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Silver Nanoparticles and their Role in Gene Expression of Motility Gene motB and Repression of AI-2-Controlled Gene

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Abstract:

Antibiotic resistance is the capability of the strains to resist or protect themselves from the effects of an antibiotic. Such a resistance towards the current antimicrobials leads to the search of novel antimicrobials. Nanotechnology has been promising in different field of science and among it is the use of nanoparticles as antibacterial agents. The gastrointestinal tract seems to be the primary reservoir of uropathogenic *E.coli* (UPEC) in humans. UPEC strains harbour the urinary tract and cause urinary tract infection. They cause serious ailments in terms of humans. They develop resistance and increase their virulence by forming biofilms. They also show a remarkable locomotory movement with the aid of autoinducer controlled genes (AI-2).

The present study is designed to investigate the expression levels of the AI-2 controlled genes and the motility genes in the presence of nanomaterials. RT PCR amplification together with the antibacterial and motility studies were done to compare the significant effect of silver nanoparticles on the pathogen. S-adenosylmethionine assay was also done to confirm the role of the treatment on the repression of the AI-2 genes.

The results showed silver nanoparticles have significant effect on the motility studies. The relative expression and repression of *motB* gene was under the control of AI-2 kinase protein. This confirms the possible role of the nanoparticles towards the pathogen UPEC. There was also an inhibition on the biofilm formation under the effect of the nanoparticle treatment. The study concludes that the silver nanoparticles could be used as a novel antibacterial agent against the UPEC strains and thereby inhibits the antibacterial resistance.

Key words: AI2, Motility genes, Nanoparticles ,SAM assay ,UPEC

Introduction:

Cytotoxic or cytostatic antibiotics against microbes allow the natural defence mechanism of the body to attack and kill them. They also inhibit the protein and transcript synthesis within the pathogen thereby stopping the growth of microbes (1). Some class of antibiotics also act on the membranes attacking the membrane pore complex and breaking the natural integrity of the cell membrane. Hence undoubtedly antibiotics are being classified into combat with the deadly and bothering pathogens and have saved more than millions. Owing to their robustness many different classes of antibiotics are discovered and used for therapeutic purposes (2).

Antibiotic resistance has been overgrowing within many strains of the pathogens and it is found they acquire such a capacity by transferring resistance-conferring genes via conjugative plasmids. As known, these extra chromosomal materials encode the genes which are needed by the bacteria for transferring the plasmid DNA. In due course of time, the recipient strain also attains the resistance power towards antibiotics (3). Antibacterial or antimicrobial agents are widely used in the textile industries, water purification plants, food and medicine processing industries. Some of these compounds which are organic in nature render toxicity to the humans and to the environment.

Hence inorganic disinfectants like nanoparticles (NPs) are used widely nowadays (4).

Bacterial infections are cosmopolitan in occurrence and are the major cause of many chronic and acute infections. The usage of antibiotics was practiced since decades owing to their effectiveness in treating the diseases. Moreover these are cost effective and known for their powerful outcomes (5). Many of the antibiotic resistance mechanisms are not applicable towards nanoparticles (NPs) as they act directly on the bacterial cell wall without even penetrating the cell. Hence biologists are focussing on these nanoparticles which are really resistant to antibiotic resistance mechanisms of bacteria. Hence more attention is being focused on using novel NP-based materials with proven antibacterial activity. Nanomaterials are those with dimensions in the nanometre scale and their basic unit exists as 3D space (6)NPs have proved in terms of antibacterial properties towards both Gram-positive and Gram-negative bacteria and exhibit a broad spectrum of range. For instance Zinc oxide NPs were effective against the **Staphylococcus aureus**, and Ag NPs were found to be potent against **Escherichia coli** and **Pseudomonas species** (7).

