



Medium-Induced Turbidity of Zinc Sulfate Heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) in Cation-Adjusted Mueller–Hinton Broth: Implications for Broth Microdilution Susceptibility Testing



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ABSTRACT

Introduction: In antimicrobial susceptibility testing, complete solubility of the tested compound is critical. This study investigated the impact of zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) on visible turbidity in cation-adjusted Mueller–Hinton broth (CAMHB) during broth microdilution assays, with and without bacteria.

Methods: Solutions of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ were prepared in sterile deionized water and mixed with CAMHB at varying concentrations (4 to 2048 $\mu\text{g/mL}$). Visually detectable turbidity was assessed immediately and after 24 hours at room temperature and 37°C. Broth microdilution was conducted as a proof-of-concept assessment using *Staphylococcus aureus* ATCC 29213 and five clinical isolates, with agar dilution used for methodological comparison.

Results: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ remained completely clear in deionized water at any concentration. In contrast, visible turbidity appeared in CAMHB at concentrations around 1000–1024 $\mu\text{g/mL}$, interfering with MIC determination. Although agar dilution mitigated turbidity issues, the findings suggest that medium-associated physicochemical changes at high concentrations of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ may potentially affect the freely dissolved or biologically available fraction.

Conclusion: In this proof-of-concept study, medium-induced turbidity of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was identified as an important methodological consideration when performing susceptibility testing at high nominal concentrations, as the nominal concentration of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ may not accurately represent its biologically available concentration in culture media. Further physicochemical characterization is required before high-concentration MIC data for zinc sulfate are interpreted with confidence. The findings should be interpreted as evidence of a potential methodological limitation in $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ susceptibility testing that should be considered in zinc susceptibility testing.

Keywords: Zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$); Antimicrobial susceptibility testing; Minimum inhibitory concentration; Broth microdilution; Cation-adjusted Mueller–Hinton broth; Turbidity

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Introduction

Broth microdilution remains the reference antimicrobial susceptibility testing (AST) for determination of minimum inhibitory concentrations (MICs) of antimicrobial agents (1).

The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommended standardized experimental conditions, including the use of cation-adjusted Mueller–Hinton broth (CAMHB), standardized inoculum density, defined incubation conditions, and unambiguous interpretation of bacterial

growth endpoints for accurate MIC determination (1-3). An essential assumption of dilution-based susceptibility testing is that the antimicrobial agent remains sufficiently dissolved throughout the assay (2). In broth microdilution, MIC determination is based on visual assessment of turbidity, where a clear well indicates inhibition of bacterial growth and a turbid well indicates bacterial growth (2). Because broth microdilution endpoints are determined by visual assessment of bacterial growth, any physicochemical property of the tested compound that alters the optical clarity of the test medium may interfere with endpoint interpretation and compromise the

accuracy of MIC determination. However, interactions between antimicrobial agents and culture media for conventional non-antibiotic agents such as metal salts, metal complexes, and natural products remain considerably less investigated.

Metal ions, particularly zinc, have emerged as promising antimicrobial agents because they target multiple bacterial processes, including membrane integrity, protein function, oxidative homeostasis, and biofilm formation. Zinc salts have demonstrated antibacterial and antibiofilm activity against a broad spectrum of Gram-positive and Gram-negative pathogens, including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp., and *Pseudomonas aeruginosa* (4-6). Unlike conventional antibiotics, the antimicrobial activity of metal ions is strongly influenced by their chemical speciation and environmental conditions. Consequently, interactions between zinc ions and constituents of microbiological media may influence antimicrobial susceptibility testing results (4, 7, 8). These interactions remain insufficiently characterized despite the growing use of zinc-containing compounds in antimicrobial research.

The influence of zinc on antimicrobial susceptibility testing is not novel. Previous investigations have demonstrated that zinc concentrations naturally present in various commercial CAMHB companies vary considerably and can significantly influence MIC values for metallo- β -lactamase-producing Gram-negative bacteria by altering the zinc availability required for enzyme activity. These observations highlight that zinc-related physicochemical properties within susceptibility testing media can substantially affect AST outcomes (7-11). Therefore, previous studies have mostly focused on ambient concentration of zinc in CAMHB rather than on the physicochemical behavior of zinc sulfate added to the medium at antimicrobial concentrations.

