METAL SUSCEPTIBILITY OF A HETERO-VANCOMYCIN-INTERMEDIATE METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATE

COLLEEN QUIGLEY, AND
REENA LAMICHHANE-KHADKA*
DEPARTMENT OF BIOLOGY,
SAINT MARY'S COLLEGE, NOTRE DAME, INDIANA

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CORRESPONDING AUTHOR

Reena Lamichhane-Khadka*
rlamichh@saintmarys.edu

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ABSTRACT

The Gram-positive bacterium *Staphylococcus aureus* is well known for its ability to develop resistance to various antimicrobial substances. Methicillin-resistant S. *aureus* (MRSA), a cause of nosocomial infections worldwide, is becoming increasingly resistant to the glycopeptide antibiotic vancomycin, one of few antibiotics used to treat serious multiple-antibiotic-resistant staphylococcal infections. With the increase in the number of MRSA strains showing reduced sensitivity to vancomycin, it has become important to investigate alternative treatment options. In this study, we examined the effects of five metals: silver, copper, arsenate, zinc, and cadmium on the growth of a clinical MRSA strain MM66 demonstrating heterogeneous intermediate-level resistance to vancomycin. Disc diffusion and gradient plate experiments were used to compare the metal susceptibility levels of strain MM66 to that of the methicillin-resistant S. *aureus* laboratory control strain. MM66 grew less successfully when exposed to metals, and showed an overall increased level of susceptibility to metals compared to the laboratory control strain. Of the metals tested, silver exerted the highest inhibitory effect on the growth of MM66.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial and community-acquired infections worldwide (18, 2, 29, 4). Since its first appearance in the early 1960s (15), MRSA has been the primary cause of nosocomial skin and bone infections as well as bacteremia (24, 6, 19, 13). MRSA infections account for about one-third of all S. *aureus* infections in the United States annually (13). In 2005, MRSA infections caused a higher mortality rate than HIV, killing an estimated 6.3 out of every 100,000 individuals.

Vancomycin was introduced for the treatment of S. *aureus* infections, including MRSA (24, 13). By 1997, more than 50% of

MRSA nosocomial infections responded only to vancomycin (24). However, MRSA strains showing reduced sensitivity to vancomycin have appeared and are now a prevalent problem (24, 2, 19).

Since the isolation of the first vancomycin-resistant *S. aureus* in 1995 (30), isolates with different levels of resistance have been recognized. High-level resistance to vancomycin is attributed to the *vanA* gene, which was detected in all three of the first vancomycin-resistant (VRSA) isolates (30). Intermediate resistance to vancomycin or vancomycin-intermediate *S. aureus* (VISA) is defined by a minimum inhibitory concentration (MIC) of greater than 3 mg/L of vancomycin (Delgado *et al.*, 2007). However, VISA can show an MIC up to 16 mg/L, and vancomycin-resistant *S. aureus* (VRSA) can show an MIC of 32 mg/L or even more (30). Hetero-vancomycin intermediate *S. aureus* (hVISA), a subtype of VISA, initially show lower vancomycin MIC (less than 2 mg/L) but grow successfully when exposed to higher levels of vancomycin, and are likely a precursor to VISA (14).

