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INCREASED TOLERANCE TO CHLORHEXIDINE FOLLOWING PROLONGED EXPOSURE IN *STAPHYLOCOCCUS* SPECIES ISOLATED FROM THE SKIN AND MUCOSAE OF DOGS

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ABSTRACT. This study aimed to investigate if canine staphylococci isolates could develop tolerance to chlorhexidine digluconate after long-term exposure. Staphylococci (*Staphylococcus pseudintermedius* and coagulase-negative staphylococci, methicillin-susceptible and methicillin-resistant), with and without genotypic chlorhexidine resistance, were investigated for phenotypic chlorhexidine tolerance by determining the MBC (minimal bactericidal concentration) at various time points. The testing was performed as follows: determination of MBC for 30 minutes and 24 hours (MBC 24h-1); exposure of isolates for seven days to concentrations of chlorhexidine equal to 1/2 MBC 24h-1; determination of MBC for 24 h after the first week (MBC 24h-2); exposure of isolates for seven days to concentrations of chlorhexidine equal to 1/2 MBC 24h-2 and rest in Mueller–Hinton broth for seven days; determination of MBC for 24 h after the second week (MBC 24h-3). The MBC for 30 minutes ranged between 16–32 μg/ml compared to the MBC 24h-1 which was between 1–8 μg/ml. The MBCs for 24 h dropped from 8 μg/ml to 0.5 μg/ml for isolate 1, from 2 μg/ml to1 μg/ml for isolate 6 and from 2 μg/ml to 0.5 μg/ml for isolate 8 after being exposed for seven days to concentrations of chlorhexidine equal to 1/2 MBC 24h-1. For one CoNS (coagulase-negative staphylococci), the MBC 24h-2 increased four times from 1 μg/ml (MBC 24h-1) to 4 μg/ml and dropped again to 1

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µg/ml after the second week. These results suggest that continuous exposure to chlorhexidine could lead to the selection of chlorhexidine-tolerant staphylococci that could withstand concentrations used during routine decolonisation procedures.

Keywords: Staphylococcus; chlorhexidine; tolerance; MBC.

INTRODUCTION

Staphylococcus pseudintermedius is a common resident of canine skin and mucosa and is considered the main coagulase positive staphylococci commensal for this anatomical site (Weese *et al.*, 2010). It is often involved in causing or aggravating pyoderma along with other mucosal and skin residents such as *Staphylococcus aureus* and *Staphylococcus schleiferi* or coagulase-negative staphylococci (CoNS) like *Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus saprophyticus, Staphylococcus sciuri and Staphylococcus warneris* (Gómez-Sanz, 2013; Schmidt *et al.*, 2014).

The pathogenic potential of S. *pseudintermedius* resides in the virulence and antimicrobial resistance factors that it possesses or may acquire from other bacteria in co-colonisation. S. pseudintermedius can possess several enzymes, toxins and adhesion factors which can facilitate its pathogenicity (Gómez-Sanz, 2013). Furthermore, acquisition of antimicrobial resistance determinants through plasmids (*mec A, smr, qac A/B*) from other bacteria that develop alongside it makes infections more challenging to treat (Hanssen *et al.*, 2004). In addition, the CoNS that may be found along with *S. pseudintermedius* are commonly antimicrobial resistant, including to methicillin (Priya *et al.*, 2014). Tolerance to topical antimicrobials and antiseptics has been reported in *S. aureus* (Chen *et al.*, 2010), but only rarely in canine *S. pseudintermedius* isolates (Couto *et al.*, 2014; Godbeer *et al.*, 2014; Murayama *et al.*, 2013).

S. pseudintermedius is a common resident bacteria of the skin and mucosa that can cause or aggravate pyoderma (Sasaki *et al.*, 2010). Along with it, in the etiopathogenesis of dermatitis, we also find coagulase-negative staphylococci that participate as commensals, such as *Staphylococcus epidermidis* (Hrițcu *et al.*, 2020).

As antiseptics are becoming more widely used in canine dermatology as topical treatments for pyoderma, and also in households to disinfect surfaces, we may be assisting in the emergence of resistance genes targeting them. Recent reports have highlighted an increase in the use of chlorhexidine as a topical treatment for skin conditions in dogs (Dulman, 2017; Hrițcu *et al.*, 2020). Chlorhexidine is an important biguanide cationic substance that is efficient against a large number of bacterial species and is used to prevent or treat pyoderma in companion carnivores, and to help disinfect the skin before surgical procedures (Corona *et al.,* 2020; Guardabassi *et al.*, 2010). The pursued effect influences the application method and usage: scrubs/shampoos act for about 5 min (Borio *et al.*, 2015), and sprays, lotions and baths offer a longer contact period (Popovich *et al.*, 2012).

