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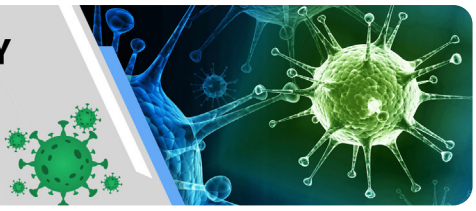

Diversity of Mupirocin-Resistance Plasmids in Nosocomial Methicillin-Resistant *Staphylococcus aureus* Clones and Their Transfer Among Clinical Strains

Marcia Giambiagi-deMarval*,[1], Ana Paula Couto Marques Cardozo[1], Viviane Coimbra-e-Souza[1], Katia Regina Netto dos Santos[1], Maria do Carmo de Freire Bastos[1], Ciro César Rossi[2]

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a prevalent cause of difficult-to-treat infections in healthcare settings, with limited treatment options. Mupirocin, an antibiotic commonly used for nasal decolonization and MRSA transmission prevention, is increasingly ineffective due to global emergence of high-level mupirocin resistance, conferred by the *mupA* gene. This study examined the diversity and transmissibility of plasmids carrying *mupA*-mediated resistance from five MRSA strains obtained from nosocomial infections in Rio de Janeiro hospitals. Plasmid origin of *mupA* was confirmed through heat stress curing, plasmid extraction, and PCR. Resistance was associated with high-molecular-weight plasmids that could transfer to methicillin-sensitive *S. aureus* and subsequently to MRSA. Diversity of the plasmids was confirmed through analysis of insertion sequence IS*257* presence, orientation, and distance from *mupA*, as well as restriction patterns. These findings highlight the need for careful monitoring of mupirocin use, given the decreasing treatment options for MRSA infections and the potential for easy transfer of mupirocin-resistant plasmids among *Staphylococcus*.

Keywords: Mupirocin; Plasmids; Antimicrobial resistance; *Staphylococcus*; Horizontal gene transfer.

Introduction

Staphylococcus aureus is a significant pathogen in healthcare settings due to its diverse virulence factors, allowing it to cause a range of diseases including skin infections, food poisoning, toxic shock syndrome, bacteremia, and endocarditis. Methicillin-resistant *S. aureus* (MRSA) is a widespread concern in hospitals, with staphylococcal infections ranging from 1-5% in Northern Europe to over 50% in South America [1]. Methicillin resistance is primarily mediated by the highly disseminated *mecA* gene, acquired through horizontal gene transfer of the mobile genetic element, staphylococcal cassette chromosome *mec* (SCC*mec*). The presence of *mecA* confers resistance to the entire beta-lactam class of antimicrobials due to its encoding of a penicillin-binding protein (PBP2a) with low affinity for beta-lactams [2].

Mupirocin is commonly used for nasal decolonization of patients and healthcare personnel in order to prevent the spread of MRSA in hospital settings [3]. The antibiotic works by binding to the isoleucyl-tRNA synthetase, which is encoded by the *ileS* gene, thereby inhibiting bacterial growth. However, resistance to mupirocin is becoming increasingly common, with a wide range of global prevalence [4]. Low-level resistance (MIC of

Affiliation:

[1]Universidade Federal do Rio de Janeiro, Instituto de Microbiologia Paulo de Góes, Rio de Janeiro, RJ, Brazil

[2]Universidade Federal de Viçosa, Departamento de Bioquímica e Biologia Molecular, Viçosa, MG, Brazil

***Corresponding author:**
Marcia Giambiagi-deMarval, Universidade Federal□ do Rio de Janeiro, Instituto de Microbiologia Paulo de Góes, Rio de Janeiro, RJ, Brazil.

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8-64 µg/mL) is typically caused by point mutations in *ileS*, while high-level resistance (MIC ≥512 µg/mL) is mediated by the *mupA* (or *ileS2*) gene. *mupA* encodes an alternative Ile-tRNA synthetase that does not bind to the drug, while the less common *mupB* gene has 65% similarity to *mupA* and 45% to *ileS* [5]. Clinical strains with MICs ≥128 µg/mL are uncommon. High-level mupirocin resistance is often encoded by plasmids larger than 25kb, although resistance above 1024 µg/mL has been linked to chromosomal *mupA* in rare cases [6, 7]. We previously demonstrated the transfer of high-level mupirocin resistance via conjugative and mobilizable plasmids from coagulase-negative staphylococci (CoNS) to *S. aureus*, potentially leading to increased resistance to drug therapy [8, 9]. Building on this, our current study focuses on the diversity and transmissibility of plasmids conferring mupirocin resistance in MRSA strains isolated from nosocomial infections in hospitals located in Rio de Janeiro.

