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Epidemiological multilevel surveillance of methicillin-resistant staphylococci in a veterinary teaching hospital and first characterization of *Staphylococcus pseudintermedius* isolates using IR biotyper®

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ABSTRACT

In a global view on Antimicrobial Resistance (AMR), Methicillin-resistant Staphylococci (MRS) are one of the most threatening pathogens in both human and veterinary medicine. The aim of this work was to assess the impact of MRS within a Small Animal Veterinary Teaching Hospital (VTH) in Italy, through a multilevel data collection on clinical, commensal and environmental isolates and a subsequent analysis through Fourier-transform infrared (FTIR) spectroscopy by IR Biotyper®. From December 2020 to May 2023, a total of 81/239 (33.9 %) MRS clinical isolates was recorded, mainly MR *S. pseudintermedius* (MRSP, 66/81, 81.5 %). High resistance rates towards most of the antimicrobials tested were observed, such as 97.5 % (79/81) for tetracycline and 84 % (68/81) for enrofloxacin. MRS prevalence in hospitalized patients' oral flora was 22 % (33/150) at admission, while in-hospital acquisition was 19.7 % (23/117). The environmental analysis showed a high frequency of MRS detection in the Intensive Care Unit area (10/34, 29.4 %), and in the personnel' shoe soles (6/7, 85.7 %) and the floor (5/7, 71.4 %). In both patients' flora and environment, the most common species were MRSP and MR *Staphylococcus haemolyticus*. Strain typing using IR Biotyper® on 96 selected isolated showed the presence of three main clusters, one of them detected at all levels (infected patients, patients' commensal flora and environment), suggesting its endemic presence within the hospital. These findings confirm the importance of MRS in small animal practice, highlighting as a multilevel surveillance program can consent to achieve an exhaustive overview that could help in the development of tailored measures of infection control.

1. Introduction

The genus *Staphylococcus* spp. comprises a variety of species (over 40) of gram-positive, non-spore forming and non-motile bacteria, that are normally part of birds and mammals' oral, nasal and skin microbiota but possess the ability to cause a variety of opportunistic infections. In the global overview of antimicrobial resistance (AMR), the emergence of Methicillin-Resistant Staphylococci (MRS) represents a significant and escalating problem in human and veterinary medicine (World Health Organization, 2024). Indeed, the acquisition of resistance towards methicillin gives the ability to alter the affinity not only to many beta-lactam antibiotics, such as cephalosporins and penicillins which traditionally serve as first-line treatments for staphylococcal infections, but it is often associated with resistance to drugs from other classes, such as fluoroquinolones, tetracyclines and sulfonamides (Loeffler et al., 2007; Nocera and De Martino, 2024), conferring a Multi-Drug Resistance (MDR) profile. Additionally, staphylococci possess a strong ability to survive in the environment, including after disinfection (Ishihara et al., 2014). In healthcare settings, this environmental persistence combined with the selective pressure exerted by antibiotic use implicates a major risk of contamination and colonization by MRS, with the consequent onset of healthcare-associated infections (Grönthal et al., 2014; Sebola et al., 2023; Weese and Van Duijkeren, 2010). Methicillin resistance is mainly mediated by the *mecA* gene, or its homologue *mecC,*

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that codify for a modified Penicillin-Binding-Protein (PBP2a). PBP2a is involved in the cell wall synthesis; by altering the target, it confers resistance to beta-lactams (Deurenberg et al., 2007), 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*) (Katayama et al., 2000). Specifically, methicillin resistance in coagulase-positive Staphylococci (CoPS) is considered more important due to their higher pathogenicity rates compared with coagulase-negatives (CoNS) (Ma et al., 2020). While in human medicine the most important, broadly described MR CoPS is Methicillin-resistant *Staphylococcus aureus* (MRSA), in small animal practice there are other CoPS more often involved in infections, such as the members of the *Staphylococcus intermedius* Group (SIG, namely *Staphylococcus pseudintermedius, Staphylococcus intermedius*) (Ross Fitzgerald, 2009). In particular, Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) is well-defined as the most common MR CoPS in dogs (Lynch and Helbig, 2021; Moon et al., 2022; Roberts et al., 2024), and causes infections such as skin disease, urinary tract infections (UTIs), otitis and surgical site infections (SSIs). Infections caused by MRS can be extremely difficult to manage in small animal practice, especially in the European Union where restrictions in the antibiotic choice in veterinary medicine are present (Schmerold et al., 2023). Additionally, although considered a sporadic event, the interspecies transmission has been described (Cuny et al., 2022; KuKanich et al., 2023; Moses et al., 2019; Rodrigues et al., 2018; Somayaji et al., 2016; Souza-Silva et al., 2022; Viñes et al., 2024), posing a risk not only for owners and veterinary workers, but also for the animals. Indeed, reverse zoonosis episodes have been reported for MRSA (Haag et al., 2019; Kasela et al., 2023; Lefebvre et al., 2009), underlining the risk for the animal to be colonized or infected with non-treatable pathogens from a human source. For these reasons, similarly to the healthcare scenario in human medicine, MRS have become a priority to face also in small animal medicine. The necessity of research, surveillance and stewardship initiatives is more urgent in large facilities such as Veterinary Hospitals, that need to manage a larger number of patients, often with complicated and previously treated infections (Walther et al., 2017a). Understanding the regional epidemiological distribution, the resistance mechanisms and the route of transmission of MRS in veterinary settings is a crucial first step to achieve an effective management strategy. Such step can be reached with surveillance programs assisted by bacteriological data. The present study aimed to obtain and analyze data about MRS isolated in an Italian Veterinary Teaching Hospital. Specifically, it focused on: i) the prevalence and the resistance patterns of clinical isolates from sick cats and dogs presented at an Italian Small Animal Veterinary Teaching Hospital (VTH) ii) the prevalence and risk factors related with oral carriage of MRS at admission and the in-hospital colonization; iii) the prevalence of MRS in samples collected from the hospital environment, including VTH's personnel; iv) a deeper epidemiological assessment using Fourier-transform infrared (FTIR) spectroscopy.

2. Materials and methods

2.1. Study design

A prospective, observational study was conducted into the VTH of the University of Bologna as a part of a pilot surveillance program from December 2020 to May 2023. The study was divided in three different parts: surveillance of MRS on clinical specimens, surveillance of MRS on patients' oral flora and surveillance of MRS on the hospital environment.

1.a. Surveillance of MRS on bacterial diagnostic samples. A longitudinal data collection was conducted on specimens from clinically diseased cats and dogs submitted for diagnostic purposes to the Laboratory of Bacteriology of the Bologna VTH. Standard microbiological procedures (listed in Table S1) were used. After incubation for 24–48 h at 37 + /−1 °C, plates that presented adequate growth were considered positive. Bacterial colonies that were macroscopically distinguishable were identified using 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 > 1.8. Only species belonging to *Staphylococcus* spp. were included in the study. Antimicrobial resistance towards 8 different antimicrobial agents listed in Table S2 was assessed through disc-diffusion test. All the discs were purchased from Oxoid (Oxoid, Milan, Italy). For each tested drug, the isolate was classified as susceptible (S), intermediate (I), or resistant (R) based on the CLSI veterinary breakpoints (Clinical and Laboratory Standards Institute, 2018). Intermediate isolates were classified as susceptible, as recommended by the European Committee on Antimicrobial Susceptibility Testing (Nabal Díaz et al., 2022). Perfect duplicates were excluded from the analysis.