Resistance to antiseptics resides in genes located in the chromosomes (nor) of bacteria or on mobile elements such

as plasmids (*qac A/B, smr*) that encode for membrane efflux pumps (Couto *et al.*, 2008). The carriage of such genes does not always correlate with a high chlorhexidine MIC (minimal inhibitory concentration), questioning the clinical significance of *qac A/B* carriage (Frosini *et al.*, 2019). The emergence of biocide-resistant *S. pseudintermedius*, particularly if methicillin-resistant, would severely limit therapeutic options, an outcome already illustrated for mupirocin (Fritz *et al.*, 2013) and chlorhexidine (Batra *et al.*, 2010) in *Staphylococcus aureus* in humans (Hrițcu *et al.*, 2020).

This study aimed to determine if the exposure of *S. pseudintermedius* and *S. epidermidis* isolates (harvested from the skin and mucosa of dogs) to concentrations of chlorhexidine equal to half the MBC (minimal bactericidal concentration), for seven days in a row, 24 hours a day, can influence the minimal bactericidal concentrations.

MATERIALS AND METHODS

Sample collection and isolates

The isolates used in this study were harvested from the skin and mucosae of dogs that presented with pyoderma and were examined in two referral hospitals, one located in the United Kingdom and one in Romania. The inclusion criterion was a staphylococcal pyoderma diagnosed based on clinical signs and cytological examination. The study group included a mixture of males and females, purebred and crossbreed dogs, with no predominance, with ages ranging from six months to eight years. The samples were harvested between June 2014–January 2016.

Staphylococcus spp. isolates were harvested, inoculated and incubated as describe Hrițcu *et al.* (2020), Sasaki *et al.* (2010) and Schmidt *et al.* (2014). The isolates were previously characterised for phenotypic (oxacillin, cefovecin, cefalexin, ampicillin, amoxicilin-clavulanic acid, clindamycin, trimetoprim-sulfamethoxazole, tetramycine, gentamicyne, chloramphenicol, ciprofloxacine, enrofloxacine, fusidic acid) and genotypic (*mec A*, *mup*, *fus B*, *fus C*, *fus D*) antimicrobial resistance, virulence (*lukI*, *expA*, *siet*, sec_{canine}, *bap*, *icaA*, *icaD*) and chlorhexidine resistance (*qac A/B – fw*: 5’-GCTGCATTTATGACAATGTTTG -3’, *rev*: 5’-AATCCCACCTACTAAAGCAG-3’ and *smr – fw:* 5’-ATAAGTACTGAAGTTATTGGAAGT-3’, *rev*: 5’-TTCCGAAAATGTTTAACGAAACTA-3’) genes (*Table 1*), as described in previous studies (Couto *et al.*, 2008; Hrițcu *et al.*, 2020). Antimicrobial phenotypic resistance was determined using the disc diffusion method on Mueller–Hinton agar plates, following CLSI 2018 recommendations. We tested for the main virulence genes that could influence the pathogenicity of *S. pseudintermedius* in pyoderma and for the antimicrobial substances used frequently in the two veterinary referral hospitals. The study focused mainly on *S. pseudintermedius*, but we also identified CoNS (*S. epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, *Staphylococcus sciuri*, *Staphylococcus warneris*) in co-culture with the *S. pseudintermedius*, which could influence the resistance patterns of this bacteria through the transmittance of resistance genes through plasmids (Mores *et al.*, 2021).

For this study, ten isolates were selected which included eight *S. pseudintermedius*, methicillin-susceptible (MSSP, n = 5) and methicillin-resistant isolates (MRSP, n = 3) and two CoNS, both *S. epidermidis*, of which one was MRS (methicillin-resistant staphylococcus) and one MSS (methicillin-susceptible staphylococcus), to investigate phenotypic

chlorhexidine resistance. All isolates were clinical isolates and no standard strains were used for comparison. The study sample included isolates of *S. pseudintermedius* with and without chlorhexidine resistance genes, multidrug-resistant or not, methicillin-resistant or not and CoNS following the same criteria, to have a heterogenous group with no predominance, but with one–two representatives of each type.

