Review article

The Anti-bacterial Effect of Colloidal silver on *Streptococcus pyogenes* and *Staphylococcus aureus in vitro*

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Abstract

The treatment of bacterial infections has been successfully achieved when antibiotics were first introduced. A short while after the introduction of antibiotics, bacterial strains became resistant to antibiotic treatment, and bacterial infections were potentially fatal once again. This challenge has led to various pursuits of antibiotic alternative treatment. The antibacterial effect of colloidal silver (18 and 20 ppm) was investigated on *Staphylococcus aureus* and *Streptococcus pyogenes in vitro* using the microdilution method. Agar disc diffusion method was used as trial run. Zones of inhibition were measured and recorded in the agar disc diffusion method to indicate bacterial inhibition. The Iodonitrotetrazolium chloride dye was added to the 96 well microplate in the microdilution method and colour changes were visually observed. An unchanged clear colour indicated bacterial inhibition. Noticeable colour changes (from a clear colour to purple) indicated bacterial growth. The results from both experimental methods showed that both 18 and 20 ppm colloidal silver had an inhibitory effect against *Streptococcus pyogenes,* but not against *Staphylococcus aureus.* This warrants further extensive research on the effect of colloidal silver as an antimicrobial.

Introduction

Streptococcus pyogenes (*S. pyogenes)* and *Staphylococcus aureus (S. aureus)* are both Gram-positive bacteria which are responsible for various communicable infections, most of which can be fatal if left untreated. The infections caused by *S. pyogenes* include pharyngitis, streptococcal tonsillitis, impetigo, cellulitis and pyoderma (Ahmad *et al.,* 2014). Streptococcal infections have been linked with increased rates of mortality in poorly developed countries, while it has declined greatly in well-developed countries (Carapetis *et al.,* 2005). Towards the end of the 20th century, there has been a decline in the incidence and severity of streptococcal infections due to the use of antibiotics; however, in the past 15years, the incidence of streptococcal infections peaked again in a more severe manner due to the increase of antibiotic resistant bacterial strains and challenges

eradicating the strains thereof (Luca-Harari *et al,* 2009). Infection by *S. aureus* is prevalent in individuals with compromised immune systems, causing scalded skin syndrome, abscesses, nosocomial pneumonia, meningitis, osteomyelitis and toxic shock syndrome (Stevens *et al.,* 2002). According to Schaumburg *et al.* (2014) about 3.28 cases per 1 000 hospital admissions in South Africa are due to *S. aureus* infection annually. In Mozambique, 178 cases per 100 000 hospital admissions are due to infection by *S. aureus* compared to the United States of America, which has about 2.3 cases per 100 000 hospital admissions yearly.

The introduction of antibiotics has successfully and effectively combated many bacterial infections; however more and more bacterial strains are becoming resistant to antibiotic drug treatment (WHO, 2015). Drug resistant bacterial strains are influenced by various factors, including but not limited to genetic mutation, bacterial strains acquiring genes conferring drug resistance (Martínez, 2012), easily accessible non-prescription antibiotics, poor compliance to antibiotics, and inadequate access to proper treatment due to the unavailability of antibiotics (WHO, 2015; Tangcharoensathien *et al*, 2018) .

Due to the many challenges with antibiotic use, most people are seeking complementary and alternative treatment for bacterial infections (Wolsko *et al.,* 2002). Colloidal silver is widely known as an alternative antimicrobial. It is known to have no interactions with other forms of treatments, therefore can be taken safely with other drugs (Iroha *et al.,* 2007). Although considered safe, the prolonged use of colloidal silver results in a permanent blue appearance, known as argyria (Owen, 2013). Many claims have been made and research studies have been conducted on colloidal silver as a potent antimicrobial, no research is known to have been done specifically on colloidal silver 18 and 20 ppm against *S. aureus* and *S. pyogenes*.

The microdilution minimum inhibitory concentration (MIC) method was used to investigate the anti-bacterial effect of colloidal silver on *S. pyogenes* and *S. aureus*. The MIC is a standardized, accurate, easy and inexpensive experiment to carry out. MIC is the concentration at which visible bacterial growth is prevented under certain growth conditions (Elshikh, *et al.,* 2016).

