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Antibacterial Activity of Exogenous Glutathione and Its Synergism on Antibiotics in Methicillin-Associated Multidrug Resistant Clinical Isolates of *Staphylococcus aureus*

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most problematic human pathogens. Antibiotic treatment of MRSA often associated with resistance to multiple classes of antibiotics is extremely challenging and urgently demands action to treat MRSA. Glutathione (GSH) is a biogenic thiol-compound that maintains an optimal intracellular redox-potential required for various normal cellular processes. Antibacterial activity of exogenous GSH has been reported in some bacterial pathogens but is largely unknown in MRSA. **Aim:** This study aimed to understand antibacterial activity of GSH, its role in antibiotic susceptibility, and a potential antibacterial mechanism in clinical isolates of *S. aureus*. **Materials and Methods:** Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), checkerboard, time-killing, and bacterial killing assays were performed for 14 clinical isolates of *S. aureus* including 10 MRSA and two type strains (ATCC 700699 and 35556). **Results:** MIC and MBC levels for the clinical and type strains were 15 - 20 mM and 25 - 40 mM of GSH, respectively. Subinhibitory concentrations of GSH synergistically enhanced susceptibility of all tested-antibiotics, resulting in sensitizing all-tested *S. aureus*. Bacterial-killing produced by GSH-mediated acidity was significantly higher than that by hydrochloric acid-mediated acidity. **Conclusion:** Overall results concluded that GSH exhibited antibacterial activity on *S. aureus* regardless of antibiotic susceptibility and synergistically enhanced antibiotic susceptibility. Additionally, GSH-mediated acidity was one of the antibacterial mechanisms. These findings suggest that GSH may be a potential antimicrobial agent or adjuvant for the conventional anti-MRSA regimens.

Keywords

Methicillin-Resistant *Staphylococcus aureus* (MRSA), Multidrug Resistance, Glutathione, Antibacterial Activity

1. Introduction

Staphylococcus aureus is a Gram-positive coccoid bacterium and a member of human microflora colonizing in mostly skin and nasopharyngeal cavity [1]. Colonization of *S. aureus* is usually a commensal and asymptomatic relationship but, if the skin and mucosal barrier are bleached, symptomatic infections will be followed; particularly, immune compromised people are the risk of more serious infections [1] [2]. The symptomatic infections result in bacteremia or sepsis, pneumonia, endocarditis, and osteomyelitis. Other infections also include such as skin and soft tissue, eyes, urinary tract, and central nervous system [2] [3].

In the United States, MRSA was first isolated from Boston City Hospital in 1968 [4]. Since then, MRSA became an endemic pathogen in mainly intensive care units (ICU) of hospitals. The percentage of MRSA among hospitals rose from 2.4% in 1975 to 29% in 1991. The annual average percentage of MRSA from 1998 to mid-2003 increased further to 51.6% for ICU patients and 42% for non-ICU inpatient isolates [5]. In 2005, MRSA rates were 59.2%, 55%, and 47.9% for isolates from non-ICU inpatients, ICU, and outpatients, respectively [6]. However, MRSA infections have declined 17% each year of hospital-onset bloodstream infection rates and declined 7% each year of community-onset bloodstream infection rates from 2005 to 2016 (The Centers for Disease Control and Prevention). In 2017, 323,700 estimated cases in hospitalized patients, 10,600 estimated deaths (33%), and $1.7 billion estimated attributable healthcare costs [7]. Similar patterns of MRSA infections before and after 2005 have been globally observed in health care settings [2] [5].

