecA* gene in the MRS population was expected, given that it is the most common gene involved in methicillin resistance in both

humans and animals. On the other hand, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA-1-like}}$ and *bla*$_{\text{TEM}}$ were confirmed to be the most common 3GCR genes in pets in Europe (139). The fact that 63% of 3GCR isolates harbored more than one resistance genes of the four investigated highlighting as gram-negative bacteria in pets are able accumulate such genes over time, that often confer higher MDR rates (up to 95%) and a higher ARGs diversity than in human isolates (38,331). CR was conferred, in the vast majority of the isolates (94%), by the *bla*$_{\text{NDM}}$ gene, reinforcing the strong suspect of a nosocomial spread. Notably, the *bla*$_{\text{NDM-5}}$ gene was found in the IncX3 plasmid of three clinical isolates submitted to WGS (see "Passive Surveillance" chapter), so the endemic presence of such gene in the hospital is very likely. Considering the high acquisition rates, it can be assumed that *bla*$_{\text{NDM}}$ has been massively acquired by patients' gut bacteria through horizontal transmission, highlighting as AMR colonization tends to be the "submerged part" of AMR infections. Nevertheless, as stated in the previous Chapter, the origin of such CR spread remains unclear, stated that carbapenems were not used in the study period and cannot be the driving force of the selection and maintenance. As hypothesized by Sellera et al. (332), there are two main ways for companion animals to acquire CR: through the contact with a colonized human host, or through contaminated environment. In our case, the possibility of a primary interspecies transmission from humans (reverse zoonosis) should be considered; additionally, the use of fluoroquinolones and potentiated penicillins such as piperacillin tazobactam could have cross-selected for CR-harboring plasmids, since the isolates also exhibited resistance to these two classes, and could have facilitated its permanence in a common environmental source of contamination within the hospital. A similar Swiss study by Nigg et al. (328), reported the acquisition of a clonal CR *E.coli* harboring *bla*$_{\text{OXA-181}}$ and *bla*$_{\text{CMY-42}}$ by patients after hospitalization (21.6%), despite only one dog was found to be positive at admission, suggesting a within-hospital spread of CR-encoding plasmids (IncX3 and IncI1). The presence of AmpC-enconding genes was assessed only in CR-GNB, due logistic limitations in the genotypical analysis. However, their prevalence was up to 25% in such isolates, indicating as in some cases the over-expression of such genes could lead to CR, as reported by other studies (124,333).

The risk factor analysis poses some further interesting points. For all the patients and all the AMROs considered, the previous hospitalization/surgery was highlighted to be a significant risk factor for AMROs carriage at admission. Furthermore, previous antibiotic use (and specifically, fluoroquinolones use) was linked with a higher chance for a dog to be AMRO (and specifically 3GCR) carrier. This is in line with previous findings from other works on pets (221,221,280,302,315,334), including one by Trott et al. that highlights as enrofloxacin use in healthy dogs increased the chance of MDR *E.coli* colonization (335). This underline as the patient' history should be considered as an important factor to check when a patient is hospitalized, in order to reduce the chance of an uncontrolled introduction of AMROs. Furthermore, species dog was statistically correlated with MRS carriage: considering that almost half of total MRS were MRSP, this finding confirms the previous statements about *S.pseudintermedius'* higher affinity for dogs rather than cats. Regarding AMRO in-hospital acquisition, the length of the hospitalization appears to be the most relevant risk factor for each AMROs, regardless of the species. Once again, this result aligns with the literature (172,221,302,315,316,336), and remarks as the hospital stay should be reduced as more as possible in order to minimize the chance of AMRO colonization. This consideration could be a bit challenging to face for small animal practice, because the "home treatment" is more difficult to achieve correctly, but it still has to be considered in order to reduce the risks. Additionally, the use of the nasogastric tube was correlated with 3GCR-GNB acquisition, suggesting iatrogenic colonization. Invasive devices are well recognized as a risk factor for HAIs (221); in our case, this finding indicates that a revision of the procedure (including when is necessary) is probably needed. Moreover, in cats the use of piperacillin-tazobactam was linked with CR-GNB acquisition. Again, this result furnishes

evidence to the suspect that a within-hospital cross-selection for CR (mediated by *bla*$_{NDM}$) due to the extensive use of such antibiotic (which use in veterinary medicine has been banned in the EU with the 2022/1255 UE regulation) is likely. In a study by Lavigne et al. (337), risk factors analysis for NDM-5 producing *E.coli* in-hospital acquisition identified the exposure to the anesthesia service, the surgery service and endotracheal intubation as significantly associated with higher chances of acquisition. Additionally, in our study the carriage of 3GCR-GNB was associated with the onset of a suspected HAI during the hospitalization, as previously reported by other study from veterinary medicine (338). This finding enforces the importance of the early detection of AMROs' carriers, in order implement specific preventive measures. Indeed, screening patients at admission not only provides data about AMROs spread in pets from the local community, but also it can be used to allow a better management of the patients, reducing the chance of in-hospital transmission in case of a positive carrier. Through the rapid communication of the results to the clinical personnel, positive patients could be properly managed with extra-preventive measures, such as contact isolation precautions. In human medicine, the 2014 European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines strongly recommend the implementation of such precautions (339). Nevertheless, different studies are arguing about the effectiveness of such precautions for 3GCR-producing Enterobacterales, due to the absence of evidence-based results able to confirm their ability to reduce in-hospital AMROs transmission and HCAIS in endemic settings (340,341). The major issues highlighted are the difficulty to afford contact isolation for too many patients and the turnaround times, often too long to allow a proper risk reduction (342,343). Furthermore, a universal screening at admission could be difficult to afford in terms of workload and resources (339). Although specific studies in small animal practice are absent, this topic could be applied also in small animal veterinary hospitals, where often economic resources are scarcer. An admission screening for AMROs carriers could be based on the risk factors analysis, targeting patients with a major risk according with their clinical history (e.g. by developing am individual "risk score"): this could reduce the costs compared with an extensive and indiscriminate screening of all patients. In a study by Kaspar et al. from human medicine (344) more than 80% of patients that were transferred to Swiss hospitals from high-risk areas were screened for AMROs unnecessarily; they instead recommend more limited screening based on identifiable risk factors.

Additionally, the conventional methods to detect AMROs, such as bacteriological culture on selective media, have several limits in terms of time and specificity (342), so then possible, the implementation of faster and more accurate methodologies could be considered to reduce turnaround times or to focus on specific resistance mechanisms.

On the other hand, sampling patients at discharge allows to evaluate the in-hospital AMROs acquisition during time. This can help not only to assess the individual risk of colonization, but also to detect potential epidemic transmissions in carriers before the onset of HAI outbreaks. In our case, a high proportion of patients apparently acquired AMROs during the hospitalization. Since animals had no direct contact during their stay, contamination through a common source, helped by selective pressure exerted by antibiotics, is the most plausible hypothesis. This transmission from the environment to the animal may be direct or through the hospital personnel. The most concerning in-hospital acquisition regards CR-GNB, suggesting that a screening of these AMROs should be prioritized. Indeed, CR mediated by *bla*$_{NDM}$ seem to be endemically present in the VTH, so the timely detection of patients that acquire CR, with a subsequent follow-up during time, might be relevant to avoid the spread of this concerning resistance in the local community, especially in relation with their importance for public health and with the chance of interspecies transmission to humans. In

Enterobacterales, the possibility of sharing resistances between humans and pets has already been demonstrated (38,54,345), so such consideration should be taken seriously.

This kind of surveillance had several limitations. First, although the way in which it was performed over time ("pulsed") allowed to reduce the costs and to obtain a general overview about the endemic situation, it did not consent to properly evaluate the temporal trend over time, especially about in-hospital acquisition. Second, although genotypical confirmatory tests were performed for the most common resistance genes, a deeper epidemiological investigation (e.g. by confirming the presence of *bla*$_{\text{NDM-5}}$ in CR-GNB) could have been conducted. Third, the number of patients enrolled (n=150) might have led to a failure to detect significant risk factors.

In conclusion, this study aimed to show how active surveillance can be used in big facilities such as VTHs for a first evaluation of AMROs spread, giving indication on how and where concentrate a tailored infection control program based on specific issues. Alone, this surveillance is not able to reduce AMROs impact, but it can be combined with other surveillance typologies (e.g. environmental or passive surveillance) to weight the risks and address the efforts of infection control. Local data from veterinary healthcare settings are very important to improve the quality of the global overview about AMR.

Ethical statement: Ethical approvement was obtained for this study. Patient's owners gave written consent for the sampling.

Chapter 5

Active surveillance on the environment

5.A. Specific aims

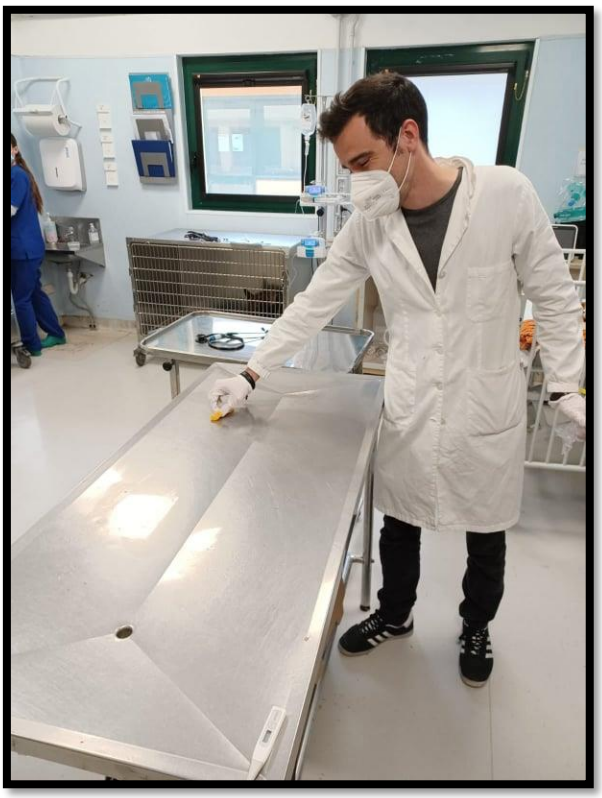
Active surveillance on the environment aimed at collecting and analyzing data over time about AMROs isolated from environmental samples collected at the Bologna VTH. Three AMROs, MRS, 3GCR and CR gram-negative bacteria (GNB) were the focus of the study. Information about AMRO frequency of detection in different areas, points and surfaces of the hospital, including the hospital personnel, were recorded during time.

5.B. Materials and methods

1. Study design. A perspective, cross-sectional and observational study was conducted into the Bologna VTH as a part of the pilot surveillance program from May 2021 to May 2023. The environmental surveillance was executed in sessions performed quarterly (pulsed surveillance), in concomitance with the sessions of active surveillance on patients.

2. Samples collection. Samples were collected from different parts of the VTH, divided according to the area (General Ward, Intensive Care Unit, Pre-Surgery Area, Surgery Area and Hospital Personnel) and points/surfaces (listed in Table X). Samples from Hospital Personnel were taken from randomly chosen veterinary workers. Samples from personnel' hands were collected by gently rubbing sterile swabs with AMIES transport media on the palms, while all the other samples were collected using sterile sponges soaked with 5 ml of sterile saline solution. After the sampling, sponges were added with 5 ml of additional sterile solution and squeezed, then the liquid transferred in a sterile tube. Samples were stored at 4°C for a maximum of 24 hours before being processed.

Figure 1. Example of sampling in the visiting table.



3. **Isolation and identification**. For the samples collected using sterile sponges, the bacterial culture was performed by directly streaking 10 microliters of the liquid in the plate with a sterile loop. Swabs were processed by rubbing the swab into selective chromogenic media, over approximately one third of the surface of each plate, and subsequently streaking using a sterile loop. All the samples were cultured on CHROMAGAR TM KPC (Chromagar^{TM} , Paris, France) for CR-GNB, on CHROMAGAR TM ESBL (Chromagar^{TM} , Paris, France) for 3GCR-GNB, and on Oxacillin Resistance Screening Agar Base (ORSAB, Oxoid, Wesel, Germany) for MRS..After 24-48 hours of incubation at 37°C in aerobic conditions, colonies chromogenically distinguishable isolated from positive cultures were subcultured on blood agar with 5% sheep blood (Oxoid) and subsequently identified through the matrix-assisted laser desorption–ionization time-of-flight mass spectrometry method (MALDI-TOF MS) using a Bruker Daltonics MBT SMART equipment, and the Biotyper Real Time Classification software v3.1 (Bruker Daltonics, Germany), following manufacturer's instructions and considering a species-level identification when the ID score was >2 (green—high accuracy). Colonies identified with a score between 1.5 and 2 were considered only at the genus level.

4. PCR and AST. Single and multiplex PCR were performed to assess the presence of resistance genes. Specifically, isolates grown on selective media for MRS were tested for the presence of *mecA* and *mecC* and following the protocol described by Stegger et al. (309); isolates belonging to the Enterobacterales family grown on selective media for 3GCR-GNB were tested for the presence of *bla*$_{CTX-M}$ group 1, *bla*$_{SHV}$, *bla*$_{OXA-1-like}$ and *bla*$_{TEM}$ following the protocols described by Dallenne et al. (310) In addition, CR-GNB Enterobacterales isolates were tested were tested for *bla*$_{NDM}$, *bla*$_{KPC\ 1-5}$, *bla*$_{VIM}$, *bla*$_{IMP}$, following the same procotol (310) and, for *bla*$_{NDM}$, the one described by Poirel et al. (311). All the GNB isolates were tested for the confirmation of the phenotypical resistances (3GCR, CR) through an AST by disc diffusion. Following CLSI breakpoints (239), 3GCR was defined as nonsusceptibility (intermediate or resistant isolate) to cefotaxime (5 μg) and ceftazidime (10 μg),

while CR was defined as nonsusceptibility to ertapenem and/or imipenem (10 µg), according to previous studies (312,313).

5. Data Analysis. Descriptive statistics was performed. For each AMRO typology, Frequency of Detection (FD) was calculated by dividing positive samples by the total of samples of the same area/point.

5.C. Results

A total of 195 samples was collected in six sessions. Forty-five (23.1%) were positive for at least oen of three AMROs investigated. Total MRS FD was 19.5% (n=38), while 3GCR-GNB FD was 7.7% (n=15) and CR-GNB 4.6% (n=9). Considering the area of collection (Table 1), Intensive Care Unit showed the highest FD for all the three investigated AMROs (29.4% for MRS, 14.7% for 3GCR-GNB and 11.8% for CR-GNB). The second highest FD was recorded for MRS in hospital personnel (22.1%).

Table 1. Distribution of the samplings according with the area. Frequency of detection for each considered AMRO is displayed.

Area	Total samplings	MRS	3GCR-GNB	CR-GNB
General ward	34	17.6%	8.8%	5.9%
ICU	34	29.4%	**14.7%**	**11.7%**
Pre-surgery	20	10.00%	10%	0.00%
Surgery	20	10.00%	3.3%	0.00%
Hospital Personnell	**77**	**22.1%**	5.2%	3.9%
TOTAL	**195**	**19.5%**	**7.7%**	**4.6%**

FOOTNOTE: CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. MRS: Methicillin-Resistant Staphylococci

Considering the point of collection (**Table 2**), the highest FD for points with at least 5 samplings were recorded for the personnel' shoe soles for MRS (85.7%), the floor for CGR-GNB and 3CR-GNB (57.1%). Personnel'clothings recorded a higher FD compared with hands for all the three AMROs investigated.

Table 2. Distribution of the samplings according with the point of collection. Frequency of detection for each considered AMRO is displayed.