For every isolate, data about patient's signalment (species, age, sex) were collected. Additionally, according with the definition given by Hacque et al. (Haque et al., 2018), the potential Healthcare-Associated (HCAI) origin of the infection was also assessed.

1.b. Surveillance of MRS on patients' oral flora. A cross-sectional study was executed through six sessions performed every four months. Following the indications given by the clinical staff, every patient (dog or cat) that was expected to be hospitalized for more than 48 h was sampled at admission (within 12 h) and before discharge or death, until reaching twenty-five patients for each session. For every sampled 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) were recorded. Oral swabs were collected using sterile swabs with Amies transport medium. The samplings were performed by gentling inserting the swab for 10–15 s into the oral cavity, lateral to the tongue. Samples were stored at 4 °C for a maximum of 24–48 h before being processed. Samples were cultured by streaking on selective media for MRS (Oxacillin Resistance Screening Agar Base, Oxoid, Wesel, Germany). After 24–48 h of incubation at 37 + /-1 °C under aerobic conditions, macroscopically distinguishable colonies from positive cultures were sub-cultured at the same conditions on tryptone-soy agar (Oxoid, Wesel, Germany) and subsequently identified by MALDI-TOF mass spectrometry as described in point 1a.

1.c. Surveillance of MRS in the hospital environment. A cross-sectional study was executed through six sessions, performed every four months in concomitance with point 1.b. Each sampling session was done in the late morning, approximately from 11 to 13 a.m. 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 VTH Personnel) and points/surfaces, including personnel' shoe soles, clothes and hands. Samples from VTH personnel were taken from randomly chosen veterinary workers from all the areas. Samples from personnel' hands were collected by gently rubbing sterile swabs with AMIES transport media on the palms of both hands for 10 s each, 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 to a sterile tube. Samples were stored at 4°C for a maximum of 24 h before being processed. Bacteriological culture, subculture and identification were performed with the same modalities described in point 1.a and 1.b.

1. **Detection of methicillin-resistance.** In accordance with CLSI guidelines (Clinical and Laboratory Standards Institute, 2020), methicillin-resistance (MR) in isolates collected from clinical samples was established in those that at the disc diffusion test exhibited phenotypical resistance to oxacillin 1 μg (for *S. pseudintermedius, S. intermedius, Staphylococcus epidermidis* and *Staphylococcus schleiferi,* disk diameter zone ≤ 17 mm) and to cefoxitin 30 μg (disk diameter

zone ≤ 21 mm for *S. aureus* and *Staphylococcus lugdunensis,* and ≤ 24 mm for other *Staphylococcus* spp. excluding *S. aureus, S. lugdunensis, S. pseudintermedius, S. intermedius, S. epidermidis* and *S. schleiferi*). For methicillin-resistance confirmation in isolates collected from patients' flora and the environment, the presence of the *mecA* and *mecC* gene was tested by multiplex PCR following the protocol described by Stegger et al. (Stegger et al., 2012).

2. **Fourier-transform infrared (FTIR) spectroscopy using IR Biotyper®.** A list of selected isolates of methicillin-resistant *S. pseudintermedius* from clinical samples, patients'oral flora and hospital environment was analyzed to detect clonal dissemination by FTIR spectroscopy using an IR Biotyper System (Bruker Daltonics GmbH & Co. KG, Bremen, Germany). IR transmission spectra were acquired from bacterial suspensions in ethanol solution, as recommended by the manufacturer, in three technical replicates of two biological replicates on independent days. Spectra acquisition, processing and analysis was performed by means of the IR Biotyper software V4.0. Data analysis was performed by HCA (hierarchical cluster analysis) and PCA (Principal Component Analysis)/LDA (Linear Discriminant Analysis).
3. **Data Analysis**. Descriptive statistics was performed. For *Staphylococcus* spp. isolates clinical samples, statistics was performed considering the specimen type, the bacterial species identified, mixed or single infection, non-susceptibility percentages towards each tested drug and MR prevalence. In active surveillance on patients, frequency of detection (FD) of MRS carriers at admission was determined in patients with a positive sample at admission, while in-hospital colonization was determined in animals with that were negative at the admission sampling and positive at discharge. A risk factors analysis for MRS carriage and in-hospital colonization was executed considering patient's anamnestic and hospitalization. The association was assessed using univariable logistic regression (enter), and variables with a $p < 0.1$ 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. Data were checked for multicollinearity with the Belsley–Kuh–Welsch technique. Statistical analysis was performed with MedCalc (version v22.009).

3. Results

Clinical isolates. A total of 239 *Staphylococcus* spp. isolates was collected and identified, of which 175 (73.2 %) in monoculture, and 64 (26.8 %) in mixed culture. One hundred and eighty-eight (78.7 %) were collected from 167 dogs (95 males and 72 females), and 51 (21.3 %) from 46 cats (27 males and 19 females). Mean age was 8.3 years (SD 4.1). One hundred and eighty-six (77.8 %) were coagulase-positive, 38 (15.9 %) coagulase-negative and 15 (6.3 %) coagulase-variable. The most common species was *S. pseudintermedius* in dogs (n = 142, 75.5 %), while it was *Staphylococcus felis* in cats (n = 20, 39.2 %)

According to the AST results, 81/239 (33.9 %) isolates (75 CoPs and 6 CoNs) were classified as MRS, of which 65/188 (34.6 %) from dogs and 16/51 (31.4 %) from cats (Table 1). The main MRS species was MRSP (n = 66/81, 81.5 %) followed by MRSA (n = 6/81, 7.4 %). Considering all the *S. pseudintermedius* isolates, methicillin-resistance was detected in almost half of the cases (n = 66/154, 42.8 %). Notably, MR CoNs represented a small proportion of the total count of MRS (n = 6/81, 7.4 %).

Compared with non-MRS, MRS isolates had a significantly higher resistance rate for mostly all the antibiotics tested (see Table 2), such as tetracycline (79/81, 97.5 %) and enrofloxacin (68/81, 84 %). Notably, 55.7 % (n = 88) of non-MRS isolates were found to be resistant to penicillin, and 33.5 % (n = 53) to clindamycin.

Considering the specimen of origin of the 239 *Staphylococcus* spp. isolates (Fig. 1), they were mainly from urines/bladder biopsy/bladder stones (n = 78/239, 32.6 %), followed by ear swabs (n = 59/239, 24.7 %) and wounds (n = 26/239, 10.9 %). The highest proportion of MRS was observed in isolates from SSIs (20/23, 87 %) and blood cultures (8/11, 72.7 %). Thirty-five isolates (14.6 %) were classified as suspected HCAIs cases, of which 28 (80 %) were MRS.

Isolates from patients' oral flora. A total of 150 patients were sampled in six sessions. One-hundred and five out of 150 (70 %, 56 males and 49 females) were dogs, while forty-five out of 150 (30 %, 36 males, 9 females) were cats. Average age was 9.1 years (95 % CI 8.5–9.9), while the average length of hospitalization was 5.3 days (95 % CI 4.6–6.1). Distribution of hospitalization data is shown in Table 3.