Table 1 shows phenotypes and genotypes of selected isolates as follows: four MSSP and two MRSP isolates with no resistance genes for antiseptics (of those screened for), one MSSP isolate carrying the *qac A/B* gene, one MRSP isolate with both *qac A/B* and *smr* genes, one CoNS carrying none of the investigated antiseptic resistance genes and one CoNS carrying the *qac A/B* gene. Both the CoNS were selected from co-cultures with *S. pseudintermedius* harvested from dogs with pyoderma.

Phenotypic chlorhexidine tolerance

A broth microdilution method to determine the MBC was used in this study as previously described by Guardabassi in 2010, with minor modifications. Isolates were stored on Microbank beads at −80 °C overnight before aerobic resuscitation at 37°C on Columbian blood agar (CAB, Oxoid, Basingstoke, UK) for pure growth.

For each isolate, 1–2 representative colonies were selected from CAB and suspended in 8 ml phosphate-buffered saline (PBS), to achieve turbidity of 0.5 McFarland, corresponding to approximately 10^8 colony forming units (CFU)/ml (Worthing *et al*., 2018). After vortexing, the bacterial suspension was then further diluted 1:100 with PBS to 10^6 CFU/ml. Doubling dilutions of chlorhexidine digluconate were made with PBS, ranging from 256 mg/l to 0.25 mg/l. To determine the MBC, 100µl of each chlorhexidine concentration was added to each well followed by adding 100µl of a bacterial suspension at 10^6 UFC/ml. The test was done in triplicate for each isolate.

After 30 min of incubation at 37°C, 10µl from each well was pipetted onto CAB. CAB plates were incubated aerobically at 37°C for 24 h to determine MBC 30 (minimal bactericidal concentration after 30 min). The microtitre plates were immediately returned to the incubator for 24 h at 37°C. Another 10µl from each well was pipetted onto CAB and incubated at 37°C for 24 h to determine MBC 24h (MBC 24h-1 = minimal bactericidal concentration for 24 h).

For interpretation of results, MBC 30 and MBC 24-1, were read as the first concentration at which bacterial growth was observed. The testing was performed in three steps as follows:

(i) Initially the MBC 30 and MBC 24-1 were determined.

(ii) Then, for the first seven days, we prepared fresh Mueller–Hinton broth solutions each day containing chlorhexidine digluconate in concentrations equal to ½ of each isolate's MBC 24-1. Following this, we took 10 µl from each isolate bacterial suspension of 10^6 UFC/ml and added it to the Mueller–Hinton chlorhexidine solution. For seven days, we took 10 µl from the one-day-old solution and added it to the new solution. After seven days, we took 10 µl from the last Mueller–Hinton chlorhexidine bacterial culture and passed it onto CAB and incubated them at 37°C for 24 h to test for bacterial growth. For the isolates that showed bacterial growth, we calculated the MBC at that time point and named it MBC 24h-2.

(iii) For the next seven days we again prepared fresh Mueller–Hinton broth solutions each day containing chlorhexidine digluconate in concentrations equal to ½ of each surviving isolate's MBC 24-2. Following this, we took 10 µl from a bacterial suspension of 10^6 UFC/ml for each isolate and added it to the new Mueller–Hinton chlorhexidine solution. For seven days, we took 10 µl from the one-day-old solution and added it to the new solution. After seven days, the isolates were

transferred for another seven days only in fresh Mueller–Hinton broth only. After these seven days passed we took 10 µl from the Mueller–Hinton chlorhexidine bacterial culture and passed it onto CAB and incubated them at 37°C for 24 h to test for bacterial growth. For the isolates that showed bacterial growth, we calculated the MBC at that time point and named it MBC 24h-3.

Each isolate and dilution was tested in triplicate. For each test, we used negative controls.

RESULTS

Our study found that higher concentrations of chlorhexidine are needed to kill staphylococci in a short amount of time – 30 min (16–32 µg/ml) than following a longer exposure – 24 hours (1–8 µg/ml). The minimal bactericidal concentration for 30 minutes (MBC 30 min) was between 4–32 times higher than that determined after 24 hours (MBC 24h-1) (*Table 2*).

The continuous exposure for seven consecutive days, to half the MBC 24h-1 (0.5–4 µg/ml) for each bacterial isolate, led to a lack of bacterial growth for six out of ten tested isolates (*Table 2*).

