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Anti-biofilm role of silver nanoparticles on microbial biofilm: An in vitro study on biofilm of heart valves

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Abstract: *Background:* Infective Endocarditis (IE) caused by microbial biofilm is associated with high degree of mortality and morbidity. Of late, silver nano-particles (AgNPs) with a wide range of antimicrobial, anti-biofilm properties are being studied to combat this drug resistance. *Objective:* Assessment of anti-biofilm role of AgNPs among the potential biofilm producer isolates retrieved by in-vitro study. *Methods:* Following inclusion and exclusion criteria, some thirty extracted heart valves were considered to be included in the study. Sample collection, transport, storage and processing of samples for aerobic and anaerobic bacteriological culture were done as per standard microbiological guidelines. Biofilm detection assay and antibiofilm efficacy of indigenously prepared carboxy methyl cellulose capped AgNPs sized 9.5nm (CMC-AgNPs-9.5) were studied using Christensen's et al. method. *Statistical Analysis:* One-way ANOVA and unpaired t-test were used. $P \leq 0.05$ obtained was considered statistically significant. *Results:* Majority were Gram-positive (75%) and 91.6% of the microbial isolates were found to be biofilm producers.CMC-AgNPs-9.5were able to effectively eradicate biofilm. *Conclusion:* AgNPs by their reactive oxygen species (ROS) generation provide new ray of hope for antimicrobial, antibiofilm and prevention strategies. However in vivo study may be done in future to substantiate the present scientific claim further.
Keywords: Silver Nano-Particle, Biofilm, Heart Valves.

Introduction

Biofilms are of considerable significance for public health because of their role in several infectious diseases, particularly a variety of device-related infections. Almost 80% of chronic and recurrent microbial infections are due to biofilm production. Systemic infections can be caused by the biofilm formed on in-situ medical implants, such as heart valves, catheters, intrauterine devices, joint prostheses, intrauterine devices, contact lenses, dental units etc., can only be cured by removing the devices thereby increasing both complications and cost of treatment [1].

Infective endocarditis (IE) is an infection of the heart valves (endocardium) caused by microorganisms entering through the bloodstream. IE occurs more commonly in patients with stenosed or incompetent heart valves, prosthetic heart valves and pacemaker leads. Earlier, rheumatic fever was the prototype predisposing factor for IE and still remains relevant in developing nations. Structurally and functionally normal heart valves are highly resistant to bacterial attachment. However, damaged heart valves and endocardium leave the tissue susceptible to bacterial attachment and colonization. As the microorganisms rapidly cross the damaged valve lining, they adhere to the surface more strongly causing infective endocarditis. Stationary phase and viable-but-nonculturable state are the ways of survival for bacterial biofilm producing colonies under antibiotic stress [2].

Successful treatment of biofilm-associated infections is difficult because of high antibiotic resistance in these bacterial

colonies. Traditional antibiotic chemotherapy is unable to completely eradicate bacteria situated in the center of the biofilm and leads to persistence of infection. Therefore, to overcome the drug resistance of biofilm producing bacteria; alternative strategies and novel antibiofilm agents are being studied [3].

Silver nanoparticles (AgNPs), are discovered to have an inherent property of inhibition of biofilm production and also have a wide range of antimicrobial action. Several in-vitro studies indicate that AgNPs have dose-dependent pan-microbicidal actions depending on the degree of intracellular access [4] both in dormant/resting phase and log phase of bacterial cell cycles because of their reduced ionic state [5]. But none are still safe for systemic use or environment friendly. However, if biocompatible stabilizing and reducing agents along with silver nano-particles were used for preparing antimicrobial nano-particles, they can emerge as an extremely effective chemotherapeutic agent. This in-vitro study aims for the assessment of antimicrobial efficacy of such bio-compatible AgNPs especially formed using carboxy-methyl cellulose as stabilizing and capping agent, dextrose as reducing agent [6].

Material and Methods

This study was conducted from January 2019 to July 2020, approximately one and half year in duration in a tertiary care hospital of West Bengal a state of eastern part of India with due approval of Institutional Ethics Committee (IEC). It was a cross sectional observational study.