Another methodological challenge in antimicrobial susceptibility testing is the evaluation of compounds with poor aqueous solubility. Several experimental antimicrobial agents, particularly hydrophobic natural products such as curcumin and flavonoids, exhibit precipitation or limited solubility in microbiological media, complicating visual MIC endpoint determination. To overcome these

limitations, several studies have employed colorimetric viability indicators, including resazurin, or spectrophotometric measurements to facilitate endpoint interpretation (12-14). Whether the nominal concentration of a poorly soluble antimicrobial compound accurately represents the biologically available concentration throughout susceptibility testing remains largely unexplored. Therefore, physicochemical compatibility between the test compound and the susceptibility medium represents an important but often overlooked aspect of AST methodology.

During primary susceptibility testing of zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), a heptahydrate form of zinc sulfate, we observed that solutions remained visually clear in sterile deionized water over a wide concentration range but developed visible turbidity immediately after mixing with CAMHB at high concentrations, even in the complete absence of bacterial inoculation. This observation raised two methodological concerns. First, intrinsic medium-associated turbidity may obscure visual discrimination between bacterial growth and compound-induced opacity during broth microdilution. Second, visible turbidity may interfere with visual discrimination between bacterial growth and compound-associated cloudiness during MIC determination. Whether the observed cloudiness also reflects changes in the physicochemical state or dissolved fraction of zinc requires separate investigation. Although the latter hypothesis requires dedicated physicochemical investigation, it has important implications for interpreting susceptibility data generated using high concentrations of zinc sulfate.

Therefore, the present study aimed to characterize the concentration-dependent turbidity of zinc sulfate in CAMHB under standardized susceptibility testing conditions and to evaluate its potential impact on MIC determination. By distinguishing compound-induced turbidity from bacterial growth, this proof-of-concept study highlights a methodological consideration that may affect the interpretation of susceptibility testing of zinc-containing antimicrobial agents.

Materials and Methods

Bacterial isolates

A total of six *Staphylococcus aureus* isolates

(one reference strain ATCC 29213 and five clinical isolates) were included to provide a proof-of-concept assessment of the methodological issue identified during $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ susceptibility testing. The isolate set was not intended to estimate the distribution of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ susceptibility among *S. aureus*. Clinical isolates were obtained from the bacterial collection of the Department of Medical Bacteriology at Tarbiat Modares University. Before antimicrobial susceptibility testing, all isolates were identified as *S. aureus* using conventional microbiological methods, including Gram staining, catalase, coagulase, DNase, oxidase, and mannitol fermentation tests (15), and were further confirmed by polymerase chain reaction (PCR) targeting the species-specific *nuc* gene (16) (data not shown).

Zinc sulfate heptahydrate preparation

A Zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) (Merck Co., Germany; Purity: 99.8%) stock solution was freshly prepared in sterile (autoclaved before use) double-distilled deionized water (Zolal Teb Shimi Co., Iran) at a concentration of 10 mg/mL (10,000 $\mu\text{g/mL}$) and 10.24 mg/mL (10,240 $\mu\text{g/mL}$) immediately before each experiment.

According to the manufacturer's specifications of double-distilled deionized water, the water had a total dissolved solids (TDS) value of 0.0 ppm (as CaCO_3), electrical conductivity (EC) ≤ 1 $\mu\text{S/cm}$, pH 6.5–7.5, chloride (Cl^-) = 0.0 ppm, and Fe^{2+} = 0.0 ppm.

The sterility of the deionized water was verified inoculating 500 μL of the water into 500 μL of sterile CAMHB (Condalab Co., Spain) (final volume = 1 mL), followed by incubation at 37°C for 24 h. No visible microbial growth was observed.