Nanotechnology-based discoveries promised for both the users and biologists in overcoming the drug resistance problems. Nanoparticles (NPs) are beneficial in the fields of medicine and veterinary science (8). Many of such nanostructures like silver and gold metallic particles are developed and employed against deadly microbial pathogens. Their effectiveness entirely depends on their interaction with the microorganisms. Recent studies reported that antibiotics when conjugated to NPs processes show a higher antibacterial activity than at individual level (9). This confirms the synergistic effect between the NPs and the antibiotics. Moreover they are more effective in terms of the antibacterial activity (10). Study done by Shahverdi in 2007 on **E. coli** and **S. aureus** in combination with penicillin G, amoxicillin, erythromycin, clindamycin, or vancomycin proved the synergistic activity between the Ag-NPs and antibiotics (11).

Urinary tract infections (UTIs) are the most widely spread nosocomial infections and are acquired in nature (12). Though they are not deadly or related to high mortality rates, they pose a serious threat to the risk of enhancing healthcare-associated costs (4). And these infections also lead to the usage of more antibiotics which aids in bacterial ecology and damage. Among such species which are commonly associated with the UTIs are uropathogenic Escherichia coli (UPEC). Recent studies have reported the multidrug-resistant UPEC strains which have increased the casualties of the infected patients (13). UPEC is found to form multi-cellular communities called biofilms on the surface of catheters and bladder walls. Bacteria stay in close proximity so as to aid in exchanging of genetic material relating to antimicrobial resistance.

Bacteria use quorum sensing mechanisms to up-regulate some of the genes which help in sensing their population density by quorum sensing molecules. Autoinducer 2 (AI-2) is expressed by LuxS which codes for protein S-ribosylhomocysteine lyase by both gram-negative and gram-positive bacteria and was found to be species-nonspecific. They aid in chemotactic movements, synthesis of flagellae and to produce virulence factors. These genes also play a vital role in formation of biofilms (14). In contrast, one autoinducer, designated AI-2, and its synthase, LuxS, are widely distributed in the bacterial kingdom, and AI-2 controls a variety of traits in different bacteria (15). These unique characteristics of AI-2 have led to the hypothesis that AI-2 is used for interspecies communication.

The present work is designed to study the effect of AI-2 controlled genes on the motility of the species with and without the treatment. Motility studies and gene expression studies were done to estimate the up-regulation or down-regulation of AI2 inducer to conclude the role of AI-2 in biofilm formation.

Materials and Methods:

Chemicals and consumables: All the chemicals used in the present study were obtained from the Sigma-Aldrich and HiMedia. The RNeasy Mini Kit (Cat No.74104), used for the total RNA extraction from cells & tissues were purchased from the Qiagen. The components like Random primers, polymerase and the 100bp DNA reference ladder, all purchased from the HiMedia. All set of primers, used in this project were designed using the primer design software, cross checked for the specificity using the Clustal W software and were purchased from the Eurofins pvt Ltd. Silver nanoparticles (10nm) were purchased from Sigma Aldrich, bangalore.

Bacterial strains: Uropathogenic *E.coli* (UPEC) (MTCC 729) was procured from the MTCC repository. The bacterial culture was maintained on nutrient agar plates at 37^0C for 24 hrs.

Antibacterial Assay: Antimicrobial activity of the silver nanoparticles (10nm) was tested against the pathogen. Overnight cultures were prepared in

Nutrient broth (NB) media by inoculation with a single colony from agar plates and incubated at 37^0C for 24 hrs. Overnight cultures were diluted with fresh NB media to approximately 10^6 colonies forming units (CFU) and used for further assays.

Antibacterial activity using the agar cup plate method: Culture grown to overnight in NB were pour plated onto Mueller-Hinton agar plates and performed for antibiotic susceptibility assay. Sterile paper discs were impregnated with varying concentrations (10,20,50,100 µg/µl) of silver nanoparticles and placed onto the plate equidistantly. The plates were then incubated at 37^0C for 24 hrs and observed for clear zone of inhibition.