Aim and objectives:

i) To study the status of biofilm from heart valve specimens which were sent to our laboratory.
ii) To study the antibiofilm role of carboxymethyl cellulose capped silver nanoparticles sized 9.5nm (CMC-AgNPs-9.5) against biofilm-producing multidrug-resistant bacteria colonizing heart valves to achieve better prevention and treatment of Device associated Infections (DAI).
iii) To study biofilm inhibiting efficacy by four-fold concentration of CMC-AgNPs-9.5 of planktonic flora on preformed in-vitro biofilm.

While performing the study, we received samples of native and prosthetic heart valves operated out from patients undergoing heart valve replacement surgery for both infective and non-infective causes. A total of fifty heart valves were retrieved during the study period. Thirty of them were included in the study as per the following inclusion and exclusion criteria. Out of them eighteen were natural heart valves and twelve were prosthetic valves.

Inclusion and Exclusion criteria: Valves extracted out due to the reasons of IE and the patients who were refractory to the conventional treatment while on antibiotic therapy for greater than ten days were included in the present study. However, valves operated out due to reasons other than infections such as valvular dysfunction, congenital anomalies and treatment responsive cases were excluded. Also, IE due to fungal causes was excluded.

Following retrieval, the heart valves included in the study were separately collected and transported immediately to microbiology laboratory in sterile screw capped universal containers containing sterile normal saline for further processing. The specimens were processed using standard microbiological procedures following the guidelines of standard text book of diagnostic microbiology [7]. One part of each valve tissue was transferred in sterile container containing brain heart infusion (BHI) broth in microbiology laboratory followed overnight aerobic incubation at 37^0C. Another part was taken for Gram staining on arrival in the laboratory. An aliquot of each incubated BHI broth was inoculated in suitable media viz. MacConkey's agar and 5% sheep blood Agar (SBA) followed by aerobic incubation.

Resultant colony, if any, were identified and antibiotic susceptibility testing were done by conventional microbiological techniques and using automated VITEK-2 compact system [8] wherever required following the guideline of Clinical and Laboratory Standards Institute (CLSI) [9]. One part of each heart valve sample was also processed anaerobically starting from collection, transport and

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inoculation in Robertson's cooked meat medium (RCM). The RCM tubes were sub-cultured after 48 hours in suitable anaerobic media viz. brucella blood agar and incubated anaerobically in Gas Pack system and checked for any anaerobic growth [10].

Biofilm status of the retrieved valves were studied using microtiter plate assay as described by Christensen et al. 1985[11] and classified according to Stepanovic et al. method as non, weak, moderate and strong biofilm formers [12]. Detection of biofilm associated genes of *Staphylococcus aureus* such as *mecA* gene was done by PCR followed gel electrophoresis [13]. Antimicrobial activities of planktonic bacterial growth by using CMC-AgNPs-9.5 was done along with antibiofilm effect of CMC-AgNPs-9.5 on the preformed biofilm was studied using standard Christensen et al. method [11].

However, for the constraints of resources and time, the present study group selected the strong biofilm producer colonies only to challenge with CMC-AgNPs-9.5 with the premise that if strong biofilms could be treated then the moderate and weak ones could also be challenged successfully. We used American Type Culture Collection (ATCC) strains of *Escherichia coli ATCC 25922, Staphylococcus aureus* ATCC 25923, methicillin resistant *Staphylococcus aureus* (MRSA) and Biofilm producing *Staphylococcus aureus* ATCC 43300, methicillin-sensitive *Staphylococcus aureus* (MSSA) ATCC 29213 as reference strains in our present study.

Preparation of nanoparticles CMC-AgNPs-9.5 [14]:

- AgNPs were prepared by chemical reduction of silver nitrate ($AgNO_3$) solution (Sigma Aldrich, USA); sodium carboxy-methylcellulose (CMC, Amrit Chemicals, India) was used as capping agents using dextrose (Sigma Aldrich, USA) as reducing agent.
- In a beaker containing a magnetic stirrer, 50 ml sodium carboxymethyl cellulose (4000 Centipoises) solution at 50 mg / L concentration was taken. To this 50 ml 0.4 mM $AgNO_3$ solution (effective concentration in 200 ml AgNPs at 10.68 mg/ L silver from 17 mg $AgNO_3$ / L) in de-ionized water was added forcefully with continuous stirring by magnetic stirrer so that effective concentration of silver in nano preparation resulted to 1mM. Then 100 ml 0.2 mM dextrose was added forcibly into the solution and stirring was continued for one hour at 50°C. The resultant mixture was stored in an amber-colored bottle, one at room temperature another at 4°C refrigerator.