All experiments were performed at least in three independent biological replicates.

Evaluation of zinc sulfate visible turbidity in CAMHB

To investigate whether $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ itself produced visible turbidity independent of bacterial growth, working solutions were prepared at final concentrations of 4, 8, 16, 32, 64, 128, 256, 512, 1024, and 2048 $\mu\text{g/mL}$. All concentrations reported in this study refer to the mass concentration of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, rather than to the concentration of elemental Zn.

For each concentration, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was first diluted in sterile deionized water and subsequently mixed with an equal volume (500 μL) of CAMHB to achieve a final volume of 1 mL.

Control tubes containing sterile deionized water alone and CAMHB alone were included.

The appearance of each tube was evaluated visually immediately after preparation (0 min) and after 15 min, 30 min, 1 h, 2 h, 16 h, 18 h, and 24 h. To evaluate the possible effect of temperature, duplicate sets were incubated at room temperature and at 37°C.

All experiments were performed at least in three independent biological replicates.

pH measurement

To evaluate whether changes in pH could be associated with the observed medium-induced cloudiness, the pH of CAMHB, the $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ stock solution (10,240 $\mu\text{g/mL}$), and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ /CAMHB mixtures containing 512–1,200 $\mu\text{g/mL}$ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ were measured using a calibrated pH meter (S20 SevenEasy™, Mettler Toledo, Switzerland). Measurements were performed in duplicate under the same experimental conditions used for the turbidity assessment. The results are reported as mean $\pm$ SD.

Broth microdilution susceptibility testing

MIC testing was performed according to CLSI recommendations using CAMHB in 96-well plate, with a final volume of 200 μL per well.

Two-fold serial dilutions of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ranging from 2048 to 4 $\mu\text{g/mL}$ were prepared and added to the wells, with 100 μL of each concentration added to each well.

A bacterial suspension equivalent to 0.5 McFarland (approximately 1.5×10^8 CFU/mL) was prepared and diluted to 1×10^6 CFU/mL. Then, 100 μL of the bacterial suspension was added to 100 μL of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ solution in each well, resulting in a final volume of 200 μL and a final inoculum of 5×10^5 CFU/mL. One well was assigned as the growth control and contained only CAMHB and the bacterial inoculum, without $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. Uninoculated wells containing the corresponding $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ concentrations were examined in parallel to distinguish compound-associated cloudiness from bacterial growth. Plates were incubated for 16–18 h at $35 \pm 2^\circ\text{C}$. Following incubation, bacterial growth was

assessed visually by comparing each inoculated well with the growth control and uninoculated wells. The MIC was defined as the lowest $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ concentration showing no visible bacterial growth. All experiments were performed at least in three independent biological replicates.

Fine concentration assessment

Because broth microdilution suggested that the MIC of *S. aureus* ATCC 29213 was located between 512 and 1024 $\mu\text{g/mL}$, additional concentrations of 600, 700, 800, 900, and 1000 $\mu\text{g/mL}$ were evaluated.

Parallel tubes without bacteria were prepared to determine the concentration at which medium-associated turbidity first appeared. All experiments were performed at least in three independent biological replicates.

Agar dilution method

Agar dilution was performed according to CLSI recommendations using Mueller-Hinton agar (Condalab Co., Spain) supplemented with $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ at concentrations ranging from 2048 to 32 $\mu\text{g/mL}$.

Each isolate was spot-inoculated (final inoculum is $\sim 5 \times 10^4$ CFU/spot) onto agar plates containing the appropriate $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ concentration. Plates were incubated for 16–18 h at $35 \pm 2^\circ\text{C}$ before growth was recorded. All experiments were performed at least in three independent biological replicates.

Results

Zinc sulfate remained clear in deionized water but became turbid in CAMHB

Sterility verification of the deionized water showed no detectable microbial growth.

Zinc sulfate was completely soluble in sterile

deionized water across all tested concentrations.

In contrast, after mixing with CAMHB, visible turbidity developed reproducibly at concentrations of approximately 1000–1024 $\mu\text{g/mL}$ and above, even in the complete absence of bacterial inoculation (Figure 1).

