

Zoonotic *Staphylococcus* spp. among domestic animals in Ukraine: antibiotic resistance and diagnostic approaches

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Staphylococcus pseudintermedius and *S. aureus* are common infectious agents with zoonotic potential, capable of acquiring antibiotic resistance and causing hospital-acquired infections. This study aimed to assess the prevalence of *S. pseudintermedius* among dogs in Ukraine and explore the possibility of using chromogenic media for accurate identification and differentiation of *S. aureus* and *S. pseudintermedius*. The data obtained indicate a higher prevalence of nasal carriage (44.8%) of *Staphylococcus* spp. in healthy dogs compared to ear carriage (28.5%). Among the isolated strains in nasal swabs, *S. pseudintermedius* was most often detected (24.1%), *S. aureus* was detected less often (5.2%). Among the samples taken from sick dogs, 36.5% were found to be *Staphylococcus* spp., of which 20.5% contained *S. pseudintermedius* and 4.5% *S. aureus*. Among the studied cats, 36.8% were found to contain *Staphylococcus* spp., *S. pseudintermedius* – 10.5%, and *S. aureus* – 5.3%. Antibiotic resistance profiles were different: *S. pseudintermedius* was resistant to the combination of trimethoprim-sulfamethoxazole, as well as to penicillin and erythromycin, while *S. aureus* was resistant to penicillin. It should be noted that only one coagulase-positive *S. aureus* isolate obtained from healthy animals was resistant to oxacillin. The use of chromogenic media demonstrated the possibility of successful differentiation of *S. aureus* and *S. pseudintermedius* based on differences in culture properties. In addition, various combinations of microbiological tests were evaluated, resulting in the development of two schemes that included specific media and additional reactions to improve identification accuracy. The combination of CHROMagar™ Orientation with the coagulase detection reaction demonstrated higher sensitivity (100%) for the detection of *S. pseudintermedius* compared to the combination of mannitol salt agar (MSA) with the coagulase detection reaction (75%). In addition, the PCR reaction was valuable for the definitive identification of coagulase-positive staphylococci (CoPS). These comprehensive results shed light on the prevalence, species distribution, antibiotic resistance patterns, and diagnostic approaches for *S. aureus* in dogs and cats in Ukraine, emphasizing the importance of developing reliable surveillance and control strategies.

Keywords: *Staphylococcus pseudintermedius*; dog; resistance to antibiotics; microorganisms; prevalence; zoonotic pathogens.

Introduction

Staphylococci are gram-positive cocci that are commonly found on the skin and mucous membranes. They are a diverse group of bacteria that includes both pathogenic and non-pathogenic species. *Staphylococcus aureus* is a pathogenic microorganism that can cause disease in various species of animals and humans. However, other members of this family, such as *S. pseudintermedius*, are becoming increasingly important in the context of human health risk assessment.

Coagulase-positive staphylococci are frequently isolated from healthy and sick companion animals. In their systematic review (Abdullahi et al., 2022), they indicate that on average, 18.3% of healthy dogs have nasal carriage of *S. pseudintermedius* and 10.9% of *S. aureus*. The presence of commensal *S. pseudintermedius* is important for healthy dog microbiota. However, this microorganism is often the cause of various infections. To date, the mechanisms by which this opportunistic pathogen initiates a pathogenic effect on the host are not known for certain (Lynch & Helbig, 2021).

Staphylococcus aureus is a well-studied pathogen with well-defined dangers to animal and human health. The risk of human colonization with *S. aureus* from other humans is higher than from dogs. Also, *S. aureus* colonizing dogs have a greater potential to colonize humans than *S. pseudintermedius* (Cuny et al., 2022). However, in recent years, there have been

increasing reports of the zoonotic potential of *S. pseudintermedius*. Moses et al. (2023) found and analyzed 49 reports of human infection or colonization with *S. pseudintermedius*. The factors that determine the zoonotic potential of this pathogen are not well understood, and the virulence factors that allow the infection process to begin in humans are also little known (Bhooshan et al., 2020). However, regardless of the zoonotic potential of animal strains of staphylococci, they will act as a source of antibiotic-resistance genes for bacteria that can cause infections in humans (Souza-Silva et al., 2022).

Staphylococci can acquire resistance to various classes of antimicrobial agents. The risk of this genus acquiring resistance to β -lactam antibiotics is particularly high. Resistance to this class of drugs can be caused by the presence of the β -lactamase enzyme or by the incorporation of the *mec* complex into the genome. The target for β -lactam antibiotics is a group of penicillin-binding proteins (PB) that are part of the bacterial cell wall. Drugs with the addition of β -lactamase inhibitors, some semi-sentient penicillins, and cephalosporin antibiotics are resistant to the enzyme (Tooke et al., 2019). However, genotypic changes in staphylococci lead to the synthesis of an altered PB2a protein, which these drugs do not affect. To detect this resistance, disks with the semi-sentient antibiotic methicillin were initially used, so all strains with the *mec* gene are called methicillin-resistant *S. aureus* or *S. pseudintermedius* (MRSA and MRSP). The *mec*

genes are contained in the mobile genetic element SCC mec, which can be transferred between different staphylococcal species (Lakhundi & Zhang, 2018). MRSA and MRSP strains can be identified by phenotypic features – resistance to the screening antibiotics oxacillin or Cefoxitin, or by detection of resistance genes by molecular genetic methods.

In a retrospective ten-year study, the proportion of MRSP isolates identified by oxacillin resistance increased significantly. The level of resistance to other antibiotic groups, except for aminoglycosides, increases to a lesser extent. Resistance to aminoglycosides, on the contrary, is decreasing. The number of multidrug-resistant isolates is almost unchanged (Burke & Santoro, 2023). Adiguzel et al., (2022) also report an increase in the number of methicillin-resistant staphylococci during the study period. The risks of antibiotic resistance differ for different families of staphylococci. In general, the *S. aureus* family has a greater potential to acquire antimicrobial resistance than *S. pseudintermedius* (Ferradas et al., 2022). As noted by Haenni et al. (2020), *S. pseudintermedius* isolates causing infectious otitis media in dogs in Europe have significant genetic diversity. Resistance genes are detected in isolates from animals that have not been previously treated with antibiotics. The strains of *S. pseudintermedius* that cause infections in dog owners are genetically similar to the bacteria that colonize pets. However, several different genotypes can colonize a single dog, and more research is needed to study the dynamics of their transmission Wegener et al. (2022). Therefore, it is important to analyze the genetic relatedness of strains within a country or region.