Evaluation of the antimicrobial activity of CMC-AgNPs-9.5: Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of CMC-AgNPs-9.5 for the planktonic growth of the isolates was accomplished by microdilution broth method [15] as follows: Bacterial suspension of 0.5 McFarland was incubated for 15-20mins in shaker incubator. 0.1ml of the bacterial inoculum is taken from the above solution and added to 9.9ml of fresh cation adjusted Mueller Hinton Broth (CAMHB) in a test tube,100µl of stock AgNP solution was taken in the 1st well of a microtiter plate and then 50µl of distilled water in subsequent wells till the desired dilution is required, 50µl of the AgNP from 1st well was transferred to the subsequent next well and so on; the last 50µl is discarded thus 2-fold dilution was made following which 50µl of the bacterial inoculum was then added to the wells and the plate was incubated for 24 hrs. at 37^{0}c. After 24hrs the MIC was determined by visual turbidity and Optical density measurement at 450nm and MBC was determined by plating on a blood agar plate.

Biofilm detection assay [11] *:(1st set experiment):* The biofilm potential of the isolates under study was detected using a microtiter plate assay by modified Christensen's method [11]. Briefly, 2µl of each fresh bacterial suspension of 0.5 McFarland standard, in BHI broth was directly added to each well of sterile microtiter plates already filled with 200 µl of the same broth per well. Three repetitions were done with each isolate and were incubated at 37°C for 24 hours. The wells were washed thrice with PBS. The plates were fixed with cold methanol, then 250 µl of 0.1% crystal violet solution was added to each well and kept for 10 minutes at room temperature.

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Each microtiter dish was tapped briskly over the waste tray to remove the crystal violet solution and then rinsed off under running tap water. Plates were allowed to air-dry and 250 µl of 95% ethanol was added to each stained well. Dye was allowed to solubilize by covering plates and incubating for 15 minutes at room temperature. The cut-off optical density [ODc] was defined as three standard deviations above the mean OD of the negative control i.e., BHI broth. Based on OD produced by bacterial films at a wavelength of 550 nm, isolates were classified as described by Stepanovic et al. in 2000 [12] as follows:

- OD <ODc = biofilm nonproducer;
- OD >ODc but < 2ODc= weak biofilm producer;
- OD>2ODc but < 4ODc = moderate biofilm producer
- >4ODc = strong biofilm producer.

CMC-AgNP-9.5 challenge test of preformed biofilm: (2nd set experiment): We tried to challenge the biofilm producing capabilities of seven strong biofilm producer isolates by CMC-AgNPs-9.5 as follows: Biofilms were grown on 96 wells microtiter plate as described above. Non-adherent bacteria on the wells were washed thrice using sterile phosphate buffer solution (PBS). To these wells with pre-formed biofilms, CMC-AgNPs-9.5at fourfold concentration of the concentration in planktonic growth by convention are added in culture media containing BHI broth with positive control being a drug free well and negative control being only broth.

Plates were then incubated for 24 hours at 37°C, then plates were rinsed thrice with PBS following which they were sonicated to remove biofilm from the walls of the well. Then sterile BHI broth was added to each well and re-incubated at 37°C, the presence of viable bacteria in a particular well was determined by turbidity both visually and measuring OD at 450nm and the growth of bacteria determined by plating on SBA from a particular well indicates regrowth of planktonic bacteria from surviving biofilm.

Biofilm detection assay on CMC-AgNP-9.5 treated preformed biofilm: (3rd set experiment): Biofilm detection assay on those preformed biofilm set simultaneously along with 2nd set experiment was done using the method described as above in 1st set of experiment.

Results

The prepared CMC-AgNPs were yellowish brown in color as observed in the supernatant after reaction with the Ag^+ ions, indicating the metal ion reduction and formation of silver nano-particles which is being depicted as Ag^0. Physical characterization revealed the maximum absorption peak as displayed by Jasco V 650 UV VIS Spectrophotometer, Japan at around 409nm with narrow distribution; their zeta potential was -28mV; the size documented by the lognormal size distribution curve obtained from DLS (Malvern Zen 3600 Zetasizer, USA), further confirmed by transmission electron microscopy (JEOL JEM 2100 HR with EELS, USA) demonstrated an average size of 9.5 nm with a triangular shape [16].