In the multivariate analysis, MRS carriage at admission was significantly associated with the dog species ($p = 0.0085$), and with hospitalization/surgery in the previous 90 days in dogs ($p = 0.0166$). In dogs, MRS acquisition was associated with the length of hospitalization ($p = 0.0009$). Four out of 150 patients (2.7 %) developed an infection sustained by a MRS (three MRSP and one MRSA), and one died. Of these four patients, two were MRS carriers at admission, while two were not, and did not acquire MRS in their oral flora during the hospitalization. The four infection cases were two surgical site infections, one bloodstream infection and one deep pyoderma.

A total of 85 MRS isolates was identified, and the genotypical analyses confirmed the presence of the *mecA* gene in all the isolates (100 %). Additionally, in 3 isolates (3.5 %) the presence of the *mecC* gene was detected. The most common species (See Fig. 2) were MRSP (n = 39, 45.9 %) and MR *S. haemolyticus* (n = 35, 41.2 %). Other species identified were, *S. intermedius* (n = 4), *S. warnerii (n = 2), S. hominis* (n = 1)

Table 1
Distribution of the 239 *Staphylococcus* spp. isolates from clinical specimens collected from dogs and cats in the study period, considering bacterial species. The number of methicillin-resistant isolates is also reported, as well as the percentage of total MRS isolates represented by each species and the percentage of MRS isolates between all the isolates of the same the bacterial species.

	Total isolates	Total isolates from dogs	Total isolates from cats	n. of MRS	% between all the MRS isolates	% of MRS within the bacterial species
S.pseudintermedius	154	142	12	66	81.5 %	42.8 %
S.intermedius	13	11	2	3	3.7 %	23.1 %
S.aureus	18	10	8	6	7.4 %	33.3 %
S.felis	20	0	20	2	2.5 %	10 %
S.warnerii	4	2	2	0	0.0 %	0.0 %
S.haemolyticus	4	4	0	2	2.5 %	50 %
S.epidermidis	5	2	3	2	2.5 %	40 %
S.schleiferi	13	11	2	0	0.0 %	0.0 %
S.simulans	3	2	1	0	0.0 %	0.0 %
S.delphini	1	1	0	0	0.0 %	0.0 %
S.sciuri	1	1	0	0	0.0 %	0.0 %
S.lentus	1	0	1	0	0.0 %	0.0 %
Staphylococcus spp.	2	2	0	0	0.0 %	0.0 %

Table 2
Antimicrobial resistance rates of the 239 *Staphylococcus* spp. isolates from clinical specimens included in the study, considering all the isolates, and MRS and non-MRS isolates separately.

Antibiotic drug tested	Total number of resistant isolates	% of resistant isolates	Number of resistant MRS isolates	% of resistant MRS isolates	Number of resistant non-MRS isolates	% of resistant non-MRS isolates
AK	5	2.1 %	2	2.5 %	3	1.9 %
CN	55	23 %	44	54.3 %	11	7 %
PEN	169	70.7 %	81	100 %	88	55.7 %
TE	115	48.1 %	79	97.5 %	36	22.8 %
ERI	118	49.4 %	66	81.5 %	52	32.9 %
CLI	118	49.4 %	65	80.2 %	53	33.5 %
ENR	80	33.5 %	68	84 %	12	7.6 %
SXT	62	25.9 %	53	65.4 %	9	5.7 %

FOOTNOTE: AK=Amikacin; CN=Gentamicin; PEN=Penicillin G; TE=Tetracycline; ERI=Erythromycin; CLI=Clindamycin; ENR=Enrofloxacin; SXT: Trimethoprim-sulfamethoxazole.

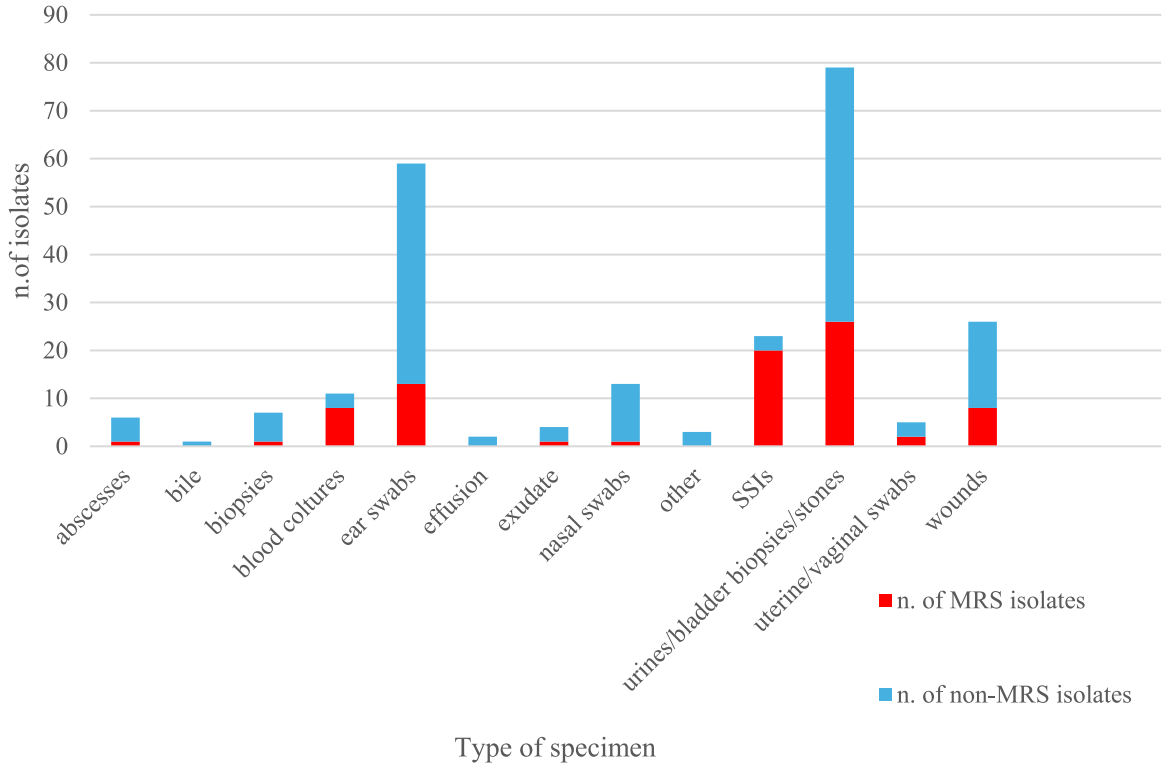


Fig. 1. Distribution of the MRS and non-MRS isolates included in the study according with the type of specimen they were from.

and *S. aureus* (n = 1), while in 3 cases (3.5 %) an accurate species-level identification was not possible.

Environmental samplings. A total of 195 samples were collected in six sessions (30 samples per session, except for the 5th and the 6th, where 33 and 42 samples were collected, respectively). Thirty-eight (19.5 %) were positive for at least one MRS species. Considering the area of collection (Table 4), the Intensive Care Unit Area showed the highest frequency of detection (n = 10/34, 29.4 %). Moreover, a significant frequency of detection was recorded in hospital personnel samplings (n = 17/77, 22.1 %). Considering the point of collection (Table 5), the highest frequency of detection was recorded for the personnel's shoe soles (n = 6/7, 85.7 %), followed by the floor (5/7, 71.4 %). Personnel's clothing recorded a higher frequency of detection compared with hands (20 % vs 11.4). A total of 41 MRS isolates collected from the environment was identified and the genotypical analyses confirmed the presence of the *mecA* gene in all the isolates (100 %). The most common species (see Fig. 2) was MR *S. haemolyticus* (n = 12, 29.3 %), followed by MRSP (n = 9, 21.9 %).

