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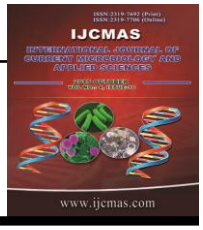

Original Research Article

Evaluation and Efficacy of In-Vitro Antibacterial Activity of Silver Nano Particles Against Multidrug Resistant Bacterial Isolates from Skin Infections of Patients at a Tertiary Care Hospital in Western Uttar Pradesh of India

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Keywords

Silver nanoparticles, MRSA, Luria- Bertani (LB) broth, Skin and Soft-Tissue Infections (SSTIs)

A B S T R A C T

Morbidity and mortality rates due to methicillin-resistant *Staphylococcus aureus* (MRSA) infection are increasing, so studies in controlling these infection sis gravely required. Silver nanoparticles (Ag-NPs) antimicrobial activity was well known since ages. Our work aimed evaluation and efficacy of antibacterial activity of Ag-NPs against MRSA isolated from patients with skin and soft-tissue infections of patients at a tertiary care hospital in western U.P. of India. MIC and MBC values of Ag-NPs for isolated Methicillin-sensitive *S. aureus* (MSSA) and MRSA strains were observed very low (i.e. in the range of 6.25-100 µg/ml),while MIC and MBC for reference strain *S. aureus* ATCC 25923 was found very low i.e. 12.5 µg/ml and 25 µg/ml, respectively; indicating very good bacteriostatic and bactericidal activity. Ag-NPs with highest concentration showed almost no growth for upto 16 hrs representing a bactericidal effect at this concentration. Effect was proportional to dose since 50.0 µg/ml was the most effective treatment (the bacterial population did not recover) and 6.25 µg/ml was the least effective. No minimum time of exposure to silver nanoparticles was needed to achieve an inhibitory effect. At the initial time point (0 h), 50.0 µg/ml of silver nanoparticles inhibited most of the bacterial populations. After 20 hours of incubation, no significant recovery was observed. Ag-NPs caused a growth delay of the bacterial cells; slope of the bacterial growth curve continuously decreased with increasing Ag-NPs concentration. Regardless of their drug-resistant mechanisms involved, Ag-NPs exhibited good in-vitro antimicrobial activity against MRSA which could be thus an alternate drug of choice.

Introduction

Infections caused by drug-resistant microorganisms result in significant increase in mortality, morbidity, and cost related to prolonged treatments. Such infections are particularly caused by some breach in the epidermis, leading to infections by the

microorganisms normally colonizing the skin like *Staphylococcus aureus* (Lopez *et al.,* 2003). Currently in clinical settings, skin and soft-tissue infections (SSTIs) particularly due to multidrug-resistant pathogens, are increasingly being encountered (Ansari *et al.,* 2011). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major problem that the world is now facing (Awadh *et al.,* 2009). Two years after the introduction of the drug to deal penicillin resistant isolates, in 1961 MRSA were first isolated (Jevons, 1961). MRSA are resistant to all β-lactam antibiotics, some to multiple antibiotic classes. Anxiety regarding the future availability of effective chemotherapeutic options resulted from resistance to all known antibiotic classes within the species, which in turn has occurred due to mutation and horizontal gene transfer. Since 1990s in many countries prevalence of MRSA has increased dramatically. In the UK, MRSA bacteraemia rose from <2% in 1990 to 43% in 2001(Johnson *et al.,* 2005) and similar trends seen in other countries including the USA (National Nosocomial Infections Surveillance System, 2002) and Japan (Izumida *et al.,* 2007).