XRD analysis confirms silver nano status. Out of the 50 samples received 30 were studied which satisfied the inclusion criteria comprising of 18 from natural and 12 from prosthetic heart valves. Among which the study group found sterile culture in 6 (4 natural and 2 prosthetic) heart valves. Hence, they were excluded from further study. Rest 24 (14 natural and 10 prosthetic) valve samples gave rise to one microorganism from each of them. No sample yielded multiple colonies. No anaerobic organism grew in the study. So, the resultant 24 microorganisms were studied further.

The isolated organisms in decreasing order of frequency were *Staphylococcus hemolyticus* (28%), *Acinetobacter baumannii* (21%), *Staphylococcus aureus* (14%), *Staphylococcus xylosus*(14%),*Pseudomonas aeruginosa* (14%), *Enterococcus fecalis* (7%) among the natural heart valves and *Staphylococcus epidermidis* (30%), *Staphylococcus aureus*(20%), *Staphylococcus hemolyticus* (20%), *Staphylococcus xylosus* (10%), *Kocuria kristanae* (10%), *Pseudomonas aeruginosa* (10%) among the prosthetic heart valves.

The antimicrobial resistance profile of the isolated microorganisms as depicted in Table 1 revealed resistance against glycopeptides was highest among *Staphylococcus aureus* (25%), rest all were sensitive whereas 50% of

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Staphylococcus aureus and *Enterococcus fecalis* were resistance against oxazolidinones; Table 2 showed resistance to carbapenems in 66% of *Pseudomonas aeruginosa*,33% of *Acinetobacter baumannii* whereas 66% of *Acinetobacter baumannii* and 33% of *Pseudomonas aeruginosa* were resistant to macrolides. Only 33% of *Pseudomonas aeruginosa* were resistant to colistin rest all were sensitive. The MIC and MBC of the Gram-positive and Gram-negative isolates against CMC-AgNP-9.5 and ionic silver were displayed in Table 3 and 4 respectively.

Table-1: Gram Positive Isolates

Antimicrobials	***Staphylococcus aureus (n=4)***	***Staphylococcus hemolyticus (n=6)***	***Staphylococcus xylosus (n=2)***	***Staphylococcus epidermidis (n=3)***	***Enterococcus fecalis (n=2)***	***Kocuriakristanae (n=1)***
Cefoxitin	4	6	2	3	-	1
Co-Amoxiclav	4	6	2	3	-	0
Penicillin	4	6	2	3	2	0
Ampicillin	4	6	2	3	2	0
High Level Gentamicin	-	-	-	-	1	-
Amikacin	3	1	1	2	-	0
Ciprofloxacin	3	1	0	2	1	0
Levofloxacin	3	0	0	2	1	0
Erythromycin	2	6	0	1	-	0
Clindamycin	3	6	0	1	-	0
Vancomycin	1	0	0	0	0	0
Teicoplanin	1	0	0	0	0	0
Linezolid	2	0	0	0	1	0

Table-2: Gram Negative Isolates

Antimicrobials	***Acinetobacter baumannii (n=3)***	***Pseudomonas aeruginosa (n=3)***
Amoxicillin- clavulanic acid	3	3
Ceftazidime	2	3
Ceftriaxone	3	3
Cefotaxime	2	3
Cefpodoxime	2	3
Imipenem	1	2
Meropenem	1	2
Doripenem	1	2
Amikacin	2	1
Gentamycin	2	1
Tobramycin	2	1
Ciprofloxacin	2	2
Levofloxacin	1	2
Colistin	0	1
Minocycline	1	-

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Table-3: MIC and MBC of Gram-positive isolates against CMC-AgNP-9.5[N=100µg/ml] and Ionic silver (equivalent concentration) [N=concentration of CMC-AgNPs-9.5]