Antibiotic-resistant *S. aureus* also demonstrate reduced sensitivity to other antimicrobial substances such as household disinfectants (16) and various metals (3). Metals such as zinc, copper, silver, cobalt, and cadmium are well-known for their antimicrobial properties, and have been used in medicine, animal husbandry, and agriculture. Metals such as copper and zinc are common in feed supplements for agricultural animals (3). Cadmium and arsenic are common pollutants in soil and water, exposing environmental bacteria to their oligodynamic action (28, 34). Copper and silver have been used more specifically for their antimicrobial activity. Both are used in making water vessels and food containers to keep the contents disinfected (17, 27, 7). Copper sulfate and other copper salts have also commonly been used as organic biocides and fungicides as well as in the medical setting as astringents (17). Copper salts along with other metals such as mercury salts and tellurium, magnesium, and arsenic oxides have also been used to treat leprosy, tuberculosis, gonorrhea, and syphilis (17). However, many bacteria including *S. aureus* are becoming increasingly resistant to several metals. Resistance of *S. aureus* to various metals has been reported in clinical cases (23), and has been linked with the widespread use of metal-containing compounds such as feed supplements and biocides. A possible link between antibiotic resistance and cross-resistance to metals and biocides has been suggested in several bacteria including *S. aureus* (21, 16). Several studies have suggested that with the development of antibiotic resistance, *S. aureus* strains may also develop decreased sensitivity to metals that were previously toxic at small concentrations (25, 11, 3, 1). Resistance to metals in MRSA has also been associated with methicillin-resistance, being displayed in the same strains (3, 25).

In this study, we examined the susceptibility of a clinical MRSA strain MM66 demonstrating heterogeneous intermediate-level resistance to vancomycin (6) to five metals: silver; copper; arsenic; zinc; and cadmium. Two laboratory control strains of *S. aureus* – a fully antibiotic-sensitive strain ATCC 25923, and a MRSA control strain ATCC 43300 – were used to determine the relative susceptibility of MM66 to the metals tested. Based on the available data from studies involving clinical strains of methicillin-resistant *S. aureus*, we hypothesized that MM66 would demonstrate a comparatively reduced susceptibility than ATCC 44330 to the metals at concentrations previously tested on similar strains. In contrast, this study revealed that MM66 has lower levels of resistance to copper, zinc, arsenic, and cadmium compared to ATCC 43300. Growth of MM66, ATCC 43300 and ATCC 25923 was completely inhibited by silver (2.5 mM).

METHODS AND MATERIALS

STRAINS, GROWTH CONDITIONS, AND REAGENTS

The clinical hVISA strain MM66 was obtained from Prof. John Gustafson's laboratory at Oklahoma State University, Stillwater, Oklahoma. This strain was first isolated in Las Cruces, New Mexico by Delgado *et al.* (2007). Two controls were used in this study; American Type Culture Collection (ATCC) 25923, a fully sensitive laboratory strain of S. *aureus*; and ATCC 43300, a laboratory MRSA strain sensitive to vancomycin. Each strain was streak-plated onto Luria Bertani Agar plate from stock cultures and maintained at 4°C. Metal solutions were prepared from metal salts (zinc chloride, copper sulfate, cadmium acetate, silver nitrate, and sodium arsenate) at a concentration of 200 mg/L and filter-sterilized. Aliquots of 1 mL each were made at 1 mg/mL, 5 mg/mL, and 10 mg/mL to be used for disc preparation. All stock solutions were stored (at 4°C) in the refrigerator. Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA), Luria Bertani broth (LB) and Luria Bertani Agar (LBA) were prepared per instructions provided on powder containers. Overnight cultures were prepared by inoculating 5mL of LB with one isolated colony of bacteria from the stock plates and incubating at 37°C at 200 rates per minute (rpm) in the shaking incubator (New Brunswick Scientific, I2500 series, Enfield, CT). All experiments were performed in triplicates starting with triplicate overnight cultures in LB. All media used in this study were obtained from BBL Difco (Franklin Lakes, NJ), and all chemicals were obtained from Sigma (Saint Louis, MO) unless indicated otherwise. The strains used in this study are listed in Table 1.

DISC DIFFUSION ASSAY

Metal susceptibility was examined by disc diffusion similarly to the method described by Poston and Saw Hee (1991). For the first trial, a 50 microliter volume of overnight culture was used to inoculate 5 mL of sterile MHB. For the second trial, this amount was decreased to 20 microliters. Tubes were incubated at 37°C, 200 rpm for approximately three to four hours until they visually matched the McFarland turbidity standard of 0.5. Meanwhile, discs were prepared by adding calculated volumes of sterile stock solution of each metal to sterile paper discs of 4 mm diameter. Total liquid volume added to each disc was adjusted to 20 microliters with sterile distilled water to ensure a uniform distribution of the metal salt on the disc.