Materials and Methods

Microorganisms and culture conditions

This study investigated five distinct clones of mupirocin-resistant MRSA strains isolated from patients in three different hospitals located in Southeastern Brazil, belonging to the bacterial collection of the Laboratory of Molecular Microbiology at the Federal University of Rio de Janeiro. All bacterial cultures were obtained by culturing frozen stocks at -80 ºC in brain heart infusion broth (BHI) at 37 ºC for 24h prior to each test. As a positive control for all tests, the mupirocin-resistant MRSA strain HU1A, containing the pMG1 plasmid, was used [10].

Confirmation of the *mupA*-related resistance

To confirm the presence of the *mupA* gene, colony PCR was performed on mupirocin-resistant strains. Cell lysis was achieved by suspending the bacterial culture in 100 µL of Tris-EDTA buffer, followed by heating at 100 °C for 15 min. The suspension was then centrifuged at 9000 g for 30 s and 10 µL of the supernatant was used as DNA template for PCR. The reaction mixture (50 µL) contained 1U of Taq DNA polymerase (Gibco, MA, USA) in the provided buffer, 0.1 mM dNTPs, and 50 pmol of primers M1 (5' GTTTATCTTCTGATGCTGAG 3') and M2 (5' CCCCAGTTACACCGATATAA 3'). PCR cycling conditions were the same as previously reported for the M1/M2 primers [11]. The presence of the 237 bp amplicon was confirmed by 1% agarose gel electrophoresis, followed by staining with ethidium bromide (0.5 µg/mL). The *S. aureus* HU1A strain [10] was included as a positive control.

Confirmation of plasmidial origin of mupirocin resistance

Plasmid curing

The plasmidial origin of mupirocin resistance was confirmed by inducing plasmid loss (curing) through temperature stress as previously described [8]. The strains were cultured in brain heart infusion (BHI) at 43 ºC with gentle agitation. Every 24 hours, an aliquot of the bacterial culture was transferred to fresh medium (1:100 dilution) for a total of 96 hours. Bacterial cells were then plated on BHI agar plates and incubated for 24 hours at 37 °C. The next day, each separate colony was transferred to two BHI replica plates: one containing 256 µg/mL mupirocin and the other with no antibiotic. After incubation, colonies growing in the absence of mupirocin but not in its presence were considered cured. Confirmation of curing was done by PCR to detect the absence of the *mupA* gene, as described above.

Antimicrobial susceptibility of the cured strains

The susceptibility of both mupirocin-cured and original strains was assessed using disc diffusion for various antibiotics (Oxoid, Ireland), including ampicillin (10 µg), cephalothin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), mupirocin (200 µg), oxacillin (1 µg), tetracycline (30 µg), and sulfamethoxazole-trimethoprim (1:19 25 µg). The experiments were conducted in triplicate according to the guidelines provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), which can be found at https://www.eucast.org/. Additionally, the minimum inhibitory concentration (MIC) of mupirocin was determined using E-Test strips (AB Biodisk, Sweden) in accordance with the manufacturer's instructions.

Analysis of plasmid transferability

Bacterial conjugations

The transmissibility of the identified plasmids was evaluated by conjugation on filter matings, following the method described by Projan and Archer [12]. Nitrocellulose membranes of 1 cm^2 were placed on BHI agar plates, onto which 100 µL of a mixture containing 10^8 CFU each of the mupirocin-resistant donor and recipient strains were dispensed. The recipient strain used was *S. aureus* RN8411, which is resistant to novobiocin, rifampicin, and tetracycline, and devoid of plasmids [12]. After 24 hours of incubation at 37 °C, the content on the nitrocellulose membranes was resuspended in 1 mL of PBS and aliquots of 100 µL were plated on selective agar plates containing mupirocin (150 µg/mL), novobiocin (10 µg/mL), and rifampicin (10 µg/mL) to promote the growth of transconjugants. Conjugation was confirmed by PCR amplification of the *mupA* gene, as described previously. To further evaluate the transmissibility of the plasmids, a second round of conjugation was performed using the newly obtained transconjugants as donors and a mupirocin-sensitive derivative strain of *S. aureus* HU1A as the recipient strain. The second group of transconjugants was selected on BHI agar plates containing tetracycline (10 µg/

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mL) and mupirocin (150 µg/mL). Conjugation frequencies were calculated as the ratio of CFU/mL of transconjugants obtained to that of the donor cells.