Materials and method

Ethical considerations

The research study was carried out after permission was granted by The Faculty Academic Ethics Committee. The experiments were done at the University of Johannesburg's Water and Health Research Centre, Doornfontein Campus, under supervision of a qualified laboratory technician and with permission granted.

Laboratory protection and safety

Upon entering the laboratory, hygiene measures, like washing hands, cleaning working area surfaces and sterilizing instruments with ethanol, were taken.

A laboratory coat, hand gloves as well body covering clothing and shoes were worn during laboratory experiments for protection of self from accidental spills.

Instrumentation and material used were handled away from self and laboratory technician for protection. Inflammable substances were used with caution.

All laboratory instruments used during experiments were washed at the end of each experiment. Disposables were discarded in relevant bins provided by the laboratory technician after use.

Materials

Reference strains of *Streptococcus pyogenes* (ATCC 12384) and *Staphylococcus aureus* (ATCC Baa-1026) were purchased in a lyophilised form. The strains were stored at 4-5°C and reconstituted in sterile saline water as required. Table 1 below summarizes the media, buffers, chemicals and consumables used in the study.

Cefepime hydrochloride monohydrate was used as positive control against both the *S. aureus* and *S. pyogenes* strains during the experiments. Two different concentrations of colloidal silver (18 ppm and 20 ppm) were used as test compound. The 18 ppm colloidal silver (Silver lab) contains ionic colloidal silver in deionized purified water. The 20 ppm colloidal silver (BIO-SIL) contains ISO 3696 distilled water and fine silver. The treatment was stored below 25°C away from direct sunlight.

Table 1 Summary of media, reagents and chemicals used for the experimental work.

Description	Catalogue number	Supplier
Unsensitized tubes	69285	Biomerieux
Saline solution	V1204	Biomerieux
Mueller Hinton agar	CM0337B	Laboratory Specialities (PTY) LTD T/A Thermo Fisher Scientific (Oxoid)
Petri dish 90mm	1C097	Clinical Science Diagnostics
96-well microplate with lid	734-2097	Monitoring & Control Laboratories (Nunc)
Swabs PS cotton in PP tube sterile	300261	Merck Chemicals (Deltalab)
P-Iodonitrotetrazolium violet	I8377	Sigma Aldrich
Neomycin sulfate hydrate	J61499.14	Monitoring & Control Laboratories
Cefepime hydrochloride monohydrate	J66237.03	Monitoring & Control Laboratories
Cartridges blank	CT0998B	Laboratory Specialities (PTY) LTD T/A Thermo Fisher Scientific (Oxoid)
Inoculating loops, 10ul	612-9354	Monitoring & Control Laboratories (VWR)
Agarose	BIO-41025	Celtic Molecular Diagnostics (Bioline)
Tris-HCl	T3253	Sigma Aldrich
Acetic acid glacial	A0011FC02500	Associated Chemical Enterprises BK

Methods

Aseptic techniques

Aseptic techniques were adhered too, to ensure that no contamination takes place, thus ensuring reliability of results.

Growth and maintenance of bacteria

The stock culture arrived freeze dried in a vial/container and needed to be hydrated before use. The unopened vial was taken out of the storage and allowed to equilibrate to room temperature. The vial was opened and sterile forceps used to remove one pellet of bacteria without removing the desiccant. Immediately, the vial was recapped and returned to the storage of 4-5°C. The pellet of bacteria was inoculated into a sterile flask containing

100 ml saline solution. A sterile swab was used to crush the pellet and it was heavily saturated in the hydrated suspension.

The saturated swab was used to inoculate the Mueller-Hinton agar culture plate, with the swab streaked over one-third of the plate. Inoculations were placed in an incubator for 24 hours at 35°C.

The streak plate method was used to subculture *S. pyogenes* and *S. aureus* onto Mueller-Hinton agar plates. Fresh cultures were prepared for each experiment.