After incubation, if suspensions were less turbid than the standard, they were placed back in the incubator so that further growth could take place. If they were more turbid, they were diluted with sterile MHB. Once all tubes matched the McFarland standard, culture was inoculated onto the entire agar surface of MHA plates with a sterile cotton swab. Prepared discs were placed on the plates with sterile forceps and gently pressed to ensure that entire disc surface contacted the agar. The plates could sit for one minute before being inverted and incubated at 37°C in a stagnant incubator overnight. Zones of inhibition were measured after 18, 20, 24, 48, and 72 hours.

METAL GRADIENT PLATE ASSAY

Gradient Plate was performed as described by Price *et al.*, 1999. Square plates (90mm x 90mm) were obtained from Fisher Scientific (Pittsburgh, PA) and placed at a 14° angle so that bottom layer of agar would solidify at

Table 1. Strains used in this study.

Strain	*Relevant background*	*Source/reference*
MM66	Clinical hVISA; vancomycin MIC 3.0 μg/ml	Delgado *et al.*, 2007
ATCC 25923	Lab *S. aureus* strain; sensitive to antibiotics	ATCC
ATCC 43300	Lab MRSA strain; sensitive to vancomycin	ATCC

hVISA: hetero-vancomycin-intermediate S. aureus
MRSA: Methicillin-resistant S. aureus

the same angle. To make the bottom layer, 40 mL of freshly prepared MHA was poured and allowed to solidify. The next day, the top layer MHA was prepared by adding calculated volumes of 200 mg/mL stock to the MHA before again pouring 40 mL of MHA, but this time pouring with the plate flat so that the top layer formed a flat surface.

Twenty microliters of overnight culture were transferred to 5 mL of sterile MHB and incubated at 37°C, 200 rpm. After incubation, the cultures were diluted to an optical density (OD) of 0.1 at 600 nm as measured by a spectrophotometer (Thermo Scientific, Waltham, MA). When OD of 0.1 was reached, culture was inoculated onto gradient plate in a line streak from lowest concentration to highest concentration with a sterile cotton swab by streaking the same line three times to ensure equal distribution of the culture along the plate. The plates were then inverted and incubated at 37°C and confluent growth was measured after 18, 20, 24, 48, and 72 hours.

RESULTS

DISC DIFFUSION ASSAY

Exposure to 10, 30, 35, and 40 micrograms of zinc chloride, copper sulfate, and sodium arsenate did not have any effect on the growth of the *S. aureus* isolates. When exposed to 60 micrograms of zinc chloride, ATCC 25923 displayed small, faint zones with visible reduction of growth but ATCC 43300 and MM66 strains remained unaffected. None of the strains showed any zone of inhibition with 60 micrograms of copper sulfate and sodium arsenate.

When exposed to cadmium acetate, the strains showed some zones of decreased growth, herein referred to as 'zones of inhibition'; however, these zones varied in size across the strain types. After 20 hours of incubation around a 40-microgram disc, MM66 displayed the largest zone of effect (35.3 mm in diameter) whereas ATCC 25923 and ATCC 43300 displayed smaller zones of inhibition (12.3 mm and 15 mm, respectively) (Figure 1). MM66 also displayed clear zones of inhibition at lower concentrations of cadmium acetate (30 and 10 micrograms); no zone of inhibition was observed in the control strains at these concentrations.