For the isolates that survived the first phase of testing, we again determined the minimal bactericidal concentration for 24 hours (MBC 24h-2). The obtained values were 2–16 times lower than those registered for the same period at the beginning of the study for three out of four isolates, all of them *S. pseudintermedius*.

Table 1 – Isolates selected for the characterisation of phenotypic resistance to chlorhexidine

Nr. crt	Species	Isolate type	Resistance genes	Resistance phenotype
1	*S. pseudintermedius*	clinical	*qac A/B*	tetracycline, fusidic acid
2	*S. pseudintermedius*	clinical	-	clindamycin, gentamicin, chloramphenicol, tetracycline, fusidic acid
3	*S. pseudintermedius*	clinical	-	fusidic acid
4	*S. pseudintermedius*	clinical	-	ampicillin
5	*S. pseudintermedius*	clinical	-	co-amoxicillin, trimethoprim-sulfamethoxazole, fusidic acid
6	*S. pseudintermedius*	clinical	*mec A*	oxacillin, co-amoxicillin, clindamycin, trimethoprim-sulfamethoxazole, gentamicin, tetracycline, enrofloxacin, fusidic acid
7	*S. pseudintermedius*	clinical	*mec A*, *fus C*	oxacillin*, clindamycin, chloramphenicol, fusidic acid
8	*S. pseudintermedius*	clinical	*mec A, smr, qac A/B*, *fus C*	oxacillin*, cefovecin, fusidic acid
9	*S. epidermidis*	clinical	*qac A/B*, *fus B*	clindamycin, tetracycline, fusidic acid
10	*S. epidermidis*	clinical	*mec A*	oxacillin, ampicillin, co-amoxiclav,

Oxacillin* = oxacillin, cephalexin, ampicillin

Table 2 – Results of the resistance test for chlorhexidine

	Nr.	STEP 1 MBC 30min	MBC 24h - 1	STEP 2 exposure 7 days	MBC 24h - 2.	STEP 3 exposure 7 days	7-day rest in MHB broth	*MBC 24h - 3.	Genotype
MSSP	1	32ug/ml	8ug/ml	4ug/ml	0.5ug/ml	0.25ug/ml		NG	*qac A/B*
	2	16ug/ml	2ug/ml	1ug/ml	NG				-
	3	16ug/ml	2ug/ml	1ug/ml	NG				-
	4	16ug/ml	2ug/ml	1ug/ml	NG				-
	5	32ug/ml	8ug/ml	4ug/ml	NG				-
MRSP	6	16ug/ml	2ug/ml	1ug/ml	1ug/ml	0.5ug/ml		NG	*mec A*
	7	32ug/ml	8ug/ml	4ug/ml	NG				*mec A*, *fus C*
	8	32ug/ml	2ug/ml	1ug/ml	0.5ug/ml	0.25ug/ml		NG	*mec A*, *smr*, *qac A/B*, *fus C*
CoNS	9	32ug/ml	1ug/ml	0.5ug/ml	4ug/ml	2ug/ml		1ug/ml	*qac A/B*, *fus B*
	10	32ug/ml	2ug/ml	1ug/ml	NG				*mec A*

MSSP – methicillin-susceptible *S. pseudintermedius*; MRSP – methicillin-resistant *S. pseudintermedius*; CoNS – coagulase-negative staphylococci; NG – no bacterial growth; MBC – minimal bactericidal concentration; *MBC 24h-3 was read after a seven-day rest in Mueller–Hinton Broth (MHB)

We registered a drop from 8 μg/ml to 0.5 μg/ml for isolate 1, from 2 μg/ml to1 μg/ml for isolate 6 and from 2 μg/ml to 0.5 μg/ml for isolate 8. For one CoNS (isolate 9), the MBC 24h-2 was four times higher than the MBC 24h-1, from 4 μg/ml to 1 μg/ml.

The only isolate that showed bacterial growth at the end of the study was the same one that had quadrupled its MBC 24h-1 (isolate 9). However, for this same isolate, the MBC 24h-3 that we determined was the same as MBC 24h-1, of 1 μg/ml.

DISCUSSION

We chose to use a 14-day exposure to concentrations of chlorhexidine digluconate calculated based on each isolate's 24 h MBC. Before this, we also determined a 30min MBC and we could already see some differences in the bactericidal concentrations for the two different time exposures. For a short time duration, we needed a higher chlorhexidine concentration to kill the selected isolates, but over 24 h, a fraction (1/32, 1/8 or 1/4) of that concentration was enough to obtain a similar effect, thus illustrating that long-term exposure is more efficient and more cost-effective than a shorter one. This could mean for example, that patients would benefit from the use of products such as sprays, mousses and lotions containing a low amount of chlorhexidine that could prolong the biocidal effect in time, for 24 h, after taking a bath with a shampoo that has a

high chlorhexidine concentration (4%) but also helps remove the organic matter that may decrease the absorption of the active substance.