The infections caused by methicillin-sensitive *S. aureus* can be treated by a variety of antimicrobial compounds. However, the infections caused by MRSA are difficult to treat because MRSA is insensitive to most β-lactam antibiotics including methicillin as well as to many other classes of antibiotics [8]. Methicillin resistance is mediated by *mecA*, acquired by horizontal transfer of mobile genetic element (*SCCmec*), which encodes an enzyme (PBP2a) responsible for bacterial cell wall synthesis and has a low affinity to β-lactams, resulting in resistance to nearly all this class of antibiotics. Several other mobile genetic elements carrying resistance to multiple classes of antibiotics such as trimethoprim, erythromycin, and clindamycin have been also acquired by MRSA [9]. In addition, MRSA can accumulate multiple chromosomal mutations with resistance to both intermediate-level vancomycin and daptomycin [10]. MRSA can also acquire a plasmid carrying the *vanA* operon responsible for high-level vancomycin resistance from vancomycin resistant *Enterococcus faecalis* [11]. Recently, the *cfr* gene encoding

the RNA methyltransferase was reported in MRSA, which confers resistance to linezolid [12]. Although several new antimicrobials against MRSA (e.g., ceftaroline, ceftobiprole, and dalbavancin) have been developed [2], resistance to the new antimicrobials already reported in clinical isolates [13] [14]. Considering the development of antibiotic resistance and the current antimicrobial treatment of MRSA which primarily recommends vancomycin, daptomycin, or linezolid [2] [3], the infections caused by MRSA are extremely difficult to treat. *S. aureus* is the second leading pathogen for human deaths associated with antibiotic resistance, and MRSA caused more than 100,000 deaths worldwide in 2019 [15], which urgently claims a new drug or strategy to treat MRSA.

Glutathione (GSH) is a tripeptide (L-glutamate, L-cysteine, and L-glycine) thiol-compound synthesized in most of Gram-negative bacteria and all eukaryotic cells [16]. In *E. coli*, GSH is synthesized by two sequential ATP-dependent reactions catalyzed by γ-glutamyl-cysteine synthetase and GSH synthetase. The molecular structure of GSH consists of a γ-peptide linkage between the carboxyl group of the glutamate side-chain and the amino group of cysteine which is attached by a normal peptide linkage to the glycine. A sulfhydryl (thiol) from cysteine is a major functional group for the GSH [17] [18]. GSH also possesses two free α-carboxyl groups but their biological functions are currently unclear. GSH exists predominantly (≥99%) in the thiol-reduced form and the remaining amount undergoes thiol oxidation to form GSH-disulfide or mixed-disulfides with target proteins. GSH is a more important intracellular redox buffer compared to NAD(P)H and other intracellular redox systems [19] [20]. In Gram-positive bacteria including *Bacillus subtilis* and *S. aureus*, a functional analogue of GSH, bacillithol (α-anomeric glycoside of L-cysteinyl-D-glucosamine with L-malic acid), was reported and its role in cellular processes are similar as that of GSH [21].

A major role of intracellular GSH is maintaining an optimal redox-potential for normal cellular processes which include activation/inactivation of redox-sensitive proteins, regulation of intracellular pH and potassium, deactivation of toxic substances, and adaptation to various stresses (e.g., oxidative stress, temperature stress, or osmotic stress) [16] [22]. Alterations of the intracellular redox potential impair the cellular processes and thus the level of intracellular GSH is strictly regulated by synthesis, degradation, and transport [16]. The role of extracellular (exogenous) GSH was also reported in bacteria. Schairer *et al.* and other investigators reported that exogenous GSH exhibited antibacterial activity in *E. coli*, *Klebsiella pneumonia*, *P. aeruginosa*, *S. aureus* [23] [24] [25] [26]. We also reported the antibacterial activity of exogenous GSH in *P. aeruginosa* and *Acinetobacter baumannii* [27] [28]. However, the roles of exogenous GSH in clinical isolates of MRSA are unclear. In addition, the antibacterial activity of exogenous GSH on antibiotic susceptibility and its molecular details are largely unknown. In this study, we explored the antibacterial activity of exogenous GSH on clinical isolates of MRSA, its role in antibiotic susceptibility, and a potential mechanism for the antibacterial activity.