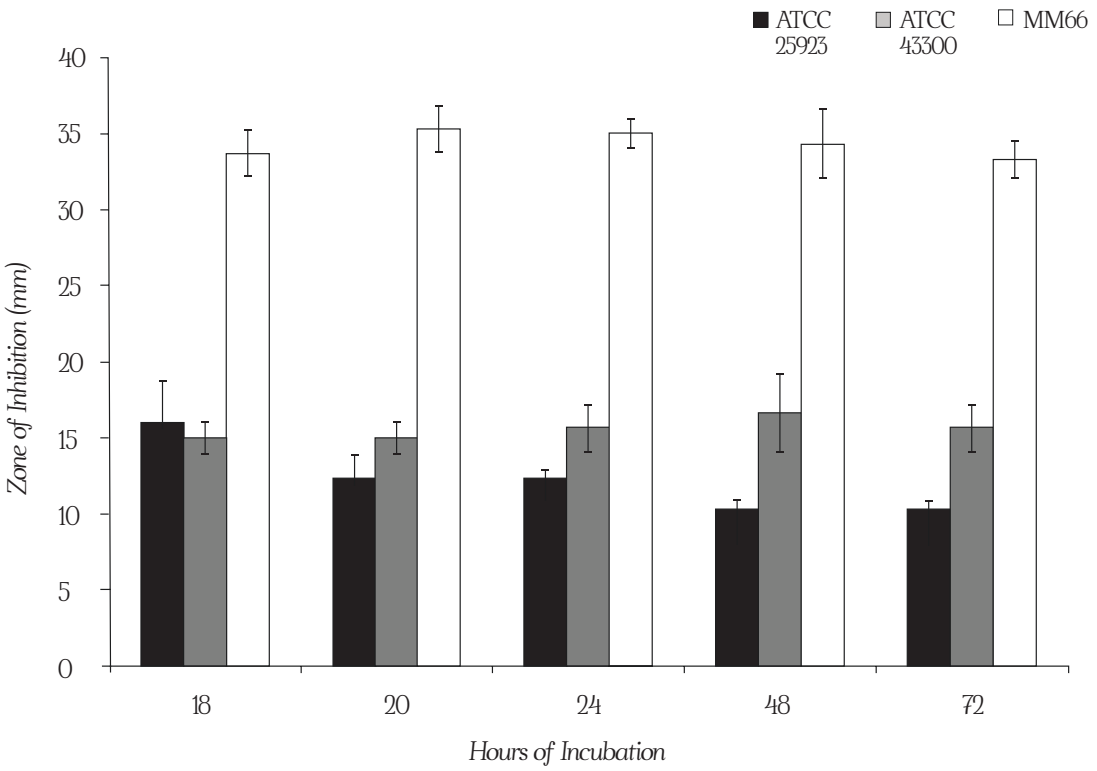

Figure 1. Comparison of the zones of inhibition of 40 µg cadmium acetate disks on S. *aureus* strains ATCC 25923 (black bars), ATCC 43300 (gray bars) and MM66 (white bars). Error bars represent standard deviation (n=3, $p<0.05$). Significantly greater zones of inhibition (connoted by *) were observed in MM66.

Clear zones of inhibition were observed in all three strains at all concentrations of silver nitrate. The zone sizes of all strains were similar (Figure 2). The results were consistent in a second disc diffusion experiment performed on triplicates of each strain.

METAL GRADIENT PLATE ASSAY

On the 5mM copper sulfate gradient plates, MM66 displayed reduced susceptibility compared to ATCC 25923 and ATCC 43300. ATCC 25923 grew up to 83.33±9.87 mm, ATCC 43300 grew with average of 80.667±2.08 mm, and MM66 grew through the entire length of the gradient (90±0 mm) (Table 2). However, when a higher concentration gradient (20 mM) was used, growth of MM66 was more reduced compared to the other strains. ATCC 25923 grew an average of 19.67±1.53 mm, ATCC 43300 grew an average of 16±1 mm, and MM66 grew an average of 6.33±5.01 mm. (Table 3).