The antibiotic era, barely 60–70 years old, is also threatened because of, increase resistance rhythm of this organism against different antibiotics (Richard *et al.,* 1998). So, studies are desperately required in finding out new antimicrobial agents against MRSA. In the past few years, the rates of morbidity and mortality are increasing due to MRSA infection, so studies in controlling these infections are gravely required. Nanobiotechnology is an important area of research that deserves all our attention owing to its potential application to fight against multidrug-resistant microbes. Nanotechnology offers opportunities to re-explore the biological properties of already known antimicrobial compounds by manipulating their size to alter the effect. Silver nanoparticles are nanoparticles of silver precursor (or) silver oxides like silver nitrate, silver citrate and silver acetate. Silver nanoparticles sizes ranges between 1 nm and 100 nm. Silver antimicrobial properties were known from antiquity, having the history with manhood dating back to 4000 BC (Chen *et al.,* 2008). Silver vessels were used to preserve water and wine. Antibacterial properties of silver are documented since 1000 BC, when silver vessels were used to preserve water. Hippocrates the father of medicine, promoted the use of silver for healing the wounds (Wesley Alexander, 2009). With the present day understanding of nanoscience, one can clearly get enlightened that these formulations contained silver nanoparticles (Kalishwaralal Kalimuthu *et al.,*2010). The mutation-resistant antimicrobial activities of silver are being used in different pharmaceutical formulations such as antibacterial clothing, burn ointments, and coating for medical devices (Ravishankar Bhat *et al.,* 2011).

Ag-NPs, which are being used increasingly as antimicrobial agents, may extend its antibacterial application to MRSA, the main cause of nosocomial infections worldwide. To explore the antibacterial properties of Ag-NPs against MRSA, the present work included an analysis of the relation between nanosilver effect and MRSA's resistance mechanisms, a study of the size dependence of the bactericidal activity of nanosilver and a toxicity assessment of nanoparticles against bacterial cells. Keeping the knowledge of silver nanoparticles in mind, this study was undertaken to analyze the evaluation and efficacy of antibacterial activity of silver nanoparticles against MRSA isolated from skin and soft-tissue infections (SSTIs) of patients at a tertiary care hospital in western uttar pradesh of India.

Material and Methods

This Prospective study of 6 months duration was conducted at a tertiary care hospital in western uttar pradesh of India. Before beginning the study clearance from Institutional Ethics Committee (IEC) was obtained. Written Informed consent from subjects involved was obtained. Skin and soft-tissue infections suspected of infected with *Staphylococcus aureus* were included in study. Other pathogens isolated from skin and soft-tissue infections were excluded from study and not taken into consideration. 26 *Staphylococcus aureus* (12 MSSA & 14 MRSA) samples isolated from skin and soft-tissue infections of patients were used. *Staphylococcus aureus* ATCC 25923 was used as control strain.

Sample collection processing, lab analysis: Clinical samples from sources like pus and other exudates were collected and processed aseptically observing the principles of "Universal Safety Procedures". Methicillin-resistant *S. aureus* and drug-susceptible *S. aureus* isolated from patients samples were cultured at 35^0C on Mueller–Hinton agar (MHA). *Staphylococcus* ATCC 25923 was used as control. A stock solution of commercially manufactured 10 nm silver nanoparticles (Reinste Nano Ventures Pvt Ltd, New Delhi) was used. The subsequent dilutions were made in Luria–Bertani broth. The antibacterial activity of the Ag-NPs was assessed by determining the minimal inhibitory concentration (MIC), the minimum bactericidal concentration (MBC), and by measuring the dynamic growth curve of the bacteria.

The MIC value corresponds to the lowest concentration of antimicrobial agents that inhibited 99% of bacterial growth and the MBC value corresponds to the lowest concentration of antimicrobial agents where 100% of the bacterial growth was inhibited, compared to the positive control (no treatment). Bacterial cell viability was also measured further.

Minimal inhibitory concentration (MIC) determination: Initially on MHA plates bacterial strains were grown overnight at 35^oC. Further using the standard broth dilution method (CLSI M07-A8), Ag-NPs antimicrobial activity was examined. Accordingly by microdilution method in Luria- Bertani (LB) broth (Hi-Media Mumbai, India) using serial two-fold dilutions of Ag-NPs in concentrations ranging from 200 to 1.5625 µg/ml, initial bacterial inoculums of 2×10^8 CFU/ml and the time and temperature of incubation being 24 h at 37°C, respectively MIC were determined. To confirm the value of MIC, the MIC measurement was done in triplicate for each tested bacteria.

Minimal bactericidal concentration (MBC) determination: From all above tubes used in MIC determination of the Ag-NPs tested, aliquots of 50 µl in which no visible bacterial growth was observed were further charged in MHA plates not supplemented with Ag-NPs and were incubated for 24 h at 35^oC.