Fourier-transform infrared (FTIR) spectroscopy. A total of 96 MRSP isolates (50 from clinical samples, 39 from patients'oral flora, and 7 from the hospital environment) were analyzed. FTIR spectroscopy-based clustering displayed three main clusters (Fig. 3): cluster 1 (purple) with 23 isolates (14 from clinical samples and 9 from patients'oral flora) of 20 patients; cluster 2 (green) with 14 isolates (7 from clinical samples and 7 from patients'oral flora) of 11 patients; cluster 3 (red) with 46 isolates (27 from clinical samples, 19 from patients'oral flora) of 36 patients, and with all the 7 isolates from the hospital environment. Temporal epidemiological trends considering clustering are shown in Figs. 4 and 5. Isolates from Cluster 3 (red) were detected in all sampling sessions of patients' oral flora and the environment, whereas Clusters 1 (purple) and 2 (green) were detected in only 3 out of 6 sessions each. Considering clinical isolates, Cluster 3 was again the only cluster continuously detected throughout all trimesters of observation, except for the last one.

4. Discussion

In the present study, we applied a surveillance system to monitor the

Table 3
Hospitalization data of the 150 patients (dogs and cats) included in the study during six different sessions, from May 2021 to May 2023.

Hospitalization data	Number of patients	%
Antimicrobial treatment in the previous 90 days	44	29.3 %
Antimicrobial treatment with more than one drug in the previous 90 days	9	6.6 %
Hospitalization/surgery in the previous 90 days	35	23.3 %
Antimicrobial treatment during the hospitalization	106	70.7 %
Antimicrobial treatment with more than one drug during the hospitalization	22	14.7 %
Opioid analgesics use during the hospitalization	93	62 %
Treatment with corticosteroids during the hospitalization	30	20 %
Hospitalization in Intensive Care Unit	100	66.7 %
Surgery during the hospitalization	59	39.3 %
Anesthesia room	73	48.7 %
Use of urinary catheter	20	13.3 %
Use of nasogastric tube	34	22.7 %
Use of other invasive devices (central venous catheter, surgical drainage tube, ureteral stent)	25	16.7 %

MRS carriage at admission was detected in 33 patients (22 %, 95 % CI 15–29, 30 dogs and 3 cats), while 23/117 patients (19.7 %, 95 % CI 12–26.9, 18 dogs and 5 cats) acquired MRS during the hospitalization.

occurrence of MRS an Italian Small Animal VTH. Specifically, we performed a multilevel data collection on clinical samples, on patient's oral flora and on the hospital environment, including the hospital personnel, analyzing spatiotemporal and epidemiological relations through FTIR spectroscopy. Considering isolates from infection, we observed a MRS prevalence of 33.9 % % between all the *Staphylococcus* spp. isolates. Although the study was conducted in a reference hospital, with potential higher resistance rates compared with first-opinion clinics from the same area, such finding aligns with the present literature from Southern Europe. Depending on the criteria used, MRS prevalence varies, ranging from 10 % in a 2018 French study on otitis cases to 33 % in Italy and Spain (Bourély et al., 2019; De Lucia et al., 2011; Marco-Fuertes et al., 2024; Menandro et al., 2019; Prošić et al., 2024; Ventrella et al., 2017). MRSP was the most isolated MRS species (81.5 %), with a prevalence of 42.8 % between all the *S. pseudintermedius* isolates. These data are

Table 4
Frequency of detection of MRS in the environment in the study period considering the area of collection.

AREA	Total samplings	Number of positive samples	% of positivity
General ward	34	6	17.6 %
Intensive care	34	10	29.4 %
Pre-surgery	20	2	10.0 %
Surgery	30	3	10.0 %
Personnel	77	17	22.1 %
Total	195	38	19.5 %

Table 5
Frequency of detection of MRS in the environment in the study period considering the point of collection.

Point	Total samplings	Number of positive samples	%
Anestesia machine	10	0	0.00 %
Carts	5	0	0.00 %
Clippers	17	1	5.9 %
Floor	7	5	71.4 %
Fluidtherapy liquids	7	0	0.00 %
Gauzes	2	0	0.00 %
Keyboards	14	2	14.3 %
Laryngoscopes	5	0	0.00 %
Personnel' clothings	35	7	20 %
Personnel' hands	35	4	11.4 %
Personnel' shoe soles	7	6	85.7 %
Portable ultrasound machine	2	1	50 %
Scale	3	2	66.7 %
Sink	6	1	16.7 %
Smartphones	10	2	20 %
Stretchers	5	2	40 %
Surgical lamp	1	0	0.00 %
Thermometers	8	1	12.5 %
Visiting table	16	4	25 %