Organism	Biofilm and Valve status	CMC-AgNP-9.5	CMC-AgNP-9.5	Ionic Silver	Ionic silver
		MIC	MBC	MIC	MBC
S.aureus strain 1	Moderate; natural	N/65536 dil.=0.0015ug/ml	N/65536dil.= 0.0015ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.aureus strain2	strong; natural	N/32768 dil.=0.003ug/ml	N/32768dil.= 0.003ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.aureus strain3	moderate; prosthetic	N/32768dil. =0.003ug/ml	N/32768dil. =0.003ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.aureus strain4	strong; prosthetic	N/32768dil. =0.003ug/ml	N/32768dil. =0.003ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.hemolyticus strain 1	moderate; natural	N/16384dil.= 0.006ug/ml	N/16384dil.= 0.006ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.hemolyticus strain 2	moderate; prosthetic	N/32768 dil.=0.003ug/ml	N/32768dil.= 0.003ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.hemolyticus strain 3	Strong; natural	N/65536 dil.=0.0015ug/ml	N/65536dil.= 0.0015ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.hemolyticus strain 4	Weak; natural	N/16384dil.= 0.006ug/ml	N/16384dil.= 0.006ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.hemolyticus strain 5	Strong; natural	N/65536 dil.=0.0015ug/ml	N/65536dil.= 0.0015ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.hemolyticus strain 6	Weak; prosthetic	N/32768 dil.=0.003ug/ml	N/32768dil.= 0.003ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.epidermidis strain 1	strong; prosthetic	N/131072 dil.=0.0007ug/ml	N/131072dil. =0.0007ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.epidermidis strain 2	Moderate; prosthetic	N/16384dil.= 0.006ug/ml	N/16384dil.= 0.006ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.epidermidis strain 3	strong; prosthetic	N/131072 dil.=0.0007ug/ml	N/131072dil. =0.0007ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.xylosus strain 1	Weak; natural	N/32768 dil.	N/32768dil.	N/2dil.=50ug/ml	Ndil.=100ug/ml
S.xylosus Strain2	moderate; natural	N/131072 dil.=0.0007ug/ml	N/131072dil. =0.0007ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
Kocuriakristanae strain 1	No biofilm; prosthetic	N/65536 dil.=0.0015ug/ml	N/65536dil.= 0.0015ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
E.fecalis strain 1	Moderate; natural	N/32768 dil.=0.003ug/ml	N/32768dil.= 0.003ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
E.fecalis strain 2	Moderate; prosthetic	N/65536 dil.=0.0015ug/ml	N/65536dil.= 0.0015ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml

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Table-4: MIC and MBC of Gram-negative bacteria against CMC-AgNP-9.5 [N=100µg/ml] and Ionic silver (equivalent concentration) [N=100µg/ml](N=concentration of CMC-AgNPs-9.5)

Organism	Biofilm and valve status	CMC-AgNP-9.5	CMC-AgNP-9.5	Ionic silver	Ionic silver
		MIC	MBC	MIC	MBC
P.aeruginosa strain1	No biofilm; natural	N/65536dil. =0.0015ug/ml	N/65536dil. =0.0015ug/ml	Ndil.=100ug/ml	Ndil.=100ug/ml
P.aeruginosa strain 2	Moderate; natural	N/16384dil. =0.006ug/ml	N/16384dil .=0.006ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
P.aeruginosa strain 3	Weak; prosthetic	N/131072dil. =0.0007ug/ml	N/131072dil .=0.0007ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
A.baumannii strain 1	Weak; natural	N/16384dil. =0.006ug/ml	N/16384dil .=0.006ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
A.baumannii strain 2	Strong; natural	N/32768dil. =0.003ug/ml	N/32768dil. =0.003ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml
A.baumannii strain 3	Weak; natural	N/16384dil. =0.006ug/ml	N/16384dil .=0.006ug/ml	N/2dil.=50ug/ml	Ndil.=100ug/ml