Another danger associated with antibiotic-resistant staphylococci is that they contaminate the clinical environment. MRSA and MRSP are among the most common pathogens of nosocomial diseases in animals (Sebola et al., 2023). In Thailand, *S. pseudintermedius* was detected in a newly constructed hospital, with strains showing resistance to disinfectants (Fungwithaya et al., 2022). Contamination of the environment and equipment of the ophthalmology office can occur with strains that cause infection or colonize other areas of the body of sick dogs (Gentile et al., 2020). Protocols for disinfecting equipment and the environment in hospitals need to be improved to effectively combat staphylococci that have the potential to become nosocomial agents.

Also, many authors note the need to consider all families of staphylococci in the One Health concept. These microorganisms pose a danger not only in the context of their pathogenic potential but also in the context of antibiotic resistance. Such strains require additional diagnostic measures for their effective treatment or maybe a source of resistance genes for pathogens that pose a high risk to humans (Dos Santos et al., 2022; Souza-Silva et al., 2022). Therefore, it is necessary to develop and improve methods for differentiating staphylococci to better understand the characteristics of each family. In addition, the growth inhibition zones for some antibiotics according to EUCAST and CLSI standards differ for *S. aureus* and other coagulase-positive species.

Phenotypic characterization of the pathogen and rapid biochemical tests are not sufficient to differentiate the *Staphylococcus intermedius* group (SIG) from the *S. aureus* complex. The introduction of MALD-TOF MS helps to distinguish between these pathogens but increases the cost of the study (Bibby & Brown, 2021). Another problem is the differentiation of SIG staphylococci from each other. The literature describes some reactions that will allow the differentiation of these species (Bhooshan et al., 2020). However, more research is needed to develop optimal and affordable diagnostic strategies. The most sensitive option for differentiating these pathogens is the use of molecular genetic methods. PCR or sequencing can be used to differentiate SIG from *S. aureus*. An additional advantage of this method is the ability to differentiate SIG within a group (Bannoehr et al., 2009). However, these methods require special devices and reagents, as well as trained personnel, which not all laboratories can afford. This study aims to determine the prevalence of *S. pseudintermedius* among dogs in Ukraine and to explore the possibility of using chromogenic media and PCR reactions to identify and differentiate *S. aureus* and *S. pseudintermedius*.

Materials and methods

The research was conducted at the Research Laboratory of Veterinary and Sanitary Expertise and Laboratory Diagnostics of the Institute of Post-

graduate Training of Veterinary Medicine Managers and Specialists of the Bila Tserkva National Academy of Sciences and the Volyn Regional State Laboratory of the State Service of Ukraine for Food Safety and Consumer Protection.

Materials from the healthy dogs in this study were collected during routine veterinary visits as part of the diagnostic work. All samples were collected and used for this analysis with the verbal consent of the owners. Materials from sick animals were collected as part of treatment measures to establish the etiologic factor of the infectious process, directly by a veterinarian. The Ethics Committee of the Bila Tserkva National Agrarian University approved the research conducted within the framework of the research topic “Study of the role of opportunistic pathogens in the etiology and pathogenesis of animal diseases”, registration number 0121U110291. All manipulations were performed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Convention, 1986) and the Law of Ukraine “On Protection of Animals from Cruelty” No. 3447-IV as amended on August 04, 2017.

Sampling took place between June 2022 and May 2023. For the purpose of diagnosing resident staphylococcal carriage, 105 ear and 58 nasal samples from healthy dogs admitted to the veterinary hospital were examined.

To detect staphylococci associated with the pathological process, 109 samples were collected from sick animals in the period March – June 2023, 107 samples were taken from skin or mucous membranes of various parts of the body, and 2 samples from milk. Samples from patients were taken from different types of lesions and sorted by localization.

Materials from animals were collected using sterile swab soaked in phosphate buffer.

For the screening of resident staphylococcal carriage, trypsin soy broth (Condalab, Spain) was used as a medium for the accumulation of trypsin. The swab was inserted into a tube with broth and pressed against the tube wall, cultivated for 24 hours at 37 °C.

Sowing in mannitol salt agar. After cultivation culture was streaked onto the surface of mannitol salt agar (Condalab, Spain) and cultured for 24–48 hours at 37 °C.

Then, the fermentation of mannitol was determined (by changing the colour of the medium from red to yellow) and the pure culture was isolated. Differentiation of mannitol fermenting cultures at the family level was carried out using biochemical reactions to detect catalase, oxidase, and coagulase.

Sowing on chromogenic medium. Two chromogenic media were used in the study. The pure culture was suspended in sterile saline and inoculated on the surface of the agar, cultivated for 24 to 48 hours at 37 °C. The results were evaluated by the cultural and morphological properties of microorganisms according to the instructions for the medium.

Catalase detection reaction on glass. A bacterial mass was taken from the surface of the agar with a bacterial pipette and applied to a glass slide. Then a drop of 5% hydrogen peroxide (Ilan Pharm, Ukraine) was applied to the bacterial mass. The formation of foam indicated the presence of the catalase enzyme, and the absence of a reaction indicated the absence of the enzyme.

Oxidase detection reaction. The bacterial mass was applied to the test zone of the OXItest (Erba, USA) strip using a plastic loop. After an application for 30 s, the colour change was observed; a change in colour to blue or blue indicates a positive reaction.

Coagulase detection reaction. Lyophilized rabbit plasma (Biolik, Ukraine) was diluted into 5 mL of sterile saline. A bacterial batch of 0.5 mL of diluted plasma was added to a test tube and cultured at 37 °C. The reaction was observed after 1, 2, 4, 18, and 24 hours. Clot formation indicates a positive reaction to coagulase.

Detection of deoxyribonuclease. The pure culture was inoculated on the surface of DNA agar (HiMedia, USA). Seeding was done by drawing a straight line in the center of the Petri dish and cultured for 24 hours at 37 °C. After that, 1 HCl solution was added to the surface of the medium, incubated for 1 min, and the remaining liquid was removed and observed on a black background. The reaction was considered positive when a zone of medium clarification appeared around the colony. Antibiotic resistance was determined by the agar diffusion method. The pure

culture was taken with a bacterial loop and suspended in sterile saline. The bacterial suspension was diluted to a concentration of 0.5 according to McFarland standard and 1 ml was inoculated onto the surface of Mueller Hinton agar medium, spread with a spatula. After the dish dried, antibiotic disks were placed on the agar surface. The disks were used in the assay: penicillin 10 units, ceftriaxone 30 mcg, oxacillin 30 mcg, gentamicin 10 mcg, erythromycin 15 mcg, tetracycline 30 mcg, trimethoprim-sulfamethoxazole 1.25/23.75 mcg, ciprofloxacin 5 mcg. The growth inhibition zone was measured and interpreted according to Performance Standards for Antimicrobial Disk Susceptibility Tests, 28th Ed. Zone diameter for the combination amoxicillin – clavulanic acid 20/10 mcg were taken from (Bzdil et al., 2021).