Cultivation of biofilms: Biofilm formation studies were done according to Larkin (2017) by ethanol acetone method. Briefly, the overnight cultures from Luria Broth were taken and diluted to a ratio of 1: 200 using Luria Broth+Glucose solution (15). The contents were then transferred to Microtitre plate and incubated at 37^0C for 24 hrs. After incubation, the wells were washed 3 times with PBS (200µl in each well) and air dried. The wells were then stained with 2% crystal violet for about 15minutes and rinsed with tap water and air dried. The crystal violet was then solubilized in 200µl of ethanol:acetone 80:20 and the absorbance was measured at 590nm. Inhibition percentage of biofilm was calculated by the formula (%inhibition= $[(OD_{control}-OD_{sample}) / OD_{control}] \times 100$

RNA Extraction: The bacterial culture in NB was used for the extraction process. The experiment was done for all treatments including the control. The culture along with the medium was used in the extraction process. Briefly the total RNA was extracted by using an RNeasy minikit. The protocol was followed according to the instructions provided by the manufacturer. The cells were further suspended in 2ml of PBS followed by centrifugation at 6000rpm for 5min. The pellet obtained was re-suspended in 560µl of A1 buffer. 560µl chilled ethanol was then added to the contents and mixed thoroughly. The contents were centrifuged again at 8000rpm for about 1min to make the RNA bind to the column. About 700µl of wash buffer was added and centrifuged at 15sec followed by 60µl of A2 buffer. The RNA was now collected in the collection tubes and stored at -20c for further. The quality of RNA was checked by the UV spectrophotometer. Total RNA obtained was used for cDNA synthesis.

RT PCR cDNA synthesis: The synthesis was carried by the RT PCR kit using Superscript TMII Reverse transcriptase, 200U/µl (Cat no: 18064). 2µg of the RNA was used as the starting reaction. The RNA concentration obtained was about 1.42µg/µl. 1.9µl of the total RNA was used for the reaction. Random primers were used in this study. 1µl of RT enzyme was added and mixed thoroughly. The contents were incubated at 25C for 10min, followed by the incubation at 70C for 50min. The obtained cDNA was used for PCR analysis(Table 1).

Table 1 : Displaying the period PCR primers, melting temperature , GC content material and sequence ,PCR amplification of motility and Autoinducer members in samples:

		Length	Tm	GC%	Sequence	Product length
motB	FW	20	58.95	50	TATGACTGCGATGATGGCCT	174bp
	RV	20	58.99	55	GTGTAATCATCACCACCGCC	
lsrk	FW	20	58.98	50	CTGTCAAAGAAACCGGCACA	241bp
	RV	20	58.99	50	ACATGAGGATTAACGCGCAC	

Primers used in this study were designed to detect expression of motility protein B and autoinducer-2 kinase (gene ID: 946402 and 946069, respectively). PCR amplification was performed, on the sample (both Control and treatment) in the thermocycler cycler (Eppendorf). The motility B primers (motB) of about 600nM concentration, were used for the amplification program, and the total reaction volume was carried out in a total volume of 50µl. The PCR mixture consisted of 10x reaction buffer with MgCl2 (1.5mM), 2µL of dNTP mix (2.5mM), 2µL each of forward and reverse primers (10picomoles/µl each primer), 0.3µL of Taq DNA polymerase (5 U/µL), and 50ng/µL of template DNA in a total volume of 20µL. The reactions were carried out in duplicates, and run in parallel with their respective negative controls (water). 1µl of the cDNA products was initially used and the PCR was performed with the following cycling profile: initial denaturation at 94°C for 3 min, followed by 30 cycles of 30s denaturation at 94°C, annealing at 62°C for 30s, and extension at 72°C for 1min. The time for the final extension step was increased to 3 min. The

same parameters were maintained for AI-2 kinase (*lsrK*)

. The PCR products amplified were then qualitatively analysed on 1% agarose gel.