Plasmid extraction from donor and recipient strains

Plasmids were extracted from 5 mL of donor and transconjugant strains cultured in BHI supplemented with mupirocin (512 µg/mL), following the protocol described by Giambiagi-deMarval et al. [13]. *S. aureus* HU1A and *S. aureus* RN7242 [12] were included as positive controls and for comparison of plasmid sizes, as they harbor high-molecular weight plasmids of 35 kb (pMG1) and 52 kb (pGO1), respectively. The plasmids were visualized by 0.8% agarose gel electrophoresis, followed by staining with ethidium bromide.

Analysis of plasmid diversity

Analysis of *mupA* genetic environment and plasmid restriction pattern

To assess plasmid diversity, we employed two methods. The first method involved PCR analysis of the *mupA* flanking region and its vicinity for the presence of the insertion sequence IS*257*, using primers 1234 and 1235 targeting this mobile element, and primers M1 and MupAR targeting *mupA*, as previously suggested [14]. The resulting amplicons were visualized in a 1.0% agarose gel stained with ethidium bromide. The second method involved restriction analysis of the plasmids using Eco*RI* and Hind*III* enzymes. Plasmid DNA was extracted, separated by gel electrophoresis, purified from gel with Wizard® SV Gel and PCR Clean-Up System (Promega, WI, USA), and digested with the restriction enzymes according to the manufacturer's instructions (Gibco, ThermoFisher, USA). The resulting DNA fragments were separated by electrophoresis and subjected to Southern blotting using a digoxigenin-labeled probe amplified with primers M1 and M2 (Table 1), using the PCR DIG Probe Synthesis kit (Roche, Germany). Hybridization was done under high stringency conditions using the DIG High Prime DNA Labeling and Detection Starter kit II (Roche, Germany), according to the manufacturer's instructions. These methods were performed using the plasmid DNA from the strain HU1A as a positive control.

Results

Mupirocin-resistant strains

This study investigated five mupirocin-resistant MRSA strains (HU10A, HU10C, HU14A, UFU5, and UFU5P) obtained from distinct patients. The presence of the *mupA* gene was confirmed in all strains, and no small plasmids were detected (Fig. 1A, top left). All strains carried at least one high-molecular weight plasmid, with strains HU10A and HU10C carrying two plasmids (Fig. 1A, bottom left).

Plasmid curing

All five mupirocin-resistant MRSA strains lost their mupirocin resistance when subjected to temperature stress. The loss of *mupA* and plasmids was observed in all strains, except for HU10A and HU10C, which maintained one of their two high-molecular weight plasmids (Fig. 1A, right). The experiment involved testing 300 to 500 colonies from each strain, and the curing frequency ranged from 1.6% (strain HU14A) to 21.6% (strain HU10C). Notably, the MIC of the selected cured derivatives (MICmup) decreased significantly, with the original strains having MICmup values ranging from 512 to >1024 µg/mL, while the cured ones had MICmup values ranging from <1 to 16 µg/mL (Table 1). Except for strains H10C and UFU5P, loss of mupirocin resistance was accompanied by loss of resistance to other antimicrobials (Table 1).

Table 1: Phenotypic characteristics of mupirocin-resistant MRSA clinical strains before and after they were submitted to plasmid curing by temperature stress.