Determining the specificity and sensitivity of diagnostic protocols. From the previously studied cultures, 40 Gram-positive and catalase-positive cocci were selected. Each culture was streaked on mannitol salt agar, Cromagar Orientation, DNA agar, and plasma coagulation was performed.

Cultures identified as *S. pseudintermedius* were confirmed by PCR. Data was analyzed according to a 2 × 2 table for diagnostic specificity and sensitivity according to (Kateete et al., 2010) (Table 1).

Table 1
Deriving sensitivity, specificity, positive/negative predictive values for the identification tests *S. pseudintermedius*

Test results	<i>S. pseudintermedius</i>	Other staphylococci	Total
Positive	a	b	a + b
Negative	c	d	c + d
Total	a + c	b + d	(a + b) + (c + d) = n

Notes: a = true positives, b = false positives, c = false negatives, d = true negatives, Diagnostic sensitivity = $d/(b + d)$, Diagnostic specificity = $a/(a + c)$, Positive predictive value (PPV) = $a/(a + b)$, Negative predictive value (NPV) = $d/(d + c)$.

PCR research. All Gram-positive and catalase-positive cocci isolated from healthy and diseased animals were tested by PCR.

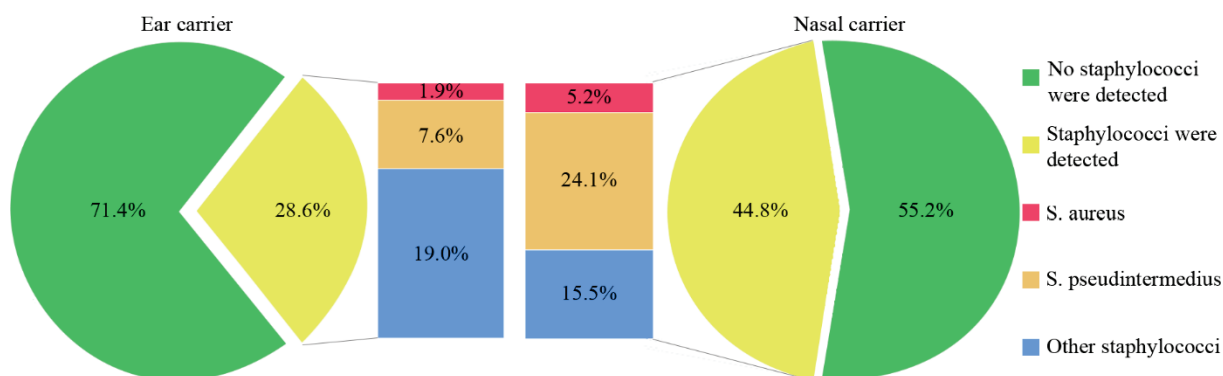


Fig. 1. Prevalence of ear and nasal carriage of staphylococci among healthy dogs

Out of 109 examined sick animals, *Staphylococcus* spp. was identified in 44 (40.3%) samples. Out of 77 dogs examined, *Staphylococcus* spp. was detected in 31 animals (40.3%), *S. pseudintermedius* in 18 (23.4%), and *S. aureus* in 4 (5.2%) dogs. Out of 32 cats studied, *Staphylococcus* spp. was identified in 13 (40.6%), *S. pseudintermedius* in 4 (12.5%), and *S. aureus* in 2 (6.3%) animals.

All isolates of coagulase-positive staphylococci formed a specific reaction product with species-specific and genus-specific primers (Fig. 2).

The largest number of samples from patients with traumas were taken from lesions in the body area – 78, bacteria of the *Staphylococcus* genus were identified in 30 samples. Out of 13 samples collected from ear lesions, *Staphylococcus* spp. was detected in 5, out of 10 samples from eye lesions – in 4, and out of 6 samples from nose lesions – in 4 (Fig. 3). Of the two samples of milk taken from the bitch, *S. pseudintermedius* was detected in one.

CoPS resistance to at least one antibiotic was observed in 10 out of 17 isolates from the skin. *Staphylococcus pseudintermedius*, the only *Staphylococcus* isolated in all samples from the ear, did not show resistance to any antibiotics. Of the six CoPS isolated from cats, 2 were found to be re-

Three primer pairs selected from the literature were used for PCR (Table 2). The study was performed using PCR with detection in an agarose gel. The amplification reaction was performed in a 25 µL reaction mixture, which included: 12.5 µL of OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs, USA), 7.5 µL of deionized water, 1 µL of F and R primers, and 3 µL of DNA. Amplification was performed in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems, USA).

Table 2
Primers used in the study

Target microorganism, m	Oligonucleotide sequence	Product size	Source
<i>Staphylococcus</i> spp.	5'-GGCCGTGTTGAACGTGGTCAAATCA-3'	370 b.p.	Martineau et al. (2001)
	5'-TIACCATTTCAGTACCTTCTGGTAA-3'		
<i>S. aureus</i>	5'-TRGGCAGTAGGATTTCGTTAA-3	359 b.p.	Sasaki et al. (2010)
	5'-CTTTTGCTCYCMFTTTGG-3		
<i>S. pseudintermedius</i>	5'-TCGCTTGCTATGATTGTGG-3	926 b.p.	(2010)
	5'-GCCAATGTCTACCATAGC-3		

Primary denaturation at 94 °C for 1 min, amplification for 30 cycles: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 68 °C for 60 s, and final elongation at 68 °C for 5 min.

The results were detected in a 2% agar gel with the addition of 0.5% ethidium bromide. The reaction was analyzed for the presence of a specific fragment of the appropriate size.

Results

In the studied dogs, nasal carriage of *Staphylococcus* spp. was more frequent at 44.8% than ear carriage at 28.5%. Of the 58 nasal swabs examined, *S. pseudintermedius* was detected in 14, *S. aureus* in 3, and other *Staphylococcus* in 9 animals. In the ear swabs taken from the ear, *S. pseudintermedius* was identified in 8, *S. aureus* – in 2, and other *Staphylococcus* – in 20 animals (Fig. 1). Of the detected coagulase-positive staphylococci, only 1 had phenotypic resistance to oxacillin.

sistant to 1 antibiotic, both samples were taken from the affected conjunctiva. The other feline isolates studied had full or intermediate susceptibility to all the drugs tested. *Staphylococcus pseudintermedius* was also detected in one milk sample (Fig. 4).