2. Materials and Methods

2.1. Bacterial Strains, Culture Conditions, and Chemicals

Two type strains of *S. aureus* (ATCC 35556: methicillin-sensitive; ATCC 700699: methicillin-resistant) were purchased from American Type Culture Collection (Manassas, VA). Fourteen clinical isolates of *S. aureus* were obtained from Pathology Department of Jacobi Medical Center (Bronx, NY). All *S. aureus* strains were routinely cultured on Luria-Bertani (LB) agar or LB broth medium. All chemicals and antibiotics were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Antibacterial Activity Measurement of Glutathione (GSH)

Antibacterial activity and synergism of some bioactive compounds such as GSH, polyamines, and epigallocatechin-3-gallate (green tea extract) have been previously studied by several investigators as described [23] [24] [25] [29] [30]. To understand antibacterial activity and synergism of GSH minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and bacterial growth rate were performed. Fresh overnight cultures of *S. aureus* were diluted in saline to an optical density at 600 nm of 0.1 to 0.15 (approximately 1×10^9 viable cells per mL). A portion of the diluted cell suspension (~10^6 cells) was inoculated into 1 mL of divalent cation-adjusted Mueller-Hinton (MH) (Oxoid, Ogdensburg, New York) broth (pH 7.0) supplemented with GSH at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 mM. The inoculum was then incubated overnight (16 to 18 hrs) at 37˚C without shaking. MIC levels were defined as the lowest concentration of GSH that completely inhibited cellular growth. MBC levels were determined by plating the cellular growth of the inoculums used for the MIC measurement on plain LB agar plates with incubation at 37˚C for 24 hours. MBC levels were defined as the lowest concentration of GSH that killed 100% of the inoculated cells. MIC and MBC levels were confirmed by three independent experiments. Bacterial growth rate with or without GSH was determined by conventional growth curves at an optical density of 600 nm. Briefly, overnight cultured cells were diluted 100-fold into MH broth (30 mL) supplemented with GSH at the subinhibitory concentration or without any additional compound. Then, the cells were cultured at 37˚C with shaking (300 rpm) and the cellular growth was measured at time intervals.

2.3. Antibiotic Susceptibility Testing

Antibiotic susceptibility with or without GSH was determined by MIC measurement as described [29]. Briefly, each antibiotic was added to MH broth (pH 7.0) to achieve serial two-fold dilutions between 0.25 and 256 μg/mL with subinhibitory concentrations of GHS or without GSH using sterile 17 × 100 mm snapped-cap Falcon culture tubes (1 mL/tube; Fisher Scientific). Fresh overnight culture of the diluted cells (~10^6 cells) was inoculated to each MH broth supplemented with antibiotics and GSH. The inoculum was then incubated overnight at 37˚C without shaking. MIC levels were defined as the lowest concentration

that completely inhibited the growth of the inoculum. MIC levels were confirmed by three independent experiments. Antibiotic resistant breakpoints were followed by Clinical Laboratory Standards Institute (CLSI; M100, 32nd edition, 2022; https://clsi.org/standards/products/free-resources/access-our-free-resources/).

2.4. Checkerboard Assay

A checkerboard assay was performed to determine the interaction between GSH and antibiotics as described by White *et al.* [31]. Briefly, an array of combinations was made between 0 to 20 mM of GSH and 0 to 1024 μg/mL of antibiotics (chloramphenicol, methicillin, and tetracycline). Fresh overnight culture of the diluted cells (~10^6 cells) as described above was inoculated to each GSH/antibiotic combination in the array. The inoculum was incubated at 37˚C for 18 hours. Fractional inhibitory concentration (FIC) was calculated as follows: FIC of GSH = lowest MIC of GSH in combination with antibiotic/MIC of GSH alone, FIC of each antibiotic = lowest MIC of each antibiotic in combination with GSH/MIC of each antibiotic alone. FIC index = FIC of GSH + FIC of each antibiotic. Synergism was defined as a FIC index of ≤0.5; indifference defined as a FIC index of ≥0.5 by ≤4; antagonism defined as a FIC index of ≥4.