Bacterial viability and growth inhibition testing on bacterial growth curve: From fresh colonies on MHA plates, inoculations were done into 100 ml of Luria-Bertani (LB) broth (Hi-Media Mumbai, India). Growth was allowed until the optical density (OD) reached 0.1 at 600 nm (OD of 0.1 corresponds to 10^8 CFU/ml of medium). Further, 2×10^8 CFU/ml of above was added to 100 ml of liquid LB media supplemented with 5, 10, 15, 20 and 25 µg/ml of Ag-NPs. All the flasks were be put on rotator shaker (150 rpm) and incubated at 37°C. Control broths were used without nano particles. Optical density was measured after every 2 hour (up to 20 h) at 600 nm using

spectrophotometer and the bacterial growth was determined. The growth inhibition percentage was obtained with respect to the positive control.

Statistical tests used for data analysis: The triplicate MIC and MBC test results was express as the mean • ± the standard errors of the mean. A Student's 't' test was used to compare these results. *P* values lower than 0.05 was considered significant. Microsoft excel was used to create the figures.

Results and Discussion

A very low (i.e. in the range of 6.25-100 µg/ml) MIC and MBC values of Ag-NPs against MSSA and MRSA strains recovered from patients with skin and soft-tissue infections from our tertiary care hospital were observed, indicating very well bacteriostatic and bactericidal activity of Ag-NPs (Table 1 & Fig. 1). Ag-NPs MIC and MBC value for reference strain *S. aureus* ATCC 25923 was too found very low i.e. 12.5 µg/ml and 25 µg/ml, respectively (Table 1).

Bacterial growth curve was monitored using growth in Luria-Bertani (LB) broth. The bactericidal activity of different concentrations of silver nanoparticles (200, 100, 50, 25, 12.5, 6.25, 3.125, 1,5625 µg/ml) was compared among the different drug-resistant strains using time-kill assays. The time-kill assays were used to analyze post-treatment bacterial viability and to define the minimum time necessary to reach an inhibitory or bactericidal effect. As shown in figure 2, by measuring the OD (at 600 nm) of the control and bacterial solutions containing different concentration of Ag-NPs, at a regular interval of 2 h (upto 20 h), bacterial growth changes in relation to time were monitored. Bacterial cell growth was indicated by increase in turbidity of the liquid medium and thus absorption also increased. With increase in Ag-NPs concentration the slope of the bacterial growth curve continuously decreased thus indicating that at concentrations used, the Ag-NPs caused delay in growth of the bacterial cells. At highest concentration used Ag-NPs showed no growth for up to 16 hrs thus indicating their bactericidal effect at this concentration (Fig. 2a & 2b). Ag-NPs treatment affected bacterial growth to different extents (Fig. 1). The effect was proportional to the dose since 50.0 µg/ml was the most effective treatment (the bacterial population did not recover) and 6.25 µg/ml was the least effective. Although 6.25 µg/ml is considerably under the MIC–MBC range of silver nanoparticles, the bacterial population did not reach normal levels of growth after 20 h of incubation. No minimum time of exposure to Ag-NPs is needed to achieve an inhibitory effect. At the initial time point (0 h), 50.0 µg/ml of Ag-NPs inhibited most of the bacterial populations. After 20 hours of incubation, no significant recovery was observed.

In the current study, we aimed to determine through different in vitro assays the antibacterial properties of Ag-NPs against drug-resistant bacteria, infectious agents that represent a constant threat in hospital and community environments i.e MRSA. To achieve this goal, we challenged clinical isolates classified as resistant to one or more antibiotics (methicillin/oxacillin/cefoxitin) with different concentrations of a nanosilver suspension and described the effect on bacterial cell viability and growth rate. To gain a more complete understanding and to attempt a preliminary approach to determining the mechanism of inhibition of Ag-NPs, a comparison was made between multidrug resistant strains and drug-susceptible strains of the same species bacteria. No significant differences in bactericidal activity were found among the different compared groups (drug-resistant

vs. susceptible), which suggests that Ag-NPs are broad spectrum antibacterial agents. These results further agree with previous findings by other research teams, where it was proven that Ag-NPs exert the same effect on drug-resistant vs. susceptible strains (Kong *et al.*, 2008; Petica, 2008).