At 2.5 mM concentration gradient of sodium arsenate, both ATCC 25923 and ATCC 43300 grew through the length of the plate, measuring an average of 90±0 mm of growth. MM66 grew an average of 39.833±4.26 mm which is significantly less than the growth of the other two

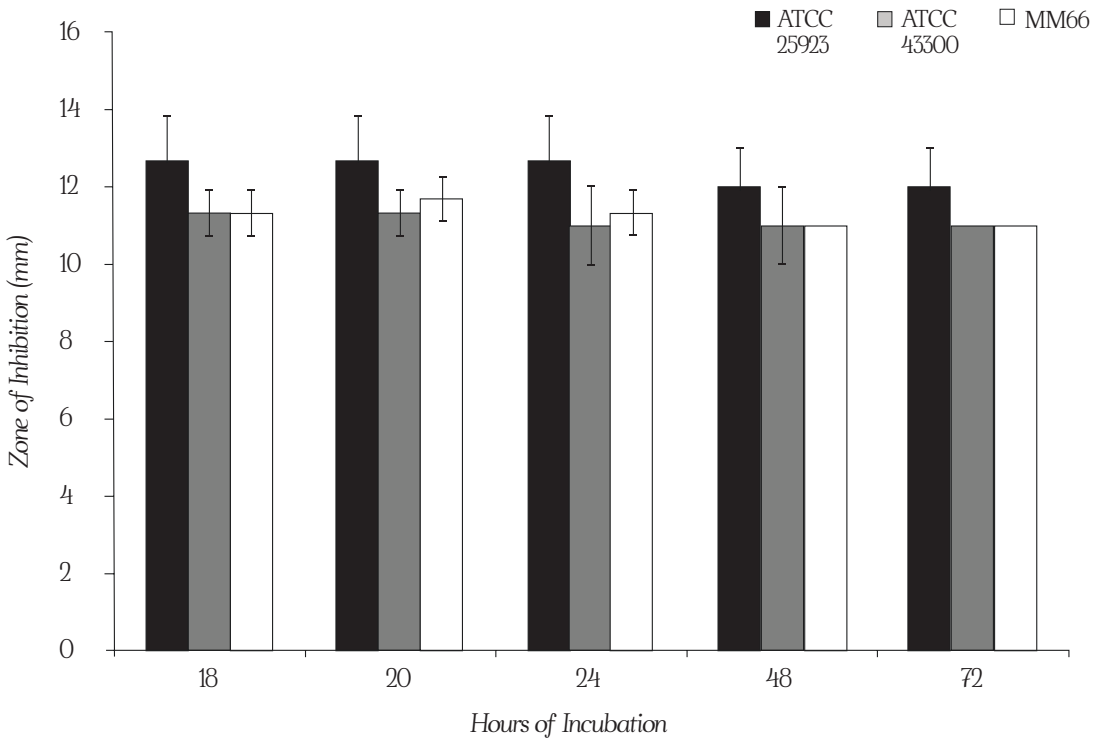

Figure 2. Comparison of the zones of inhibition of 40 µg silver nitrate disks on S. *aureus* strains ATCC 25923 (black bars), ATCC 43300 (gray bars) and MM66 (white bars). Error bars represent standard deviation (n=3, *p*=0.05). No significant differences were observed in zone size.

strains (Table 2). At 5 mM concentration gradient, ATCC 25923 still grew the full length of the gradient, measuring 90±0 mm. However, ATCC 43300 grew an average of 85.33±3.21 mm and MM66 did not grow at all. In a third experiment using 20 mM sodium arsenate gradients, MM66 did not demonstrate any growth, ATCC 25923 grew an average of 40±1 mm and ATCC 43300 grew an average of 23.667±5.68 mm. ATCC 43300 grew significantly less than ATCC 25923 (data not shown in table).

On cadmium acetate, MM66 failed to grow on a gradient of 2.5 mM; ATCC 25923 grew to an average of 30±1 mm at 2.5 mM gradient and failed to grow on 5 mM; and ATCC 43300 grew to an average of 72.667±5.51 mm on a 2.5 mM gradient of cadmium acetate and 16.667±4.51 mm on 5 mM gradient (Tables 2 and 3). The growth of both controls was significantly reduced at a higher gradient of cadmium acetate (10 mM); ATCC 25923 grew to an average of 10.667±9.29 mm and ATCC 43300 grew to an average of 16.333±1.15 mm, and growth of MM66 was completely inhibited.