No visible turbidity was observed at concentrations of 512 $\mu\text{g/mL}$ or lower.

Visible turbidity appeared instantly and stayed consistent during 24 hours of monitoring.

Incubating at 37°C resulted in slightly higher visible turbidity than at room temperature for concentrations of ≥ 1024 $\mu\text{g/mL}$, while no clear temperature-related differences were noted at lower concentrations.

Medium-associated turbidity complicated broth microdilution endpoint interpretation

Broth microdilution demonstrated visible turbidity in wells containing $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ concentrations of ≥ 1024 $\mu\text{g/mL}$ irrespective of bacterial inoculation.

Consequently, it was not feasible to visually distinguish between bacterial growth and visible turbidity caused by the compound within this concentration range.

This limitation affected interpretation of MIC endpoints for isolates requiring high $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ concentrations.

pH measurements

The CAMHB control had a pH of 7.35 ± 0.07 , whereas the $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ stock solution (10,240 $\mu\text{g/mL}$) prepared in water had a pH of 5.70 ± 0.06 and remained completely clear. The pH of the $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ /CAMHB mixtures was generally lower than that of the CAMHB control and varied within the range of 6.49–7.17 across the tested concentrations. Although an overall decrease in

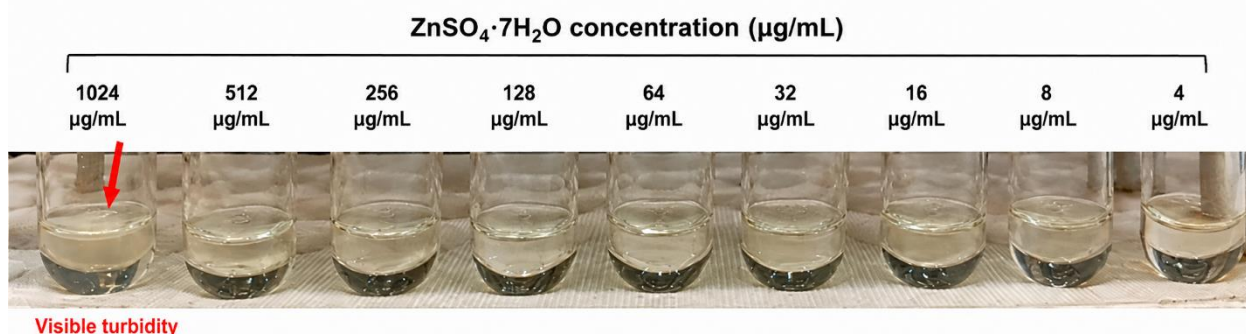


Figure 1. Visual assessment of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ -associated cloudiness in cation-adjusted Mueller–Hinton broth (CAMHB). Representative appearance of uninoculated CAMHB containing increasing concentrations of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ solutions after 24 h of incubation at 37°C . Visible cloudiness was first observed at approximately 1024 $\mu\text{g/mL}$ of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in uninoculated CAMHB, whereas lower concentrations remained visually clear under the conditions shown.

Table 1. pH of CAMHB containing different concentrations of ZnSO₄·7H₂O

ZnSO ₄ ·7H ₂ O concentration (µg/mL)	pH, mean ± SD
CAMHB (control without ZnSO ₄ ·7H ₂ O)	7.35 ± 0.07
ZnSO ₄ ·7H ₂ O stock (10,240) (control without CAMHB)	5.70 ± 0.06
512	7.17 ± 0.03
600	7.09 ± 0.03
700	6.96 ± 0.01
800	6.80 ± 0.08
900	6.81 ± 0.03
1,000	6.60 ± 0.07
1,024	6.49 ± 0.02
1,200	6.49 ± 0.02

Abbreviations: CAMHB; cation-adjusted Mueller-Hinton broth

pH was observed with increasing ZnSO₄·7H₂O concentration, the relationship was not strictly monotonic; for example, the mean pH increased slightly from 6.795 at 900 µg/mL to 6.81 at 1,000 µg/mL before decreasing to 6.60 and 6.49 at 1,024 and 1,200 µg/mL, respectively (Table 1).