2.5. Time-Killing Assay

Time-killing assay was performed to determine synergism between GSH and antibiotics as described by White *et al.* [31]. Briefly, three conditions of MH broth (1 mL for each) supplemented with a subinhibitory concentration of GSH alone, the subinhibitory concentration of antibiotics alone, and both GSH and the antibiotics at the same subinhibitory concentrations were prepared in sterile 17 × 100 mm snapped-cap Falcon culture tubes. Fresh overnight cultures of each isolate (~10^6 cells) were inoculated in each condition of the three prepared MH broth. The inoculated cells were incubated at 37˚C without shaking, and aliquots (100 μL) were withdrawn at specific time intervals (0, 4, 8, and 12 hours) and spread the cells on plain LB agar plates with an appropriate dilution in case. Colony forming units (CFUs) on the LB agar plates were counted after 24 hours' incubation at 37˚C. Synergism was defined as a ≥2log10 decrease in CFUs per milliliter during the time-period (12 hours).

2.6. Bacterial Killing Assay

The bacterial killing pattern was determined by CFUs per milliliter at different concentrations of GSH and at different pH levels. Briefly, fresh overnight culture of cells was diluted to about ~10^8 in 1 mL of MH broth containing GSH (0, 5, 10, 15, 20, and 25 mM) and HCl to be pH levels of 7.1, 5.9, 5.2, 4.4, 4.1, and 4.0 which are equivalent pH levels of the concentrations of GSH, respectively. The cells with GSH or HCl were incubated without shaking for 18 hours at 37˚C and spread on plain LB agar plates with an appropriate dilution in case. CFUs on the LB agar plates were counted after 24 hours' incubation at 37˚C.

3. Results

3.1. Antibacterial Activity of Exogenous GSH on *S. aureus*

Antibacterial activity of GSH was evaluated by determining MIC and MBC levels for fourteen clinical isolates of *S. aureus* including 10 MRSA and two type strains of ATCC35556 (methicillin-sensitive) and ATCC700699 (methicillin-resistant). Results revealed that MICs and MBCs were 15 to 20 mM and 25 to 40 mM of GSH, respectively (Table 1). To clarify the results, bacterial growth rate with or without GSH was determined for the type strains. The growth rate of the type strains significantly decreased with subinhibitory (10 mM) and inhibitory (20 mM) concentrations of GSH when compared to that without GSH (data not shown here). These results suggest that GSH exhibits antibacterial activity on both methicillin sensitive and resistant *S. aureus*.

Table 1. Antibacterial activity of glutathione (GSH) in clinical isolates of *S. aureus*.

Strain	Methicillin susceptibility[a]	Bacterial growth[b]	
		MIC (mM)	MBC (mM)
ATCC35556	S	20	40
ATCC700699	R	20	40
JMC0034	R	20	40
JMC0340	R	20	40
JMC0939	R	15	30
JMC1298	R	15	25
JMC1418	R	15	25
JMC5362	S	15	25
JMC5500	S	15	25
JMC6920	R	20	40
JMC7520	S	15	25
JMC7955	R	15	25
JMC9523	R	15	30
NCBH1584	R	15	30
NCBH2045	R	15	30
NCBH5154	S	20	40

[a]Methicillin susceptibility was based on CLSI breakpoints (M100, 32nd edition, 2022; https://clsi.org/standards/products/free-resources/access-our-free-resources/). R: methicillin-resistance (MIC ≥ 4 μg/mL); S: methicillin-sensitive (MIC ≤ 2 μg/mL). [b]Bacterial growth was measured as MIC and MBC as described in Materials and Methods and repeated three times with identical results. MIC values denote no growth at the indicated or higher concentrations in MH broth; MBC values denote no visual colony at the indicated or higher concentrations in MH agar plate.