Still, within these 24 hours, various factors can interfere to either decrease the local chlorhexidine concentration or to decrease the permeability for this substance (organic mixture specific for the skin) (Worthing *et al*., 2018).

The 30 min MBC values that we obtained (16–32 ug/ml) were similar or lower to those reported by other studies if we consider that Corona, in 2020, reported a 30 min MBC of 32 ug/ml for MRSP and 64 ug/ml for MSSP and Guardabassi, in 2010, reported 30 min MBC of 64 and 128 ug/ml (Corona *et al*., 2020; Guardabassi *et al*., 2010).

The MBC 24h-1 (1–8 ug/ml) was similar or lower to the concentrations reported to penetrate the upper layers of the epidermis (7.88 ug/mg) after exposing the skin to chlorhexidine for 24 h (Karpanen *et al*., 2008). This could mean that those concentrations could be effective on some bacterial isolates found within the crevasses and folds of the skin, but also that the deeper penetration of the biocide into the epidermal layers could ensure a longer residual action.

The chosen dilutions were similar to those used in other studies that calculated the MBC for this antiseptic (Corona *et al*., 2020; Guardabassi *et al*., 2010; Wothing *et al*., 2018), and we found that after a seven-day exposure to half the MBC 24h-1 very few isolates (4 out of 10) manifested bacterial growth. Of these, only one had increased its 24 h MBC up to four times (an isolate of *S. epidermidis* harbouring the *qac A/B* resistance gene), whilst the others had decreased their MBC by 2, 4 or 16 times.

After another seven-day exposure to the recalculated concentration based on the newly obtained MBC 24h-2, only the *S. epidermidis* isolate showed bacterial growth on MH agar, but its new MBC 24h-3 had returned to the initial value (MBC 24h-1) of 1 ug/ml.

These results suggest that isolates that do not harbour efflux pump resistance genes could still manifest some degree of tolerance to long-term exposure to low concentrations of chlorhexidine digluconate, and even surpass residual activity and stress (one MRSP – isolate 6). These isolates may possess other resistance or tolerance factors that we did not investigate in this study (*qac C*, *qac G*, *qac J*).

The other two isolates that carried efflux pump resistance genes *qac A/B* or *smr* (one MSSP and one MRSP) remained sensitive when we repeated the exposure, suggesting that these genes do not always offer protection for the bacteria (Frosini *et al*., 2019; Wothing *et al*., 2018).

All three *S. pseudintermedius* isolates that showed bacterial growth after the first seven days did not withstand the subsequent exposure, even though the chlorhexidine concentrations that we used were much lower. The tolerance to biocide exposure over a short period is sometimes considered to be a consequence of a low metabolic rate auto-induced by the bacteria as a reaction to the unfriendly environment (Cieplik *et al*., 2019).

The *S. epidermidis* isolate which survived through the entire test and

showed bacterial growth after the seven days of rest, could illustrate a well-balanced tolerance that permits fluctuations of the MBC depending on the stimuli that the environment provides. Additionally, considering that the *qac A/B* gene is located on mobile elements (plasmids), it could be a source of tolerance capacity to other bacteria of the same species, or even belonging to other species (LaBreck *et al.*, 2018), via plasmid transfer, when found in co-culture and under similar stress conditions.

There is enough evidence regarding some residual antimicrobial action of chlorhexidine preparations depending on the type of product, contact time and excipients. It seems that scrubs have the lowest residual activity, followed by aqueous-based solutions and ethanol-based solutions, even when the first ones have a higher concentration of the active substance (Bhooshan *et al.*, 2020; Sogawa *et al.*, 2010). Regarding contact time, some studies show that a longer contact period (24 h) ensures a higher penetrability of the substance along with higher concentrations in the upper layers of the epidermis, but also a low concentration in the deeper layers, without ever reaching the dermis (Karpanen *et al.*, 2008). It is important to consider this aspect as commensal bacteria of the skin may be present not only on the surface of the epidermis but also within the dermis, inside the hair follicles, meaning that they can recolonise the skin immediately after disinfection (Karpanen *et al.*, 2008).