Importantly, the ZnSO₄·7H₂O stock solution had a lower pH (5.70 ± 0.06) than all of the ZnSO₄·7H₂O/CAMHB mixtures tested, yet remained completely clear. Thus, the observed turbidity in CAMHB cannot be explained simply by the acidic pH of the ZnSO₄·7H₂O solution. The findings are more consistent with a role for the CAMHB matrix and its ionic constituents in the observed turbidity, although the present measurements do not establish a causal relationship between pH and turbidity formation.

Fine concentration testing identified the onset of medium-induced turbidity

Intermediate concentrations of ZnSO₄·7H₂O ranging from 512 to 1024 µg/mL were examined to refine the definition of the transition zone. Tubes without bacteria remained visually clear up to 900 µg/mL.

Visible turbidity first appeared at 1000 µg/mL and persisted at increased concentrations.

For *S. aureus* ATCC 29213, bacterial growth was observed up to 600 µg/mL, whereas inhibition occurred at ≥ 700 µg/mL. This value was obtained for the reference strain under the specific experimental conditions of this study and was not intended to represent a general MIC value for *S. aureus*. Accordingly, the broth MIC for the reference strain was determined as 700 µg/mL under the conditions of the fine-

concentration assessment. Importantly, the uninoculated control remained visually clear at 700 µg/mL, whereas visible cloudiness was first observed at approximately 1000 µg/mL. Thus, the 700 µg/mL MIC endpoint was below the concentration at which medium-induced cloudiness became detectable.

Reliable MIC determination was not possible for any of the five clinical isolates using broth microdilution. Visible bacterial growth persisted at zinc sulfate concentrations below 1000 µg/mL, whereas intrinsic turbidity attributable to the interaction between zinc sulfate and CAMHB was consistently observed at concentrations of 1000 µg/mL and above in uninoculated controls. This overlap precluded reliable visual interpretation of the MIC endpoint. Consequently, the broth microdilution assay could only indicate that the MIC exceeded the highest concentration that remained free of medium-induced turbidity, while the precise MIC value could not be determined under these experimental conditions.

Agar dilution results

For *S. aureus* ATCC 29213, agar dilution showed growth at 512 µg/mL and no growth at 1024 µg/mL, indicating an MIC within the interval of >512 to ≤1024 µg/mL. Since intermediate concentrations were not tested in the agar dilution assay, the broth microdilution MIC of 700 µg/mL is consistent with this interval and reflects the higher concentration resolution of that experiment rather than a true discrepancy between methods.

All five clinical isolates continued to grow at the highest tested concentration (2048 µg/mL), indicating MIC values ≥ 2048 µg/mL.

Unlike broth microdilution, agar dilution eliminated uncertainty arising from visual broth turbidity.

Nevertheless, because ZnSO₄·7H₂O produced concentration-dependent visible cloudiness after incorporation into nutrient media, the agar dilution results should be interpreted separately from the visual interference observed in broth microdilution. The present study did not determine whether the physicochemical state or dissolved fraction of ZnSO₄·7H₂O is comparable between agar and broth.

Discussion

In this proof-of-concept study, ZnSO₄·7H₂O

produced reproducible visible cloudiness in uninoculated CAMHB at high nominal concentrations (approximately 1000–1024 µg/mL and above), whereas the corresponding zinc sulfate solutions remained visually clear in sterile deionized water. The principal methodological finding is that this compound-associated cloudiness occurred in the absence of bacterial growth and therefore interfered with visual interpretation of broth microdilution endpoints. Because MIC determination by broth microdilution relies on visual discrimination between bacterial growth and a clear inhibition endpoint, visible cloudiness generated by the test compound may make a turbid well difficult to distinguish from a growth-positive well. Thus, the primary implication of the present study is a limitation in visual MIC endpoint interpretation at high $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ concentrations.