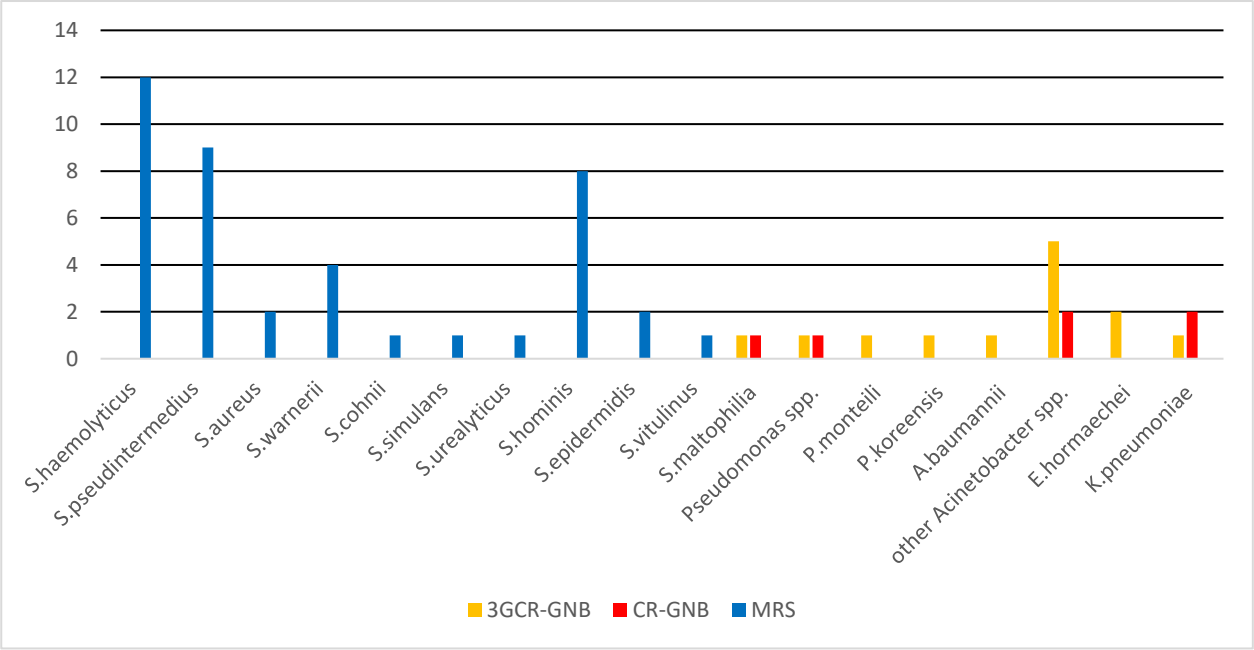
Point	Total samplings	MRS	3GCR-GNB	CR-GNB
Keyboards	14	14.3%	7.1%	0.00%
Clippers	17	5.9%	0.00%	0.00%

Fluidtherapy liquids	7	0.00%	0.00%	0.00%
Gauzes	2	0.00%	0.00%	0.00%
Thermometers	8	12.5%	0.00%	0.00%
Smartphones	10	20%	0.00%	0.00%
Visiting table	16	25%	12.5%	0.00%
Scale	3	66.7%	66.7%	0.00%
Personnel' hands	35	11.4%	0.00%	0.00%
Personnel' clothings	35	20%	8.6%	2.9%
Personnel' shoe soles	7	85.7%	14.3%	28.6%
Laringoscopes	5	0.00%	0.00%	0.00%
Stretchers	5	40%	0.00%	0.00%
Carts	5	0.00%	0.00%	0.00%
Anestesia machine	10	0.00%	0.00%	0.00%
Surgical lamp	1	0.00%	0.00%	0.00%
Sink	5	16.7%	16.7%	16.7%
Floor	7	71.4%	57.1%	57.1%
Portable ultrasound machine	2	50%	50%	50%

FOOTNOTE: CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. MRS: Methicillin-Resistant Staphylococci

Bacterial identification and phenotypical/genotypical analysis. A total of 65 isolates (41 MRS and 24 3GCR-GNB, including 9 CR-GNB) was identified (see Figure 2). Between the MRS, the most common species were MR *S.haemolyticus* (n=12), followed by MRSP (n=9) and MR *S.hominis* (n=8). Considering GNB, *Acinetobacter* spp. was the most frequent (n=5) in the 3GCR, and *Pseudomonas* spp. (n=4) in the CR GNB, while three Enterobacterales (2 CR *K.pneumoniae* and one 3GCR *E.hormaechei*) were found. Genotypical analysis (see Table 1) on MRS confirmed all the isolates possessed the *mecA* gene, while the *mecC* gene was not detected. Considering 3GCR and CR resistance genes detection on Enterobacterales, *bla* $_{NDM,}$ *Bla*$_{CTX-M}$, *bla*$_{TEM,}$ *bla*$_{SHV}$ and *bla*$_{OXA-1-like}$ were detected in the two CR *K.pneumoniae* isolates, while 3GCR *E.hormaechei* did not show any positivity for the genes searched. All the GNB confirmed the resistances (3GCR and CR) at the AST.

Figure 2. Species distribution of the 65 AMROs isolates identified from the environmental samplings.



FOOTNOTE: CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. MRS: Methicillin-Resistant Staphylococci

5.D. Discussion

Active surveillance on the environment allows to evaluate the degree of contamination in healthcare facilities, the general compliance of hospital workers and the high-risks areas and "hot spots" to target with specific measures. AMROs environmental contamination has been linked with an increased risk of HAIs in human medicine (346,347), and its importance should be considered especially in relation to persist for long period of time (up to years), acting as reservoir of infection (348). Low infection prevention and control standards have been linked with a high AMROs contamination (349), so implementing a successful strategy becomes necessary. Additionally, a contaminated environment not only increase the chance to contaminate animals, but also the staff itself, with a potential spread from the hospital to the local community. Walther et al. (81) reports that veterinary hospital workers have a major risk of being colonized by AMROs. As a first step, surveillance helps in obtaining a general overview and individuating the priorities. Veterinary hospitals and large clinics are considered a higher risks compared with first opinion practices (81), so they should be incentivized to start specific written protocols focused on the primary role of the personnel and on devices reported to have high prevalences. Currently, in small animal practice there is no legislation about minimum cleaning and disinfection standards, so every facility normally follows internal regulations. In our study, the VTH considered had internal protocols of routine cleaning and disinfection for the different areas, including cleaning of floors once daily, disinfection of surfaces (keyboards, visiting table) and equipment (portable ultrasound machine, clippers…) on a need-by-need basis. However, a general FD of 23.1% was observed in the six sessions, all performed during non-outbreak periods, which is comparable to other studies from small animal practice (350,351).

Comparing the results with the active surveillance on patients (see Chapter 4), we observed a generic dyscrasia on AMROs proportion, with a higher FD for MRS (19.5%) compared with 3GCR (7.7%) and CR-GNB (4.6%). Among other facts, this could be due to the low capacity of gram-negative bacteria (especially Enterobacterales such as *E.coli* and *K.pneumoniae*) to survive in the environment compared with MRS during non-outbreak periods. A carbapenemases-producing Enterobacterales prevalence f 11% (mainly in the floor, tables with shower system, water taps and centrifuges) was observed in a study by Schmitt et al. (350), but notably it was during a OXA-48

producing Enterobacterales outbreak. Similarly, another study from Switzerland reports an outbreak of MDR *K.pneumoniae* linked with environmental contamination (352). Studies on MRSA in small animal hospitals report prevalences of 13.5% and 16% (174,353), individuating in surfaces touched by multiple people and patients (doors, floor, carts and visiting tables) the most contaminated points. A large Canadian study on 101 small animal hospitals reports as all of them presented at least one positive sampling for MRS, with a total prevalence of 9% for MRSA and 7% for MRSP (354). Another Swiss study (349) reports an environmental prevalence of ESBL-producing Enterobacterales of 0-2%. In our case, although we did not perform sampling on the doors, high MRS FD were found in the floor, stretchers, personnel' shoe soles and clothing, confirming the fact that direct and indirect transmission of MRS is more likely in points/surfaces that are frequently used by multiple animals and workers, with the latter acting as mechanical vectors in the dissemination during the day.

Regarding the areas sampled, the Intensive Care Unit (ICU) was the one with the highest AMRO FD. Considering that ICU patients are generally critical patients, and that the use of multiple antibiotics is more likely, such finding was predictable but at the same time is worrisome, because it threatens more susceptible patients with the chance of being infected with MDR bacteria, such as the OXA-48 producing Enterobacterales detected in multiple ICU surfaces (floor, bath tubes, sinks) by Haenni et al. (127). Another study by Schmitt et al. (355) describes a 3% prevalence of ESBLs in the ICU surfaces.

Considering the different points/surfaces, the floor and the personnel' shoe soles registered the highest FD. The importance of shoe soles in the transmission of AMROs has not still been well established (356), but the high FD detected suggest they can be the reflection of the high floor contamination, acting as potential vectors from different areas. Some studies (174,357,358) have highlighted high prevalence of AMROs in the floor, although a study in an equine hospital (359) described no significant reduction in the floor microbial load after the use of footwear hygiene practice. Compared with human medicine, where the hospital floor is not considered as a high risk point due to the low chances of direct contact with patients, in small animal practice it could play a more relevant role, as patients (especially dogs) tend to have much more contact with the floor, with a subsequent higher exposure.

Looking at the hospital personnel, their potential role as vectors of resistance should be further investigated. MRS were the most frequent AMROs, with a higher FD in clothing (20%) compared with hands (11.4%). A study by Schmitt et al (350) reports a similar prevalence (10%) of MRSA in the hospital personnel' hands. In human medicine, hand hygiene is recognized as one of the most powerful factor to reduce AMR and HAIs incidence (360). Indeed, contaminated hands can directly transfer AMROs to patients, but also contaminate fomites and devices. With a proper hand hygiene executed before and after patient contact and contact with high-risk surfaces, the risk of transmission would be reduced (225). Several studies report that a simple and straightforward process, taking only a few seconds to clean hands helps prevent HAIs and save lives, reduce morbidity, and minimize health care costs (361,362). Specifically, hand hygiene with alcohol-based hand rub prior to patient contact should be emphasized, as well as attention to performing hand hygiene prior to leaving the room/ area in order to reduce the risk of cross-contamination (363). Staff compliance is essential, and can be achieved by direct observation and by measuring the use of alcohol-based hand-rub solution (364), or by periodic samplings (e.g. twice a year) on frequently touched surfaces. In a observational study from 2014 on companion animals veterinarians (363), compliance rates was only 14%, and only 3% of veterinarians performed hand wash both before and after patient contact. On the other hand, also contaminated clothing (scrubs and coats) can help the AMR dissemination (365,366),

especially in veterinary medicine where it is often needed to manipulate or hold a patient with the help of the body. The use of appropriate Personal Protective Equipment (PPE) reduce the risk of such contamination (106). Current evidence from human medicine shows that wearing short-sleeved uniforms can be more beneficial; additionally, in-hospital or industrial laundry services are preferred over home laundering, single-use protective gowns for contact precautions and daily change of uniforms can significantly reduce AMR contamination (365,367). In our case, in-hospital laundry was not guaranteed for all the workers, and there were no specific rules of wearing for the different areas, except for the Surgery. Providing specific guidelines on home laundering practices (this will also reduce the chance of home contamination), and implementing rules of wearing for every specific area (e.g. defining high-risk area where specific hospital clothing must stay in) can be useful options to prevent AMROs diffusion, and subsequently HAIs spread (366). As previously stated, the role of veterinary workers in AMR must be taken seriously also in relation with the risk of occupational disease.

This study also identified specific points that need to be specifically targeted, such as the portable ultrasound machine, the stretchers, and the scale. All these points have in common that they are regularly exposed to multiple patients and workers, even from different areas, during the routing activities of the hospital, so they should be object of specific protocols for cleaning and disinfection, and regular controls to monitor their contamination rate. In order for a disinfectant to work properly, the surface or item must first be clean (free of visible organic material) and the product must be applied at the manufacturer's suggested dilution and contact time (amount of time the disinfectant is in contact with the item before being removed). Disinfectants should be selected based on several criteria, including the product's spectrum of activity, susceptibility to inactivation by organic matter, and potential pathogens in the environment.

Considering the most common bacterial species isolated, the MRS reflect the distribution on the patients (see Chapter 4), with a high prevalence of MR *S.haemolyticus* and MRSP. Notably, MRSA presence seems to be very low, suggesting that this pathogen is not particularly diffused in the hospital, as confirmed by passive surveillance (see Chapter 3). When looking at gram-negative bacteria, the low FD of 3CGR and CR Enterobacterales, despite their high prevalence on patients 'gut flora, has been already discussed. It could be related to their lower ability to survive in the environment, but also to the inability of surveillance to properly identify the common environmental source. Notably, two CR *K.pneumoniae* were isolated (from the floor and from the portable ultrasound machine) and were confirmed to possess the same resistance genes of the isolates found in the active surveillance in patients, strongly suggesting an horizontal contamination. On the other hand, *Acinetobacter* spp. and *Pseudomonas* were isolated more frequently. As reported by Zendri et al. (351), those bacteria can be frequently found in veterinary hospitals, due to their ability (especially *Acinetobacter* spp.) to survive even in dry surfaces. Unfortunately, the presence of 3GCR and CR resistance genes was not assessed in such species, but their phenotypical resistance was confirmed. However, the genus *Pseudomonas* spp. often display different resistance mechanisms rather than beta-lactamases production, such as porin loss (368), so its importance in the diffusion of enzymes-encoding genes could be inferior. On the other hand, *Acinetobacter* spp. possess both a natural resistance through the production of enzymes such as AmpC and oxacillinases, and the ability to acquire resistance genes (157,369), If compared with passive surveillance (Chapter 3) and active surveillance in patients (Chapter 4), where the *Acinetobacter* spp. prevalences were low, this result highlight as this genus could play a major role in the environmental maintenance of AMR, rather than in patients or as a primary infectious agent.

The limitations of this stud include the absence of a deeper genotypical and epidemiological investigation, that could have helped in the analysis of the clonal or plasmidic spread of AMROs and ARGs. Another limit is the limited number of samplings and the lack of a specific focus on high-frequency touched surfaces/points (e.g. doors, fridge..), that could have given a more complete overview on the role of the personnel. Regarding the absence of a temporal trend analysis of the different sampling sessions, it was not performed due to: i) the high variability of the degree of contamination depending on the day/time (355); ii) the variability of the collection points in each session.

In conclusion, active surveillance on the environment furnished the basis to develop tailored measures to control the environmental spread of AMR. Through periodic samplings combined with passive and active surveillance on patients, a hospital-specific risk analysis can be done, allowing to detect the most important hazard points and to develop specific preventive measures.

Chapter 6

Comparison of active surveillance between two different VTHs

6.A. Specific aims

An active surveillance program (on patients and environment), with the same modalities as the one performed in the Bologna VTH, was executed also in the VTH of the Veterinary Faculty of the Complutense University, Madrid, Spain, during the six-months period that the candidate spent there. This kind of action was made to obtain and analyze data from a similar healthcare facility, to compare the results in order to underline the points in common and the differences between the two VTHs.

6.B. Materials and methods

1. **Study design.** From October 2022 to February 2023, a perspective longitudinal observational study was conducted into the Madrid VTH. The study included: i) pulsed active surveillance on patients; ii) active surveillance on environment; and iii) feedback reports.

    1a. Pulsed active surveillance on patients. Active surveillance on patients was performed in periodic sessions (pulsed surveillance), with the same modalities described in Chapter 4. For every patient, demographic data (species, sex) and the length of hospitalization were recorded.

    1b. Active surveillance on environment and staff. Environmental surveillance was performed monthly for four times by sampling surfaces, devices listed in table 1. With the same modalities as described in Chapter 5, samples from randomly chosen hospital staff (hands, clothes, and shoe soles) were taken.

    1c. Feedback reports. Clinical, surgical, and microbiological personnel was involved in results communication, feedback reports and discussion of single cases through videocalls and chat groups.

2. **Sampling.** Same as Chapter 4 and 5.

3. **Isolation and identification.** Same as Chapter 4 and 5.

4. Resistance detection by phenotypic methods. To confirm the resistance pattern of every isolate, a phenotypic confirmatory test was performed by disc diffusion method. Mueller-Hinton Agar (Oxoid) was inoculated with the tested isolates at standard concentration (0.5 Mc Farland), and different antimicrobials were used to test the resistance patterns. Non-susceptibility to both cefotaxime and ceftazidime was used to confirm 3GC resistance, according to EUCAST guidelines (370) (inhibition zones: <23 mm for cefotaxime, <18 mm for ceftazidime). Furthermore, ESBL production in confirmed 3CGR Enterobacterales was evaluated with a combination disc test (CDT) with cephalosporins (ceftazidime 30 µg, cefepime 30 µg, and cefotaxime 30 µg) alone and in combination with clavulanic acid 10 µg, following EUCAST guidelines (EUCAST, 2017). An additional confirmatory phenotypic test to discriminate the expression of AmpC β-lactamases was performed through a CDT with discs containing the cephalosporins (ceftazidime 30µg, cefepime 30 µg, and cefotaxime 30µg) and both cloxacillin 200 µg and clavulanic acid 10 µg, according to EUCAST. Carbapenem resistance in screened CR-GNB isolates was tested with the commercially available kit for synergy test, (Liofilchem, Roseto degli Abruzzi, Italy), while MRS isolates were evaluated with the phenotypic confirmatory disc diffusion test described by EUCAST guidelines (cefoxitin 30 µ and oxacillin 30 µg disc diffusion test). For negative control, strains *S. aureus* ATCC 29213 and *E.coli* ATCC 25922 were used for MRS and for 3GCR/CR GNB, respectively. For positive control, genotypically characterized strains from the VTH internal collection were used.

Data Analysis. Descriptive statistics was performed. In patients, frequency of detection (FD) at admission was assessed by dividing the number of patients with a positive sample for the total number of patients included, while in-hospital acquisition was determined by dividing the number of patients with a new AMRO species detected at discharge, by the number of total patients. Comparation with Bologna VTH results (average length of hospitalization, environmental FDs, FD at admission and in-hospital acquisition) was performed.