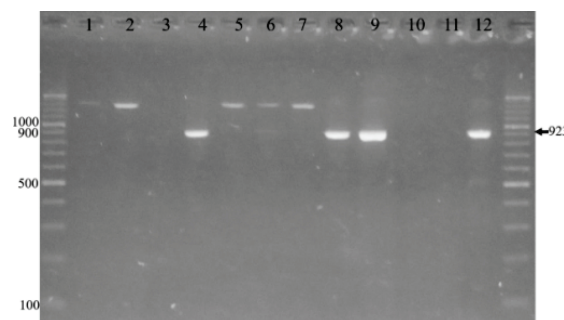


Fig. 2. Electrophoresis of amplification products with primer digestion for detection *S. pseudintermedius*: 4, 8, 9, 12 – *S. pseudintermedius*; 3, 10 – *S. aureus*; 1, 2, 5, 6, 7, 11 – other *Staphylococcus*

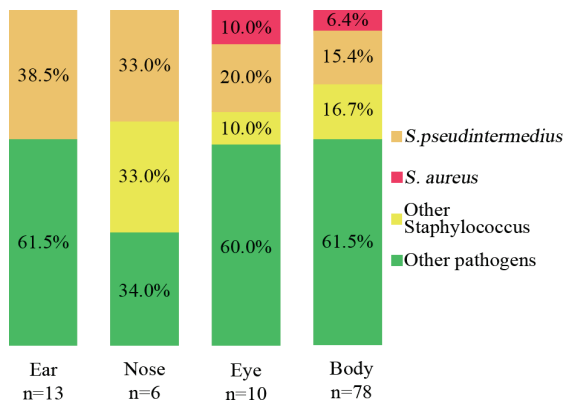


Fig. 3. Distribution of the pathogen by body parts

Affection	AMR profile									
	OXA	AMC	CTR	PEN	GEN	SXT	TET	ERY	CIP	
<i>S. pseudintermedius</i> from dogs										
Body	S	S	S	S	S	S	S	S	S	S
Body	S	S	S	S	S	I	S	S	S	ND
Body	S	S	S	S	S	S	S	S	S	ND
Body	S	S	S	R	S	S	S	R	S	S
Body	S	S	S	R	R	R	I	R	S	S
Body	S	S	S	R	S	R	S	S	S	S
Body	S	S	S	S	S	S	S	S	S	ND
Body	S	S	S	S	S	3	S	S	S	ND
Body	S	S	S	S	S	R	S	S	S	S
Body	S	S	S	S	S	R	S	S	S	S
Body	S	S	S	R	S	R	S	R	S	S
Ear	S	S	S	S	S	S	S	S	S	R
Ear	S	S	S	S	S	S	S	S	S	ND
Ear	S	S	S	S	S	I	S	S	S	S
Ear	S	S	S	S	S	S	S	S	S	S
Eye	S	S	S	S	S	R	S	S	S	S
Milk	S	S	S	S	S	S	S	S	S	S
Nose	S	S	S	S	S	R	S	R	S	S
<i>S. pseudintermedius</i> from cat										
Body	S	S	S	S	S	S	S	S	S	ND
Ear	S	S	S	S	S	I	S	S	S	ND
Eye	S	S	S	S	S	S	S	S	S	I
Nose	S	S	S	S	S	I	I	S	S	ND
<i>S. aureus</i> from dogs										
Body	S	S	S	R	S	R	S	R	S	ND
Body	S	S	S	R	S	S	S	S	S	R
Body	S	S	S	R	S	S	S	S	S	S
Body	S	S	S	S	S	I	S	S	S	S
<i>S. aureus</i> from cat										
Body	S	S	S	S	S	S	S	S	S	S
Ear	S	S	S	S	S	I	S	S	S	I

Fig. 4. Antimicrobial resistance profile of CoPS isolated from sick animals: S – sensitive, I – intermediate, R – resistant; PEN – penicillin, OXA – oxacillin, AMC – amoxicillin/clavulanic acid, CTR – ceftriaxone, GEN – gentamicin, ERY – erythromycin, TET – tetracycline, SXT – trimethoprim/sulfamethoxazole, CIP – ciprofloxacin

Table 4

Comparison of the frequency of *S. pseudintermedius* detection using different combinations of microbiological tests

Microbiology test	Test result	<i>S. pseudintermedius</i> n (% of 20)	Other <i>Staphylococcus</i> n (% of 20)	Subtotal
MSA	MF	20 (100)	9 (45)	29
	Other reaction	0 (0)	11 (55)	11
MSA+Coag	MF and Coag+	20 (100)	6 (25)	24
	Other reaction	0 (0)	15 (75)	16
MSA+ DNAsa	MF and DNAsa +	19 (95)	6 (30)	25
	Other reaction	1 (5)	14 (70)	15
MSA+ DNAsa +Coag	MF and Coag+ or DNAsa +	20 (100)	6 (30)	26
	Other reaction	0 (0)	14 (70)	14
ChrO	Pink colour	20 (100)	7 (35)	27
	Other reaction	0 (0)	13 (65)	13
ChrO+Coag	Pink colour and Coag+	20 (100)	0 (0)	19
	Other reaction	0 (0)	20 (100)	21
ChrO+ DNAsa	Pink colour and DNAsa +	19 (95)	0 (0)	19
	Other reaction	1 (5)	20 (100)	21
ChrO+Coag+ DNAsa	Pink colour Coag+ or DNAsa +	20 (100)	0 (0)	20
	Other reaction	0 (0)	20 (100)	20

Notes: MSA – mannitol salt agar, ChrO – CHROMagar Orientation, Coag – coagulase, DNase – deoxyribonuclease, PPV – positive and NPV – negative predictive value.

7 isolates of *S. pseudintermedius* were not resistant to any of the tested antibiotics, and 5 isolates were resistant to one antibiotic, 3 to two, 1 to three, and 1 to four drugs. One isolate of *S. aureus* was not resistant to antibiotics, and 1 isolates were resistant to one, and 1 to four antimicrobial agents (Table 3). The study of the culture properties of CoPS showed that the growth pattern of *S. aureus* and *S. pseudintermedius* on chromogenic media is significantly different. On CHROMagar Orientation medium, *S. aureus* colonies were yellow in colour (Fig. 5b), and on CHROMagar *Staphylococcus* medium, they were pink in colour (Fig. 5a1). Whereas *S. pseudintermedius* on CHROMagar *Staphylococcus* had colonies of light blue to blue colour (Fig. 5a2,3, 5c, 6), and on CHROMagar Orientation medium from light pink to purple (Fig. 5c, 7).