Broth microdilution is considered the reference method for MIC determination by CLSI and EUCAST because of its high degree of standardization and reproducibility. Although these documents provide detailed recommendations regarding inoculum preparation, incubation conditions, and quality control, they do not specifically address situations in which the tested compound itself produces visible cloudiness that interferes with visual endpoint interpretation (1-3). Our findings therefore identify a methodological circumstance that is not explicitly considered in current susceptibility testing recommendations but may become increasingly relevant as metal-based antimicrobials, nanoparticles, antimicrobial peptides, and natural products are incorporated into AST workflows.

The observed medium dependence provides a plausible basis for considering the role of the CAMHB matrix. CAMHB is a chemically complex susceptibility-testing medium containing peptides, amino acids, phosphate, and divalent cations. Such components can influence the behavior and availability of metal ions under experimental conditions (1, 4, 7). Previous investigations have shown that the concentration of zinc naturally present in different commercial CAMHB formulations can substantially influence susceptibility testing of metallo-β-lactamase-producing organisms by altering zinc availability within the medium. However, these studies evaluated background zinc concentrations

intrinsically present in the broth rather than the physicochemical behavior of exogenously added zinc salts at antimicrobial concentrations (7, 8, 10). In the present study, we extend these observations by demonstrating that $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ itself may produce visible turbidity after incorporation into CAMHB, which can interfere with susceptibility endpoint interpretation.

An important distinction in interpreting the present findings is between the demonstrated visual interference and possible physicochemical explanations for its origin. The present experiments establish that $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ can produce visible cloudiness in CAMHB in the absence of bacterial growth, which is sufficient to interfere with visual MIC endpoint interpretation. If the observed cloudiness reflects precipitation, aggregation, or other physicochemical changes, the freely dissolved or biologically available fraction of zinc could potentially differ from the nominal concentration. However, the experiments were not designed to determine whether this cloudiness resulted from precipitation, colloidal aggregation, complex formation, or another physicochemical process and this possibility was not directly evaluated in the present study. These mechanisms should therefore be regarded as alternative hypotheses rather than explanations demonstrated by the present study (4). This possibility raises an important methodological consideration, as MIC determinations are generally based on prepared test concentrations and do not routinely account for changes in the freely dissolved fraction of compounds during incubation (17). However, confirmation of this hypothesis requires dedicated physicochemical analyses and was beyond the scope of the present investigation.

This concept may also be relevant to antimicrobial susceptibility testing of other experimental compounds with limited aqueous solubility. Hydrophobic phytochemicals, including curcumin and related flavonoids, are known to present formulation and solubility challenges that may complicate antimicrobial susceptibility testing. To facilitate endpoint interpretation, several studies have employed metabolic indicators such as resazurin or spectrophotometric measurements. Although these approaches may improve discrimination between bacterial growth and optical interference, they do not directly assess the dissolved fraction

or physicochemical state of the antimicrobial compound during incubation. Consequently, improved endpoint visualization should not necessarily be interpreted as evidence that the nominal test concentration remained fully biologically available throughout the susceptibility assay (4, 13, 14, 17, 18).

For this reason, the methodological implications of the present findings differ between broth- and agar-based susceptibility testing. Agar dilution avoids the specific visual ambiguity encountered in broth microdilution because bacterial growth is assessed by colony formation rather than by changes in broth clarity (1, 17). Thus, agar dilution can circumvent the visual endpoint interference demonstrated in the present study. However, the present study did not determine whether the physicochemical state or dissolved fraction of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ is equivalent in agar and broth. Therefore, the agar dilution results should not be interpreted as establishing equivalence in zinc availability between the two methods. Direct comparison of dissolved and particulate zinc under the respective testing conditions would be required to address this question.