6.C. Results

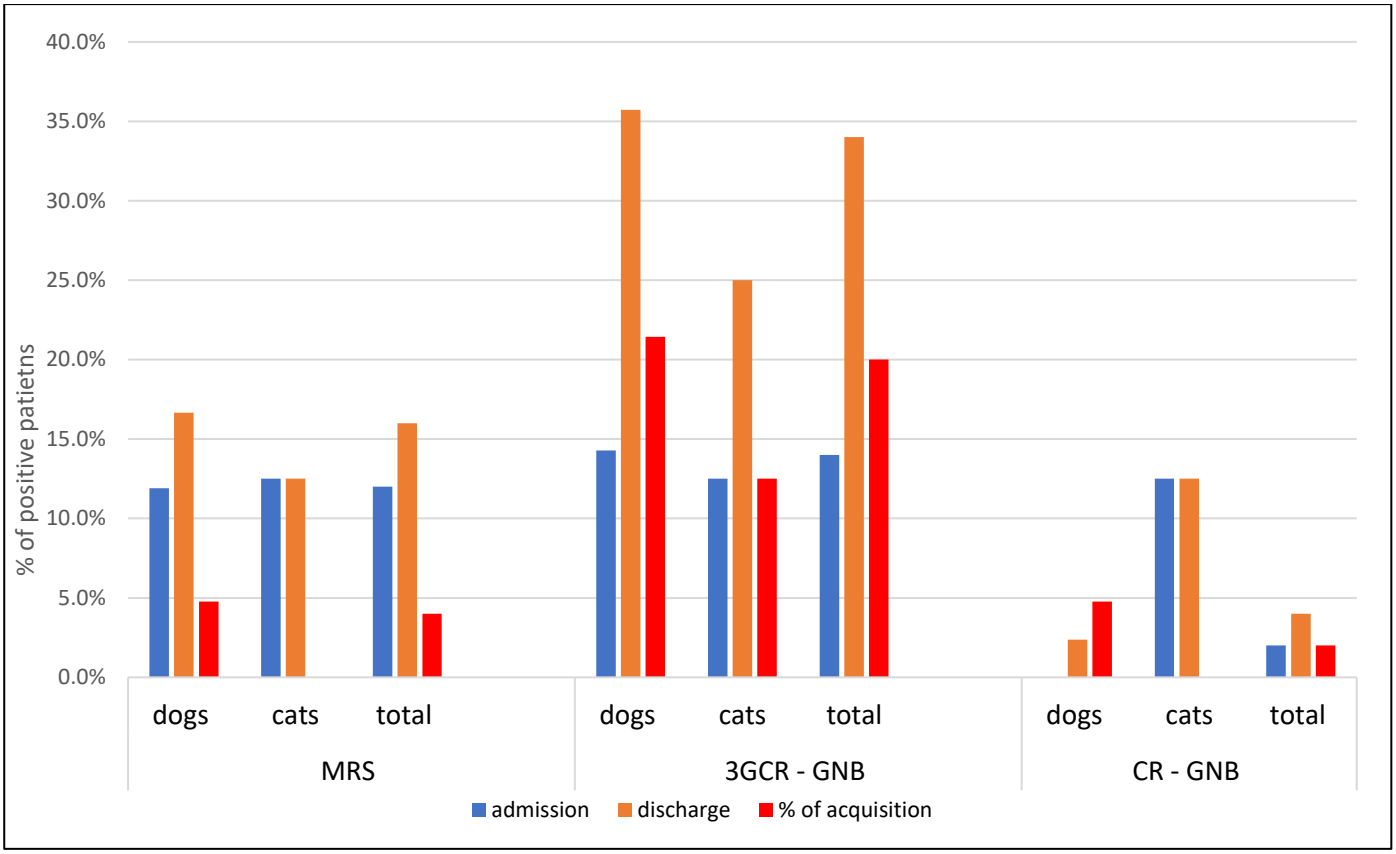
Active surveillance on patients. A total of 50 patients was sampled in two sessions. 42 (84%) were dogs and 8 (16%) cats. 27 were males and 23 females, while the average length of hospitalization was 3.8 days. A total of 49 investigated AMROs was isolated from 24 patients. All the 14 MRS isolates (100%) were confirmed at the phenotypical CDT analysis, while the phenotypically confirmed 3GCR-GNB and CR-GNB were 96.7 % (30/31) and 75% (3/4), respectively. Considering confirmed 3GCR-Enterobacterales (n=29), ESBL or AmpC production was confirmed by the CDT for all the isolates. In total, 24% of patients (n=12, 9 dogs and 3 cats) were carriers at admission of at least one of the three investigated AMROs, and 4% (n=2, two dogs) were carriers of two. Patients that acquired at least one of the three investigated AMROs during the hospital stay were 14 (28%, 13 dogs and one cat). One patient (2%, one dog) acquired two AMROs (3CGR *K.pneumoniae* and CR *P.aeruginosa*).

Positivity percentages are shown in figure 1. Considering MRS screening, detection frequency was 12% (6 patients, 5 dogs and one cat) at admission, while 8 patients (16%, 7 dogs, one cat) were positive for MRS screening before discharge. Three dogs (6%, 3/50) acquired confirmed MRS during hospitalization (one was positive at admission but acquired a new MRS species).

Considering confirmed 3GCR-GNB, detection frequency was 14% (7 patients, 6 dogs and 1 cat) at admission, while 18 patients (36%, 16 dogs and 2 cats) were positive at discharge. Eleven patients (22%, 10 dogs, one cat) acquired confirmed 3GCR-GNB during hospitalization.

Considering CR-GNB detection, one cat (2%) was positive admission, while 2 patients (4%, one dog and one cat) were positive at discharge. One patient (2%, one dog) acquired confirmed CR-GNB during hospitalization.

Figure 1. Detection frequency and in-hospital acquisition of MRS, 3GCR-GNB and CR-GNB in the commensal flora of the 50 patients hospitalized for more than 48 hours at the Madrid VTH and included in the study (from October 2022 to February 2023), sampled with oral and perirectal swabs both at admission (<12 hours) and before discharge.



FOOTNOTE: CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. MRS: Methicillin-Resistant Staphylococci.VTH: Veterinary Teaching Hospital.

Active surveillance on environment and staff. From a total of 98 samples collected, 34 isolates of the investigated AMROs were detected. All the MRS isolates (n=22) were confirmed at the phenotypical CDT analysis, while the phenotypically confirmed 3GCR-GNB and CR-GNB were 2/8 and 2/4, respectively. No confirmed 3GCR-Enterobacterales were detected. Total detection frequency was 22.4% for MRS, and 2 % for 3GCR-GNB and CR-GNB. Detection frequencies for every sampling site are shown in table 1. MRS were found to be more present in clinical staff' shoe soles (6/12, 50%) and hands (4/12, 33%). 3GCR-GNB and CR-GNB were found in the shoe soles, but also in the hair clipper of the hospitalization area.

Table 1. Environmental and staff surveillance sampling sites included in the study, and number of positive samples for each site for the detection of MRS, 3GCR-GNB, CR-GNB. Samples were taken monthly from October 2022 to February 2023.

Area	Sampling sites	Total samplings	MRS positives (phenotypically confirmed by CDT)	3GCR-GNB positives (phenotypically confirmed by CDT)	CR-GNB positives (phenotypically confirmed by CDT)
Waiting room	Chairs	4	2	0	0
	Dog scale	2	1	0	0
Hospitalization area	Exam tables	4	1	0	0
	Thermometers	4	0	0	0
	Urgency phone	4	1	0	0
	Keyboards	4	1	0	0
	Hair clipper	4	0	1	1
	Stretcher wheels	4	1	0	0
	Stretcher	4	1	0	0
	Portable ultrasound machine	4	0	0	0
	Personnel' hands	12	4	0	0
	Personnel' working clothes	12	1	0	0
	Personnel ' shoe soles	12	6	1	1
Consultation rooms	Exam tables	8	0	0	0
X-Ray room	Lead gowns in X-ray room	4	0	0	0
	Table in X-ray room	4	2	0	0
Laboratory of bacteriology	Laboratory of bacteriology	8	1	0	0
TOTAL		**98**	**22**	**2**	**2**

% of positive samples	**22.4%**	**2%**	**2%**

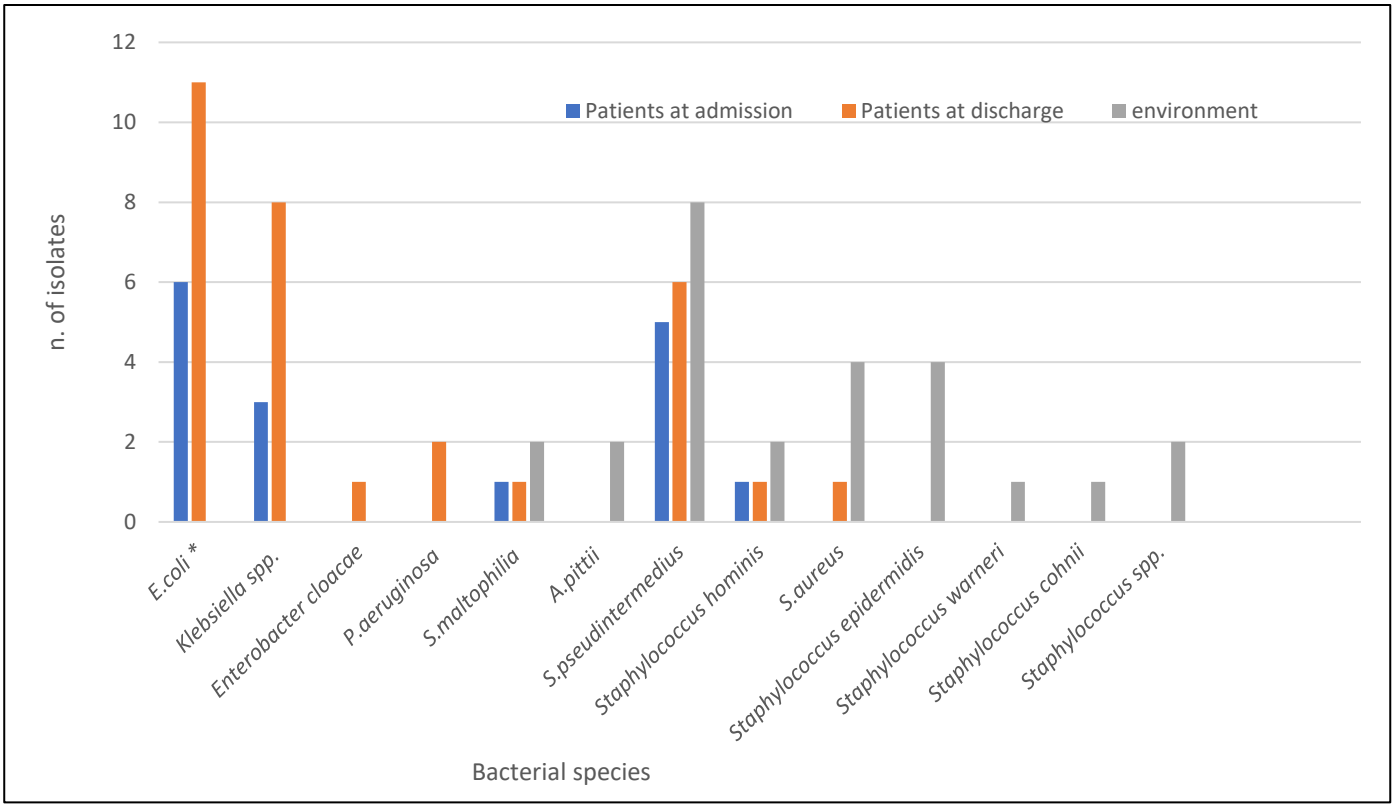
FOOTNOTE: CDT: Combination Disc Test. CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. MRS: Methicillin-Resistant Staphylococci.VTH: Veterinary Teaching Hospital.

Feedback reports. A total of three videocalls (one at the beginning of the project, one at the halfway and one at the end) were done to highlight the most critical points, to define how to improve some parts and to discuss single cases. Specifically, we report three anecdotical cases discussed:

- During the first session of active surveillance on the environment, of particular concern was the isolation of 3GCR and CR *Acinetobacter pittii* from one of the hair clippers used in the hospitalization area. The result was rapidly communicated, and a specific disinfection method was added for the clipper. This method included a first general cleaning, followed by a disinfection of the blade with 96% alcohol soak for 1 minute after every use. Furthermore, the body of the clipper must be disinfected once a day with peroxygenic acid, with a waiting time of 10 minutes. In the subsequent samplings, no more AMROs were isolated from the clippers.
- During the first session of active surveillance on patients, a cat with an history of previous hospitalizations in a different setting was admitted at the hospital and sampled. The patient had a diagnosed urinary tract infection caused by a multi-resistant *P.aeruginosa*. The AST from urine performed at the referring clinic showed resistance to all tested antimicrobials except for amikacin, imipenem, and ceftazidime. At the admission, the patient was under treatment with imipenem. After 24 hours of incubation, the perirectal sample realized at admission revealed positivity for CR *Stenotrophomonas maltophilia.* The result was communicated to the staff and with the suspect of the development of resistance towards imipenem confirmed by the presence of commensal CR *S. maltophilia* at the perirectal swab, the antimicrobial treatment was changed to ceftazidime, with a rapid improvement of clinical signs.
- A dog with history of chronic skin disease was hospitalized for six days during the first session, and the acquisition of 3GCR-GNB *E.coli* and *K.pneumoniae* during the stay was recorded. During the second session (74 days later), the patient was hospitalized again, and the perirectal swab at the admission revealed the same 3GCR-GNB pattern.

Species identification. Results are shown in figure 2. 3GCR-*E.coli* was the most isolated species in patients at admission (n=6) and at discharge (n=11). The three CR-GNB isolated from patients were identified as *S. maltophilia, P.aeruginosa* and *K.pneumoniae.* MRSP was the most frequent species found in the environment (n=8), followed by MRSA and *Staphylococcus epidermidis* (n=4).

Figure 2. List of bacterial species isolated and identified with MALDI-TOF, divided considering the isolation site (patients at admission, patients at discharge and environment/staff).



FOOTNOTE: *isolates identified as *E.coli* are not distinguible from *Shigella* spp. with MALDI Biotyper technology.

Comparison of the results. See Table 2.

Table 2. Comparison of the main results from active surveillance (both on patients and the environment) in the two Veterinary Teaching Hospitals (Madrid and Bologna).

Active surveillance on patients	**Madrid**	**Bologna**
Total patients sampled	50	150
Average duration of session (25 patients)	45 days	32 days
Average hospitalization	3.8 days	5.3 days
General FD at admission	24%	36.7%
General in-hospital acquisition	28%	40.7%
MRS FD at admission	12%	22.0%
MRS in-hospital acquisition	6%	19.7%
3GCR-GNB FD at admission	14%	24.7%
3GCR-GNB in-hospital acquisition	22%	36.3%
CR-GNB FD at admission	2%	11.3%
CR-GNB in-hospital acquisition	2%	25.6%

CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. MRS: Methicillin-Resistant Staphylococci.

Active environmental surveillance	**Madrid**	**Bologna**
Total samples	98	195
MRS FD	22.4%	19.5%
3GCR-GNB FD	2%	7.7%
CR-GNB FD	2%	4.6%
MRS on personnel' hands	33%	11.4%

MRS on personnel' clothing	8%	20%
MRS on personnel' shoe soles	50%	85.7%

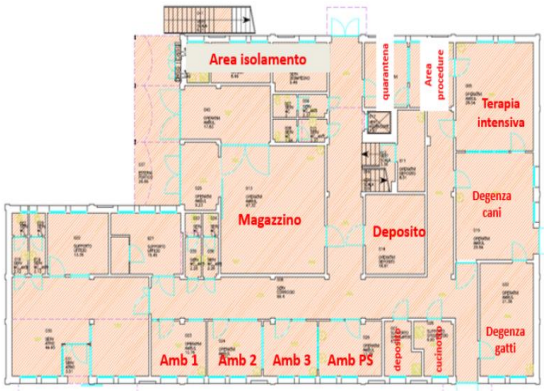
CR-GNB: Carbapenem-resistant gram-negative bacteria. 3GCR-GNB: third-generation cephalosporins resistant gram-negative bacteria. FD: Frequency of detection. MRS: Methicillin-Resistant Staphylococci.

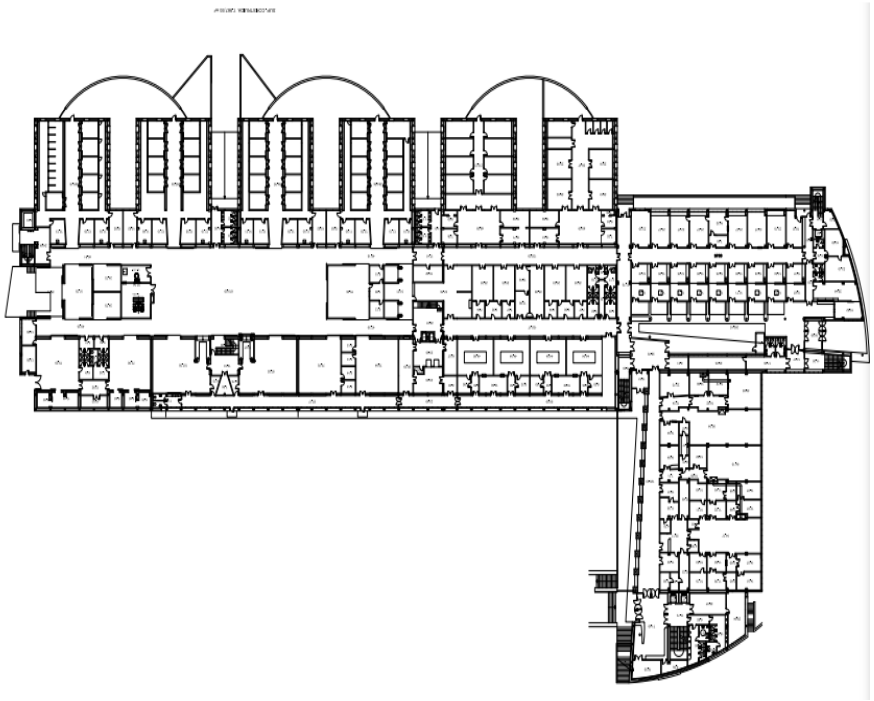
6.D. Discussion

The results from active surveillance on patients show as in general, a lower FD of AMRO in patients admitted to the hospital was present in the Madrid VTH, for each typology (MRS, 3GCR-GNB, CR-GNB) considered. Although reports from human medicine show that the situation between Italy and Spain is similar in terms of resistance rates (263), this finding could be primarily due to epidemiological and local geographical differences, always an important factor when performing this kind of comparison. Another explanation of the difference in the FD at admission can be found in the sample size, that was three times higher in Bologna. Additionally, it has to be considered that although in both cases the patients were sampled after maximum 12 hours from the hospital admission, the author anecdotally reports that in the Madrid VTH the samplings were performed more often directly by the personnel in the moment of the admission, while in Bologna was more frequently performed after the admission by the laboratory personnel. This could have led, as previously mentioned, to an overestimation of the FD at admission in the Bologna VTH.