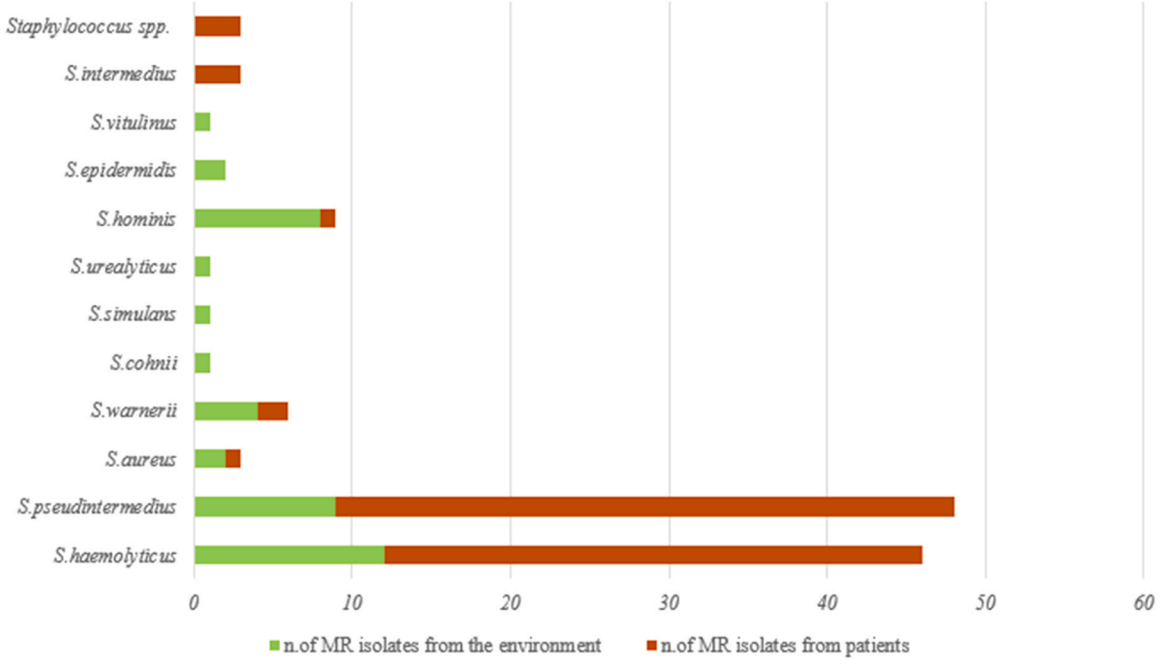
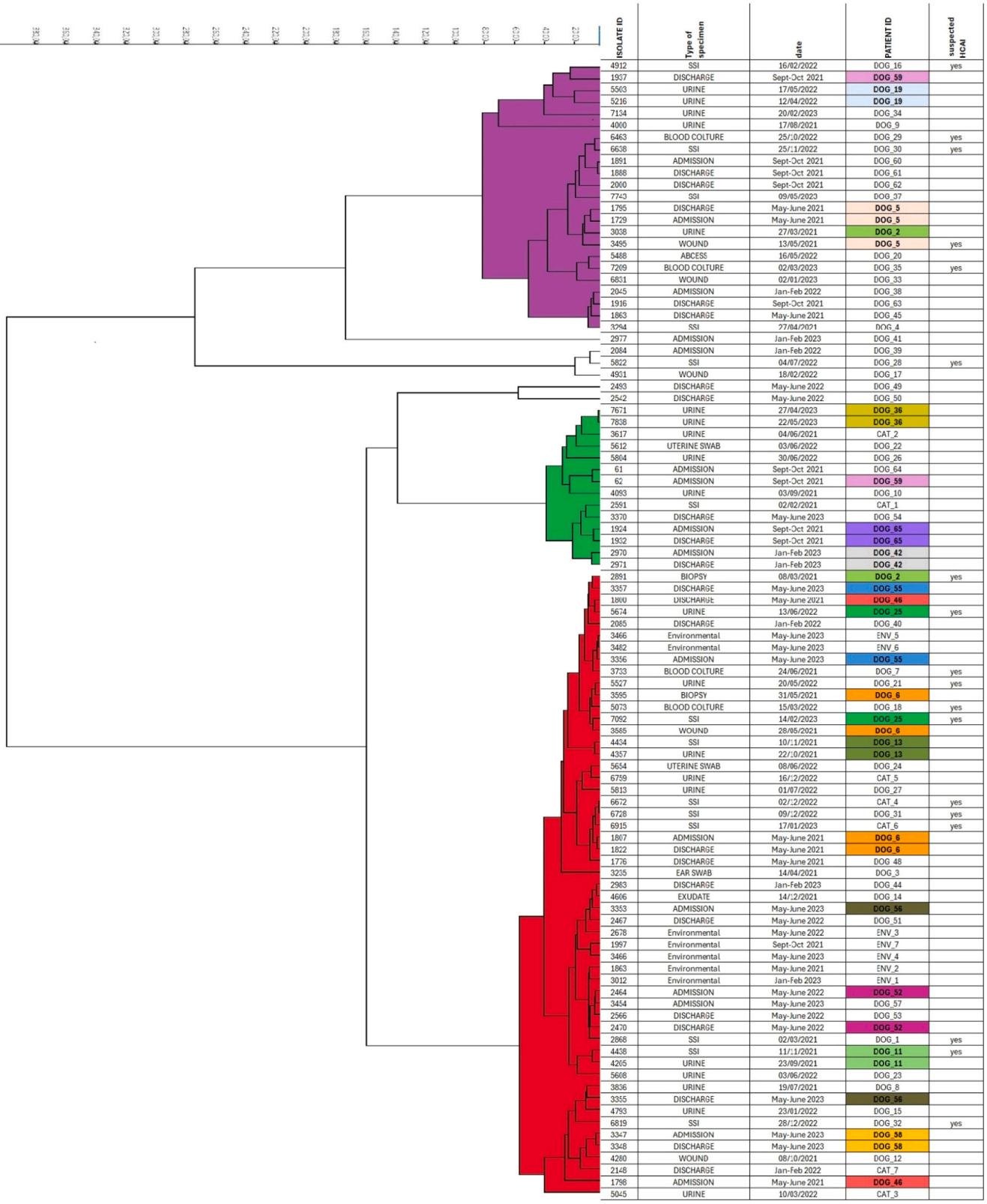


Fig. 2. Distribution of the MRS isolates collected from patients'oral flora (n = 85) and the environment (n = 41).



ISOLATE ID	Type of specimen	date	PATIENT ID	suspected HCAI
4912	SSI	16/02/2022	DOG_16	yes
1937	DISCHARGE	Sept-Oct 2021	DOG_59	
5503	URINE	17/05/2022	DOG_19	
5216	URINE	12/04/2022	DOG_19	
7134	URINE	20/02/2023	DOG_34	
4000	URINE	17/08/2021	DOG_9	
6463	BLOOD COLTURE	25/10/2022	DOG_29	yes
6638	SSI	25/11/2022	DOG_30	yes
1891	ADMISSION	Sept-Oct 2021	DOG_60	
1888	DISCHARGE	Sept-Oct 2021	DOG_61	
2000	DISCHARGE	Sept-Oct 2021	DOG_62	
7743	SSI	09/05/2023	DOG_37	
1795	DISCHARGE	May-June 2021	DOG_5	
1729	ADMISSION	May-June 2021	DOG_5	
3038	URINE	27/03/2021	DOG_2	
3495	WOUND	13/05/2021	DOG_5	yes
5488	ABCESS	16/05/2022	DOG_20	
7209	BLOOD COLTURE	02/03/2023	DOG_35	yes
6831	WOUND	02/01/2023	DOG_33	
2045	ADMISSION	Jan-Feb 2022	DOG_38	
1916	DISCHARGE	Sept-Oct 2021	DOG_63	
1863	DISCHARGE	May-June 2021	DOG_45	
3294	SSI	27/04/2021	DOG_4	
2977	ADMISSION	Jan-Feb 2023	DOG_41	
2084	ADMISSION	Jan-Feb 2022	DOG_39	
5622	SSI	04/07/2022	DOG_28	yes
4931	WOUND	18/02/2022	DOG_17	
2493	DISCHARGE	May-June 2022	DOG_49	
2542	DISCHARGE	May-June 2022	DOG_50	
7671	URINE	27/04/2023	DOG_36	
7838	URINE	22/05/2023	DOG_36	
3617	URINE	04/06/2021	CAT_2	
5612	UTERINE SWAB	03/06/2022	DOG_22	
5804	URINE	30/06/2022	DOG_26	
61	ADMISSION	Sept-Oct 2021	DOG_64	
62	ADMISSION	Sept-Oct 2021	DOG_59	
4093	URINE	03/09/2021	DOG_10	
2591	SSI	02/02/2021	CAT_1	
3370	DISCHARGE	May-June 2023	DOG_54	
1924	ADMISSION	Sept-Oct 2021	DOG_65	
1932	DISCHARGE	Sept-Oct 2021	DOG_65	
2970	ADMISSION	Jan-Feb 2023	DOG_42	
2971	DISCHARGE	Jan-Feb 2023	DOG_42	
2891	BIOPSY	08/03/2021	DOG_2	yes
3357	DISCHARGE	May-June 2023	DOG_55	
1800	DISCHARGE	May-June 2021	DOG_46	
5674	URINE	13/06/2022	DOG_25	yes
2065	DISCHARGE	Jan-Feb 2022	DOG_40	
3466	Environmental	May-June 2023	ENV_5	
3482	Environmental	May-June 2023	ENV_6	
3356	ADMISSION	May-June 2023	DOG_55	
3733	BLOOD COLTURE	24/06/2021	DOG_7	yes
5527	URINE	20/05/2022	DOG_21	yes
3595	BIOPSY	31/05/2021	DOG_6	
5073	BLOOD COLTURE	15/03/2022	DOG_18	yes
7092	SSI	14/02/2023	DOG_25	yes
3585	WOUND	28/05/2021	DOG_6	
4434	SSI	10/11/2021	DOG_13	
4357	URINE	22/10/2021	DOG_13	
5654	UTERINE SWAB	08/06/2022	DOG_24	
6759	URINE	16/12/2022	CAT_5	
5813	URINE	01/07/2022	DOG_27	
6672	SSI	02/12/2022	CAT_4	yes
6728	SSI	09/12/2022	DOG_31	yes
6915	SSI	17/01/2023	CAT_6	yes
1807	ADMISSION	May-June 2021	DOG_6	
1822	DISCHARGE	May-June 2021	DOG_6	
1776	DISCHARGE	May-June 2021	DOG_48	
3235	EAR SWAB	14/04/2021	DOG_3	
2983	DISCHARGE	Jan-Feb 2023	DOG_44	
4606	EXUDATE	14/12/2021	DOG_14	
3353	ADMISSION	May-June 2023	DOG_56	
2467	DISCHARGE	May-June 2022	DOG_51	
2678	Environmental	May-June 2022	ENV_3	
1997	Environmental	Sept-Oct 2021	ENV_7	
3466	Environmental	May-June 2023	ENV_4	
1863	Environmental	May-June 2021	ENV_2	
3012	Environmental	Jan-Feb 2023	ENV_1	
2464	ADMISSION	May-June 2022	DOG_52	
3454	ADMISSION	May-June 2023	DOG_57	
2566	DISCHARGE	May-June 2022	DOG_53	
2470	DISCHARGE	May-June 2022	DOG_52	
2868	SSI	02/03/2021	DOG_1	yes
4438	SSI	11/11/2021	DOG_11	yes
4265	URINE	23/09/2021	DOG_11	
5608	URINE	03/06/2022	DOG_23	
3836	URINE	19/07/2021	DOG_8	
3355	DISCHARGE	May-June 2023	DOG_56	
4793	URINE	23/01/2022	DOG_15	
6819	SSI	28/12/2022	DOG_32	yes
3347	ADMISSION	May-June 2023	DOG_58	
3348	DISCHARGE	May-June 2023	DOG_58	
4280	WOUND	08/10/2021	DOG_12	
2148	DISCHARGE	Jan-Feb 2022	CAT_7	
1798	ADMISSION	May-June 2021	DOG_46	
5045	URINE	10/03/2022	CAT_3	