Cell lysate: The bacterial cells before and after treatment with the nanoparticles were collected by centrifuging at 8000rpm for 10 minutes at 4°C. The pelleted cells were homogenized in 1-2mL of cold PBS (pH 6.8). The contents were then centrifuged at 10,000 rpm for 15min at 4°C. The supernatant was collected and stored on ice until further use. S.adenosyl methionine assay was carried according to B L Hanzelka al (1996)(16).

S-adenosyl methionine (SAM) containing 0.1% BSA was used as standard. SAM and S-adenosylhomocysteine (SAH) were measured as described by(17). The SAM-BSA standard was aliquoted into 7 tubes labelled as 1-7. Varying concentrations (0-100µg/ml) of nanoparticles were added into test tubes. 200µL of 0.1 mol /L sodium acetate, pH 6.0, and 160µL 40% trichloroacetic acid was added and incubated for 30 min on ice. The tubes were mixed thoroughly and SAM and SAH were detected at 254nm using UV spectrophotometer. The samples with and without treatment collected earlier were assayed in the same method. The concentration of SAM left behind was calculated to assay the autoinducer activity.

Statistical analysis: All the experiments which are statistically significant were analysed using the MS excel 2010, and the experiments were done in triplicates. The results were statistically significant and remembered at

$P \leq 0.05$.

Results:

Antibacterial activity using the agar cup plate method: From the disk diffusion assay it was observed that the bacterial culture was susceptible to the silver nanoparticles treatment. AgNPs treatment was showed to be dose- dependent with maximum inhibition noticed at 100µg/µl AgNPs (Fig.1). Since 100µg/µl was found to show maximum activity, 50µg/µl of concentration was chosen as optimum for the study.

Figure 1: Image showing the antibacterial assay by disc diffusion method. C: control. 4: 100µg/µl.

Cultivation of biofilms: from the biofilm studies it was observed that the results are in accordance with the antibacterial disc diffusion assay. The inhibition percentage was found to be 8, 28, 63 and 89% for 10, 20, 50 and 100µg/µl respectively. Inhibition percentage was taken as 0% for the control. OD of the control was found to be 1.21. The results were dose dependant(Fig. 2 and 3).

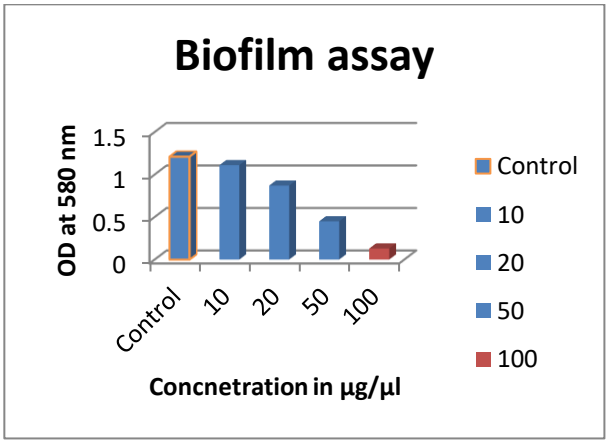

Figure 2: Graph showing the OD values for the biofilm assay. Control without treatment. All the values were average of triplicates. Values are expressed as value ± s.e (standard error)

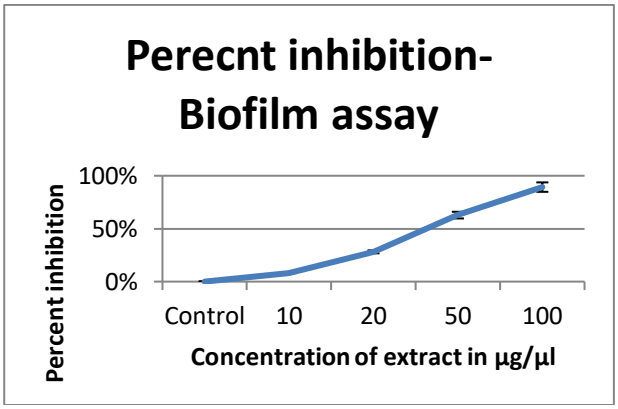

Figure 3: Graph showing the OD values for the biofilm assay. Control was without treatment. All the values were average of triplicates. Values are expressed as value ± s.e (standard error)