Considering in-hospital acquisition, again the results show a higher percentage of patients that acquired AMROs in the Bologna VTH than in Madrid. Specifically, the acquisition of CR-GNB was much higher (25.6% vs 2%). In this case, other additional explanations can be found from a management point of view. First, the average length of hospitalization, already demonstrated to be an important risk factor for in-hospital acquisition, was higher in the Bologna VTH (5.3 days versus 3.8). Second, the Bologna VTH presented a higher number of patients hospitalized for more than 48 hours, as demonstrated by the lower average duration of the sessions (32 days vs 45 days): this may increase the risk of environmental contamination and patient-to-patient transmission. Third, although a deeper analysis of antibiotics consumption was not performed, differences in the stewardship policies were present; anecdotally the author reports that antibiotics use in the Madrid VTH did not include, for example, piperacillin-tazobactam, demonstrated to be risk factors for CR-GNB acquisition in the Bologna VTH. Fourth, the two VTHs presented different structures (see Figure 3), with wider and more compartmentalized spaces in the Madrid VTH, with a subsequent lower risk of overcrowding and cross-contamination.

Figure 3. Planimetry of the two VTH (Bologna first). The Madrid VTH was part of a bigger structure, that includes also the equine hospital and the Surgery Area. In Bologna, the Surgery and Pre-Surgery Area were located in another external structure, not shown here.





When looking at environmental surveillance, again a higher FD was found in the Bologna VTH for CR-GNB and 3GCR-GNB, while MRS rates were similar. Given that even in this case the differences in the sample size represent a partial explanation, also the difference in some sampling sites (e.g. the floor, the surgical/pre-surgical areas were not sampled in the Madrid VTH) could be an additional reason of such differences. On the other hand, some zones such as the portable ultrasound machine and the hair clipper showed in both facilities to be frequently contaminated by AMROs, suggesting as they should be considered critical points to be targeted by infection control policies. The samplings on the hospital personnel highlighted as the FD on the hands was higher in Madrid, while was lower in clothing and shoe sole. Given all the premises about the sample size, the first finding potentially be related with a lower compliance in handwashing of the Madrid VTH's personnel; nevertheless, this speculation does not match with a higher in-hospital acquisition on patients, neither with a higher environmental contamination. The second finding (on clothing contamination) could be explained with the fact that, in the Madrid VTH, a specific area was designed to let workers change cloths. The third finding on shoe soles could be associated with specific preventive measures present at the Madrid VTH, that allow the entrance to determined areas (e.g. ICU and isolation area) only with single-use overshoes, limiting the risk of contamination. Nevertheless, the floor was not sampled in Madrid, so a further analysis of its importance as environmental reservoir is not possible.

This comparative analysis has several limitations. First, the smaller sample size could have masked or overlooked some aspects and did not allow a risk factors analysis on patients. Second, a genotypical characterization was not performed, impeding a comparison between the AMROs' resistome in the two facilities. Third, a comparative description of the antimicrobial consumption in the two VTH could have allowed a more exhaustive analysis of the reasons behind the differences in the AMR rates.

In conclusion, this comparative, descriptive research allowed to better understand the points in common and the differences between two similar healthcare facilities located in two European countries. The use of the same *modus operandi* was crucial in order to obtain comparable data, enabling to focus on the specific needs and priorities of each VTH, and to contextualize the importance of the results.

Chapter 7. Outbreak surveillance

7A. Specific aims

The outbreak surveillance aimed at collecting and analyzing data over time about environmental AMROs during suspected outbreak periods at the Bologna VTH. Additionally, it aimed at evaluating the effectiveness of the outbreak management protocol, by measuring and comparing determined outcomes.

7B. Materials and methods

1. **Study design.** A perspective, cross-sectional and observational study was conducted into the Bologna VTH as a part of the pilot surveillance program from December 2020 to May 2023. Outbreak surveillance was performed when data from passive and active surveillance displayed the onset of a potential HAI outbreak, with *ad hoc* samplings depending on the bacterial agents and patient area involved. The outbreak management protocol was divided into four phases:
   - **i) Alarm:** started when data from passive surveillance showed that an unusual number of closely related cases of potential HAIs (e.g., caused by the same bacterial species) was recorded in a short period of time. Then, a rapid communication of the findings to the hospital personnel (see Chapter 8) was performed, with a discussion of the cases and, if considered necessary, a subsequent decision on how and where perform the samplings.
   - **ii) Investigation:** execution of targeted samplings, analysis and communication of the results to the hospital personnel.
   - **iii) Response:** depending on the results from the investigation phase, discussion and decision of the measures needed to limit the outbreak.
   - **iv) Evaluation of the response:** execution of control samplings, enhanced patients monitorization and revision/upgrade of the hospital preventive measures.

2. **Samples collection**. Samples from environment and workers were collected from different areas of the VTH depending on the discussion of the "Alarm" phase, with both sterile swabs and sterile sponges, as described in the "Materials and methods" section of Chapter 5.
3. **Isolation and identification**. Samples were processed into selective media, that changed depending on the specific aim. For the samples collected using sterile sponges, the bacterial culture was performed by directly streaking 10 microliters of the liquid in the plate with a sterile loop. Swabs were processed by rubbing the swab into the media, over approximately one third

of the surface of each plate, and subsequently streaking using a sterile loop. After 24-48 hours of incubation at 37°C in aerobic conditions, colonies isolated from positive cultures were subcultured on blood agar with 5% sheep blood (Oxoid) and subsequently identified through the matrix-assisted laser desorption–ionization time-of-flight mass spectrometry method (MALDI-TOF MS) using a Bruker Daltonics MBT SMART equipment, and the Biotyper Real Time Classification software v3.1 (Bruker Daltonics, Germany), following manufacturer's instructions. Colonies identified with a score between 1.5 and 2 were considered only at the genus level.

4. Data Analysis. Descriptive statistics was performed for four parameters directly measurable: i) timing (time passed since the first case to the "Response" phase), ii) clinical outcomes (number of deaths/critical infections); iii) number of patients involved; iv) length (days) and severity (access restriction/complete close) of restrictions needed to control the outbreak. Such parameters were then compared with retrospective data from one outbreak occurred before the start of the surveillance program at the Bologna VTH.

7C. Results

Cases description. During the observation period, two suspected outbreaks were registered, both associated with the *E.cloacae* complex (ECC).

- **First case.** On February 12th, 2021 a SSI in a dog caused by a MDR *E.cloacae* was recorded. In the following 16 days, two more MDR *E.cloacae* infections (one SSI and one prostatic infection in two dogs hospitalized in the ICU), with similar resistance patterns, were registered; these findings triggered the "Alarm" phase. A targeted surveillance ("Investigation" phase) on the ICU and Surgery environment and workers was performed (see Table S6), that highlighted the presence of *E.cloacae* on one ICU worker's hands, and in the cage of one of the hospitalized patients with the infection, after the routine cleaning. Results were communicated to the personnel that performed an additional disinfection of all the ICU cages. In the next 19 days, three more cases of *E.cloacae* infections (two SSIs and one UTI from three dogs) were recorded, so after the discussion with the personnel, it was decided to temporarily restrict patient admission for 3 days in order to perform a deep disinfection of the ICU area with hydrogen peroxide; furthermore a sensibilization on the importance of a correct sanitization procedure and hand washing, including a video of example, was performed ("Response" phase). The subsequent environmental sampling on ICU environment and workers showed no *E.cloacae* detection, and no further *E.cloacae* infections were registered in the following 4 weeks.

- **Second case.** On July 22nd, 2022, a MDR *E.cloacae* was isolated from a SSI in a dog with a previous hospitalization history. In the following 13 days, five more MDR *E.cloacae* isolates from four patients, in specimens from suspected HAIs (one blood culture, two SSIs, one abdominal effusion and one prostatic infection) were recorded, triggering the "Alarm" phase. Given that all the patients considered underwent surgery, a targeted surveillance ("Investigation" phase) was performed on the surgical and pre-surgical areas and workers (Table S7). Isolates identified as *Enterobacter hormaechei* and *Enterobacter* spp. were found in the portable ultrasound machine of the pre-surgery area, and – in higher quantity - on a surgical staple device (LigaSure™) used in one surgery room. Furthermore, 3CGR *Pseudomonas* spp. was found in the same LigaSure™ device. A deep disinfection of the surgical room and the equipment was performed with hydrogen peroxide; furthermore, the

disinfection and sterilization procedure of the LigaSure™ device was changed and a specific procedural indication was added for the portable ultrasound machine ("Response" phase, see Annex 1). A subsequent sampling on the surgical and pre-surgical areas did not show any further *E.cloacae* isolates, and no more clinical cases of MDR *E.cloacae* (although one non-MDR *E.cloacae* was isolated from an outpatient) were recorded in the following 4 weeks.

Data analysis. The analysis of the effectiveness parameters are shown in Table 1, in comparison with retrospective data of a *S.marcescens* outbreak occurred before the beginning of the surveillance program, in 2019.

Table 1. Comparison of the management of three outbreaks occurred in the Bologna VTH between 2019 and 2022. In the first one, caused by *S.marcescens*, there was no surveillance program present. In the second and the third, caused by *E.cloacae* and *E.cloacae* complex, the described surveillance program was present. Four parameters were selected to evaluate the effectiveness of the management.

Parameter	*S. marcescens* (with no surveillance) 2019	*E.cloacae* February 2021	*E.cloacae* complex July 2022
Timing since first case	**6 months**	**35 days**	**15 days**
Clinical outcomes	**Severe (3 sepsis, 1 euthanasia)**	**Moderate (no sepsis/deaths)**	**Moderate (1 sepsis)**
Number of involved patients	**12**	**6**	**6**
Length/severity of patient restriction measures	**7 days (complete close)**	**3 days (restrictions in ICU admissions)**	**0 days**

7.D. Discussion.

The outbreak surveillance is a typology of surveillance that is executed only during the occurrence of suspected bacterial HA outbreaks. Although the patient-to-patient transmission in healthcare

settings is possible, the environment (including the personnel) has a critical importance in the maintenance and transmission of the infection chain. With this type of surveillance, a deep, specific investigation based on suspects from clinical evidence was possible, that ultimately allow to detect the most important hazards and to limit the spread (and so the damage) of the outbreak. In our case, we observed two suspected HA outbreaks during an observational period of 30 months (1 outbreak every 15 months). Such outbreak frequency is in line with the one described for VTHs by Benedict et al. in 2008 (195) in a survey that reported that out of the 38 VTHs included in the study, 82% reported at least one outbreak in the five year prior to the interview, and 45% more than one. Such percentages could be an underestimation, due to the inability of most VTHs to detect and report the outbreaks (e.g. the ones without a surveillance system) and, even when detected, to the tendency of neglection for the fear of the bad reputation that reporting would give to the facility.

In the first case, the surveillance allowed to detect that ICU worker 'hands and a cage where one infected patient was staying (even after routine disinfection) were a major risk for transmission. Those two results leaded to a large sensibilization on the importance of hand washing and a correct disinfection within the hospital personnel. While the first point has already been discussed in the Chapter 5, and the increased use of alcohol based hand rubs has been associated with a decrease in HAIs incidence in human hospitals (371), also the importance of a correct device and equipment disinfection, with substances with a demonstrated effectiveness against the specific pathogen and a correct respect of the waiting time, must be underlined. In the second case, the outbreak was related with the surgical and pre-surgical area. Specifically, surveillance highlighted as the portable ultrasound machine of the pre-surgical area and the LigaSure™ device were contaminated. Again, such results allowed to modify and implement specific measures. The contamination of the ultrasound machine could be due to the lack of disinfection procedures between patients' examinations, so a specific procedure to guide clinicians in its use (See Annex 1) was added. The LigaSure™ device was routinely cold sterilized by immersion in peracetic acid for 30 minutes before their use, but possibly not always with at right concentration or with a correct cleaning after use, that could have let to the survival of 3GCR *Enterobacter* spp. and *Pseudomonas* spp. A Canadian study (372) on cold sterile solutions from 90 small animal clinics reports a 13% prevalence of bacterial isolates, mainly opportunistic gram-negatives. This suggest that factors such as inappropriate dilution, pH or contamination with organic matter may play a role in facilitating bacterial survival. For these reasons, a written procedure for cold sterilization was implemented with specific indications (see Annex 1). It is possible that other environmental factors not detected by surveillance contributed to the ECC spread (for example the floor, as the results from environmental surveillance – Chapter 5 – showed that 3GCR ECC isolate was found), but considering that in both cases no more suspects were detected in the following months, it could be speculated that the measures were effective to limit the outbreaks.

Notably, both the outbreaks reported were associated with the *E.cloacae complex* (ECC). The ECC include different *Enterobacter* species (*E.asburiae, E.cloacae, E.hormaechei, E.kobei, E.ludwigii, E.nimipressuralis, E. xiangfangensis*) a well-recognized agent of nosocomial infections in human medicine (Sanders et al., 1997; Wisplinghoff et al., 2004 da Annavajhala), already described as a pathogen present in the environment in veterinary hospitals (351), and it is characterized by intrinsic resistance to beta-lactams (including to some potentiated penicillins such as amoxicillin-clavulanate) and the ability to acquire resistance genes for multiple antibiotic classes (373). Its success in relation with hospital survival could be related with various factors, including resistance to heavy metals (374), cell stress response systems (375) and the ability to survive to disinfectants (376,377). In our case, the findings suggest that the ECC isolates possessed the ability to resist to

disinfectants (e.g. through biofilm production), given that ECC was found in a cage after routine disinfection (first case) and in a daily cold sterilized LigaSure™ device (second case). Furthermore, given that ECC was related with HA outbreak twice after more than a year of distance, it can be speculated that it was probably endemically present in the VTH environment, and becomes evident in specific circumstances that facilitated its spread. Indeed, it is well known that HAI agents can silently remain in hospitals, even during years (173,175).

In our case, the main limits of this kind of surveillance have been the absence of a genotypical characterization of the isolates, in order to have a better overview of the epidemiology and the factors that promoted the occurrence of the two outbreaks. Nevertheless, these considerations can be the object of future studies. Additionally, in the second outbreak surveillance was focused only on the environmental contamination, without investigating the rate of ECC colonization in patients. As previously stated, normally more patients are colonized than infected, and infected patients are usually colonized before the onset of the disease (depending on where the pathogen enters the body as well as on the individual immune status). Although Wilson et al. (378) found that the number of new patients colonized with MRSA seemed not to be reduced by the use of enhances cleaning procedures, failure in the recognition of the proportion of colonized patients can lead not only to an underestimation of the extent of the outbreak, but also to an increased risk of dissemination of AMROs in the local community (172). Additionally, it impedes to implement useful strategies to limit the spread, such as patient cohorting and the "search-and-isolate" policy already described in small animal practice(172). In our case, the active surveillance sessions on patients did not coincide with the occurrence of the outbreaks, but possibly the "search-and-isolate" policy could have been difficult due to logistic impediments (impossibility of isolation of a high number of infected patients).

The efficacy of the outbreak containment protocol was assessed by comparing the two outbreak with a previous one, caused by *S.marcescens* in 2019, without an active surveillance program. All the parameters chosen (timing, clinical outcomes, number of patients involved and length of restrictions) considerably improved. Although the differences could be due also to the different etiological agents and to other non-measured parameters, it can be speculated that the institution of the whole surveillance program, and specifically the containment protocol helped in the management of both outbreaks.

In conclusion, this kind of emergency surveillance is a useful tool to limit and contain pathogen spread in case of HA outbreaks, and ultimately to detect and correct procedural mistakes and critical points. As previously mentioned by citing a famous phrase by Langmuir, **"***Good surveillance does not necessarily ensure the making of the right decisions, but it reduces the chances of wrong ones***."**(203)**.**

Chapter 8. Education and communication

8.A. Specific aims

The communication system was developed to create and reinforce a positive collaboration between veterinary professionals from different niches, and to increase the knowledge and the awareness of veterinary workers about AMR, HAIs in general and within the Bologna VTH.

8.B. Materials and methods

The communication part was an integrated section of the surveillance program, and it was carried out with three main ways:

1) **Chat group.** Communication of every suspected case of HAI detected from the laboratory, to assist clinicians in the daily management of complicated infections.
2) **Discussion meetings.** Periodic meetings of approximately two hours each, with the presence of the surgical and clinical personnel, microbiology staff, equine perinatology team and professionals from human medicine.
3) **Dissemination.** Through seminars/webinars, oral and poster presentations in congresses/conferences and publications in scientific journals.