Table 3

Sensitivity of CoPS isolated from sick animals

Antibiotic	<i>S. pseudintermedius</i> , n=22		<i>S. aureus</i> , n=6	
	S	I/R	S	I/R
OXA	100%	-/0%	100%	-/0%
AMC	100%	-/0%	100%	-/0%
CTR	100%	-/0%	100%	-/0%
PEN	81.8%	-/18.2%	50%	-/50%
GEN	95.5%	0%/4.5%	100%	-/0%
SXT	45.5%	18.2%/36.3%	50.0%	33.3%/16.7%
TET	90.9%	9.1%/0%	100%	0%/0%
ERY	81.8%	0%/18.2%	83.3%	0%/16.7%
CIP	85.8%	7.1%/7.1%	60%	20%/20%

Notes: S – Sensitive, I – Intermediate, R – Resistant; PEN – penicillin, OXA – oxacillin, AMC – amoxicillin/clavulanic acid, CTR – Ceftriaxone, GEN – gentamicin, ERY – erythromycin, TET – tetracycline, SXT – trimethoprim/sulfamethoxazole, CIP – ciprofloxacin.

In the analysis of the comparison of methods for the identification of *S. pseudintermedius*, 40 cultures of catalase-positive and gram-positive cocci were studied. Their belonging to the family *Staphylococcus* spp. was confirmed by PCR. Thus, 20 isolates were identified as *S. pseudintermedius*. Six isolates, which were not identified as *S. pseudintermedius* and showed coagulase and DNase activity, were identified by PCR as *S. aureus*. In total, 29 isolates fermented mannitol and caused a change in MSA colour from pink to yellow. 27 of the studied staphylococci had a pink colour on CHROMagar Orientation (Table 4). The growth characteristics of all *S. pseudintermedius* isolates on both media were in line with the expected results. In general, 26 staphylococci showed a positive coagulase reaction, and 25 showed DNase activity. One isolate of *S. pseudintermedius* did not have DNase activity.

The sensitivity, specificity, positive and negative predictive values were determined using the formulas given in Materials and Methods. The sensitivity of MSA for the detection of *S. pseudintermedius* was 10% lower than that of chromogenic medium. The introduction of additional plasma coagulation reactions or detection of DNase activity significantly increases the sensitivity of both MSA and chromogenic medium. In this case, the specificity and NPV of both media in combination with additional reactions were above 90%.

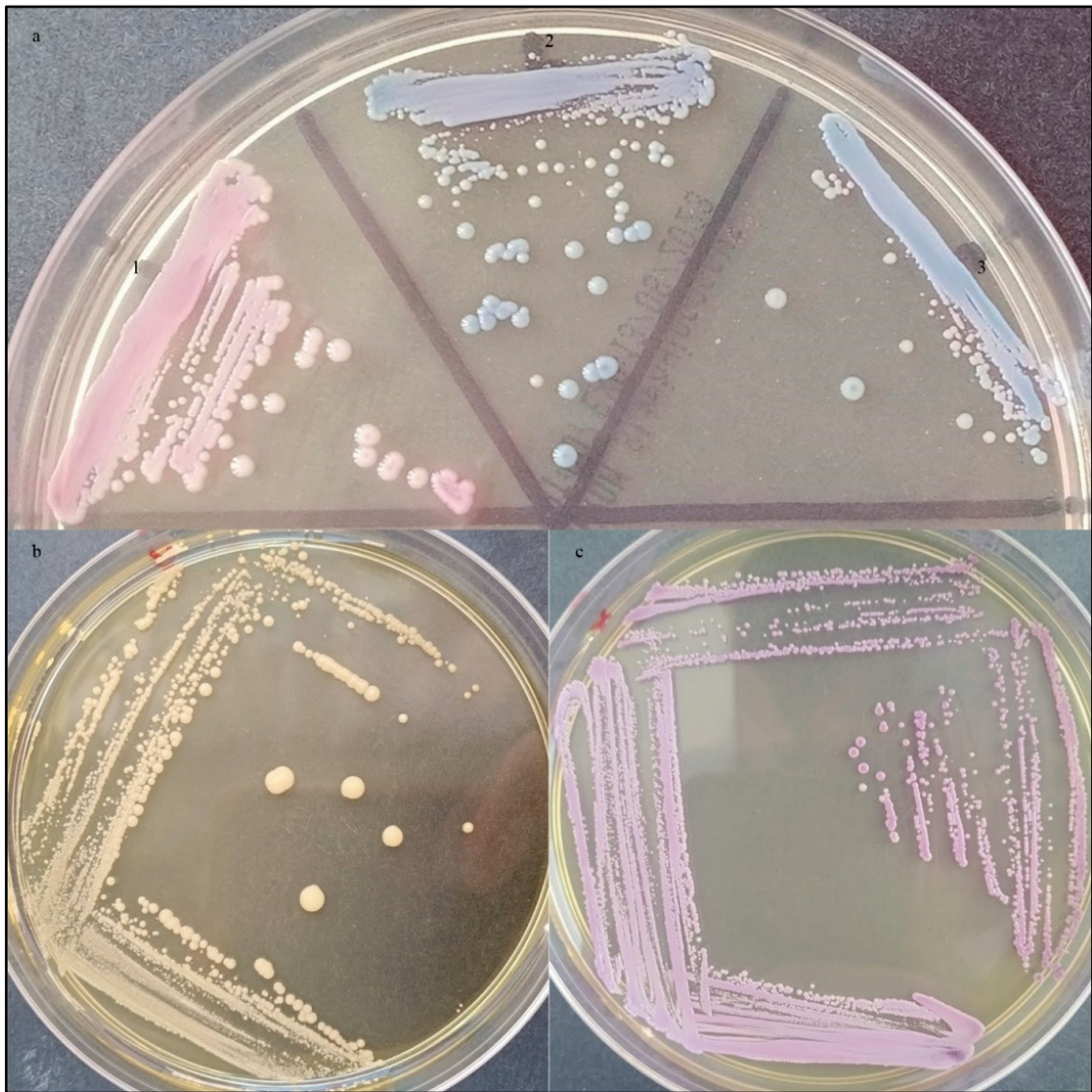


Fig. 5. *Staphylococcus aureus* colonies on the CHROMagar *Staphylococcus* (a1); *S. pseudintermedius* colonies on the CHROMagar *Staphylococcus* (a2–3); *S. aureus* colonies on the CHROMagar Orientation (b); *S. pseudintermedius* colonies on the CHROMagar Orientation (c)