The mechanism of the bactericidal effect of Ag-NPs remains to be elucidated. Several studies have proposed that Ag-NPs bind to the surface of the cell membrane, disrupting cellular permeability and the respiration functions of the cell. Smaller Ag-NPs having a large surface area available for interaction have a greater bactericidal effect than larger silver nanoparticles (Kvitek *et al.*, 2008). It is also possible that Ag-NPs not only interact with the surface of the membrane, but also penetrate inside the bacteria and inactivate DNA replicating ability (Morones *et al.*, 2005) causing the devastation of the cell.

On the other hand, silver nanoparticles target protein synthesis, nucleic acid synthesis, and Gram positive cell wall synthesis, which explains why these bacteria were more susceptible (although not significantly more) to silver nanoparticles. Indeed, silver nanoparticles attach to the surface of the cell membrane and disturb its function, penetrate bacteria, and release silver ions (Sondi *et al.*, 2004; Lok *et al.*, 2006). Sondi et al (2004) found that silver nanoparticles target the bacterial membrane, leading to a dissipation of the proton motive force (Lok *et al.*, 2007). Consequently silver nanoparticles need to reach the cell membrane to achieve an antibacterial effect.

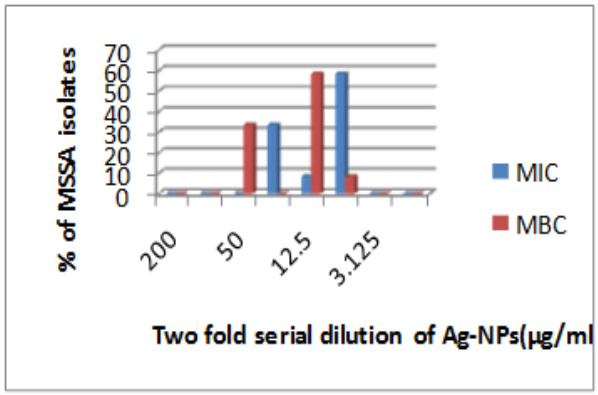
In our study, MIC of Ag-NPs against MRSA was estimated (50 µL). In their study Ansari *et al.* (2011) stated, the values of MIC and MBC of Ag-NPs against all clinical isolates of MSSA, MRSA and single strain of *S. aureus* ATCC25923 were found in the range of 12.5-50 µg/ml and 12.5-100 µg/ml, respectively (Ansari *et al.*, 2011). Findings of Martinez-Castanon *et al.* (2008) were similar to our findings. They reported that Ag-NPs were inhibitory at concentration of 16.67 µg/ml against *S. aureus* ATCC 25923, but they used the Ag-NPs of size 29nm which was larger than the size used by us (10nm). Our results of antibacterial activity of Ag-NPs against *S. aureus* ATCC 25923 are exactly in accordance with results shown by Ansari *et al.* (2011) and Fernandez *et al.* (2008).They showed MIC and MBC values of Ag-NPs of 12.5 µg/ml and 25 µg/ml, respectively for *S. aureus* ATCC 25923, which is in agreement of our finding where the value of MIC and MBC of our Ag-NPs was near about same as shown by them. Our results showed better antibacterial activity as compared to earlier work of (Ayala-Nunez *et al.*, 2009). They have reported MIC and MBC values of Ag-NPs 1800 µg/ml and 2700 µg/ml, respectively. The growth curve of standard strain of *S. aureus* ATCC 25923 and MRSA were plotted in the presence of 0, 5, 10, 15, 20, and 25 µg/ml concentration of Ag-NPs. Figure 2 clearly indicates that as the concentration of Ag-NPs increases, reduction in bacterial growth was observed and this was even continued for 16 hrs. There was clear inhibitory action of Ag-NPs on *S. aureus* ATCC 25923 and MRSA at all concentrations. Our results were better as compared to study done by Shrivastava *et al.* (2007) probably because the size of nanoparticles was smaller in our study. The finding of Li *et al.* (2010) showed a complete growth inhibition for *S. aureus* ATCC 6538P at 20 µg/ml, while in case of our study no growth was observed up to 16 hrs at 25 µg/ml of Ag-NPs (Fig. 2). Thus, our result shows that there was very little difference between antibacterial activities of Ag-NPs against standard strain and methicillin-resistant strain, i.e. both were

equally sensitive. The fact that the drug-resistant and drug-susceptible strains were affected by Ag-NPs in the same manner indicates that the drug-resistant proteins that give bacteria the capacity to avoid antibiotics do not affect the efficacy of nanosilver.