The present study has several limitations. First, the chemical and physical basis of the medium-induced cloudiness was not characterized; therefore, precipitation, colloidal aggregation, complex formation, and other possible mechanisms cannot be distinguished from the present data. Second, dissolved and particulate zinc concentrations were not quantified after mixing $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ with CAMHB. Third, zinc speciation, particle size, and solid-phase composition were not assessed. Only a single zinc salt formulation was evaluated. Finally, the microbiological assessment included only one reference strain and five clinical isolates and was designed as a proof-of-concept rather than a representative susceptibility survey. Therefore, the MIC observations cannot be generalized to the broader *S. aureus* population. Future studies incorporating larger and systematically selected isolate collections, combining susceptibility testing with direct physicochemical characterization will be required to determine the mechanism of cloudiness and whether any change in the dissolved zinc fraction accompanies the observed phenomenon.

Collectively, the present findings indicate that

compound-associated visible cloudiness should be considered when interpreting visual broth microdilution endpoints for $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ at high nominal concentrations. Collectively, the present findings indicate that compound-associated visible cloudiness should be considered when interpreting visual broth microdilution endpoints for $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ at high nominal concentrations. The demonstrated issue is one of visual endpoint interpretation; the physicochemical mechanism responsible for the cloudiness and its possible relationship to dissolved zinc remain unresolved. Accordingly, assessment of compound-medium compatibility before MIC testing may help identify concentrations at which visual interpretation becomes unreliable, while dedicated physicochemical studies are needed to determine whether the observed cloudiness is associated with changes in zinc speciation or availability.

Conclusion

Zinc sulfate heptahydrate remained completely soluble in sterile deionized water but developed reproducible visible turbidity after incorporation into CAMHB at concentrations of approximately $\geq 1000\text{--}1024 \mu\text{g/mL}$, independent of bacterial growth. This medium-induced turbidity directly interfered with visual interpretation of broth microdilution MIC endpoints and represents an important methodological limitation when testing $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ at high concentrations.

Beyond endpoint interpretation, our findings suggest that physicochemical interactions occurring after incorporation of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ into microbiological media may reflect physicochemical changes that could alter the freely dissolved fraction of zinc; however, this possibility was not directly evaluated in the present study and requires confirmation by appropriate physicochemical measurements. These findings suggest that physicochemical interactions occurring after incorporation of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ into microbiological media may alter the freely dissolved fraction of zinc available to bacterial cells. However, this hypothesis was not measured in the present study.

Future studies integrating microbiological susceptibility assays with physicochemical characterization of dissolved and particulate zinc species will be essential to determine how medium-induced physicochemical changes

influence antimicrobial activity measurements. Until such evidence becomes available, MIC values obtained at concentrations associated with medium-induced turbidity should be interpreted with appropriate caution.

Given the limited number of isolates examined, the present findings should not be interpreted as evidence of the general zinc susceptibility of *S. aureus*; rather, they provide a methodological proof-of-concept demonstrating the need to assess compound–medium compatibility before interpreting high-concentration zinc susceptibility results.

Author's contribution

N.B: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Supervision, Project administration, Resources, writing – original draft, Writing – review & editing.

P.D: Investigation, Data curation, Validation, Formal analysis.

A.M-M: Resources (providing laboratory bench for some experiments).

All authors have read and approved the final manuscript.

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Data Availability

Data are available on request from the corresponding author.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Tarbiat Modares University (Approval No. IR.MODARES.REC.1404.129). The study involved the secondary use of previously archived bacterial isolates, and no new biological samples were collected from participants. The use of the archived isolates for the present study was reviewed and approved by the Ethics Committee.

Consent for publication

Not applicable.

Conflict of Interest

The authors declare no conflict of interest.

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Artificial intelligence (AI)-assisted technology

During the preparation of this manuscript, the authors used ChatGPT (OpenAI, GPT-5.5) for assistance with language refinement, improving clarity and readability, and providing suggestions for scientific organization of the manuscript. The tool was not used to generate research data, perform data analysis, interpret experimental results, or create scientific findings. All experimental procedures, data generation, data interpretation, and final decisions regarding manuscript content were performed and verified by the authors. The authors take full responsibility for the accuracy, originality, and integrity of the manuscript.

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