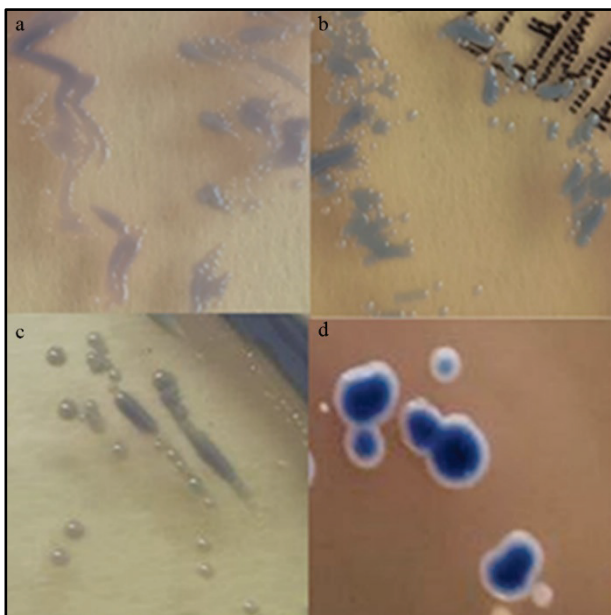


Fig. 6. Colonies (a–d) of different strains of *S. pseudintermedius* on CHROMagar *Staphylococcus*

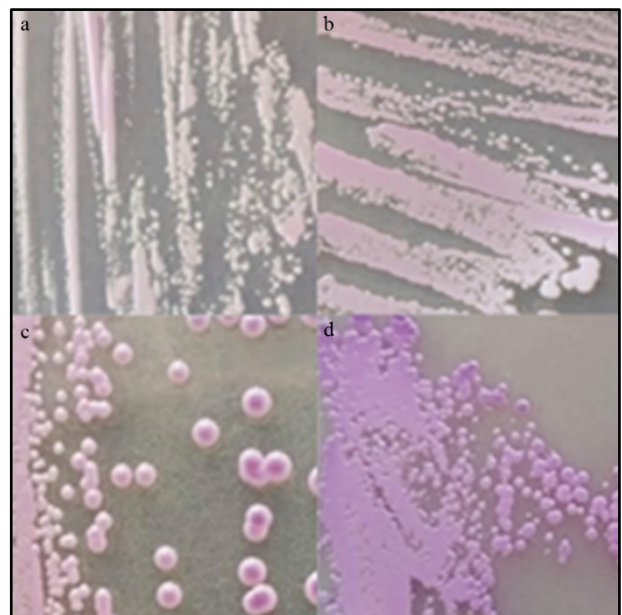


Fig. 7. Colonies (a–d) of different strains of *S. pseudintermedius* on CHROMagar Orientation

Table 5

Sensitivity, specificity, negative/positive predictive values of different combinations of microbiological tests for finding *S. pseudintermedius*

Test	Sensitivity, %	Specificity, %	PPV, %	NPV, %
MSA	55	100	69	100
MSA+Coag	70	100	77	100
MSA+DNase	70	95	76	93
MSA+DNase+Coag	70	100	77	100
ChrO	65	100	74	100
ChrO+Coag	100	100	100	100
ChrO+Dnasa	100	95	100	95
ChrO+Coag+Dnasa	100	100	100	100

Notes: MSA – mannitol salt agar, ChrO – CHROMagar Orientation, Coag – coagulase, Dnasa – deoxyribonuclease, PPV – positive predictive value, NPV – negative predictive value.

Additional reactions in combination with MSA have lower sensitivity and positive predictive value. The use of both reactions in combination with chromogenic medium led to the highest values for all indicators described in the study (Table 5).

Based on the results of the study, we proposed schemes for the identification of *S. pseudintermedius* and differentiation of this pathogen from *S. aureus* (Fig. 8). The scheme using MSA is aimed at differentiating *Staphylococcus* spp. from other genera of microorganisms. The plasma coagulation reaction is performed after obtaining Mannitol+ colonies to exclude coagulase-negative staphylococci. Chromogenic medium is used in the scheme to differentiate *S. aureus* from *S. pseudintermedius*. The diagnostic capabilities of the second scheme differ depending on the chromogenic medium used in the first step. For example, CHROMagar *Staphylococcus* (CroS) inhibits the growth of non-staphylococcal microflora. After the initial inoculation on both media, it is possible to distinguish *S. aureus* colonies from other members of the genus. CHROMagar Orientation (CroO) can be used to identify more pathogens than those listed in the instructions provided with the patent material. On CroO, the colour of *E. coli* colonies is also pink. Microscopy and staining allows distinguishing coccil microorganisms from rod-shaped ones. The plasma coagulation reaction allows distinguishing *S. pseudintermedius* colonies from CoNS.

Discussion

Coagulase-positive staphylococci are part of the opportunistic microflora of dogs and colonize a large part of the population.

Thus, a study of nasal resident staphylococcal carriage found that among a healthy dog population, *S. pseudintermedius* (24.1%) is a more common pathogen than *S. aureus* (5.2%), and carriage of methicillin-resistant strains is inherent in a low percentage of the dog population. Our results correlate with the data of the authors Abdullahi et al. (2022), who in their review determined the prevalence of nasal carriage of *S. aureus* in 2.1–48.0% of healthy dogs, with an average of 10.9%. Of the 22 publications that reported carriage of staphylococci, two reported no carriage. In 28 sources, the results of MRSA detection were described, in 12 studies such isolates were not detected. In the reports of carriage of these strains, their level ranged from 0.13% to 47.7%. For *S. pseudintermedius*, these figures are in the range of 11.0–74.7% of dogs, with an average of 18.3%. Three out of 13 publications on the prevalence of this pathogen, two reported its absence in the studied dogs. There were 18 studies aimed at detecting MRSP, in 8 of which no relevant isolates were isolated. The carriage rates ranged from 0.85% to 51.1%. In a study of nasal carriage of staphylococci in dogs in Malaysia, *S. pseudintermedius* was detected in 9.3% of domestic dogs, including 1.7% of MRSP. *Staphylococcus aureus* was not detected in the dogs studied (Afshar et al., 2023). In another study (Tang et al., 2020), *S. pseudintermedius* was isolated more often 14.6% than in our study 7.6%. However, according to Frosini et al. (2022), a nasal swab may not be sufficient to identify a carrier. Combining swabs from multiple body sites increases the likelihood of detecting *S. pseudintermedius* in dogs.