Table.1 MIC and MBC of Ag-NPs tested against clinical isolates of MSSA, MRSA and references train *S. aureus* ATCC25923

MSSA Isolates (12)				MRSA Isolates (14)		
Number of isolates	**MIC (µg/ml)**	**MBC (µg/ml)**		**Number of isolates**	**MIC (µg/ml)**	**MBC (µg/ml)**
1	6.25	6.25		2	6.25	12.5
6	6.25	12.5		1	12.5	50
1	12.5	12.5		2	12.5	12.5
4	25	50		6	12.5	25
***S. aureus* ATCC25923**	12.5	25		2	25	25
				1	50	100

Figure.1 Clinical isolates of MSSA and MRSA showing MIC and MBC treated with serial two fold dilution of Ag-NPs



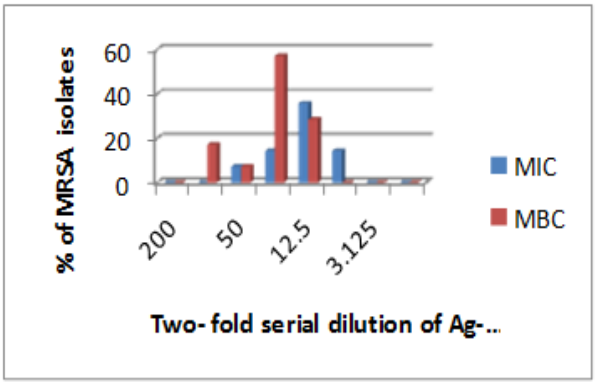
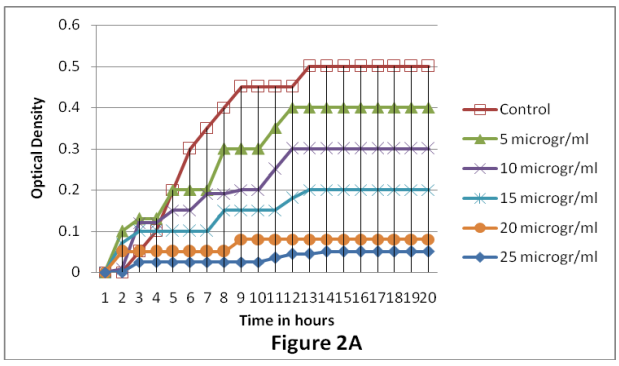
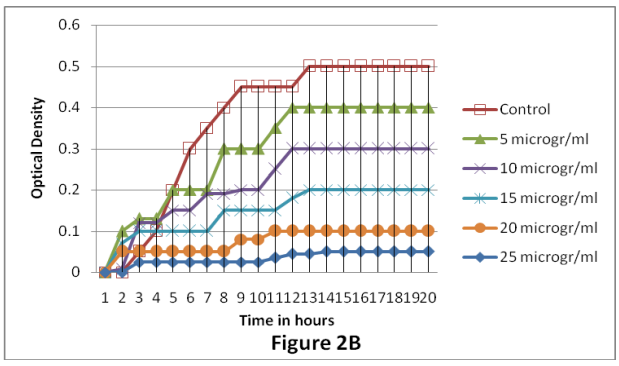
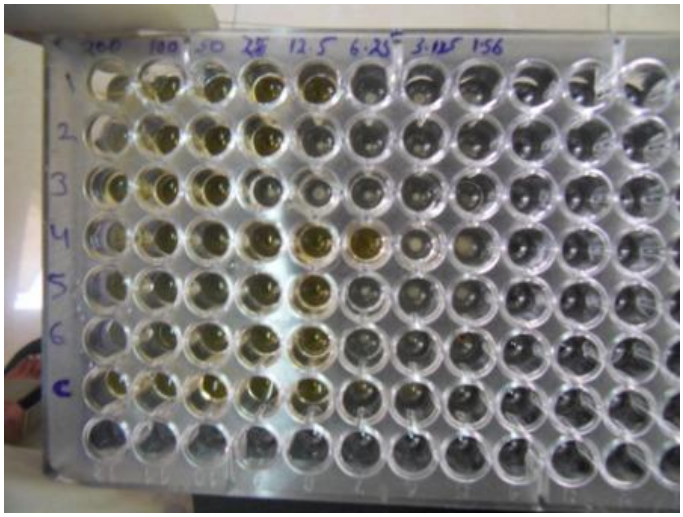