3.2. Effect of GSH on Antibiotic Susceptibility

To test the effect of GSH on antibiotic susceptibility subinhibitory concentrations of GSH were combined with several antibiotics for MIC measurement of the type strain *S. aureus* ATCC 700699. Results showed that, without GSH, the type strain exhibited high levels MICs (≥32 μg/mL) for all tested *β*-lactam antibiotics and MICs of 8 μg/mL (chloramphenicol), 32 μg/mL (ciprofloxacin), 256 μg/mL (gentamicin), 32 μg/mL (tetracycline), and 4 μg/mL (vancomycin). In contrast, MIC levels of all tested-antibiotics in combination with subinhibitory concentrations of GSH (1, 6, and 12 mM) were decreased in a dosage dependent manner up to 1000-fold (MIC falls from ≥256 to 0.25 μg/mL methicillin at 12 mM GSH). Cellular growth with GSH alone (12 mM) was slightly lower than that without GSH; MIC levels to methicillin, oxacillin, chloramphenicol, ciprofloxacin, gentamicin, tetracycline, and vancomycin were all less than 1.0 μg/mL at 12 mM GSH (Table 2). To corroborate the effect of GSH fourteen clinical isolates including 10 MRSA (MIC ≥4 μg/mL) were used to determine methicillin susceptibility with or without GSH. As shown in Table 3, methicillin resistant isolates were also resistant to other class(s) of antibiotic(s). MIC levels of clinical isolates both methicillin-sensitive and -resistant were all decreased to ≤1 μg/mL of methicillin in combination with subinhibitory concentrations of GSH (7.5 to 10 mM). These results suggest that the subinhibitory concentration of GSH enhances methicillin susceptibility regardless of antibiotic susceptibility in *S. aureus*.

Table 2. Antibiotic susceptibility of *S. aureus* ATCC 700699.

Antibiotics	MICs (μg/mL)[a] with GSH indicated concentrations			
	none	1 mM	6 mM	12 mM
Aztreonam	≥256	≥256	128	8
Carbenicillin	≥256	256	32	4
Ceftazidime	≥256	256	128	4
Meropenem	32	32	16	2
Methicillin	≥256	≥256	256	0.25
Oxacillin	≥256	256	64	0.5
Chloramphenicol	8	4	1	≤0.25
Ciprofloxacin	32	32	16	0.5
Gentamicin	256	256	64	1
Tetracycline	32	32	4	≤0.25
Vancomycin	4	2	1	≤0.25
None	+++[b]	+++	+++	++

[a]MIC measurement was repeated three times with identical results. [b]+++: growth levels as an optical density of ≥1.5 at OD_{600}; ++: growth levels as an optical density of ≥1.0 at OD_{600}.

Table 3. Effect of glutathione (GSH) on methicillin susceptibility and antibiotic susceptibility of clinical isolates of *S. aureus*.

Clinical isolate	MIC (μg/mL)[a]					
	Meth	Meth plus GSH (mM)[b]	Cip	Ery	Tet	Van
ATCC35556	2	≤0.25 (10)	≤0.25	0.5	≤0.25	0.5
JMC0034	16	≤0.25 (10)	16	≥32	≤0.25	1
JMC0340	32	≤0.25 (10)	16	32	≤0.25	4
JMC0939	16	≤0.25 (7.5)	≥32	≥32	≤0.25	1
JMC1298	32	1 (7.5)	≥32	≥32	1	1
JMC1418	32	0.5 (7.5)	16	32	1	1
JMC5362	2	≤0.25 (7.5)	≤0.25	32	≤0.25	1
JMC5500	2	≤0.25 (10)	≤0.25	≥32	≤0.25	1
JMC6920	≥32	1 (10)	0.5	≥32	16	1
JMC7520	1	≤0.25 (7.5)	≤0.25	≤0.25	2	≤0.25
JMC7955	32	0.5 (7.5)	≥32	16	0.5	1
JMC9523	4	≤0.25 (7.5)	16	≤0.25	≤0.25	1
NCBH1584	16	≤0.25 (7.5)	32	16	4	1
NCBH2045	16	0.5 (10)	16	16	0.5	4
NCBH5154	1	≤0.25 (10)	2	0.5	32	1

[a]MIC measurement was repeated three times with identical results. [b]Subinhibitory concentrations of GSH (7.5 to 10 mM). Note: Cip: ciprofloxacin; Ery: erythromycin; Meth: methicillin; Tet: tetracycline; Van: vancomycin.