None of the strains grew on 2.5 mM, 5 mM, and 10 mM gradients of silver nitrate. This result was consistent with all the triplicates. Comparative results of gradient plate assay for all strains are presented in Tables 2 and 3.

Table 2. Metal gradient plate experiment results (at lower concentrations of metals).

Strain	*Metal Gradient*				
	Zn 0→2.5 *mM*	*Cu* 0→5 *mM*	*Cd* 0→2.5 *mM*	*Ag* 0→2.5 *mM*	*Asa* 0→2.5 *mM*
ATCC 25923	32 ± 1.0	83.33±9.8	30±1.0	0	90±0
ATCC 43300	79.3±4.2	80.7±2.1	72.7±5.5	0	90±0
MM66	38.67±2.3*	90±0*	0*	0	49.3±4.5*

Numbers represent mm grown on 90 mm gradient plates and standard deviations (n = 3).
**connotes significant difference compared to MRSA control strain ($p < 0.05$).*

Table 3. Metal gradient plate experiment results (at higher concentrations of metals).

Strain	*Metal Gradient*				
	Zn 0→5 *mM*	*Cu* 0→10 *mM*	*Cd* 0→5 *mM*	*Ag* 0→5 *mM*	*Asa* 0→5 *mM*
ATCC 25923	17 ± 1.7	90±0	0	0	90±0
ATCC 43300	36.3±1.5	90±0	16.7±4.5	0	90±0
MM66	14.5±1.6*	64.2±0*	0*	0	0*

Numbers represent mm grown on 90 mm gradient plates and standard deviations (n = 3).
**connotes significant difference compared to MRSA control strain ($p < 0.05$).*

DISCUSSION

The results of the disc diffusion test suggested a lack of sensitivity in both the laboratory and clinical MRSA strains to zinc, copper, and arsenate. Compared to the laboratory MRSA strain ATCC 43300, MM66 showed increased sensitivity to cadmium acetate. Although the growth of all strains was affected by cadmium acetate, growth of MM66 was the most inhibited; the control strains had smaller zones of inhibition surrounding the cadmium acetate discs. Clear zones of inhibition were only present on MM66 plates, indicating an increased sensitivity of this strain to cadmium. Distinct zones of inhibition were observed around the silver nitrate discs at all concentrations used. This suggested that the MRSA strains are highly susceptible to silver.

Overall, similar effects of the metals on the growth of the strains were observed by both the disc diffusion and gradient plate methods. Therefore, the findings of both experiments supported one another. The gradient plate experiments provided a more comprehensive comparison of susceptibility among the strains. Unlike disc diffusion experiments in which discs with specific concentrations of the metals were used, the gradient plate assays

used continuous concentration gradients of metals. Therefore, bacterial growth starting from the lowest concentration to the highest concentration of the metal could be observed.

In the gradient plate experiments, the hVISA strain MM66 showed reduced growth in presence of zinc compared to the lab MRSA strain ATCC 43300. Zinc is required by the bacteria at a low concentration to carry out various metabolic reactions as catalysts, structural stabilizers, and as coenzymes or cofactors (1). Therefore, growth of the strains at the zero end of the concentration gradient was expected. As the concentration becomes higher, the cells cease to grow due to the toxic effects of zinc. In our experiments, ATCC 43300 could grow at higher levels of zinc compared to MM66, suggesting that the clinical hVISA strain is probably compromised in its ability to resist higher levels of zinc.

The strains showed highly reduced susceptibility to copper sulfate; all three strains grew to almost the entire length of the gradient plates at 5 mM copper sulfate concentration gradients. Strain MM66 displayed the longest distance of growth, suggesting a more reduced susceptibility of this strain to copper compared to the MRSA control strain. Interestingly, in higher concentration gradients of copper (10 mM and 20 mM), MM66 displayed shorter distances of growth compared to its control counterparts. Copper, like zinc, is necessary in trace amounts for the metabolic functions of the bacteria (1). It is possible that MM66 grows more successfully at a lower concentration of copper sulfate, but is affected more rapidly when the concentration gets higher, as seen in the 10 mM and 20 mM gradients. Further research investigating the molecular basis of copper resistance in these strains may provide more information in this aspect.