Fig. 3. Dendogram showing the clustering of the 96 selected Methicillin-Resistant *Staphylococcus pseudintermedius* (MRSP) isolates based on the IR Biotyper® spectra. Spectra were pre-processed by LDA applied at isolate level (to minimize the not informative technical and biological variance), using 30 principal components. The dendrogram was generated using Euclidean distance and Ward's algorithm as linkage type. The three main clusters detected are marked with different colors (purple=cluster 1; green=cluster 2; red=cluster 3). The type of specimen (infection site for clinical isolates, admission/discharge/environmental in case of commensal or environmental isolates) is also shown, as well as the date of sampling and the patient ID. Multiple isolates from the same patient are shown with the same color. Clinical isolates suspected to be healthcare-associated infections (HCAIS) are marked with "yes". FOOTNOTE: SSI=Surgical Site Infection.

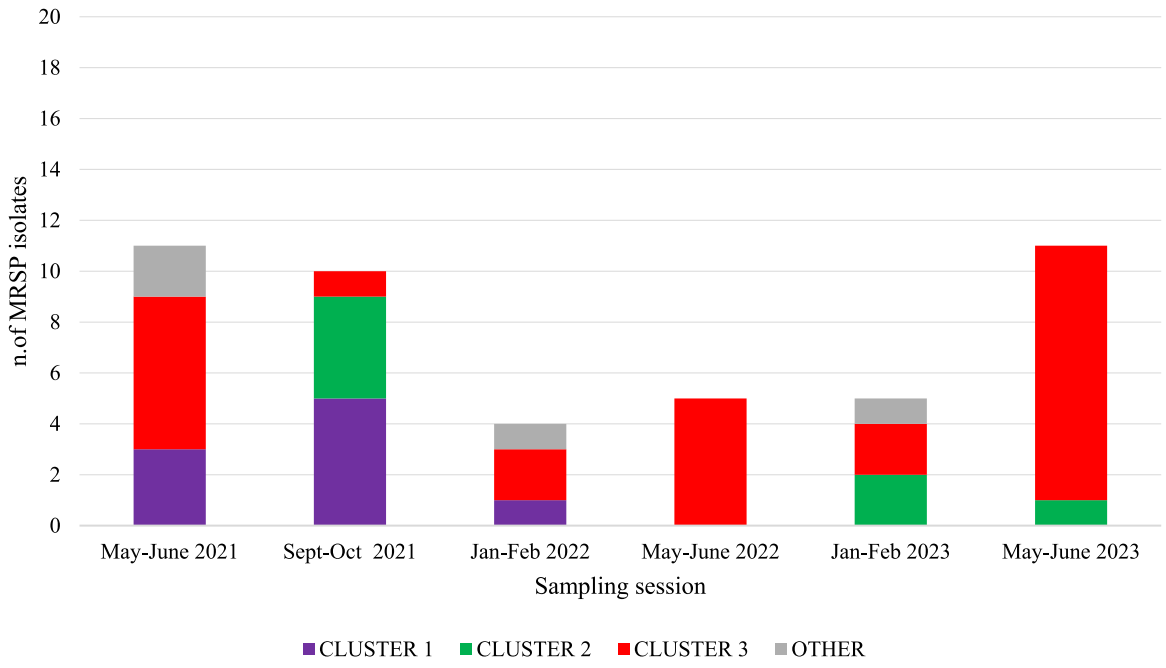


Fig. 4. Temporal epidemiological trend of the 46 selected Methicillin-Resistant *Staphylococcus pseudintermedius* (MRSP) isolates from the environment and patients' commensal oral flora, considering the sampling session and the assigned cluster based on the IR Byotyper® spectra.

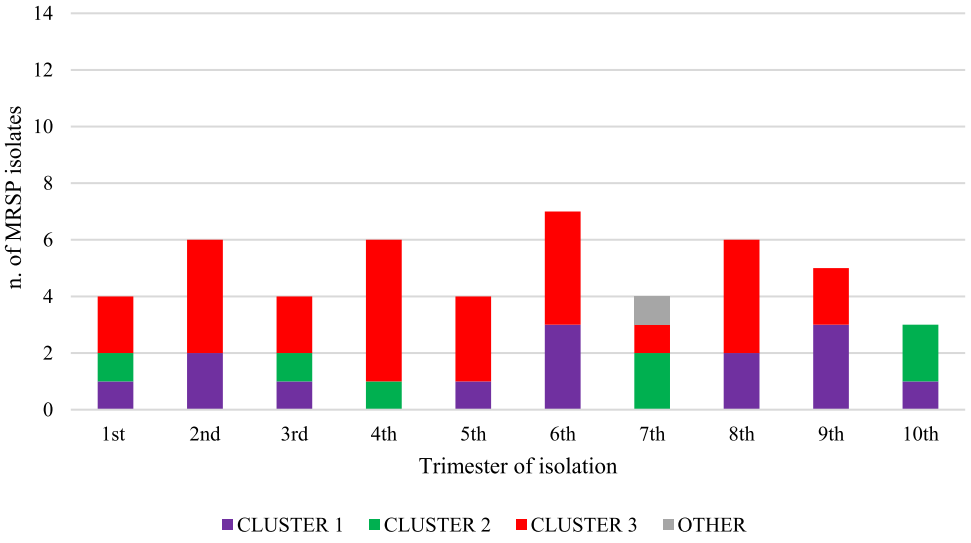


Fig. 5. Temporal epidemiological trend of the 50 selected Methicillin-Resistant *Staphylococcus pseudintermedius* (MRSP) isolates from clinical samples, considering the date of isolation and the assigned cluster based on the IR Biotyper® spectra.