8.C. Results

Chat group. The chat group was mainly used for the routinary discussion of complicated clinical cases and suspected HAIs. Every clinical case that was considered a suspected HAI was promptly communicated by the laboratory staff to the clinicians and vice-versa, with the subsequent discussion of the actions needed. The same approach was used for the outbreak surveillance (see Chapter 7), with the rapid communication of the sampling results.

Discussion meetings. A total of 22 meetings were made, with a written resume of each one about the topics faced (Table 1). In November 2023 a new biosecurity manual for the whole Department of Veterinary Sciences was redacted by the new-formed biosecurity workgroup. It included a Chapter on the surveillance program, and with some of the suggestions/considerations inspired by the meetings, such as the flows management and the risk-based categorization of the areas.

Table 1. Description of the main topics faced in each of the 22 discussion meeting organized from January 2021 to May 2023.

Meeting	Main topics
n.1	Institution of a biosecurity workgroup, planning of active surveillance, ideas to measure handwashing compliance.
n.2	Patients flow management; written program for PPE use; reform of the waiting area.
n.3	Analysis of blood cultures, detailed description of the *E.cloacae* outbreak; Planification of a risk-based score for patients admitted to the hospital.
n.4	Analysis of the management of the *E.cloacae* outbreak , institution of two workgroups: prevention and containment.
n.5	Clinical cases discussion; general rules of antimicrobial stewardship in surgery.
n.6	Presentation of the written plans of the two workgroups.
n.7	Revision of endovenous lines management; implementation of keyboards protection; first involvement of the equine perinatology team.
n.8	Discussion of trichotomy importance in human medicine vs veterinary; presentation of the first results from active surveillance; first involvement of veterinary technicians.
n.9	Discussion on the new project for the ICU area; presentation of the results of a thesis on syndromic surveillance; clinical case discussion.
n.10	Presentation on equine perinatology situation about AMR; presentation of the result from passive surveillance; evaluation of the efficacy of the "Emergency AST" on blood cultures
n.11	Detailed planification of the patient flow management; Risk-based categorization of the areas
n.12	Discussion on asymptomatic bacteriuria; presentation of the results on the risk-based score for patients at admission; clinical case discussion.

n.13	Clinical cases discussion; discussion on UTIs; virologic surveillance.
n.14	Presentation of the results of active surveillance; discussion on sepsis markers in horses.
n.15	Presentation of the results of surveillance in foals; discussion on how to increase the awareness on AMR; categorization of disinfectant and antiseptics
n.16	Revision of risks in the equine perinatology unit; proposal of a clinical audit
n.17	Discussion on canine microbiota and strategies of modulation; presentation of a *Salmonella* spp. outbreak occurred in the equine hospital
n.18	Description of the *ECC* complex outbreak; revision of the surgical staples' disinfection methods; discussion on how to improve clothing management; discussion on the importance of the floor
n.19	Presentation of the results of active surveillance in horses; discussion on SSIs management
n.20	Presentation of the results of active surveillance in the Madrid VTH and comparison with the latest results from Bologna.
n.21	Discussion on SSIs management and stewardship, with comparison between human/equine/small animal medicine.
n.22	Discussion on upper respiratory infections management; presentation of the final results of the surveillance program

FOOTNOTE: AMR=Antimicrobial Resistance; AST=Antimicrobial Susceptibility Testing; ECC=*Enterobacter cloacae* complex; ICU= Intensive Care Unit; PPE=Personal Protective Equipment; SSI=Surgical Site Infections; UTI= Urinary Tract Infections; VTH= Veterinary Teaching Hospital.

Dissemination. At present (January 2024), a total of 5 papers was published in scientific journals (see below), with three more in elaboration. A total of 4 seminars (including one in Madrid) were organized during the 30 months, and six oral presentations and one poster were presented at seven different national and international congresses (see below).

Publications

1- Cocca, G., Piva, S., Magno, S. D., Scarpellini, R., Giacometti, F., Serraino, A., & Giunti, M. (2021). Prevalence and patterns of antimicrobial resistance among Escherichia coli and Staphylococcus spp. in a veterinary university hospital. *Veterinary Sciences*, *8*(12), 308.

2- Piva S, Scarpellini R. (2022). Healthcare associated infections in companion animals. The role of the microbiology lab in the surveillance system | Infezioni correlate all'assistenza negli animali da compagnia: Il ruolo del laboratorio di microbiologia nella sorveglianza sanitaria. *Veterinaria,* 36(3), pp. 113–124.

3- Scarpellini, R., Assirelli, G., Giunti, M., Esposito, E., Mondo, E., & Piva, S. (2023). Monitoring the Prevalence of Antimicrobial Resistance in Companion Animals: Results from Clinical Isolates in an Italian University Veterinary Hospital. *Transboundary and Emerging Diseases*, *2023*.

4- Scarpellini, R., Giunti, M., Pontiero, A., Savini, F., Esposito, E., & Piva, S. (2023). Two cases of bloodstream infections associated with opportunistic bacterial species (*Enterococcus hirae* and *Enterobacter xiangfangensis*) in companion animals. *BMC Veterinary Research*, *19*(1), 63.

5- Scarpellini, R., Giunti, M., Bulgarelli, C., Mondo, E., Esposito, E., Assirelli, G., & Piva, S. (2024). First isolation of *Yersinia pseudotuberculosis* from the blood of a cat. *Frontiers in Veterinary Science*, *10*, 1261925.

Seminars

1- December 21st, 2021. Seminar for the professionals of the Department of Veterinary Medical Sciences of the Bologna University. “Healthcare-associated infections: are they our problem?”
2- April 9th, 2022. Webinar for the Italian Society of Veterinary Internal Medicine "Antibiotic resistance in veterinary settings: the experience of the University Veterinary Hospital in Bologna”
3- November 17th, 2022. Seminar for the students of the Veterinary Faculty of the Complutenze University, Madrid. “Antibiótico-resistencia y infecciones nosocomiales: Importancia de un plan de vigilancia”
4- June 12nd, 2023. Seminar for the students of the Department of Veterinary Medical Sciences of the Bologna University. “Healthcare-associated infections”

Oral presentations/posters

1- 74th Congress of the Italian Society of Veterinary Sciences (Virtual Edition): June 22nd-25th, 2021. Oral presentation “Management of two healthcare-associated infections outbreaks of *Serratia marcescens* and *Enterobacter cloacae* in a Veterinary University Hospital, and implementation of a surveillance system”

2- Workshop online “ELEPHANT workshop WP4: One Health in Action I. October 25th-29th, 2021. Oral presentation “Surveillance of bacterial hospital-acquiredinfections in the Bologna University Hospital – Small Animals section”.

3- 75th Congress of the Italian Society of Veterinary Sciences. June 15-18th, 2022, Lodi. Oral Presentation “PULSED ACTIVE SURVEILLANCE FOR THE EVALUATION OF COMMENSAL ANTIMICROBIC RESISTANT BACTERIA IN A SMALL ANIMAL VETERINARY UNIVERSITY HOSPITAL: PRELIMINARY RESULTS”

4- 6th Congress of European Association of European Veterinary Diagnosticians (EAVLD). October 24th-26th, 2022, Seville, Spagin. Oral presentation “PULSED ACTIVE SURVEILLANCE FOR THE EVALUATION OF COMMENSAL ANTIMICROBIC RESISTANT BACTERIA IN A SMALL ANIMAL VETERINARY UNIVERSITY HOSPITAL: PRELIMINARY RESULTS”. Awarded as the Second Best Oral Presentation.

5- 76th Congress of the Italian Society of Veterinary Sciences. June 21st-23rd, 2023, Bari, Italy. Oral presentation “Development of a passive surveillance system for the monitoring and the management of potential hospital-acquired infections (HAIs) in a Veterinary University Hospital (VTH)”.

6- 5th International Conference of European College of Veterinary Microbiology. September 21st-23rd, 2023, Bled (Slovenia). Poster: “Active Surveillance as a tool to fight Antimicrobial Resistance in Small Animal Practice: comparison between an Italian and Spanish Veterinary University Hospital.

7- Event “Top Ten 2023 of Medical Sciences” organized by the Accademia delle Scienze of Bologna, December 7th, 2023. Oral presentation “Antibiotic-resistance: a shared threat between humans and animals”. Co-awarded as the Best Oral Presentation.

8.D. Discussion

In this Chapter we described the less measurable, but not less important, part of the surveillance program. The institution of an efficient communication system, through different pathways, is a crucial part of every surveillance program. The continuous, evidence-based results presentation to the clinical staff, including about their compliance, can enhance their awareness about the problem, and help to detect the most common practice deficiencies (379,380). Veterinarians play a key role in the daily correct management of antibiotics, the execution of hygienic good practices and the education of the owners, that are more likely to understand the AMR phenomenon in animals if a trustful relationship with the vet is established (381). For these reasons, a cascade mechanism that starts from the education of the veterinarians can have a positive impact in the rational use of antibiotics, in the infection control policies and in the increase of people's awareness (382).

The chat group, mainly used for routinary communications, enhanced the rapidity of the response in case of outbreaks. The discussion meetings, started within few people, progressively broadened their audience, until they became sharing moments between professionals from different disciplines. They increased the knowledge and the awareness about AMR and HAIs, but also allowed to build up a specific biosecurity workgroup, that implemented practical, effective measures included in the redaction of the new biosecurity manual. The constant dissemination of the results aimed at sharing our findings to the scientific community and to the students, in order to contextualize them and to allow an external, more objective evaluation.

Chapter 9

Conclusions

9.A. Key findings of the project

1. **Passive surveillance**

- Urines and ear swabs are frequent specimens in small animal infections submitted at the VTH.
- *E.coli, S.pseudintermedius* and *S.canis* are the most frequently isolated bacterial species.
- Multi-drug resistance was observed in 41.6% of the isolates. Gram-positive bacteria displayed higher AMR/MDR percentages than gram-negatives.
- Worrying non-susceptibility percentages (considering their importance for human medicine) were registered for clindamycin, amoxicillin-clavulanate, ceftiofur, piperacillin-tazobactam and enrofloxacin.
- Whole Genome Sequencing analysis on some selected MDR Enterobacterales isolates showed the presence of multiple worrisome ARGs, including the carbapenemase-encoding gene $bla_{\text{NDM-5}}$, probably selected by the use of piperacillin-tazobactam.
- Antibiotic treatment, previous use of invasive devices, previous hospitalization/surgery and species dog are risk factors statistically associated with higher AMR or/and MDR rates. The length of hospitalization, the previous use of invasive device and previous/current antibiotic were statistically associated with isolates from suspected HAIs.
- Bacterial species of the ESKAPE group displayed higher AMR/MDR percentages, especially when associated with suspected HAIs.

- Suspected HAIs (mostly blood cultures, urines from HA UTIs and SSIs) accounted approximately for 13% of total positive specimens, with a stable trend of the cases over time.
- AMR/MDR percentages of the isolates from suspected HAIs were considerably higher (90.9% and 71.7%, respectively).
- *E.coli* was frequent in every suspected HAI specimen, *S.pseudintermedius* and *Enterobacter* spp. were more common in SSIs/surgical implants, and *K.pneumoniae* and *E.faecium* in suspected HA UTIs.

2. Active surveillance on patients

- Dogs were more frequently hospitalized for >48 hours than cats. Average length of hospitalization was 5.3 days. Around 70% of patients received antibiotics during the hospital stay. Nasogastric tube was the most used invasive device.
- More than one third (36.7%) of the patients included was carrier of at least one AMRO (MRS, 3GCR-GNB or CR-GN) at admission. The highest frequency was detected for 3GCR-GNB (24.7%).
- In-hospital AMROs acquisition was frequent (40.6%). The most worrisome percentage was recorded for CR-GNB (25.6%). MRSP and 3GCR/CR *K.pneumoniae* and *E.coli* were the commonest species identified.
- $bla_{\text{CTX-M}}$, $bla_{\text{OXA-1-like}}$, bla_{TEM} were ARGs frequently found in 3GCR, while bla_{NDM} in CR GNB. In 3GCR Enterobacterales, the co-presence of one of more ARG in the same isolate was frequent (63%).
- Species dog was associated with MRS carriage at admission; previous hospitalization/surgery, and previous quinolones treatment were associated with general AMROs carriage and 3GCR-GNB carriage. Previous antibiotic treatment was associated with general carriage and 3GCR-GNB carriage in dogs.
- The length of hospitalization was associated with general AMROs in-hospital acquisition, MRS acquisition in dogs and 3GCR/CR-GNB acquisition; the use of nasogastric tube was associated with 3GCR-GNB acquisition, and the use of piperacillin-tazobactam was associated with CR-GNB acquisition.

3. Active surveillance on the environment.

- MRS were more frequently found in the environment than 3GCR/CR-GNB
- The ICU was the area with the highest AMROs frequency of detection (FD).
- The floor, the portable ultrasound machines, the scales, the visiting tables and the sinks were the points with the highest AMROs FD.
- Considering the personnel, the shoe soles and the clothing displayed higher AMROs FD than the hands.
- MRSP, MR *S.haemolyticus,* MR *S.hominis* and 3GCR *Acinetobacter* spp. were the most frequent environmental AMROs identified.

4. Comparison of active surveillance results between an Italian and a Spanish VTH

- A lower FD, general (24%) and for each AMRO considered, was observed in the Spanish VTH.
- A general lower in-hospital acquisition was observed in the Spanish VTH, especially regarding CR-GNB (2% vs 25.6%).
- Environmental contamination was similar for MRS, but lower for 3GCR/CR-GNB.

- Comparing with the Italian VTH, personnel'hands were more frequently contaminated by AMROs, vice-versa for clothing and shoe soles.
- MRSP, 3GCR *E.coli* and *K.pneumoniae* were the most common species isolated from patients; MRSP, MRSA and MR *S.epidermidis* were the most common species isolated from the environment.
- Differences between the two VTHs are attributable to the sample size, to epidemiological and geographical factors, but also to structural and management reasons (antimicrobial policies, density of patients, average length of hospitalization, spaces distribution).

5. Outbreak surveillance

- A protocol constituted by four parts (alarm, investigation, response, evaluation of the response) was implemented.
- Two suspected outbreaks, both associated with the *E.cloacae* complex, were recorded in the observation period.
- The application of the protocol leaded to: 1) changes in some procedural deficiencies; 2) improvement, if compared with a previous outbreak, in some management parameters (timing, clinical outcomes, number of patients involved, length of restrictions).

6. Communication

- The institution of a communication system through different pathways (chat group, discussion meetings, and dissemination of the results) was an integrated part of the surveillance program. It involved professionals from different fields (including equine and human medicine).
- It helped in the redaction of a new, departmental biosecurity manual, that included the surveillance program.
- A total of twenty-two meetings, involving professionals from different areas, was done. Five publications in Q1 scientific journals, four seminars, six oral presentations and one poster were published/presented.

9.B. Suggestions for guidelines of a tailored Infection Control Program

1) Long-term surveillance

- Definition of a surveillance practitioner.
- <u>Passive surveillance</u>:
  - Only on the "customized" ESKAPE bacteria group (*Enterococcus* spp., *S.aureus/intermedius/pseudintermedius, K.pneumoniae, Acinetobacter baumannii, P.aeruginosa, Enterobacter* spp.);
  - Only on isolates from suspected HAIs (according with the definition).
  - Monitoring of new suspected HAI cases (total, and "internal" cases), and their trend over time. Definition of an expected percentage of reduction of the "internal" cases.
  - Development of a protocol to follow-up SSIs cases.

- <u>Active surveillance on patients:</u>
  - Samplings executed by clinicians at admission.
  - Risk-based score (see an example in Annex 2) when a patient is admitted to the hospital, and screening at admission for the carriage of MRS/3GCR-

GNB/CR-GNB presence if the score is higher than the cut-off. If the sampling turns positive, preventive precautions (contact isolation, use of additional PPE) are executed.
- CR-GNB screening at discharge for every patient with determined risk factors (prolonged hospitalization, use of potentiated penicillins during the stay) and subsequent communication with the owners and follow-up checks for CR-GNB positives. Genotypical analysis of the CR-GNB isolates.
- Twice a year, pulsed surveillance to all the patients hospitalized >48 hours during a month.

- Active surveillance on the environment:
  - Pulsed surveillance (every three months) on highly touched and high-risk points (doors, stretchers, portable ultrasound machine, scale, sinks, visiting tables) from all the areas considered (ICU, Surgery, Pre-surgery, General Ward).
  - Pulsed surveillance (three times per year) on randomly chosen personnel (hands, clothing, shoe sole).

2) Prevention policies

- Definition of a Infection Control Practitioner (ICP) responsible for the correct execution of the biosecurity manual.
- Definition of an expected reduction in the average hospitalization length.
- Continuous measurement of the consumption of alcohol-based hand-rub solution.
- Execution of prevention measures (contact isolation, use of additional PPE) for patients hospitalized positive for >1 of the investigated AMROs.
- Mandatory use of disposable overshoes to enter in the ICU Area.
- Internal laundry system mandatory for every worker, or implementation of specific guidelines on home laundering practices.
- Specific, written procedures for cleaning and disinfection of stretchers, scale, portable ultrasound machine (see example in Annex 2).
- Revision of the procedures/indications for nasogastric tube use.

3) Outbreak management

- Implement and optimize the written protocol for outbreak management (see an example in Annex 3).