When exposed to 5 mM arsenate, MM66 grew to almost half the length of the concentration gradient. However, this growth was significantly reduced when compared to the growth of control strains, indicating that MM66 has reduced susceptibility to arsenate compared to the control MRSA strain. This pattern was consistently observed in other concentrations of arsenate (Table 3). Despite the toxicity of arsenic, resistance to high levels of arsenic has been observed in isolates of *S. aureus* (1, 34, 33). Bacteria that are resistant to arsenate compounds contain efflux systems that expel the arsenates out of the cells. Arsenates are analogs of phosphates which are essential for cellular functions, and bacteria may take the arsenates in by phosphate transport mechanisms. Once in the cells, they are converted to arsenites and pumped back out of the cell via efflux pumps (33). The growth of the strains in arsenate gradients indicates that a similar mechanism may be present in these strains. Further research may be necessary to determine the arsenite efflux capabilities of these bacterial strains.

The results of the cadmium gradient plate assay were consistent with the results of the disc diffusion test. In 10 mM gradient of cadmium acetate, the growth of MM66 was significantly reduced compared to that of the control strains. Cadmium is a highly toxic pollutant found in the environment. This may limit its therapeutic use despite its ability to significantly inhibit the growth of MM66. Cadmium kills *S. aureus* by generating oxidative stress and inhibiting thiol metabolism (28). *S. aureus* isolates that are resistant to cadmium possess the *czr*C gene and the *cad* operons that enable them to resist the negative effects of cadmium (3, 5, 8). Methicillin–resistance in *S. aureus* has been linked with cadmium–resistance and the presence of the *czr*C gene in many MRSA isolates (3). The increased susceptibility to cadmium in MM66 indicates the presence of a previously unidentified mechanism in the hVISA strain. This suggests the need of further studies to understand the genetic basis of this phenomenon in MM66.

Silver gradients, like the silver discs, completely inhibited the growth of all three strains of S. *aureus* at the tested concentrations. The oligodynamic action of silver compounds is a known phenomenon, and silver compounds are commonly used as antimicrobial agents, such as 1 % silver nitrate to prevent opthalmia neonatorum and as silver sulfadiazine to treat infected burn wounds (27, 9). Therefore, reduced growth of the S. *aureus* strains in disc diffusion and gradient plate assays was not completely unexpected. What was unexpected was that the growth of all strains, including the clinical MRSA strain, was completely inhibited even at lower concentrations. Even though no differential growth among the strains was observed and hence no data on the relative susceptibility of the MM66 strain could be obtained, it can be said that the clinical hVISA strain MM66 appears to be susceptible to even low concentrations of silver. Silver inactivates bacterial enzymes and damages DNA, and helps in the accumulation of reactive oxygen species in the cells, leading to the death of bacteria (20). Our results are in favor of the proposition that silver compounds could be considered as alternatives to traditional topical antibiotics for the treatment of infections caused by antibiotic-resistant bacteria (27). Since S. *aureus* is implicated in a variety of skin infections, using silver compounds could be a viable option for treating skin infections. Therefore, the possibility of using silver as an effective alternative to antimicrobials in treating MRSA infections should be further investigated.

In conclusion, this research provides interesting new information about the metal susceptibility characteristics of a clinical MRSA strain that was reported as a hetero-vancomycin-intermediate S. *aureus* (6). Despite the high occurrence of cross-resistance to metals in methicillin-resistant S. *aureus* isolates, MM66 does not seem to show these tendencies. Our results suggest an overall reduced metal susceptibility in the clinical hVISA strain, with silver exerting the highest inhibitory effect on growth. The findings of this research underline the need of further investigation on the use of metals as therapeutic options in treating drug-resistant S. *aureus* infections.

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