Fig-1: Biofilm status among Natural heart valves

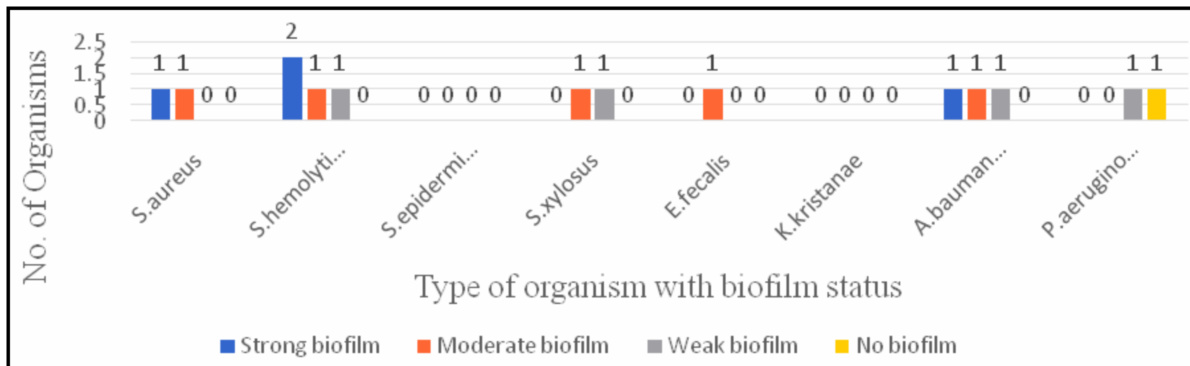
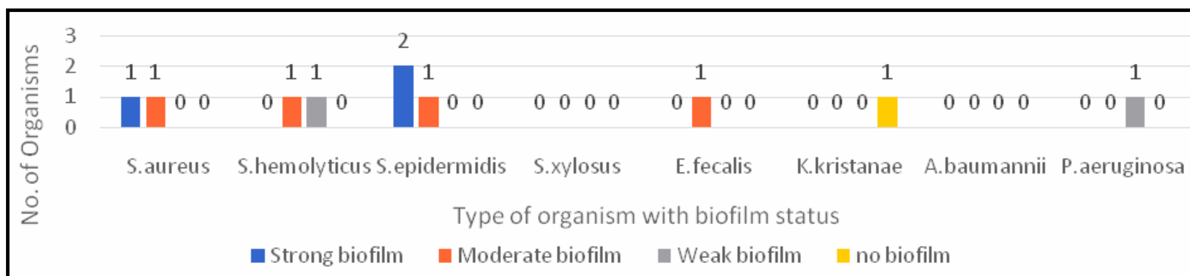


Fig-2: Biofilm Status among Prosthetic Heart valves



The potency of anti-microbial action of CMC-AgNPs-9.5 (nano-silver) is determined to be several-fold higher in MIC of compared to $AgNO_3$ (ionic form) containing 106.8µg silver/mL in undiluted form ranging between 16384 to 131072 folds. There was significant difference between the CMC-AgNPs-9.5 and Ionic silver groups ($P < 0.0001$) determined by using Graph pad Prism 9 unpaired t-test. Data are expressed as means ± SD. All the isolated organism when tested for biofilm production were found to be biofilm producers excepting two. Consequently, the rest 22 (13 in natural & 9 in prosthetic) microorganisms were

categorized as strong (30.7% & 33.3%), moderate (38.4% & 33.3%) and weak (30.7% & 33.3%) biofilm producer as depicted in Fig. 1 and Fig. 2. In nutshell, seven were strong biofilm producers; whereas, eight and seven isolates were emerged to be moderate and weak biofilm producers respectively. All the 4 clinical isolates of *Staphylococcus aureus* retrieved were found to be mecA gene positive as depicted in Fig.-3.

Fig-3: Detection of biofilm-associated mec-A gene by PCR

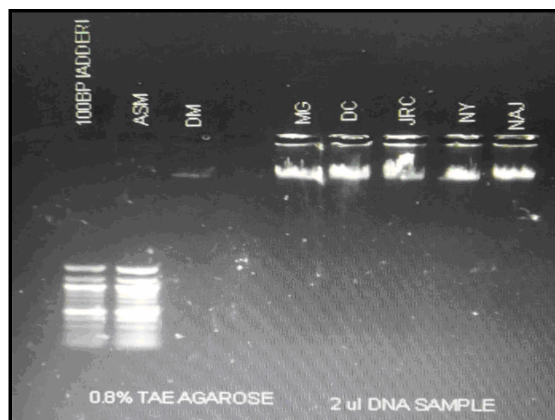


Fig-3: Image of mec-A gene on 0.8% TAE agarose gel. 1st sample and 2nd sample is DNA ladder of 100 kb, 3rd sample is ATCC 29213 methicillin-sensitive Staphylococcus aureus (MSSA) as a negative control, 4th sample is ATCC 43300 methicillin-resistant Staphylococcus aureus (MRSA) positive control.5,6,7,8 samples are clinical isolates tested from our study.