Strain	Source	Curing frequency	MIC_{mup} (µg/mL)	Antimicrobial resistance*
HU10A	Tracheal swab		>1024	amp, cph, cli, chl, ery, gen, kan, mup, tet, oxa, sft
HU10A^c		2.30%	16	cli, gen, tet, oxa
HU10C	Skin lesion		>1024	amp, cph, cip, cli, chl, ery, gen, kan, mup, tet, oxa, sft
HU10C^c		21.60%	8	amp, cph, cip, cli, chl, ery, gen, kan, tet, oxa, sft
HU14A	Lymph node biopsy		512	amp, cph, cip, cli, chl, ery, gen, kan, mup, tet, oxa, sft
HU14A^c		1.60%	<1	chl
UFU5	Urine		>1024	amp, cph, cip, cli, chl, ery, gen, kan, mup, tet, oxa, sft
UFU5^c		6.30%	<1	amp, cph, cip, cli, chl, ery, gen, kan, oxa, sft
UFU5P	Perineum surgery		>1024	amp, cph, cip, cli, chl, ery, gen, kan, mup, tet, oxa, sft
UFU5P^c		4.30%	1	amp, cph, cip, cli, chl, ery, gen, kan, tet, oxa, sft

^{c}The overwritten letter indicate each strain after they were mupirocin-cured. *Antibiotics: ampicillin (amp), cephalothin (cph), ciprofloxacin (cip), clindamycin (cli), chloramphenicol (chl), erythromycin (ery), gentamicin (gen), kanamycin (kan), mupirocin (mup), oxacillin (oxa), tetracycline (tet) and sulfamethoxazole-trimethoprim (sft).

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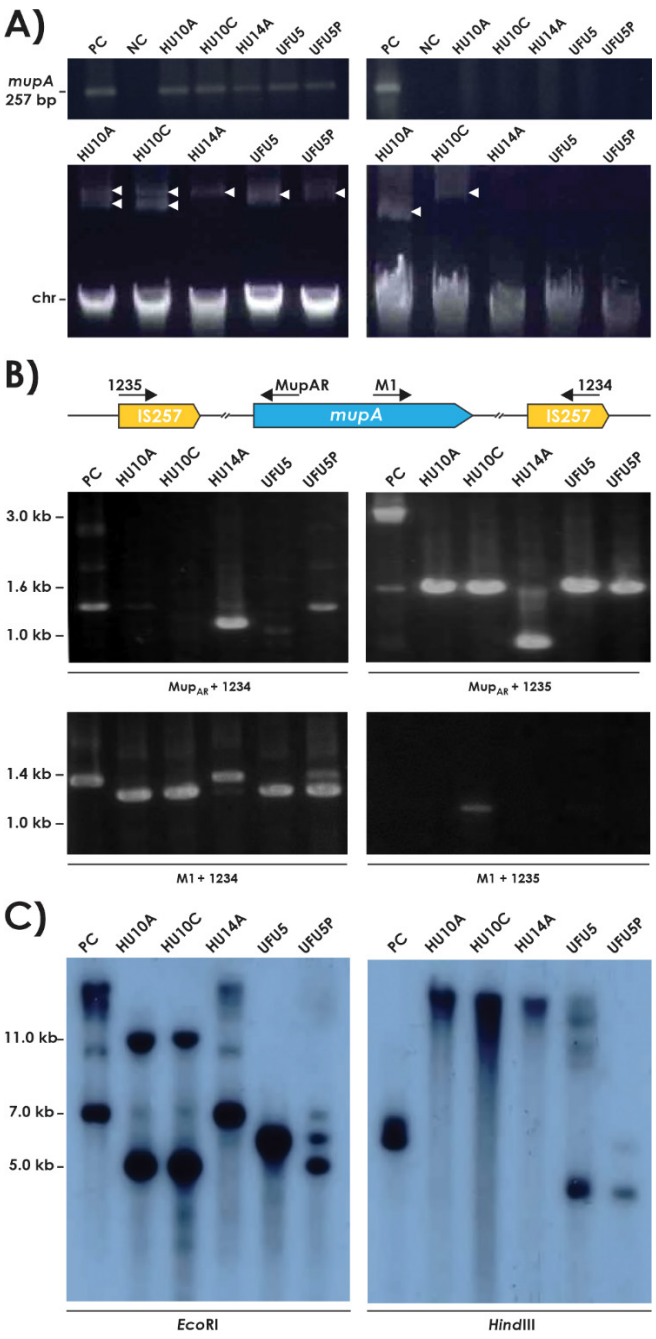