3.3. Synergistic Interaction of GSH on Antibiotics

The type strain, ATCC 700699, was used for interaction between GSH and antibiotics by determining FIC indices. As shown in **Table 4**, FIC indices for GSH in combination with chloramphenicol, methicillin, and tetracycline were 0.126, 0.066, and 0.258, respectively, which were all less than 0.5 ranged in synergism as described [31]. These results suggest that GSH synergistically enhances the susceptibility of antibiotics. The synergism of GSH on the antibiotics was confirmed by time-killing assays for the same type strain with the same antibiotics. Results showed that the type strain grew with the subinhibitory concentration of either GSH or antibiotics similar as the same strain without any additional compound within 12 hours. In contrast, the same subinhibitory concentrations of both GSH and each of the antibiotics killed ≥99% of initial inoculums within 12 hours (**Figure 1**). The killed-cell numbers were ≥2log10 within the time-period, which also ranged in synergism between GSH and the antibiotics as described [31].

3.4. pH Effect of GSH on Antibacterial Activity

GSH is composed of three amino acids with two free α-carboxyl groups from

Table 4. Fractional inhibitory concentration (FIC) indices in *S. aureus* ATCC 700699.

Compound	MIC (μg/mL)		FIC[a]	FICI[b]
	Alone	Combination		
Chloramphenicol	8	0.5	0.063	0.126
GSH (mM)	20	1.25	0.063	
Methicillin	256	1	0.004	0.066
GSH (mM)	20	1.25	0.062	
Tetracycline	32	0.25	0.008	0.258
GSH (mM)	20	5	0.25	

[a]FIC (fractional inhibitory concentration) was defined as the ratio of the MIC of a compound used in combination to the MIC of the compound tested alone. The values represent the majority of three independent tests. [b]FICI (FIC index) was calculated from summing the FIC of each compound as described in Materials and Methods.