A sterile cotton swab was used to transfer bacteria onto agar plates for subculturing. Bacteria were spread onto plates in a zigzag pattern using sterile plastic inoculating loops. A new loop was used between each step, and the used swabs and loops were discarded for safe disposal. The agar plates were then incubated for 24 hours at 35°C and used to prepare the bacterial suspension required for the experiments.

Preparation of media

Mueller-Hinton broth was used as media in the study. It consists of beef infusion from casein acid hydrolysate and starch. To prepare the media, 21 g of the powder was suspended in 1 000 ml distilled water. This suspension was heated to boil to dissolve the medium. For sterilization, the solution was autoclaved at 121°C for 15 minutes. The media was cooled to about 50°C and poured into sterile plastic petri dishes. After solidification of the agar, the plates were stored at 4°C.

Preparation of the Iodonitrotetrazolium chloride solution

A 0.4 mg/ml working solution of the Iodonitrotetrazolium chloride (INT) was prepared using sterile water and stored at 4°C until needed. The solution was used in the sections below.

Disc diffusion method

This method was used to as trial run. Bacterial strains were prepared and subcultured grown as described above, and used to create the bacterial lawns on the Mueller-Hinton agar plates. The bacterial lawns were created by transferring colonies from the agar plate with subcultured bacteria into the test tube containing saline. The bacterial suspension was adjusted with saline to reach a solution correlating to a 0.5 McFarland standard. A

sterile cotton swab was immersed in this suspension and the total area of the agar plate covered with the bacterial suspension.

Sterile discs were placed onto the media with sterile forceps according to pre-designed grid to allow the placement of multiple discs on each plate. Following this, 20 µl of each test solution, blank control (sterile water added), solution control (saline solution) and positive control (Cefepime) was added onto the discs.

These plates were covered and incubated for 24 hours at 35°C. Following incubation, the plates were taken out and placed on a sterile working surface. To assist in the visualization of the bacterial-inhibition, an agarose overlay containing INT (final electron acceptor) was added onto the plates. The INT overlay was prepared by mixing it with 1% (w/v) agarose solution (prepared in Tris-Acetic-EDTA buffer, pH 8.3), to a final INT concentration 0.2 mg/ml. The plates were incubated at 37°C until a bright pink or dark pink colour developed, indicating bacterial growth, i.e. no bacterial inhibition.

Microdilution method

The microdilution method was used as an antibiotic susceptibility test to determine the sensitivity of the bacteria (*S. aureus* and *S. pyogenes*) against colloidal silver. The bacterial suspension used for this method, corresponding to a 0.5 McFarland standard, was prepared as described above. The 96 well plates were divided into two halves to accommodate the two bacteria used.

The first half (1-6) was used for *S. aureus* and the second half (7-12) was for *S. pyogenes.*

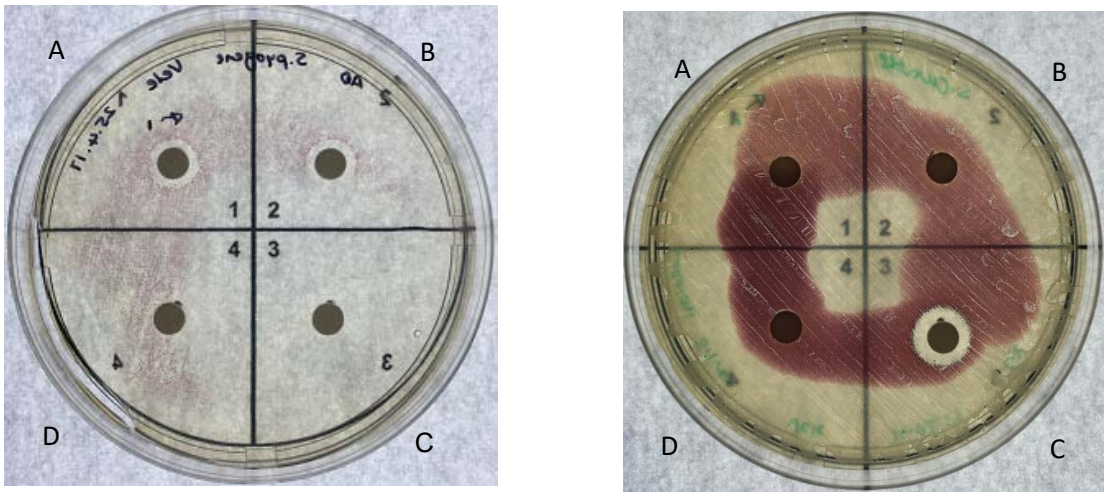
All wells were filled with 100 µl Mueller Hinton broth. Fifty microliters of either test compound, cefepime (positive control), media (media control to test sterility), water (negative or growth control) were added to the first well in each row. The compounds were serially diluted in the next well by transferring 50 µl from the first well, mixing properly, and by transferring 50 µl into the next well. This was continued to the last well (6^{th} well) and the last 50 µl removed was safely discarded. Lastly, 50 µl of the required bacterial suspension was added to each of the wells, excluding the media control wells that received 50 µl saline solution. The plates were then closed and incubated for 24 hours at 35°C.