consistent with a 2016 multicenter study on urinary infections in pets, that highlighted *S. pseudintermedius* as the main (94 %) *Staphylococcus* spp. species in Italy, with a MRSP prevalence of 50 % (Marques et al., 2016). The role of MRSP as the major MRS pathogen in pets rather than MRSA, that constituted only 7.4 % of total MRS isolates, is confirmed by our data. MRSA is mainly a human-associated pathogen, and its presence in infection in companion animals is sporadic, normally due to an anthropozoonotic transmission (Haag et al., 2019; Weese, 2010). On the other hand, MRSP is extremely common in pets, more in dogs than in cats (Burke and Santoro, 2023; Pérez-Sancho et al., 2020), due to a higher adherence of *S. pseudintermedius* to canine corneocytes compared with feline corneocytes (Woolley et al., 2008). Meticillin-resistance not only confers resistance to all the currently available beta-lactams, but it is often associated with multi-drug resistance (MDR) including for antibiotic classes such as fluoroquinolones, aminoglycosides, tetracyclines or lincosamides, with percentages up to 85 %. That represents a serious risk for the patients since they are all commonly used in small animal practice (De Briyne et al., 2014). Specifically, the high percentage of resistance towards enrofloxacin in MRS (84 %) is concerning, and similar to another Italian study on 70 *S. pseudintermedius* isolates from canine pyoderma (Casagrande Proietti et al., 2012), in which it was 94 %. Notably, some important resistance rates were found also between non-MRS isolates for penicillin (55.7 %), clindamycin (33.5 %) and erythromycin (32.9 %). This means that the absence of the *mec* gene complex is not necessarily associated with a full susceptibility profile, since other independent resistant mechanisms can be involved (Ba et al., 2014; Lade et al., 2022; Myrenås et al., 2024; Sommer et al., 2021). Considering the specimen type, the most common were urines, ear swabs and skin lesions. Such results mirror the most common infections caused by *Staphylococcus* spp. in pets, that are mainly UTIs,

otitis and pyodermas (Lynch and Helbig, 2021; Moon et al., 2022; Usui et al., 2025). Interestingly, the highest proportion of infections caused by MRS was found in SSIs and blood cultures (87 % and 72 %, respectively). This confirms as these severe and potentially healthcare-associated infections are frequently associated with MRS, especially with MRSP and MRSA, as reported in other works (Viegas et al., 2022; Windahl et al., 2015).

The active surveillance on patients' oral flora was developed to obtain information about the carriage of MRS between hospitalized animals, both at admission and the in-hospital acquisition. Monitoring MRS presence in its oral microbiota can be a useful indicator to better understand MRS mechanisms of transmission and possibly to serve as a basis for specific infection control policies. This type of surveillance was performed in periodic sessions, in agreement with Anderson et al. (Anderson et al., 2019). In a real-life scenario, extensive and continuous active surveillance requires a substantial economic effort, that not many veterinary settings are able to apply. Consequently, a periodic and time-determined approach could reduce the related costs, still maintaining the ability to furnish precious information. Our results on both MRS carriage at admission (22 %) and in-hospital acquisition (19.7 %) are in line with other works The first varied from 1.3 % in the US to 51.1 % in Nigeria (Beck et al., 2012; Bergström et al., 2012; Dazio et al., 2021; Hamilton et al., 2013; Han et al., 2015; Moses et al., 2019; Ortiz-Díez et al., 2020; Rana et al., 2022; Santana et al., 2023; van Balen et al., 2013), while the second from 1.4 % in the US to 26.4 % in Spain (Dazio et al., 2021; Hamilton et al., 2013; Ortiz-Díez et al., 2020; Santana et al., 2023; Scarpellini et al., 2023b). The variations could be attributed to several factors, including methodology, geographical specificity or differences in terms of hospital management and structure, so a crude comparison could be inaccurate. Notably, MRS infections in the sampled patients were recorded only in four cases, confirming that MRS can colonize animals, and potentially spread through them, even without the clinical evidence of an infection. This point remarks on the importance of active screening to detect the silent spread of AMR bacteria, that would otherwise be missed. Another interest related fact is that only two of the four patients (50 %) with MRS infections were carriers or acquired MRS during the hospitalization. This contradicts the general conviction that patients become colonized with AMR bacteria before that an AMR infection rises (Jarvis, 1996), highlighting that in some cases MRS could stay localized in the infection site.

The species analysis confirms that MRSP is the most common MRS in small animal practice, not only as an infectious agent but also as commensal colonizer. Particular attention should be given to MR *S. haemolyticus,* a CoNS that is not frequently involved in infections (2.5 % of total MRS cases) but can play an important role in the maintenance and dissemination of MR, as demonstrated by its high prevalence in patient's flora in our study. This prevalence is in contrast with others studies in which the most common MR CoNS found in dogs were *S. schleiferi* (Teixeira et al., 2019) and *S. epidermidis* (Miszczak et al., 2023). On the other hand, MRSA was rarely found, confirming that *S. aureus* is not a common commensal species. 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 (Guardabassi et al., 2013; John Jr, 2020).

The risk factor analysis poses some further interesting points. In line with previous findings from other works on pets (Dazio et al., 2021; Elmoslemany et al., 2021; Loncaric et al., 2019; Pomba et al., 2016; Santana et al., 2023), a previous hospitalization/surgery was highlighted to be a significant risk factor for MRS carriage at admission, underlying as the patient' history should be considered as an important factor to check when a patient is hospitalized, in order to reduce the risk of an uncontrolled introduction of AMR organisms. Furthermore, the species dog was statistically correlated with MRS carriage: considering that almost half of total MRS were MRSP, this finding confirms the previous statements about the higher affinity of *S. pseudintermedius* for dogs rather than cats. Regarding in-hospital acquisition, the length of the hospitalization appears to be a relevant risk factor in dogs, in accordance with the literature (Dazio et al., 2021; Grönthal et al., 2014; Hamilton et al., 2013; Pomba et al., 2016; Santana et al., 2023). To reduce the chance of colonization, the duration of the hospital stay should be limited to absolute minimum, although it could be more arduous to achieve in small animal practice, due to the difficulties related with a correct "home treatment" for some owners.