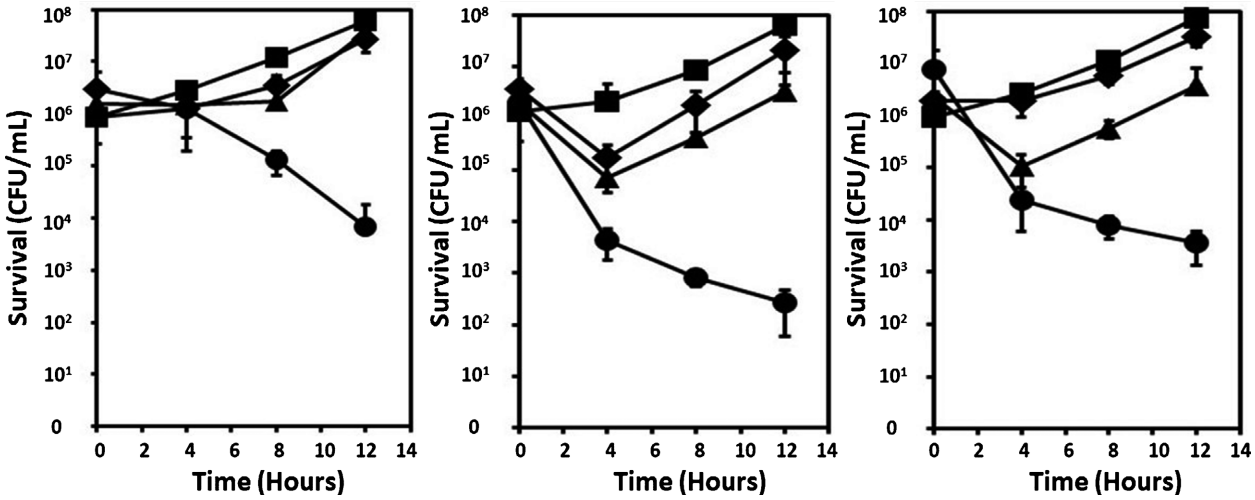

Figure 1. Killing patterns of methicillin-resistant *S. aureus* ATCC 700699 in the presence of Glutathione (GSH) and/or antibiotics. The bacterial killing assay performed as described in Materials and Methods. (a) is bacterial killing patterns in the presence of methicillin (2 μg/mL; diamond), GSH (10 mM; triangle), and both methicillin (2 μg/mL) and GSH (10 mM) (circle). (b) is bacterial killing patterns in the presence of tetracycline (2 μg/mL; diamond), GSH (10 mM; triangle), and both tetracycline (2 μg/mL) and GSH (10 mM) (circle). (c) is bacterial killing patterns in the presence of chloramphenicol (2 μg/mL; diamond), GSH (10 mM; triangle), and both chloramphenicol (2 μg/mL) and GSH (10 mM) (circle). The square markers in each bacterial killing pattern were growth patterns without any additional compound during the growth period. The standard deviation was calculated by three independent experiments.

glutamate and glycine [16]. The free *α*-carboxyl groups of exogenous GSH may decrease pH levels of the culture medium (MHB), which is possibly associated with the antibacterial activity of GSH. To test this possibility pH levels of MHB supplemented with GSH at different concentrations were measured. MHB containing GSH at concentrations of 0, 5, 10, 15, 20, 25, and 30 mM exhibited pH levels of 7.1, 5.9, 5.2, 4.4, 4.1, 4.0, and 4.0, respectively. These results indicate that exogenous GSH acidifies the culture medium in a dosage dependent manner. To understand the pH effect of GSH on antibacterial activity, bacterial killing assays

of the type strain ATCC 700699 were performed by MHB supplemented with GSH at 0, 5, 10, 15, 20, and 25 mM; in parallel, bacterial killing assays of the same strain were performed by the same medium adjusted to the equivalent pH levels with HCl (pH; 7.1, 5.9, 5.2, 4.4, 4.1, and 4.0). As shown in Figure 2, the killing effect of GSH was significantly increased at ≥5 mM (equivalent to ≥pH 5.9) and killed ≥ 99% of all inoculum at 25 mM (equivalent to pH 4.0). The killing effect of pH (by HCl) was also increased at ≥ pH 5.2; however, a significant amount of cells (~10^4 cells per mL) survived at pH 4.0 (equivalent to 25 mM GSH) (Figure 2). These results indicate that the killing effect of GSH is higher than that of HCl-mediated acidity.

4. Discussion

Antibiotic resistance is a global healthcare concern. In 2019, a comprehensive assessment comprising 204 countries and territories revealed that 4.95 million deaths were associated with antibiotic resistant infections and 1.27 million deaths were directly related to antibiotic resistant bacterial pathogens. In this report, MRSA was one of the most problematic pathogens and caused the highest number of deaths (≥100,000 deaths) [15]. CDC also reported that estimated cases of MRSA in hospitalized patients were 323,700 and estimated deaths were 10,600 in 2017, which was 1.7 billion estimated attributable health care costs [7]. These observations are clear enough that antibiotic resistance is an urgent healthcare problem and claim a new antibiotic or strategy to treat MRSA infections. In this study, we explored GSH as a potential compound to treat MRSA.

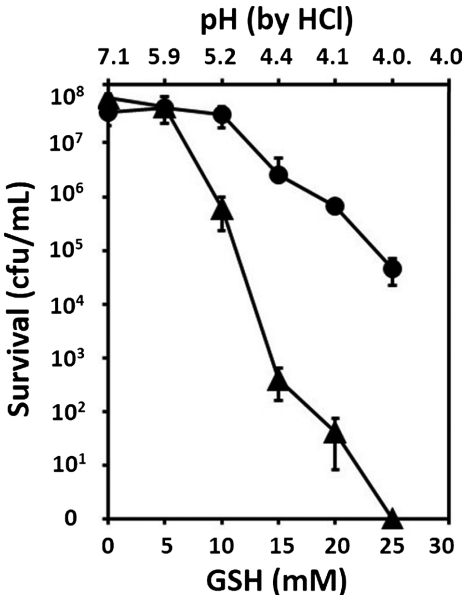

Figure 2. Killing effect of exogenous glutathione (GSH) and HCl on *S. aureus* ATCC 700699. Bacterial killing effect determined as described in Materials and Methods. Circles are killing patterns for HCl-mediated acidity and triangles are killing patterns for exogenous GSH which acidifies equivalent levels of the pH (by HCl). The standard deviation was calculated by three independent experiments.