Following incubation, 100 µl of the INT solution was added to each well and incubated for 30 minutes at 35°C. The development of a bright pink of dark pink colour indicated bacterial growth, i.e. no bacterial inhibition.

Results

Agar disc diffusion method

The agar disc diffusion method, also known as the Kirby Bauer disc diffusion method, was used to test whether or not the two compounds could inhibit bacterial growth for both *S. aureus* and *S. pyogenes*. The results were visually inspected, and zones of inhibition were measured. Examples of typical results obtained for both bacteria is shown in Figures 4.1.



(i) (ii)

Figure 4.1 Agar plates showing typical results obtained for the colloidal silver solutions against *S. pyogenes* (i) and *S. aureus* (ii), as well as bacterial lawns using the agar disc diffusion method.

The quarters in figure 4.1 represent the 18 ppm colloidal silver solution (A), 20 ppm colloidal silver solution (B), Cefepime control (C) and distilled water control (D). By visual inspection of the result images in figure 4.1, both concentrations of colloidal silver inhibited the bacterial growth of *S. pyogenes,* but not that of *S. aureus;* Cefepime control antibiotic exhibited an inhibitory effect on both bacteria used; and distilled water control showed no effect against both *S. pyogenes* and *S. aureus.*

Microdilution method

This method was used for confirmatory purposes after results have been obtained with agar disc diffusion method. Figure 4.2 below shows the typical 96 well plate layout used to test the compounds using the microdilution method. The dilutions, stains and well composition is shown in the figure. The 18 ppm colloidal silver solution is shown as C1 and 20 ppm colloidal silver solution as C2.

Wells that changed colour from clear to purple after the addition of the INP showed growth and thus no inhibition of bacterial growth by treatment compounds. The wells with an unchanged clear colour after addition of test compound show bacterial inhibition by the compound.

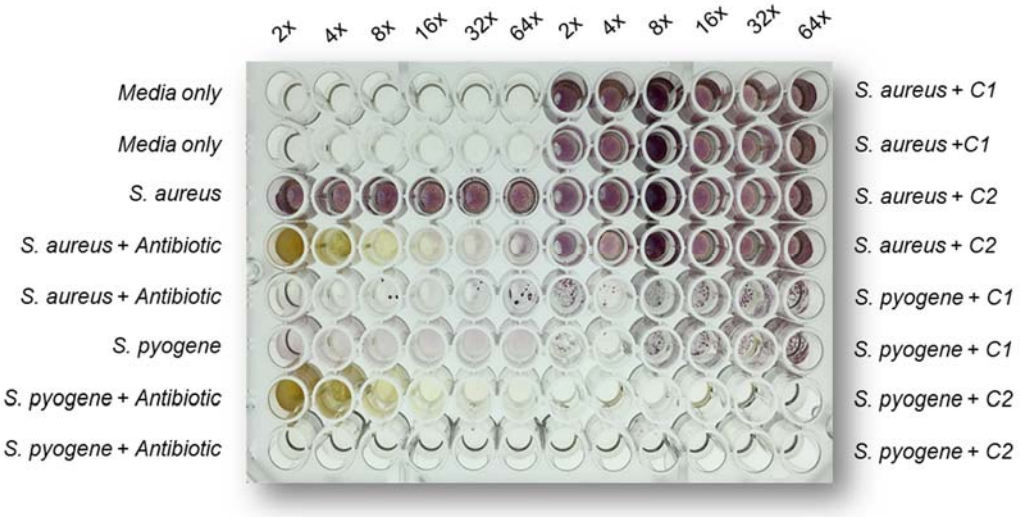


Figure 2 The typical microdilution method results of 96 well plate layout used to test the compounds using the microdilution method.