In our study, 56.3% of the isolated staphylococci from sick dogs belonged to the *S. pseudintermedius* species. 83.3% of *S. aureus* isolates and 54.4% of *S. pseudintermedius* isolates were isolated from lesions in the body area. Another 22.7% of *S. pseudintermedius* isolates were isolated

from ear lesions, and it was the only staphylococcal species present in these materials. A similar study of the microflora of dogs in Bangladesh showed that in healthy animals, the carriage of *S. aureus* was 16%, and *S. pseudintermedius* – 45.3%. At the same time, *S. aureus* was isolated from dermatological lesions more often – 28.6% than *S. pseudintermedius* – 25.0% of cases. In animals with wounds and otitis media, on the contrary, *S. pseudintermedius* was isolated more often (50.0% and 38.3%) than *S. aureus* (12.5% and 27.1%), which is consistent with the data obtained by us. MRSA isolates were much more common 46.4% than MRSP 8.1% (Rana et al., 2022). Bourély et al., (2019) also found *S. pseudintermedius* as the most common pathogen, identified in 33% of canine otitis media. *Staphylococcus aureus* caused the disease in 3.9% of the dogs studied. A Czech study of *Staphylococcus* spp. isolated from sick animals indicates a high prevalence of 72.7% of *S. pseudintermedius* among domestic carnivores. *Staphylococcus aureus* was isolated in only 1.3% of animals, while other coagulase-positive staphylococci were isolated in 11.4% of cases. Also, it was identified that 0.93% of isolates were MRSP, while MRSA was 0.04% (Bzdil et al., 2021).

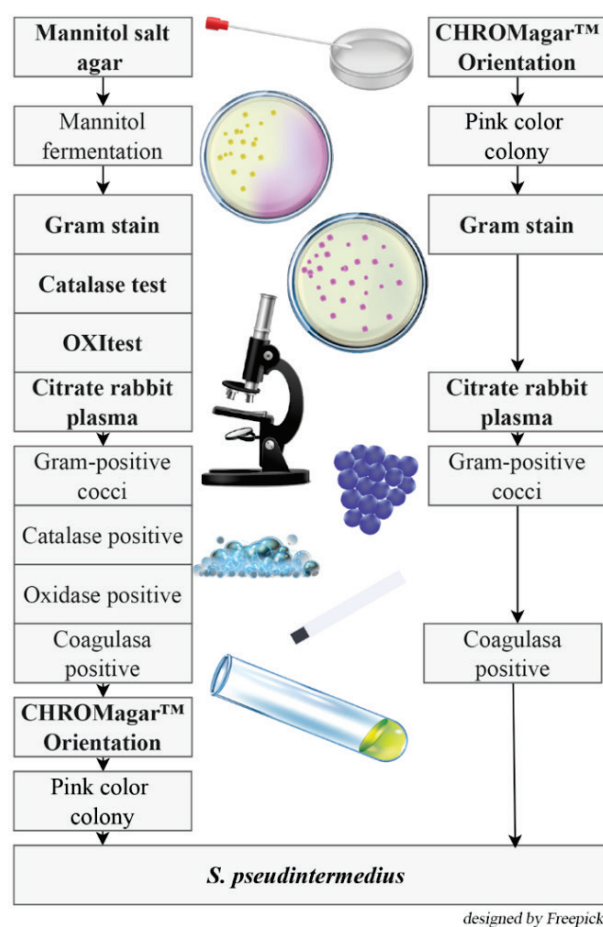


Fig. 8. Scheme of differentiation of *S. aureus* from *S. pseudintermedius* using chromogenic medium

Most isolates of *S. pseudintermedius* in our study were resistant to the combination of trimethoprim/sulfamethoxazole. A smaller number of isolates showed resistance to penicillin and erythromycin antibiotics. Only 1 tested isolate was resistant to gentamicin and tetracycline. We did not detect MRSP or MRSA strains, all isolates were sensitive to oxacillin, amoxicillin/clavulanic acid and in other studies of isolates obtained from animals with skin and mucosal lesions, the data on antibiotic resistance varied somewhat. But in all reports, the acquisition of penicillin resistance is inherent in a large number of isolates. According to Bourély et al. (2019), *S. pseudintermedius* was most often resistant to penicillin 68.5% and erythromycin 29.8% and 12.2% of isolates were resistant to the combination of trimethoprim/sulfamethoxazole. In the study de Jong et al. (2020), isolates from cats and dogs had the highest resistance to penicillin 15.2–15.5% and trimethoprim/sulfamethoxazole 12.1–12.3%. A fairly large

number of 45.8% of MRSA isolates were resistant to trimethoprim/sulfamethoxazole and gentamicin 20.8% (Bzdil et al., 2021). *Staphylococcus aureus* showed high resistance to penicillin. These data are in line with the high resistance to this antibiotic in the studies by Bourély et al. (2019) 70.9% and de Jong et al. (2020) 75.8% and 69.4%. Out of 25 samples collected from companion animals in Kyiv, CoPS was detected in 8%. Both isolates were resistant to penicillin and one to oxacillin (Vishovan et al., 2021).

CHROMagar Orientation (ChrO) and CHROMagar *Staphylococcus* (ChrS), which we use for the isolation and identification of staphylococci, have high sensitivity and specificity and reduce the time required to perform the test. These media contain indoxyl glycosidase substrates, which change colour after interaction with staphylococcal enzymes. Since these substrates are insoluble in water, after the reaction they settle in the colony and do not change the colour of the environment. CHROMagar *Staphylococcus* medium contains a substrate aimed at detecting a specific phosphatase produced by *S. aureus*. CHROMagar Orientation medium contains substrates that interact with the β -Galactosidase enzyme produced by *E. coli* (Orenga et al., 2009). This enzyme is present in *S. pseudintermedius*, but not in *S. aureus* (Bhooshan et al., 2020). We assume that the interaction with the substrate targets the β -galactosidase enzyme and causes the pink coloration of *S. pseudintermedius* colonies.