All the seven preformed strong biofilm producers were found to be sterile following treatment with CMC -AgNP-9.5 as described in second-set experiment. Those seven preformed strong biofilms when subjected to biofilm detection assay as per Stepanovic et al method showed fall in OD value as supported in Table 5 as described in third set experiment. The statistical analysis was done using one-way Anova (Graph pad Prism 9) software showed there is significant difference (p-value=0.0000; F-stat=40.8095; Critical value=4.747) between two groups i.e., before and after treating the preformed biofilm with CMC-AgNps-9.5. Data are expressed as means ± SD.

Table-5: Biofilm detection assay on Strong preformed biofilm isolates after treating them with CMC-AgNPs-9.5 using Spectrometer at 550nm.

Sl no.	Isolate tested	Negative control (media control)	OD value before challenging CMC-AgNp	OD value after challenging CMC-AgNp
1	*S.aureus strain 2*	0.26	4.07	1.3
2	*S.aureus strain 4*	0.26	5.5	1.23
3	*S.hemolyticus strain 3*	0.26	7.15	1.53
4	*S.hemolyticus strain 5*	0.26	6.27	1.3
5	*S.epidermidis strain 1*	0.26	3.56	0.89
6	*S.epidermidis strain 3*	0.26	4.9	1.12
7	*A.baumannii strain 2*	0.26	2.89	1.09

Discussion

Colloidal solution of AgNPs prepared using CMCas the stabilizing agent and dextrose as the reducing agent are considered safe, as CMC even at higher concentrations are used as topical eye drops [17]. MIC values of CMC-AgNPs-9.5 and equivalent ionic silver against different multi-drug resistant isolates determined by microdilution method showed much higher fold sensitivity for CMC-AgNPs-9.5. The term “bonus effect” was applied here as the “biological marker” of the nano-antimicrobial, mainly due to added antimicrobial action of Ag^0 by creating intra-cellular oxidative stress [18].

The mechanism of action of these nanoparticles is that they can bind to the bacterial surface mainly by electrostatic forces and split bi-layered lipid membrane with special affinity to negatively charged lipo-polysaccharide of Gram-negative bacteria [19] which in turn, causes greater permeability, osmotic disbalance and release of cytoplasmic contents. Silver atoms attack the respiratory chains at mesosome level of the bacterial cell

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membrane, resulting in cell death. Such antibacterial activities are mainly mediated by reactive oxygen species (ROS) [20] causing dephosphorylation of peptides on tyrosine residues and promotes cell death by influencing bacterial signal transduction [21].

In this study we found that CMC-AgNPs-9.5 did not show any considerable difference in MIC values between ATCC strains and planktonic MDR strains as AgNPs doesn't affect the multidrug resistance mechanisms instead they act by ROS generation irrespective of its MDR status. The antimicrobial efficacy using CMC-AgNPs-9.5 for the isolates were in the range of N/16384 dil. to N/131072 dil. compared to its ionic silver in equivalent concentration, also both MIC and MBC of all isolates using CMC-AgNPs-9.5 were same suggesting their greater and unique antimicrobial efficacy compared to conventional antimicrobials.

Methicillin-resistant *Staphylococcus aureus* (MRSA) contains SCC*mec,* a large mobile genetic element that includes the *mecA* gene, which mediates the upregulation of natural competence genes including extracellular toxins and surface structures especially under biofilm growth conditions are effective in the induction and continuance of infection in the host [22]. In our study we did find *mecA* gene by using PCR followed by gel electrophoresis in all the MRSA and biofilm positive isolates retrieved collaborating with the above statement.

AgNPs effectively prevent the formation of biofilms and kill bacteria in established biofilms, which suggests that AgNPs could be used for prevention and treatment of biofilm-related infections [23]. This study also showed excellent antibiofilm properties of CMC-AgNPs-9.5 of the preformed biofilm adherent to microtiter plate and using 4-fold MIC dilution of planktonic flora which was measured by optical densities (OD) of stained adherent bacteria at 550 nm.

Conclusion

It is a novel study involving 'out of the box' materials to show their antimicrobial and a considerable degree of anti-biofilm properties. All the strong biofilm producers were made sterile successfully in vitro as found in this study. However, as this is an in-vitro study, the extrapolation of results in living system needs further investigations preferably in animal model. The present study group is looking forward to continue this work with involvement of suitable animal model and considerable ethical issues in upcoming days.

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