These findings suggest that in order to ensure the destruction of potentially pathogenic bacteria, we would need to at least find a way to create a long-term antimicrobial effect on the surface of the skin and increase penetrability.

Products containing chlorhexidine that are meant to offer a prolonged antimicrobial action are usually used either in oral hygiene or in surgery. One study showed that daily baths with 2% chlorhexidine gluconate offer residual antimicrobial activity even three days after the procedure stops and that chlorhexidine is still detectable on the skin after 24 h from the last bath (Popovich *et al.*, 2012). Another important factor is that this residual activity is triggered by contaminants found in a liquid vehicle which will ensure the resolubilisation of chlorhexidine on the skin (Joseph Rutter and Macinga, 2013).

The usage of daily topical treatments with products containing chlorhexidine is considered very effective both in human and canine patients in reducing the bacterial population of the skin (Borio *et al.*, 2015; Popovich *et al.*, 2012).

Another application for long-term exposure to chlorhexidine is the use of special materials that are used in orthopaedics and that slowly release a limited amount of substance, over several days, thus ensuring a constant, local antimicrobial effect (Alves *et al.*, 2021).

Still, little research has been conducted regarding the tolerance of various bacterial species to this type of exposure. We already know that there are some genes coding for membrane efflux pumps (*qac* gene family) that can offer some protection against the action of chlorhexidine when expressed or activated. *Qac A* offers higher protection than *qac B*, and *qac J* offers higher

resistance than *qac G* or *smr*, but most studies have shown that the bacterial isolates harbouring these genes have only a slightly increased MIC than those that do not possess them or show no difference, which leads the authors to prefer the term biocide tolerance, rather than resistance (Frosini *et al.*, 2019; Worthing *et al.*, 2018).

Some studies followed the effects of long-term or repeated exposure to chlorhexidine for *S. aureus* (Alves *et al.*, 2021; Fritz et al., 2013), *S. epidermidis* (Alves *et al.*, 2021; Kampf, 2019), *S. pseudintermedius* (Corona *et al.*, 2020). Most of them focused on the determination of minimal inhibitory concentration (MIC) or short-term (5 min, 30 min) minimal bactericidal concentration (MBC).

However, some authors consider that the dilutions of chlorhexidine used for the calculation of MICs and MBCs are not directly correlated to those achieved on the skin, after drying, but only in bodily fluids (Horner *et al.*, 2012).

Additionally, studies have shown that broth-grown staphylococci may appear more susceptible to chlorhexidine, compared to those cultured on agar, as this biocide appears to be much more effective when in a liquid form than when in dry form, and also because it has a low diffusion rate in agarose gel (Horner *et al.*, 2012).

Still, if we consider that the residual activity of chlorhexidine is present at a much lower concentration and has a longer action, we should also consider the effect that it may have, in the long term, on commensal or pathogenic bacteria of the skin.

CONCLUSIONS

Veterinary practitioners need to limit the use of chlorhexidine strictly to situations when it is therapeutically indicated so that we can help maintain its antimicrobial effect for as long as possible, and at the same time, protect the commensal bacterial population of the skin and the natural microflora of the environment. The alternative would be to formulate strict protocols that reduce to the minimum the risk of selecting chlorhexidine-tolerant isolates, like periodic testing for resistant bacteria and the efficacy of decolonisation campaigns.

The emergence of resistance genes targeting antiseptics is an important aspect when we also consider the fact that many of these genes are located on mobile elements, such as plasmids, which may be exchanged between bacterial strains and even bacterial species when needed (Mores *et al.*, 2021).

Still, the genes that offer resistance to the action of antiseptic substances, such as chlorhexidine, may not always be expressed, being incapable of ensuring actual protection.

Despite wide and prolonged use, chlorhexidine resistance amongst staphylococci isolates remains low. Furthermore, topical therapy should overcome resistance as long as there are no barriers to drug penetration. Treatment protocols should therefore account for such barriers, e.g. hair/coat, organic matter and biofilm, but also for the different permeability that various excipients offer, to ensure continued efficacy and prevent resistance selection.

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AbbreviationsȘ MSS – methicillin-susceptible staphylococcus; MRS – methicillin-resistant staphylococcus

Ethics approval and consent to participate
The animal study was reviewed and approved by University of Liverpool Ethics Committee, the Romanian Faculty of Veterinary Science Ethics and Deontology Committee. Written informed consent was obtained from the owners for the participation of their animals in this study.

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