4) Antimicrobial Stewardship

- Definition of an expected reduction of prescriptions/use of some selected important drugs (amoxicillin-clavulanate, ampicillin-sulbactam, fluoroquinolones), and measurement of their consumption over time.
- Expansion of the number of drugs tested on the routinary AST (e.g. by including nitrofurantoin, marbofloxacin, ertapenem).
- Development of rapid diagnostics tools (e.g. with MALDI-TOF technology) to early detect the most common resistance mechanisms in clinical isolates (e.g. CTX-M/OXA-1/NDM-5 production).
- Surveillance based suggestions for the treatment choice:

- when indicated and needed, consider empiric tetracyclines use in infections caused by gram-negative bacteria, and specifically for *Enterobacter* spp. infections;
- consider potentiated sulfonamides (trimethoprim-sulfamethoxazole) in urinary infections empiric treatment (relatively low non-susceptibility percentages, and not a CIA);
- avoid amoxicillin-clavulanate, ampicillin-sulbactam, fluoroquinolones as first line or empiric treatment;
- avoid amoxicillin-clavulanate and 3rd generation cephalosporins as first line or empiric treatment in SSIs/surgical implants.

5) Communication

- Continuation and expansion of the discussion meetings;
- Dissemination of the results in highly cited scientific journals;
- Organization of webinars/seminar for students and veterinary professionals.

9.C. Final considerations and future perspectives

With this project, we tried to shed light on the role of small animal practice (and specifically VTHs) in a global AMR overview. The current literature on the topic show as, after being neglected for years, research on AMR in companion animals is increasing in interest. Their increasing presence in households, the possibility of direct contact with humans, the frequent use of the same antibiotics and the uncertainty about the direction of the transmission are all factors that are leading to such increasing interest. Specifically, the onset of healthcare-associated infections (the "tip" of the AMR iceberg) is a matter of concern. In fact, a part of their high mortality/AMR rates and the economic burden, HAIs in small animal practice are harder to manage, with less therapeutic options, and represent a public health concern due to the human-animal interface. For these reasons, the institution of a surveillance program to collect and analyze data on AMR and HAIs in pets is the first step for healthcare facilities such as VTHs, in order to increase the knowledge and possibly develop tailored Infection Control Programs constituted by long-term surveillance, prevention policies, outbreak management, antimicrobial stewardship policies and communication systems. The surveillance program we developed during these 30 months was a combination of passive and active surveillance, with the integration of a communication system. The results show as companion animals represent an important source of AMR and must be object of further analysis, especially on their role as reservoirs for AMR towards antibiotics preserved for human medicine, such as carbapenems. They did not demonstrate the effectiveness of surveillance programs alone in the reduction of AMR and HAIs impact but suggest how large veterinary settings can approach the topic, with an initial plan built to assess the risks and indicate where to concentrate the efforts. Local data from settings like veterinary hospitals are very important to improve the quality of the global overview about AMR. Specifically, VTHs seem to be high-risk places that need a special address and control measures. Future perspective should focus on the monitoring and evaluation of the human-animal interface in such facilities, for example:

i) by evaluating AMROs presence in the gut flora of veterinary workers/owners and their owned pets;
ii) by integrating data on AMR and HAIs between local veterinary and human hospitals;
iii) by a deep, complete genotypic investigation of the isolates, with a special focus on the assessment of the potential cases of reverse zoonosis.

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Supplementary material

Table S1. List of culture media, condition and temperature used depending on the specimen type.

Specimen type	**Media** [a]	**Incubation**
Urine [b]; ear swab	Blood Agar, Cled, Mac Conkey	Aerobic conditions
vaginal/uterine swab; wound; respiratory tract lavage; surgical site infection	Blood Agar, Cled, Mac Conkey	Aerobic conditions
	Columbia Agar	Capnophilic conditions
	Columbia Agar	Anaerobic conditions
bile; biopsy; blood culture [c]; abdominal/pleural/peritoneal effusion, exudate [d], abscess.	Blood Agar, Cled, Mac Conkey	Aerobic conditions
	Columbia Agar	Capnophilic conditions
	Columbia Agar, Wilkins-Chalgren Agar	Anaerobic conditions

FOOTNOTE:

[a] All the culture media were purchased from Oxoid, Germany, and prepared following manufacturer's instructions. Blood Agar and Columbia were made with 5% horse blood.

[b] Obtained both by cystocentesis and catheterization.

[c] Only for blood cultures found to be positive after an initial incubation at 37 °C for 7 days in a blood culture bottle (Signal Blood Culture System; Oxoid, Milan, Italy), following manifacturer's instructions.

[d] Abcess/exudate were defined by the submitting veterinarian based on macroscopic aspect

TABLE S2. List of tested antimicrobials divided for antimicrobial class.

Aminoglycosides	Amikacin 30 µg Gentamicin 10 µg (120 µg for *Enterococcus* spp. isolates)
Penicillins +/- beta-lactamases inhibitors	Ampicillin 10 µg Amoxicillin- clavulanate 30 µg Piperacillin-tazobactam 110 µg
Cephalosporins	Cefazolin/cephalothin 30 µg Ceftiofur 30 µg
Tetracyclines	Tetracycline 30 µg
Macrolides	Erythromycin 15 µg
Lincosamides	Clindamycin 2 µg
Fluoroquinolones	Enrofloxacin 5 µg
Sulfonamides + dihydrofolate reductase inhibitors	Trimethoprim- sulfamethoxazole 1.25/23.7 µg

Table S3. List of the 32 Enterobacterales isolates selected for further phenotypical assessment and their resistance profile towards the tested antibiotics. Isolates were collected from 14/12/2020 to 23/8/2022. Previous and current antibiotic (Ab) use at the time of the sampling is indicated, as well as if the specimens were associated with suspected Healthcare associated infections (HAIs). MIC values (mg/L) for each tested antibiotic are reported; values in red indicate resistance according to CLSI breakpoints (1), while values in green indicate susceptibility, and values in yellow refer to intermediate isolates.

ID	Species	Specimen	Patient	suspected HAI	Previous Ab use	Current Ab use	MIC TZP	A/S2	AMI	AMP	AXO	AZT	C/T	CIP	CZA	DOR	ETP	FAZ	FEP	GEN	IMI	LEVO	MERO	MIN	NIT	SXT	TAZ	TET	TGC	TOB
2154	*E.coli*	urine	dog	no	0	0	32	>16/8	<8	>16	4	<1	<2/4	<0.25	< 2/4	< 0.5	< 0.25	>16	<2	< 2	<1	<0.5	<0.5	>8	<32	<2/38	2	>8	<1	<2
2684	*E.cloacae*	SSI	dog	yes	AMC	AMC	<8	>16/8	<8	>16	<0.5	<1	<2/4	<0.25	<2/4	<0.5	<0.25	>16	<2	<2	<1	<0.5	<0.5	2	<32	<2/38	<1	<4	<1	<2
2811	*E.cloacae*	pleural effusion	dog	yes	A/S	MRB	16	>16/8	32	>16	>32	>16	<2/4	2	<2/4	< 0.5	< 0.25	>16	16	<2	<1	<0.5	<0.5	8	64	>2/38	16	>8	<1	>8
2905	*E.coli*	urine	dog	yes	MRB	0	16	>16/8	<8	>16	>32	8	<2/4	>2	<2/4	<0.5	<0.25	>16	4	<2	<1	>8	<0.5	4	<32	<2/38	8	>8	<1	<2
3031	*K. pneumoniae*	urine	dog	no	0	0	16	>16/8		>16	<0.5	<1	< 2/4	<0.25	< 2/4	< 0.5	< 0.25	16	<2	<2	<1	<0.5	<0.5	>8	<32	<2/38	<1	>8	<1	<2
3129	*E.coli*	urine	dog	no	TZP	0	512	>16/8	<8	>16	>32	16	16/4	>2	<2/4	<0.5	0.5	>16	8	<2	<1	>8	<0.5	2	<32	<2/38	>16	<4	<1	<2
3390	*E.coli*	urine	dog	yes	0	0	256	>16/8	<8	>16	16	>16	>16/4	>2	16	<0.5	0.5	>16	>16	<2	<1	>8	<0.5	>8	<32	>2/38	>16	>8	<1	>8
3454	*K.pneumoniae*	urine	dog	yes	ENR, CXD	TZP, MRB	128	>16/8	<8	>16	>32	>16	16	>2	16	2	8	>16	>16	4	2	>8	4	>8	>64	>2/38	>16	>8	<1	4
3464	*K.pneumoniae*	urine	dog	no	0	AK	256	>16/8	16	>16	>32	>16	4	>2	<2/4	<0.5	4	>16	>16	8	<1	>8	1	>8	>64	>2/38	>16	>8	2	>8
3554	*K.oxytoca**	vaginal swab	dog	no	MRB	MRB	1024	>16/8	<8	>16	8	>16	<2/4	>2	<2/4	<0.5	<0.25	>16	<2	4	<1	>8	<0.5	>8	<32	<2/38	<1	>8	<1	>8
3568	*K.pneumoniae*	urine	dog	no	AMC	AMC	16	>16/8	<8	>16	>32	>16	<2/4	>2	<2/4	<0.5	<0.25	>16	4	>8	<1	8	<0.5	2	<32	>2/38	16	>8	<1	>8
3677	*E.coli*	Broncho alveolar lavage	dog	yes	AMC	TZP	<8	16/8+	<8	>16	<005	<1	<2/4	<0.25	<2/4	<0.5	<0.25	4	<2	<2	<1	<0.5	<0.5	<1	<32	>2/38	<1	<4	<1	<2
3969	*K.pneumoniae***	urine	dog	yes	AMC	AMC	>1024	>16/8	<8	>16	>32	>16	>16/4	>2	>16/4	>4	>8	>16	>16	>8	>8	8	>8	2	<32	>2/38	>16	>8	<1	>8

4006	*E.xiangfangensis*	blood culture	cat	yes	MRB	MRB	256	>16/8	<8	>16	>32	16	8	>2	<2/4	<0.5	1	>16	4	>8	<1	8	<0.5	>8	<32	>2/38	>16	>8	<1	4
4032	*K.pneumoniae***	urine	dog	yes	AMC	0	>1024	>16/8	<8	>16	>32	>16	>16/4	>2	>16/4	>4	>8	>16	>16	>8	>8	8	>8	8	<32	>2/38	>16	>8	<1	>8
4045	*E.coli*	biopsy	dog	no	ENR, CXD	0	256	>16/8	<8	>16	4	<1	<2/4	>2	< 2/4	<0.5	< 0.25	<1	<2	>8	<1	>8	<0.5	>8	<32	>2/38	<1	>8	<1	>8
4104	*K.pneumoniae*	urine	dog	yes	MRB	0	>1024	>16/8	<8	>16	>32	>16	>16/4	>2	>16/4	>4	>8	>16	>16	>8	>8	>8	>8	>8	64	>2/38	>16	>8	2	>8
4149	*E.coli*	abdominal effusion	dog	yes	TZP, TAZ	TZP, TAZ	>1024	>16/8	16	>16	>32	>16	>16/4	>2	>16/4	>4	>8	>16	>16	<2	>8	>8	>8	>8	<32	>2/38	>16	>8	<1	>8
4158	*K.pneumoniae***	urine	dog	no	AMC, AMI	0	>1024	>16/8	<8	>16	>32	>16	>16/4	>2	>16/4	>4	>8	>16	>16	>8	>8	8	>8	8	<32	>2/38	>16	>8	<1	>8
4410	*K.pneumoniae*	ear swab	dog	yes	AMC, MRB	0	512	>16/8	<8	>16	>32	>16	>16/4	>2	>16/4	>4	>8	>16	>16	>8	>8	8	>8	8	<32	>2/38	>16	>8	<1	>8
4519	*E.coli*	abdominal effusion	dog	yes	MRB	MRB	>1024	>16/8	<8	>16	d	>16	>16/4	>2	>16/4	>4	>8	>16	>16	>8	>8	>8	>8	4	<32	<2/38	>16	<4	<1	>8
4526	*E.hormaechei*	urine	cat	yes	0	AMC	16	>16/8	<8	>16	8	4	<2/4	>2	<2/4	<0.5	<0.25	>16	<2	<2	<1	>8	<0.5	2	<32	>2/38	>16	<4	<1	8
4566	*E.coli***	urine	dog	no	RIX	0	256	>16/8	<8	>16	<0.5	<1	< 2/4	>2	< 2/4	<0.5	< 0.25	16	8	>8	<1	>8	<0.5	>8	<32	>2/38	<1	>8	<1	>8
4579	*E.coli*	urine	dog	no	AMC	0	32	>16/8	<8	>16	<0.5	<1	<2/4	<0.25	<2/4	<0.5	<0.25	8	<2	<2	<1	<0.5	<0.5	<1	<32	<2/38	<1	<4	<1	<2
4593	*E.cloacae*	SSI	dog	yes	AMC, ENR	A/S	1024	>16/8	<8	>16	>32	>16	>16/4	>2	< 2/4	<0.5	8	>16	<2	<2	<1	>8	1	8	64	>2/38	>16	8	<1	8
4615	*K.pneumoniae*	urine	dog	no	0	0	>1024	>16/8	<8	>16	>32	>16	>16/4	>2	<2/4	1	>8	>16	<2	<2	2	>8	4	>8	>64	>2/38	>16	8	2	>8
4721	*K.pneumoniae*	urine	dog	no	AMC, ENR	AMC	1024	>16/8	<8	>16	>32	>16	>16/4	>2	>16/4	>4	>8	>16	>16	>8	>8	>8	>8	8	>64	>2/38	>16	>8	<1	>8
5178	*K.oxytoca**	urine	dog	no	0	0	>1024	>16/8	<8	>16	8	>16	<2/4	>2	<2/4	<0.5	<0.25	>16	<2	4	<1	>8	<0.5	>8	<32	<2/38	<1	>8	<1	>8
5546	*E.coli***	urine	dog	no	0	0	>1024	>16/8	<8	>16	<0.5	<1	< 2/4	>2	< 2/4	<0.5	< 0.25	16	8	>8	<1	>8	<0.5	>8	<32	>2/38	<1	>8	<1	>8
5800	*K.pneumoniae*	urine	dog	no	0	0	>1024	>16/8	<8	>16	>32	>16	>16/4	>2	<2/4	<0.5	1	>16	<2	<2	<1	>8	<0.5	4	>64	>2/38	>16	<4	<1	8
5998	*E.cloacae*	SSI	dog	yes	A/S	A/S	64	>16/8	<8	>16	16	4	<2/4	>2	<2/4	<0.5	0.5	>16	<2	<2	<1	>8	<0.5	4	64	>2/38	8	<4	<1	<2
6088	*K.pneumoniae*	urine	cat	yes	TZP, AMC	0	>1024	>16/8	<8	>16	>32	>16	>16/4	>2	>16/4	>4	>8	>16	>16	>8	>8	8	>8	8	<32	>2/38	>16	>8	<1	>8

6088	*K.pneumoniae*	urine	cat	yes	TZP, AMC	0	>1024	>16/8	<8	>16	>32	>16	>16/4	>2	>16/4	>4	>8	>16	>16	>8	>8	8	8	<32	>2/38	>16	>8	<1		>8

FOOTNOTE: A/S=Ampicillin-sulbactam (2=2:1 ratio); AMC= Amoxicillin-clavunalate; AMI=Amikacin; AMP=Ampicillin; AXO=Ceftriaxone; AZT=Aztreonam; C/T=Ceftolozane/Tazobactam; CIP=Ciprofloxacin; CXD=Cefodroxil CZA=Ceftazidime-Avibactam; DOR=Doripenem; ENR=Enrofloxacin; ETP=Ertapenem; FAZ=Cefazolin; FEP=Cefepime; GEN=Gentamicin; IMI=Imipenem; LEVO=Levofoxacin; MERO=Meropenem; MIN=Minocycline; MRB=Marbofloxacin; NIT=Nitrofurantoin; RIX=Rifamixin; SXT=Trimetophrim/Sulfamethoxazole; TAZ=Ceftazidime; TET=Tetracycline; TGC=Tigecycline; TOB=Tobramycin, TZP=Piperacillin-Tazobactam.

Table S4. List of the ten isolates selected for whole genome sequencing, and the resistance genes found in each (blue boxes).