According to the CHROMagar *Staphylococcus* instructions, *S. aureus* colonies are purple to pink in colour. CHROMagar *Staphylococcus* is a chromogenic medium used to detect *S. aureus* colonies, which are purple to pink. Several studies compared its sensitivity and specificity with other media and identification schemes. Carricajo et al. (2001) found that combining CHROMagar *S. aureus* with Gram staining resulted in a sensitivity of 98.5% and a specificity of 100% when used with the coagulase test. In contrast, the traditional scheme using nutrient agar with Gram staining, catalase, coagulase, and latex agglutination had a lower specificity of 91.8%. Gaillot et al. (2000) compared CHROMagar *S. aureus* with morphology-based identification and a standard scheme using blood agar, catalase test, and latex agglutination. CHROMagar *S. aureus* showed higher sensitivity (95.5%) and specificity (99.4%) compared to the standard scheme (sensitivity – 81.9%, specificity – 98.9%). Sirobhushanam et al. (2019) indicate that when identifying *S. aureus* in human biomaterial, false-generation, and false-positive results are possible for both CHROMagar (12.9% and 30.4%) and mannitol salt agar (11.5% and 39.3%) based on culture properties alone. The growth and coloration patterns of *S. pseudintermedius* are not determined by the medium instructions. In our study, we noted the light blue colour of the colonies. The study by Beça et al. (2015) also reported the colour of *S. pseudintermedius* colonies to be purple to blue, while *S. aureus* colonies were purple to pink. Gaillot et al. (2000) reported that coagulase-negative species *S. saprophyticus* and *S. haemolyticus*, also acquire a blue colour on this medium. The CHROMagar *Staphylococcus aureus* medium is produced by the same manufacturer as CHROMagar *Staphylococcus* medium and likely contains a similar substrate. Our study using ChrS yielded results consistent with the morphological features of pathogen colonies described in the aforementioned reports.

We did not find any reports from other authors on the use of CHROMagar Orientation for the differentiation of staphylococci. This chromogenic medium allows for a clear differentiation of *S. pseudintermedius* from *S. aureus*. This is important because *S. pseudintermedius* is more important in the context of veterinary medicine. To differentiate between CoPS and CoNS, additional reactions aimed at detecting pathogenicity factors, such as coagulase or DNAase, should be used. For example, the use of a chromogenic medium in combination with the rabbit plasma coagulation reaction leads to an increase in the specificity of detection of *S. pseudintermedius* from 65% to 100%. The specificity of the MSA medium changed from 55% to 70%. Halophilic and mannitol-fermenting staphylococci, including both *S. aureus* and *S. pseudintermedius*, can grow on MSA. On ChrO, some CoNS species also had similar culture properties to *S. pseudintermedius*, but they were easily defended by additional reactions. The specificity index indicates the probability of false negative identification of other staphylococcal species as *S. pseudintermedius* and may decrease when other members of the SIG group are included in the study. We do not know what their cultural properties are on chromogenic media. The sensitivity and NPV for both media and the combination of

media with coagulase were maximal. That is, there is no possibility of not detecting *S. pseudintermedius* in the sample under study. Carricajo et al. (2001) also noted an increase in the specificity of *S. aureus* when using a coagulase test with citrated rabbit plasma. The advantage of ChrO medium over ChrS is that it is possible to grow some types of non-staphylococcal microflora. This feature can be used in clinical laboratories to detect a wider range of microorganisms. The use of simple biochemical reactions such as catalase, oxidase, and coagulase tests is time-consuming and indicates that microorganisms belong to the *Staphylococcus* genus. In the schemes presented here, we recommend using citrated rabbit plasma rather than DNA agar because the first option is cheaper, does not require preparation and sterilization of the medium, and does not require the reaction results to be developed with HCl. It should be noted that the use of chromogenic media is more expensive than non-chromogenic media. Therefore, for monitoring purposes, in order to save resources, mannitol salt agar can be used for primary inoculation and to confirm the presence of coagulase-positive staphylococci in the material. And chromogenic medium can be used to differentiate pathogens if molecular genetic methods are not available. The sensitivity and specificity of other tests, such as acetone production, hyaluronidase, and Polymyxin B antibiotic excretion, should also be studied in more detail (Bannoehr & Guardabassi, 2012).

The species *S. pseudintermedius* is difficult to identify by microbiological methods, however, the use of chromogenic media can simplify this procedure. Once the microorganism is identified on chromogenic media, additional confirmation by other methods is required (Sirobhushanam et al., 2019). As we wrote earlier, chromogenic media are aimed at detecting specific enzymes that may be present in other types of non-target bacteria. Therefore, in our study, the final confirmation of the isolates belonging to the genus *Staphylococcus* was performed using PCR. The optimization and validation of the protocol for the primer aimed at the detection of *Staphylococcus* spp. have been described previously (Shevchenko et al., 2022). The use of this method allows for the differentiation of genera and species with similar cultural and biochemical properties (Sasaki et al., 2007). However, it requires appropriate equipment and trained personnel.

Conclusions

Nasal carriage of *S. pseudintermedius* was present in 24.1% and ear carriage in 5.2% of dogs, while nasal and ear carriage of *S. aureus* was 7.6% and 1.9%. *Staphylococcus pseudintermedius* was the most common *Staphylococcus* species, isolated from sick dogs at 20.5% and cats at 10.5%. *Staphylococcus aureus* as an infectious agent was identified less frequently at 4.5% in dogs and 5.3% in cats. Among healthy dogs, we found one isolate of *S. pseudintermedius* that was resistant to oxacillin, while no such isolates were found in the sick animals. *Staphylococcus pseudintermedius* most often acquired resistance to trimethoprim/sulfamethoxazole at 36.3%, penicillin, and erythromycin at 18.2% each. *Staphylococcus aureus* was resistant to penicillin at 50%. Four isolates of CoPS were resistant to 3 or more antibiotics, all of which were isolated from wounds. Colonies of *S. pseudintermedius* on CHROMagar Orientation medium acquire a pink to purple colour and on CHROMagar *Staphylococcus* colour ranges from light blue to dark blue. The combination of the chromogenic medium with the coagulase detection reaction is more specific than the use of mannitol agar for this reaction. At the same time, the sensitivity of both media is high. The advantage of CHROMagar Orientation is its versatility. One medium makes it possible to detect and differentiate pathogens already in the primary culture. The application of the provided schemes using chromogenic media can be used for the primary differentiation of *S. aureus* and *S. pseudintermedius*. However, the use of molecular genetic methods, such as PCR, is necessary to definitively confirm the belonging of staphylococci isolated from animals to certain species of the CoPS group. Additional research is needed to determine the sensitivity and specificity of other biochemical reactions, as well as to study a larger sample of animals to determine the distribution of MRSP and MRSA strains.

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The authors declare that they have no conflict of interest.

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