Exogenous GSH was used to understand antibacterial activity on a few *S. aureus* [23] [25]. However, its roles in clinical MRSA isolates are unclear. In this study, we confirmed the antibacterial activity of GSH on 14 clinical isolates including 10 MRSA and one MRSA ATCC strain (ATCC 700699). Three experimental approaches (MIC, MBC, and bacterial growth rate) consistently supported for the antibacterial activity of GSH, which was unrelated to the susceptibility of antibiotics including methicillin and other classes of antibiotics. These observations suggest that exogenous GSH exhibits antibacterial activity in clinical isolates of *S. aureus* regardless of antibiotic resistance or susceptibility.

The effect of GSH on antibiotic susceptibility is also currently unclear. This study clarified the effect of GSH on antibiotic susceptibility. We used the type strain ATCC 700699 (MRSA) which is also resistant to many other classes of antibiotics. We found that the subinhibitory concentration of GSH (12 mM) in combination with several conventional antibiotics including methicillin and vancomycin sensitized the type strain with MICs of ≤1.0 μg/mL. We further clarified the same effect of GSH on methicillin susceptibility for 14 clinical isolates including 10 MRSA and methicillin sensitive type strain ATCC 35556. Checkerboard and time-killing assays demonstrate that GSH and the antibiotics are synergistically interacted to enhance antibiotic susceptibility. These results suggest that the subinhibitory concentration of GSH in combination with conventional antibiotics can be used to treat MRSA infections.

Although the antibacterial activity of exogenous GSH has also been reported in other bacteria such as *Pseudomonas aureus* [24] and *Acinetobacter baumannii* [23] [27] [28], molecular details of the antibacterial activity are currently unclear (or unknown). Theoretically, extracellular GSH, a small thiol-compound, can be introduced into the cytoplasm and disrupt optimal intracellular redox potential with increasing lethality of bacterial cells. The optimal intracellular redox-potential in bacteria requires for a variety of normal cellular processes as described [16] [22]. In addition, two free α-carboxyl groups of exogenous GSH can protonate with increasing acidity in the extracellular environment (e.g., culture media), which may also be associated with antibacterial activity. Indeed, Das *et al.* reported that GSH-mediated acidity disrupted biofilm formation and thereby improved antibiotic efficacy on biofilm-formed bacterial species [32].

In this study, we examined the acidity of exogenous GSH on the culture medium (MHB). Then, bacterial killing effect of the GSH-mediated acidity was compared to that of the acidity of hydrochloric acid (HCl) in the same culture medium. We found GSH increased acidity of the culture medium by decreasing pH levels from 7.1 to 4.0 in a dosage-dependent manner from 0 to 25 mM of GSH, respectively. We also observed that higher acidity from GSH or HCl killed more bacterial cells. However, the killing effect of the GSH-mediated acidity was significantly higher than that of the HCl-mediated acidity. The killing effect of the HCl-mediated acidity may be caused by only hydrogen ions. Therefore, the higher killing effect of the GSH-mediated acidity may have an additional un-

identified killing mechanism(s) except for the acidity. The killing mechanism(s) of GSH, either the acidity or the unidentified killing mechanism(s), is apparently associated with the synergistic effect on antibiotic susceptibility as shown in this study. We are currently elucidating the additional killing mechanism(s) of GSH.

Overall, this study concluded that: 1) exogenous GSH exhibited antibacterial activity on clinical isolates of *S. aureus* regardless of antibiotic susceptibility, 2) the antibacterial activity of GSH synergistically enhanced susceptibility of conventional antibiotics, and 3) GSH-mediated acidity was substantially associated with the antibacterial activity of GSH. These findings may provide an idea to treat MRSA.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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