Figure.2 Dynamic growth curve of *S. aureus* ATCC 25923 (a) and MRSA (b) in the presence of different concentrations of Ag-NPs in liquid LB broth





Picture.1 Microtitre plate showing MIC detection by Microbroth dilution method



Ag-NPs inhibited bacterial growth of both MRSA and MSSA in a bactericidal rather than a bacteriostatic manner (MBC/MIC ratio ≤ 4). For all strains, the average ratio of the minimum bactericidal concentration to the minimum inhibitory concentration indicated that Ag-NPs have a bactericidal rather than bacteriostatic effect on the tested bacteria. In theory, a bactericidal agent is preferred clinically because bacterial killing should produce a faster resolution of the infection, improve clinical outcome, and reduce the likelihood of the emergence of resistance and the spread of infection. If pathogens are killed rather than inhibited, resistance mutations that might otherwise emerge as the result of antibiotic pressure are eliminated (French *et al.*, 2006).

One of the principal elements of bacteria's infectivity is their rapid reproduction time, a characteristic that could be a good target for impeding a viable infection. As shown by time-kill assays, Ag-NPs were effective in inhibiting bacterial growth in a dose and time dependent manner.

Besides their bactericidal activity and immediate antibacterial effect against a wide variety of drug-resistant bacteria, Ag-NPs have particular characteristics provided by the silver itself. This noble metal tends to induce low bacterial resistance (Ip *et al.*, 2006) and has low toxicity and minimal side effects when ingested since at most 2–4% is retained in tissues after absorption by the body. A notable health effect has been argyria, an irreversible pigmentation of the skin that is mostly an aesthetic concern (Drake *et al.*, 2005). At the same dose range, 10 nm nanoparticles were the most effective since they are supposed not to affect HeLa's cell viability while inhibiting a considerable percentage of MRSA growth. Ag-NPs are effective bactericidal agents that are not affected by drug-resistant mechanisms of MRSA. Nanosilver size mediates MRSA inhibition and the cytotoxicity to human cells being smaller, nanoparticles the ones with a better antibacterial activity and nontoxic effect can be used.

The bactericidal activity of Ag-NPs against multidrug-resistant bacteria could be used in conjunction with advances in impregnation techniques and polymer technology to expand the range of applications of these nanoparticles in the preservation of food, disinfection of medical supplies and equipment, and decontamination of the

surfaces of items such as toys and kitchenware (Matsumura *et al.*, 2003).

The data presented here are novel in that they prove that Ag-NPs are effective bactericidal agents regardless of the drug-resistance mechanisms that exist in multidrug-resistant bacteria and show the importance of Ag-NPs in the nosocomial and community environment. Therefore, Ag-NPs can be recommended as an effective broad spectrum bactericidal agent. 't'value was found to be 0.76 and P>0.05. There is no significance difference in MIC in antibacterial activity of Ag-NPs on MSSA & MRSA, thus Ag-NPs can be used as broad spectrum antibacterial agent regardless of their drug sensitivity pattern.

In conclusion, Ag-NPs of approximately 10 nm inhibit Methicillin Resistant *Staphylococcus aureus* (MRSA) growth *in vitro* at noncytotoxic concentrations, supporting their potential use as antibacterial agents with a wide number of biomedical and therapeutic applications. Since drug resistance does not interfere with the bactericidal effect of nanosilver, they may prove useful in manufacturing pharmaceutical products and medical devices that may help to prevent the transmission of drug-resistant pathogens, but toxicological limitations for eukaryotic cells should be taken in account since nanosilver is not a target specific antibacterial agent (Nilda Vanesa Ayala-Nunez *et al.*, 2009).

Nanobiotechnology owing to its ability to fight against multidrug-resistant microbes is an important area of research and hope. To evaluate the efficacy of Ag-NPs as a bactericidal agent to be used in-vivo further studies are necessary to assess the toxic effects in human cells and microorganisms.

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