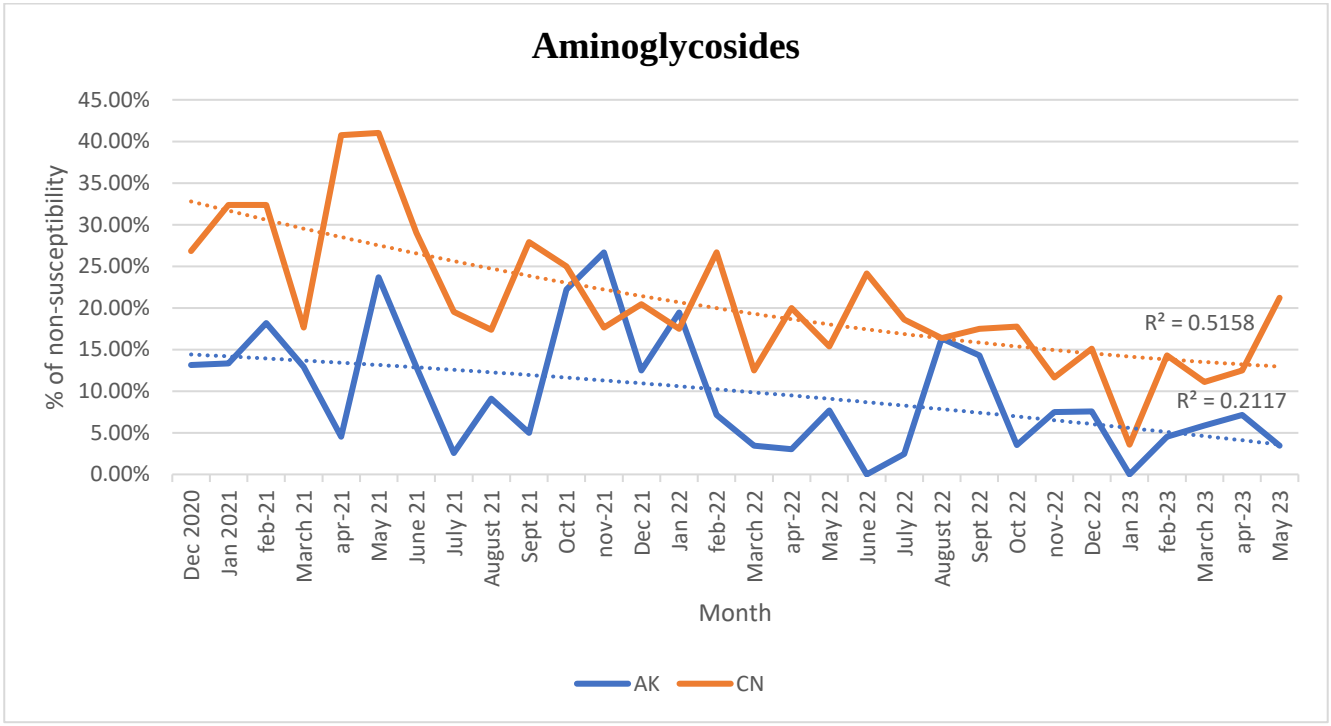
ID	4615	4593	3129	4045
MIC TZP	**>1024**	**1024**	**512**	**256**
Species	*K.pneumoniae*	*E.cloacae*	*E.coli*	*E.coli*
ST	340	114	167	88
ARR-3_4	■	■		
aac(3)-IIa_1				
aac(6')-IIa_1				
aac(6')-Ib-cr_1	■	■		
aadA16_1				
aadA2_1	■			
ant(2'')-Ia_1				■
ant(3'')-Ia_1		■		
aph(3'')-Ib_5				
aph(6)-Id_1				
blaACT-16_1		■		
blaCMY-2_1			■	
blaCTX-M-15_1				
blaDHA-1_1	■	■		
blaNDM-5_1				
blaOXA-1_1	■	■		■
blaOXY-2-8_8				
blaSHV-106_1				
blaSHV-182_1	■			
blaTEM-1B_1				
blaTEM-35_1				■
catA1_1				■
catB3_2	■	■		
dfrA14_5	■			
dfrA1_10				■
dfrA1_8		■		
floR_2				■
fosA6_1	■			
fosA_2				
fosA_7		■		
mdf(A)_1			■	■
mph(A)_2	■			
oqxA_1	■	■		
oqxB_1	■	■		
qnrB1_1				
qnrB4_1	■	■		
sul1_5	■	■		■
sul2_2				■
tet(A)_6				
tet(B)_2	■			■
tet(D)_1				

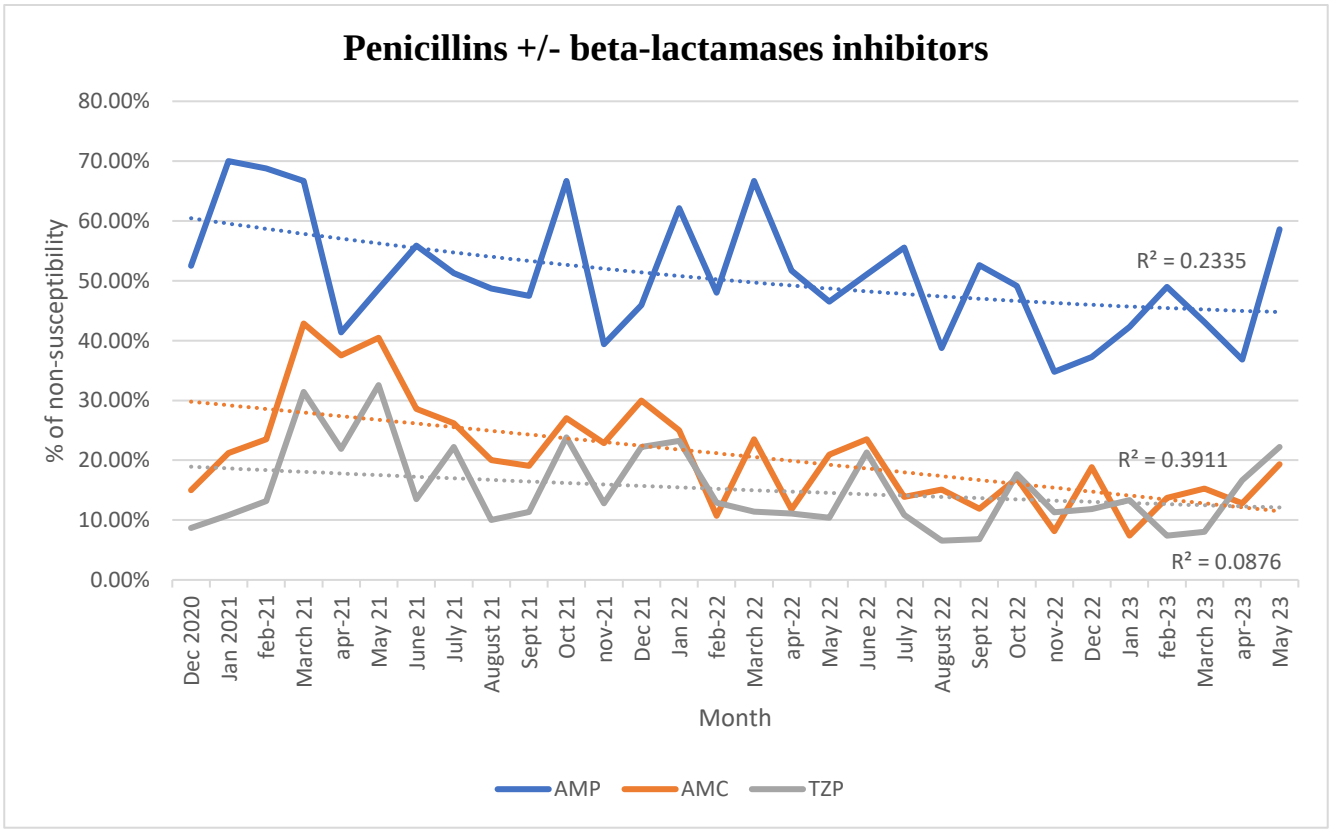
4519
>1024
E.coli
648
6088
>1024
K.pneumoniae
307
4104
>1024
K.pneumoniae
307
5800
>1024
K.pneumoniae
340
5546
>1024
E.coli
88
5178
>1024
K.oxytoca
34

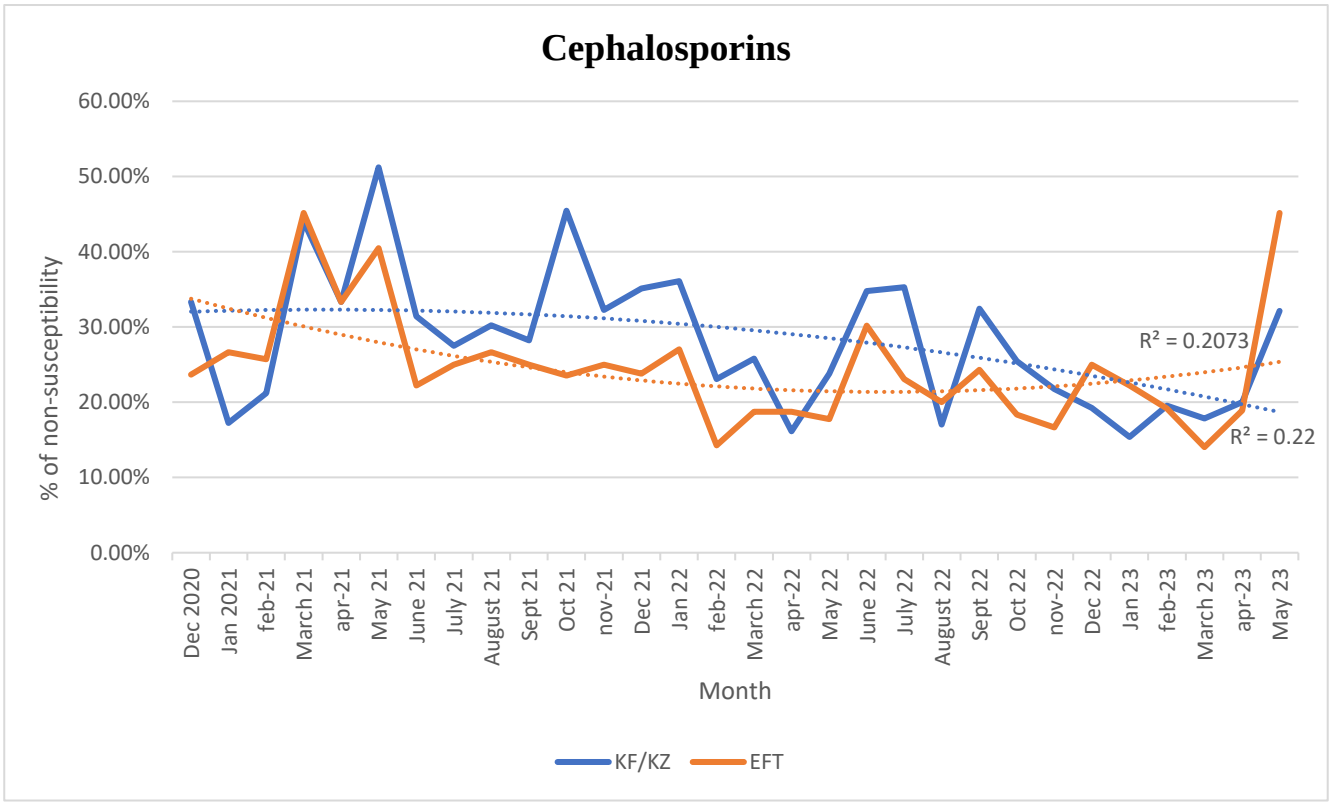
246

247

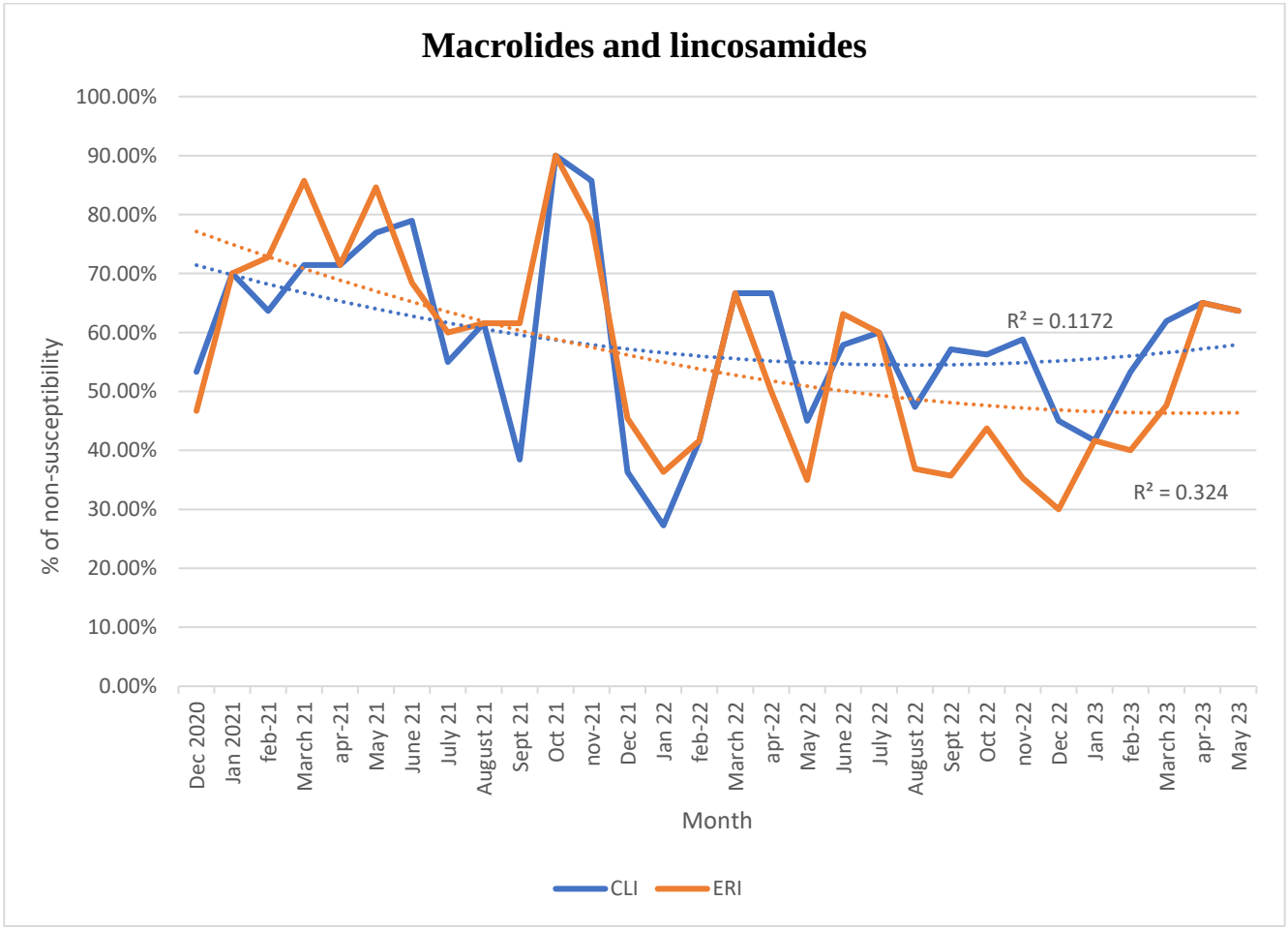
Table S5. Temporal trend of non-susceptibility percentages registered for every tested drug based on monthly records. The dotted line represents the polynomial trendline of the values. Also the value of R^2 is reported.



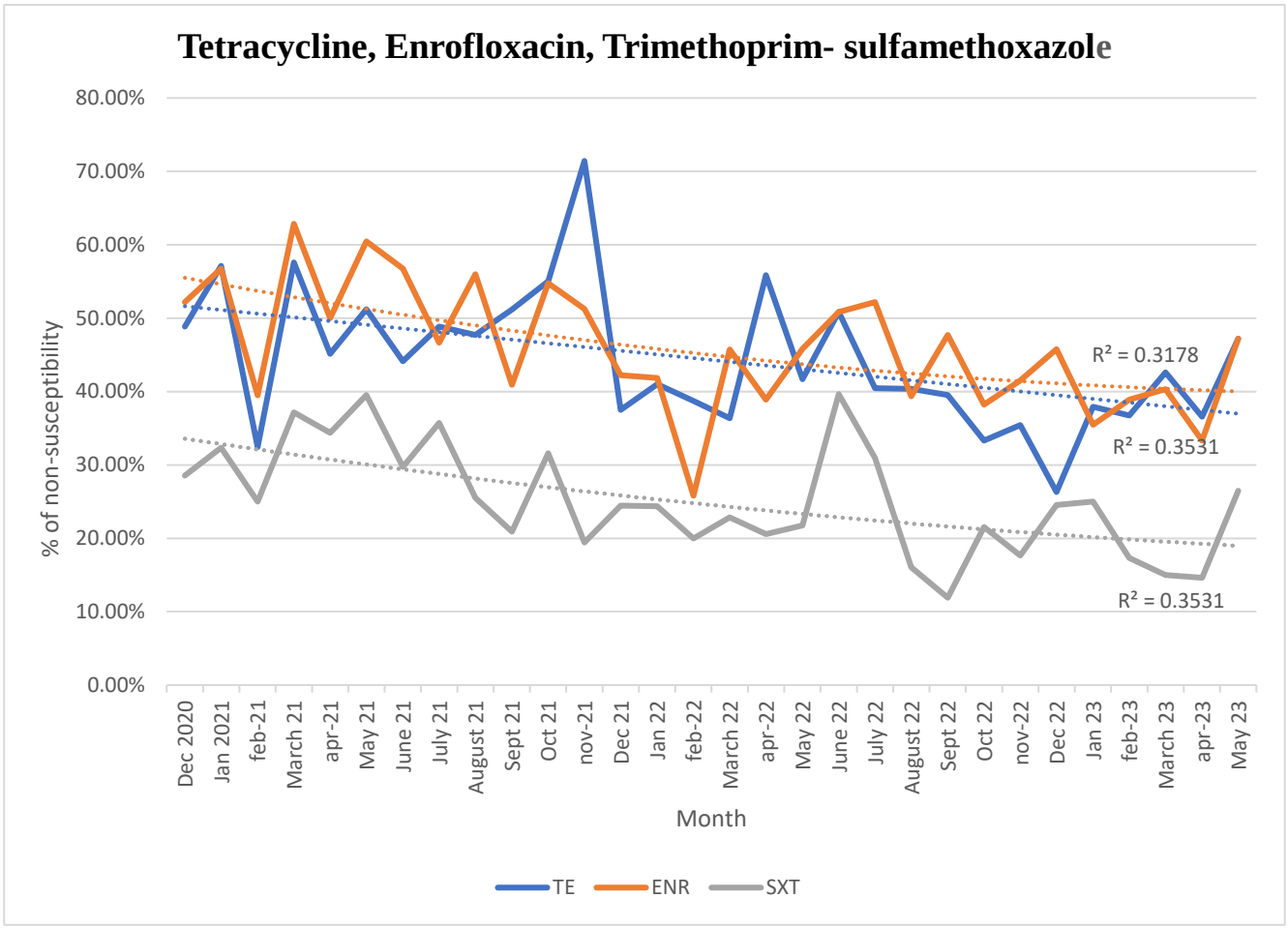



Cephalosporins
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% of non-susceptibility
R² = 0.2073
R² = 0.22
Dec 2020
Jan 2021
feb-21
March 21
apr-21
May 21
June 21
July 21
August 21
Sept 21
Oct 21
nov-21
Dec 21
Jan 22
feb-22
March 22
apr-22
May 22
June 22
July 22
August 22
Sept 22
Oct 22
nov-22
Dec 22
Jan 23
feb-23
March 23
apr-23
May 23
Month
KF/KZ
EFT

4254


Macrolides and lincosamides
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% of non-susceptibility
R² = 0.1172
R² = 0.324
Dec 2020
Jan 2021
feb-21
March 21
apr-21
May 21
June 21
July 21
August 21
Sept 21
Oct 21
nov-21
Dec 21
Jan 22
feb-22
March 22
apr-22
May 22
June 22
July 22
August 22
Sept 22
Oct 22
nov-22
Dec 22
Jan 23
feb-23
March 23
apr-23
May 23
Month
CLI
ERI

4255



FOOTNOTE: AK=Amikacin; CN=Gentamicin; AMP=Ampicillin; AMC=Amoxicillin-clavulanate; TZP=Piperacillin-tazobactam; KF/KZ=Cephalotin/Cefazolin; EFT=Ceftiofur; TE=Tetracycline; CLI=Clindamycin, ERI=Erithromycin; ENR=Enrofloxacin; SXT= Trimethoprim–sulfamethoxazole.