Active surveillance on the environment allows to evaluate the degree of contamination in healthcare facilities, although its real usefulness in supporting infection control is still a matter of debate (Burgess, 2024). Environmental contamination has been associated with an increased risk of healthcare-associated infections in human medicine (Odoyo et al., 2023; Weber et al., 2013), and its importance should be considered especially in relation with the ability of MRS to persist in the hospital environment for long period of time (Kramer et al., 2006). Given that a veterinary healthcare setting with low infection control standards have a higher rate of environmental contamination (Schmidt et al., 2020), implementing a successful strategy becomes necessary. Additionally, a contaminated environment poses a risk also for the veterinary workers (Rey et al., 2022; Rodrigues et al., 2018; Walther et al., 2017b). Currently, in small animal practice there is no legislation that standards cleaning and disinfection, so every facility follows internal regulations. In our study, the VTH involved had internal protocols of routine cleaning and disinfection for the different areas, including cleaning of floors twice daily, disinfection of surfaces (keyboards, visiting table) and equipment (portable ultrasound machine, clippers) on a need-by-need basis. However, a frequency of MRS detection of 19.5 % was observed in the six sessions, all performed during non-outbreak periods. Such rate is comparable to other studies from small animal practice, in which ranged from 5 % to 16 % for MRSA (Ishihara et al., 2010; Loeffler et al., 2005; Murphy et al., 2010; Sato et al., 2018; van Balen et al., 2013), and from 4.1 % to 16 % for MRSP (Feßler et al., 2018; Ishihara et al., 2010; Perkins et al., 2020; Scarpellini et al., 2023a; Van Duijkeren et al., 2011). The Intensive Care Unit (ICU) area was the one with the highest frequency of detection. 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. On the other hand, the surgery and pre-surgery areas registered the lower frequency of detection (10 %), similar to a study on MR CoPS from Thailand (Fungwithaya et al., 2021), in which it was 6.9 % (2/29). High MRS rates 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 (van Balen et al., 2013). Although the real impact of shoe soles in the transmission mechanism has still not been clarified (Wojtacka et al., 2022), a high MRS detection rate could reflect a high MRS contamination of the floor, and their role as vectors between different areas. In small animal practice, the floor could play an even more relevant role, as patients (especially dogs) are often at closer and direct contact with it. Moreover, MRS were found more frequently in personnel's clothing (20 %) compared with hands (11.4 %). A study by Schmitt et al. (Schmitt et al., 2022) reports a similar prevalence (10 %) of MRSA in the hospital personnel' hands. In our study, the findings on hands are supported by the relatively low prevalence of MRS on frequently touched equipment such as clippers and thermometers, suggesting that, when assisted by specific hygiene protocols, MRS dissemination via hands can be limited. In human medicine, hand hygiene is recognized as one of the most powerful factors to reduce AMR incidence (Allegranzi and Pittet, 2009; Widmer et al., 2007). 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 (Anderson et al., 2014). The level of compliance of

hand hygiene can be monitored by measuring the use of alcohol-based hand-rub solution (Elia et al., 2022), or by systematic samplings on frequently touched surfaces. In a observational study from 2014 on companion animals veterinarians (Anderson et al., 2014), 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 (Horn-Lodewyk et al., 2023; Lena et al., 2021; Singh et al., 2013), especially in veterinary medicine where it is often needed to manipulate or hold a patient using the body. The use of appropriate Personal Protective Equipment (PPE) reduce the risk of such contamination (Stull and Weese, 2015). 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 (Chiereghin et al., 2020; Lena et al., 2021). In our case, in-hospital laundry was not guaranteed for all the workers, and there were no specific rules of clothing for the different areas, except for the surgery area. 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 MRS diffusion (Horn-Lodewyk et al., 2023). Interestingly, a low prevalence of MRS was detected in wet points such as sinks and fluid therapy liquids, suggesting as MRS could primarily be more capable of colonizing skin and dry surfaces. Considering the species isolated, the environment reflects the distribution on the patients, with a high prevalence of MR *S. haemolyticus* and MRSP. Notably, MRSA presence was very low, suggesting that this pathogen is not particularly diffused in the hospital, as confirmed by surveillance on clinical isolates.

The FTIR spectroscopy performed through IR Biotyper® on selected MRSP isolates highlighted other interesting considerations in terms of epidemiology, letting to assess the real relationship between clinical, commensal and environmental isolates. To our knowledge, this is the first study in which this technology was used to assess relationships between *S. pseudintermedius* isolates. This phenotypic methodology have been already demonstrated to be an easy, fast and cost-effective tool for strain typing, comparable to other genotypic techniques such as the pulsed-field gel electrophoresis (PFGE) and the multi-locus sequence typing (MLST)(Hong et al., 2022). In our study, isolates belonging to Cluster 3 were consistently present over time not only in clinical isolates, but also in all the sampling sessions on patients'oral flora and hospital environment (Figs. 4 and 5). Specifically, all the environmental isolates analyzed (n = 7) belonged to the cluster 3: this suggests that such cluster could be endemic in the VTH. The fact that some suspected, temporally close HAI cases were caused by cluster 3 isolates placed very close in the dendrogram supports such hypothesis. Additionally, commensal MRSP analysis showed that in most of the cases, MRSP found at admission were very close to MRSP found at discharge, except for three cases in which there was considerable distance in the dendrogram. In one of these cases (See Fig. 3, Patient ID "DOG_59), the MRSP at discharge belonged to a different cluster to the MRSP at admission; this suggests that MRS colonization could occur even in patients already MRS carriers, as confirmed by Ortìz-Diez et al. (Ortiz-Díez et al., 2020). Similarly, MRSP isolates from recurrent infections in the same patient were often placed close in the dendrogram, except for one case ("DOG_2") in which two MRSP from two different clusters were found in the same patient. Another interesting aspect regards a single case of a patient ("DOG_5") with MRSP at admission and discharge, who developed an MRSP infection. The three MRSP isolates were very close each other in the dendrogram, remarking that, in this situation, MRSP colonization preceded infection.

The present research has some limitations. First, the use of a quantitative AST in clinical isolates could have led to a more accurate estimation of resistance rates. Second, the absence of other types of epidemiological analysis, such as MLST or WGS, prevented us from comparing our data with other studies, limiting the biological significance of the report. Third, the environmental analysis lacked a focus on touched surfaces/points (e.g. doors) that could have given a more complete overview on the role of the personnel.

In conclusion, this study aimed to explore the diffusion of MRS at an Italian Veterinary University Hospital through the execution of an integrated surveillance program that acted at different levels. Through a combined data collection (on clinical isolates, patients' commensal flora and on the environment) supported by epidemiological assessment, a complete overview of the internal situation can be obtained. This not only adds information about the local epidemiological situation but also furnishes the basis to develop tailored measures of infection control and antimicrobial stewardship, that consider the hospital's priorities and peculiarities.

CRediT authorship contribution statement

Miriam Cordovana: Writing – review & editing, Validation, Supervision, Software, Resources, Methodology, Data curation. **Raffaele Scarpellini:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Simone Ambretti:** Visualization, Validation, Resources, Conceptualization. **Elisabetta Mondo:** Visualization. **Erika Esposito:** Visualization. **Silvia Piva:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Data curation, Conceptualization. **Massimo Giunti:** Visualization, Validation, Supervision, Methodology, Conceptualization.

Ethical statement

Ethical approvement for the samplings on the animals was obtained for this study from the Ethical Committee of the Veterinary Faculty (Protocol n. 201303,26/8/2021). Patient's owners gave written consent for the sampling.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2025.106469.

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Glossary

AMR: Antimicrobial Resistance
MRS: Methicillin-Resistant Staphylococci
FTIR: Fourier-transform infrared