The microdilution method was used to determine the Minimum Inhibitory Concentration (MIC) for the compounds against the two bacterial strains.

The results obtained with the microdilution method confirmed that both the 18 ppm and 20 ppm colloidal silver solution had no effect on the *S. aureus* strain as the colour turned from clear to purple; however, the colloidal silver 20ppm demonstrated an inhibitory effect of *S. pyogenes* as the colour remained.

Discussion

The results demonstrated that *S. pyogenes* growth was inhibited by both the 18 ppm and 20 ppm colloidal silver solutions tested and which was confirmed by agar disc diffusion method and microdilution method. None of the colloidal silver solutions had any inhibitory effect on the *S. aureus* strain used. This attributed to various factors, including antibiotic resistance, the concentration of colloidal silver used and the method of choice.

S. aureus is more virulent than *S. pyogenes* (Acharya, 2015) and therefore prone to be resistance to treatment. It may have either adapted to the experimental environment and developed resistance or genetic mutation of the strain could have occurred, resulting in resistance.

Gutierrez *et al.* (2013) mentioned that Subinhibitory concentrations of antimicrobials may provoke cellular changes in bacteria. Both 18 and 20 ppm colloidal silver concentrations might have been too low that they caused *S. aureus* to change its structure to adapt to the environment and develop resistance to colloidal silver. Doss *et al.* (1993) further confirms that low concentrations of antibiotics also induce genetic transformation as well as the expression of virulence genes, thus exhibiting resistance to antimicrobials. The study published by Concepcion *et al.* (2007) showed that with their colloidal silver mixtures, only higher concentrations of colloidal silver (30 ppm) were effective against *S. aureus*, suggesting that the two compounds tested may have had too low a concentration of the active compound. Colloidal silver 18 ppm and 20 ppm were chosen based on the availability by manufacturing companies (Silverlab and Biosil), and on the fact that no studies are known to have been done using these concentrations.

Concepcion *et al* (2007) and his team evaluated the antimicrobial activity of colloidal silver (10 ppm, 20 ppm, and 30 ppm) compared with ophthalmic antibiotics including trobamycin, lomefloxin, ampicillin, and moxifloxacin. These were tested against *Escherichia coli, S. aureus, Staphylococcus epidermis,* and *Bacillus subtilis* using the antibacterial-activity testing (ABAT) and Kirby Bauer methods. The Kirby Bauer disc diffusion method (agar disc diffusion method) demonstrated that the bacteria tested was sensitive to 30 ppm colloidal silver, with the zone of inhibition more pronounced than that of trobamycin and ampicillin against *S. aureus.* No zone of inhibition was noted for all three concentrations of colloidal silver on all bacteria for the ABAT method, highlighting the need for using the correct methods.

Conclusion

The results demonstrated that *S. pyogenes* growth was inhibited by both the 18 ppm and 20 ppm colloidal silver solutions tested and which was confirmed by agar disc diffusion method and microdilution method. None of the colloidal silver solutions had any inhibitory effect on the *S. aureus* strain used. As discussed in sections above, either the *S.aureus* strain could have developed resistance to the colloidal silver solutions, or the colloidal silver solution may have had too low concentration of the active compound to be effective against this strain.

In conclusion, colloidal silver (18 ppm and 20 ppm) have an anti-bacterial effect on *S. pyogenes,* but not on *S. aureus.* This research has added to the developing volume of information to better understand the antibacterial effect and efficiency of colloidal silver mixtures. Further research needs to be conducted to determine whether or not the obtained results are conclusive.

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