Table S6. Sampling scheme and results of the first active outbreak surveillance executed in 2021. Samples were subsequently cultured on Mac Conkey Agar, and every macroscopically distinguishable isolate was identified with MALDI-TOF technology. Only isolates identified as *Enterobacter* spp. were considered positives.

Area	**Points sampled**	**Positivity**
Surgery	Alcoholic-solution dispensers	
	Gauzes	
	Keyboards	
	Vacuum machine	
	Medical trollleys	
	Staplers	
Intensive Care Unit	Hospitalization cages (after routine disinfection)	*E.cloacae*
	Personnel' hands	*E.cloacae*
	Rectal swabs of hospitalized patients	

Table S7. Sampling scheme and results of the second active outbreak surveillance executed in 2022. Samples were subsequently cultured on CHROMAGAR TM ESBL (Chromagar ^{TM}, Paris, France) selective media, and every macroscopically distinguishable isolate was identified with MALDI-TOF technology.

Area	**Points sampled**	**Positivity**
Emergency Surgery	Visiting table	
	Anesthesia Machine	
	Ligasure device	*Enterobacter* spp., *Pseudomonas* spp.
General ward	Personnel' hands	
	Personnel' cloths	
	Hair clippers	
Intensive Care Unit	Portable Ultrasound Machine	*Enterobacter* spp.
	Visiting table	
	Personnel'hands	
	Personnel'cloths	
	Hair clippers	
Anesthesia room	Visiting table	
	Hair clippers	
	Portable Ultrasound Machine	
	Stretchers	
	Personnel'hands	
	Personnel'cloths	
Radiology room	Lead jackets	
	Personnel'hands	
	Personnel'cloths	

ANNEX 1

Example of sanitization procedures

LigaSure™ Device

Preparation: measure and add 20 g of the solution powder (LH Peracetic II™) in one liter of water at 25-30 °C (2% concentration).

Before the use: With proper PPE (gloves), cold sterilization by immersion in the peracetic acid solution for 30 minutes, subsequently flushing with water.

After the use: Cleaning by removing visible contamination with proper PPE (nytril gloves), and leave in a clean, closed tank that must be daily disinfected.

Portable ultrasound machine

Before/during the use: Prevent contamination by limiting direct patient contact with equipment as much as possible, using PPE (gloves) as indicated and additional contact precautions for high-risk cases (patients with a diagnosed MDR infection).

After the use: Cleaning with wiping equipment to remove visible contamination (especially the keyboard and the screen); specific disinfection with GD90™ (quaternary ammonium salts), with particular attention on the ultrasound probes. Waiting time: 5 minutes.

ANNEX 2

Risk-based score for AMRO carriage

General information

Species: ☐ Dog ☐ Cat

Sex: ☐ M ☐F ☐SM ☐SF

Age: ……..

Chronic comorbities: ……..

☐ The patient lives with one or more animals that received antibiotic in the past 90 days (1 pt)

☐ The owner received antibiotics in the past 90 days (1 pt)

History

☐ Previous hospitalization/surgery in the past 90 days (2pts)

Number of days : …….. (0,25 pts for each day)

☐ Antibiotic treatment in the past 90 days (2 pts for each cycle)

- Number of cycles : ……

-Name of the drug(s) : …….

☐ Treatment with corticoids/chemotherapy in the past year (1 pt)

☐ Presence of surgical implants 1 pt)

☐ Use of invasive devices in the past 90 days (2 pts)

Remote medical history

☐ The owner work in a field where antibiotics are used (including veterinary) (1 pt)

☐ The patient had contact with an hospitalized person in the last year (0.5 pt)

☐ The patient usually consumes raw meat (1 pt)

TOTAL:

☐ >6 points = High-risk → SCREEN + PREVENTIVE MEASURES

☐ 3- 6 points = medium risk

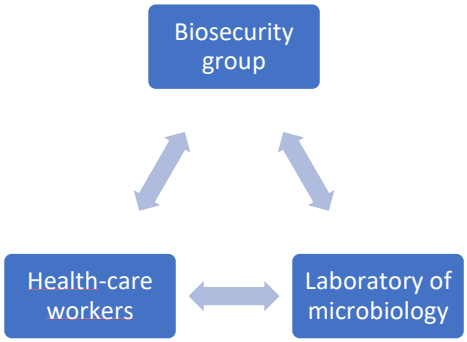
☐<3 points = Low risk

ANNEX 3

Protocol for outbreak management

1. **Alarm phase:** individuation of potential individual cases/clusters/outbreaks through the comparison of clinical findings and the results from passive surveillance (an unusual number of bacterial species/resistances detected). The suspect of an isolated case/cluster/epidemic outbreak is submitted to the biosafety group. The clinical suspects (cluster/epidemic alert) are formulated by the Senior Veterinary Doctor of the operating unit (intensive care, surgery, internal medicine).
2. **Investigation**. Depending on the suspect, the microbiology laboratory executes active surveillance (environmental sampling - healthcare workers - patient screening). Depending on the results, the suspect is confirmed by the Biosafety group. Once the biosafety group confirms the suspicion of an isolated case/cluster/epidemic outbreak, it informs the personnel operating in the VTH, providing all necessary information for the containment of the cluster or outbreak and determining its severity level (isolated case of MDR, Cluster, Epidemic outbreak).

Figure 1. Bidirectional communication between the biosafety group, healthcare workers, and the microbiology laboratory in case of infectious suspicion.



3. **Response.** The biosafety group establishes which sanitization practices to implement in relation to the pathogen and its severity.
    a. **Isolated case.** The patient is placed in isolation in a quarantine area or under healthcare containment. Personnel entering the area attend to the patient while wearing appropriate Personal Protective Equipment (PPE) (non-sterile disposable gown, non-sterile vinyl/nitrile/latex gloves, and, if necessary, non-sterile disposable shoe covers). Access to the quarantine area is limited to personnel involved in managing the clinical case and to patients colonized/infected with the same microorganism or different microorganisms but with the same resistance profile (no access for owners and other patients). Limit patient transport and movements outside the room to diagnostic activities only. When patient transport is necessary, dedicated personnel must wear disposable PPE and discard them immediately after transport. The quarantine area must be cleaned and disinfected (sanitization level I) daily by the veterinary technician, who must wear appropriate PPE and use dedicated equipment.

    b. **Outbreak.** Restrict access for personnel and patients to the area identified as the cluster center (Intensive Care, Non-Intensive Care, Surgical Block, Imaging Diagnostics,

Internal Medicine, Day Hospital). Reduce hospital activities by postponing non-urgent clinical activities and canceling major scheduled surgeries. Signal the presence of the outbreak and infected patients with appropriate signage. Personnel access to the affected area is through badges. Positive and/or colonized patients are isolated in the quarantine area and, if necessary, in a procedure room. Only personnel involved in case management can access these areas while wearing specific PPE (non-sterile disposable gown, non-sterile vinyl/latex/nitrile gloves, and disposable shoe covers). Owners and other animals are not allowed to enter. Limit patient transport and movements outside the area to diagnostic activities only. When patient transport is necessary, dedicated personnel should, if possible, transport the patient on a stretcher while wearing PPE, which should be discarded at the end of the transport. Patients in isolation should not be taken for a walk or allowed to urinate/defecate in areas where other animals are taken. Implement hygiene measures in the involved areas with sanitization level II, which will be carried out daily by the veterinary technician wearing appropriate PPE (non-sterile disposable gown, non-sterile vinyl gloves, and disposable shoe covers) and using dedicated equipment. Upon discharge of patients, a level III sanitization will be carried out. If recommended by the biosecurity group, close the hospital for a period of 3-7 days to allow a level III sanitization of all areas, and screening of personnel involved in the management of positive patients.

According with the results from outbreak surveillance, the biosecurity group indicates which procedures/equipment/points represented a biosecurity risk, and suggest effective measures to be implemented to improve their management.

4. **Evaluation of the response**
   - Control samplings after the sanitization performed by the laboratory personnel according with the indication of the biosecurity group.
   - Surveillance focused on the specific micro-organism.

Cleaning, sanitization, disinfection. Cleaning, sanitization, and disinfection are carried out by staff following the protocols. Each environment has an optimal standard based on its intended use and surrounding flows. The equipment used for cleaning, disinfection, and sanitization is dedicated to each specific area and must not be used in other premises. Detergents and disinfectants should be diluted and used in accordance with the manufacturer's recommendations and the protocols in useSanitization operations are categorized as:

• **Sanitization level I**: ordinary/daily sanitization. The personnel responsible for cleaning and disinfecting the area must wear appropriate personal protective equipment (PPE).

• Remove potentially contaminated material from surfaces (unattended and exposed materials)

• Remove organic material from surfaces using a detergent.

• Use disinfectants according to contact times.

• Clean surfaces (furniture, power outlets, handles), floors, and walls using detergents. • Clean materials/devices used in the environment based on the level of criticality

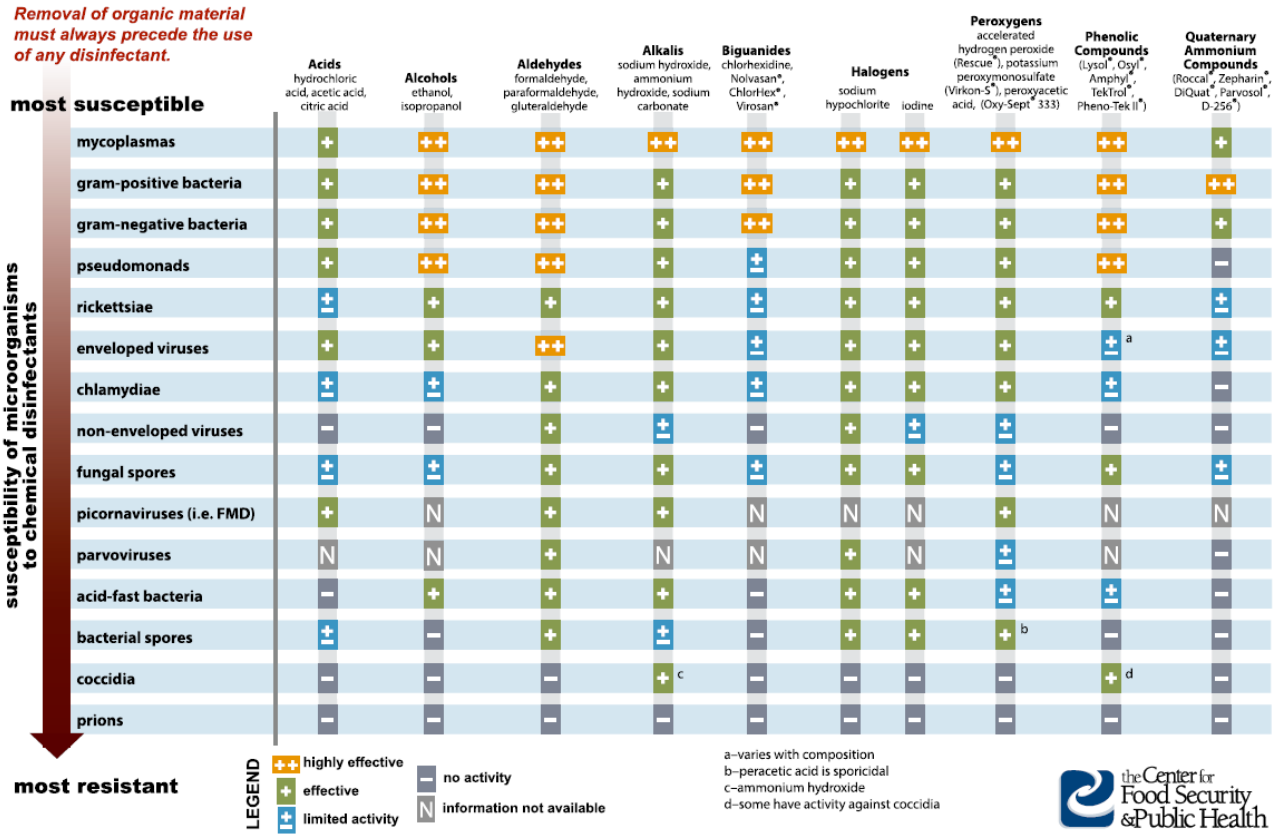
• Use disinfectants according to classification

• Empty trash bins.

• **Sanitization level II**: sanitization based on the microorganism present in the environment affected by the epidemic cluster and to be associated with possible environmental sanitization with hydrogen peroxide. This activity must be recorded through specific forms. The personnel responsible for cleaning and disinfecting the area must wear appropriate personal protective equipment (PPE).

- Remove potentially contaminated material from surfaces (unattended and exposed materials) in the contaminated environment.
- Remove organic material from surfaces. • Use disinfectants according to contact times.
- Clean surfaces (furniture, power outlets, handles), floors, and walls.
- Clean materials/devices used in the environment based on the level of criticality.
- Use high-level disinfectants according to the classification in relation to the pathogen (See table 2)
- Empty trash bins.
- Evaluate with the biosafety team the association with vaporized hydrogen peroxide.

Table 2. Spectrum of selected disinfectants. Adapted from Stull et al. (2018). 2018 AAHA Infection Control, Prevention, and Biosecurity Guidelines. *Journal of the American Animal Hospital Association* 54(6):297-326.



• **Sanitization level III**: sanitization based on the microorganism responsible for the epidemic outbreak associated with environmental sanitization with hydrogen peroxide. This activity must be recorded through specific forms. These interventions can be contracted to external companies

or carried out by veterinary technicians upon request from the area coordinator. The personnel responsible for cleaning and disinfecting the area must wear appropriate PPE.

- Remove potentially contaminated material from surfaces (unattended and exposed materials) in the contaminated environment.
- Remove organic material from surfaces.
- Use disinfectants according to contact times.
- Clean surfaces, floors, and walls.
- Clean materials used in the environment based on the level of criticality.
- Use disinfectants according to classification.
- Empty trash bins.
- Final environmental sanitization with vaporized hydrogen peroxide using Clean Cube and Ox-AIR.: For this activity, proceed with the cleaning and sanitization of the affected area following the Specific Area Disinfection Protocol, for surgical block, isolation area, and quarantine, or the Cleaning and Disinfection Protocol for other areas (such as outpatient facilities and inpatient areas).
    - Close doors, windows, and any gaps to prevent the dispersion of the nebulized product.
    - Fill the Clean Cube with Ox-AIRE
    . • Wet the hydrogen peroxide wipes with running water and place them at the 4 corners of the room or in the points considered most critical.
    - Set the cubic meters (m3) of the area to be sanitized; if the area to be sanitized is considered to have a high biological risk, increase the m3 up to doubling them.
    - Activate the Clean Cube, leave the room, and guard the entry points to that area, preventing the entry of people.
    - At the end of the delivery, it is necessary to let the product act for 15-20 minutes; after this time, it is possible to re-enter the room.
    - Verify that the hydrogen peroxide wipes have adequately changed color, indicating that the product has reached optimal concentrations in all points of the sanitized area.
    - Allow personnel and patients to re-enter the sanitized area. • Record the sanitization event using the appropriate documentation.