Figure 1: Characterization of mupirocin plasmids from MRSA. (A) Detection of the *mupA* gene (up) and plasmids (down) in MRSA strains before (left) and after (right) they were subjected to plasmid curing by stress temperature. PC: positive control, MRSA strain HU1A; NC: negative control (no DNA added to the reaction); chr: chromosome. (B) Amplification of the flanking regions between the *mupA* gene and the insertion sequence IS257. The upper figure shows the annealing sites of the primers used in different combinations, Mup$_{AR}$, M1, 1234 and 1235; the gels below show the PCR products for each primer combination. (C) Southern blotting of the plasmids, digested by the restriction enzymes *Eco*RI or *Hind*III and probed with a digoxigenin-labeled *mupA* amplicon. PC: positive control, MRSA strain HU1A.

Transfer of mupirocin resistance

Two plasmid transfer experiments were conducted to investigate horizontal gene transfer of the *mupA* gene. The first experiment involved transferring plasmids from the MRSA clinical strains to the recipient RN8411 strain lacking plasmids, while the second experiment involved transferring plasmids from new RN8411 transconjugants to the previously cured clinical multidrug-resistant HU1A strain. In all attempts, transconjugants were recovered, and the transfer frequencies ranged from $6.5\pm2.2\times10^{-8}$ to $4.7\pm2.3\times10^{-6}$ (Table 2). The transconjugants exhibited increased MICmup, ranging from 256 to 2048 µg/mL, compared to the original recipient strains, which had MICmup of <1 µg/mL (RN8411) or 8 µg/mL (HU10A MupS). All transconjugants received the *mupA* gene, as confirmed by PCR (data not shown).

Mupirocin plasmids diversity

To assess the diversity of plasmids, we examined the presence and orientation of IS257 flanking the mupA gene, as well as the plasmid's restriction profile. All plasmids were found to have IS257 on both sides of mupA, with varying numbers, orientations, and distances observed across different strains (Fig. 1B). For instance, strains HU10A, HU14A, and UFU5P exhibited two copies or fragments of IS257 upstream of mupA, in both direct and inverted orientations, as confirmed by the 1234 or 1235 primer combinations with

Table 2: Phenotypic characteristics of *Staphylococcus aureus* strains RN8411 and HU1A Mup^S, both sensitive to mupirocin, before and after they were received plasmids from the 5 clinical MRSA studied in this work.

Strain	Transfer frequency	MIC$_{mup}$ (µg/mL)
Recipients		
RN8411	-	<1
HU1A Mup^S	-	8
First transfer (Clinical MRSA → RN8411)		
RN8411 + plasmid from HU10A	$5.6 \pm 3.7 \times 10^{-7}$	2048
RN8411 + plasmid from HU10C	$2.8 \pm 1.0 \times 10^{-6}$	2048
RN8411 + plasmid from HU14A	$6.5 \pm 2.2 \times 10^{-8}$	256
RN8411 + plasmid from UFU5	$4.4 \pm 2.6 \times 10^{-7}$	2048
RN8411 + plasmid from UFU5P	$3.3 \pm 1.7 \times 10^{-7}$	2048
Second transfer (RN8411 → HU1A)		
HU1A Mup^S + plasmid from HU10A	$1.4 \pm 0.5 \times 10^{-7}$	2048
HU1A Mup^S + plasmid from HU10C	$4.7 \pm 2.3 \times 10^{-6}$	2048
HU1A Mup^S + plasmid from HU14A	$6.0 \pm 2.1 \times 10^{-7}$	256
HU1A Mup^S + plasmid from UFU5	$3.9 \pm 3.2 \times 10^{-7}$	2048
HU1A Mup^S + plasmid from UFU5P	$2.9 \pm 3.6 \times 10^{-6}$	2048

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the oligonucleotide MupAR, resulting in amplicons ranging from 1.0 kb to 1.6 kb. Similarly, strain HU10C produced two fragments downstream of mupA, corresponding to the two possible orientations of IS257, detected by combining the primer M1 with 1234 or 1235 (Fig. 1B). To further characterize the plasmids, we cleaved them with *Eco*RI, resulting in fragments that hybridized with the *mupA* probe, with sizes ranging from 5.0 to 11.0 kb. Notably, plasmid fragments generated from strains HU10A, HU10C, HU14A, and the positive control shared the same size (Fig. 1C). In contrast, plasmids from UFU5, UFU5P, and the positive control were cleaved with *Hind*III, producing fragments smaller than 7.0 kb.

Discussion

Mupirocin topical use is one of the preferred methods to prevent MRSA spread in the nosocomial setting. However, its usage has also contributed to the emergence of resistant strains, further compounded by the resistance that has emerged to commonly-used antibiotics for staphylococcal infections. Mupirocin resistance is globally prevalent, ranging from as little as 1.0% in some regions (e.g, Australia, India, France, and Iran) to over 50% in other areas (e.g, USA, Egypt, and other parts of India) [4]. In Brazil, studies conducted on nosocomial MRSA have shown that mupirocin resistance ranges from 2% [15] to 33% [16]. The emergence of mupirocin-resistant MRSA is a significant challenge and concern for controlling staphylococcal infections, particularly given that MRSA is a high priority for the development of new antimicrobial agents [17]. Hence, this study aimed to characterize the plasmidial location of the *mupA* gene - the major genetic determinant of mupirocin resistance in such strains - as well as its transmissibility and the diversity of mupirocin plasmids.

Previous studies have revealed that other *Staphylococcus* species, including *S. haemolyticus* and *S. epidermidis*, may harbor conjugative or mobilizable plasmids that can be transferred to *S. aureus*. These plasmids often contain resistance genes for other antimicrobials, in addition to mupirocin (as observed in this study), thereby further enhancing *S. aureus*' ability to resist drug therapy [8, 9]. The characterization of *mupA*-harboring staphylococcal plasmids has been limited to a few studies [18, 19], and even fewer plasmids have been fully sequenced, such as the 64 kb long plasmid pRM27 (Genbank ID KT780704.1), isolated from an MRSA strain carrying a type IV SCC*mec* [18]. This highlights the importance of characterizing *mupA* plasmids in understanding the risks associated with horizontal transfer of this gene among staphylococci. In this study, we found that all mupirocin-resistant MRSA strains carried at least one high-molecular weight plasmid, which is common among staphylococci and more likely to be transferred through conjugation or mobilization than small plasmids [20].

Staphylococcal plasmids are known to exhibit a high degree of mosaicism, resulting from recombination events between repetitive elements, which allow them to acquire genes from several ancestral genetic sources [21]. As a consequence, these plasmids often carry resistance genes to multiple antibiotics. In our study, we observed that the plasmids in our strains likely carry genes encoding resistance to antibiotics other than mupirocin, as their loss, induced by heat stress, was associated with the concurrent loss of resistance to other antibiotics. Furthermore, the low frequency of plasmid loss under these conditions suggests that they may persist even in stressful environments.

Our findings strongly suggest that the plasmids examined in this study were mainly transferred via conjugation, as they were able to self-transfer from plasmid-free transconjugants to a second recipient strain. However, we cannot rule out the possibility that other transfer mechanisms, such as transduction, may also have contributed to plasmid acquisition in these strains. Further studies are needed to determine the exact mechanisms involved in plasmid transfer and their potential contribution to the spread of antibiotic resistance in staphylococcal populations [22]. The presence, orientation, and distance of IS*257* from *mupA* allowed us to assess the diversity of the plasmids carrying the gene. IS*257* is known to be associated with the mobility of other antimicrobial resistance genes and is commonly used as a molecular marker to assess plasmid diversity [23]). Our results showed that all five plasmids were different, even though their restriction profile indicated that some of them may have common origins. Additionally, it is worth noting that *mupA* is usually flanked by insertion sequences, such as IS*257*, which makes it possible for the gene to integrate into the chromosome, thus stabilizing resistance. In fact, chromosomal copies of *mupA* have been detected in *Staphylococcus*, being responsible for both high [7] and low [24] levels of mupirocin resistance.

Conclusion

This study highlights the diversity of circulating high-molecular weight plasmids carrying *mupA* gene, easily transferred from and to MRSA isolated from nosocomial infections. The high degree of mosaicism found in staphylococcal plasmids, along with their potential to carry genes encoding resistance to multiple antibiotics, underscores the urgent need for careful monitoring of their use, as well as the development of new antimicrobial agents.

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Statements and Declarations

Competing interests: None to declare

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