RNA Extraction and PCR amplification of the gene members: The culture in NB was used for the extraction process. The experiment was done for all the treatments including the control. The culture along with the medium was used in the extraction process. 2µg of the RNA was used as the starting reaction. The RNA concentration obtained was about 1.42µg/µl. Hence 1.9µl of the total RNA was used for the reaction. When the cDNA was used for amplification of the gene members, the results were found to be in accordance with the biofilm assay. In case of motB gene expression, the expression was found to be decreased when compared to the control under the treatment (50µg/µl). The intensity of the band was used for the comparison of the gene members. Similar results was noticed with the AI2 gene member as well. The intensity was found to be less when compared to the control. AI2 gene member was found to be more in control and very less for the treatment. This confirms that the nanoparticle treatment was found to have an inhibitory effect on the growth of the UPEC strains. This can be concluded by the under expression of the motility genes *mot*B and AI2 expression members(Fig.4 and 5).

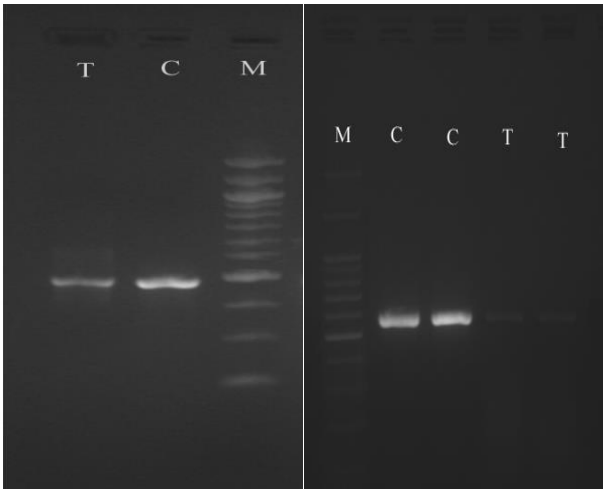

Figure 4: Left: Gel showing the image of motB gene expression. M: Molecular marker (50bp); C: control; T: Treatment (50µg/µL). Right: Gel showing the image of AI-2 gene expression. M: Molecular marker (50bp); C: control; T: Treatment (50µg/µL). 1.5% agarose gel was used in the run.

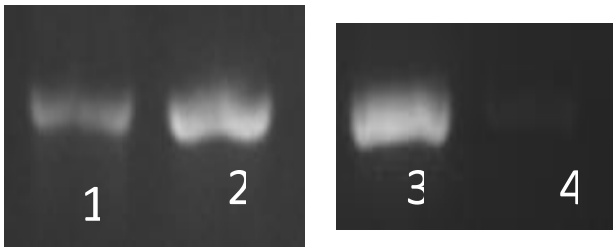

Figure 5: gel images showing the expression of the members only. Left: control and treatment of motB gene; Right: control and treatment of AI2 gene.

SAM assay: The left over SAM was calculated from the standard curve derived with ($y = 0.0044x$ $R^2 = 0.9859$). The concentration of SAM left behind was calculated to assay the autoinducer activity. The concentration of the SAM left over was found to be decreased with the dosage of treatment. The concentration of the SAM was found to be 117.69, 96.37, 63.22 and 18.23µM for control, 20, 50 and 100µg/µl respectively. As the dosage increased the SAM value reduced which corresponds to the reduction in autoinducer activity which ultimately leads to the inhibition of biofilm formation(Fig 6).

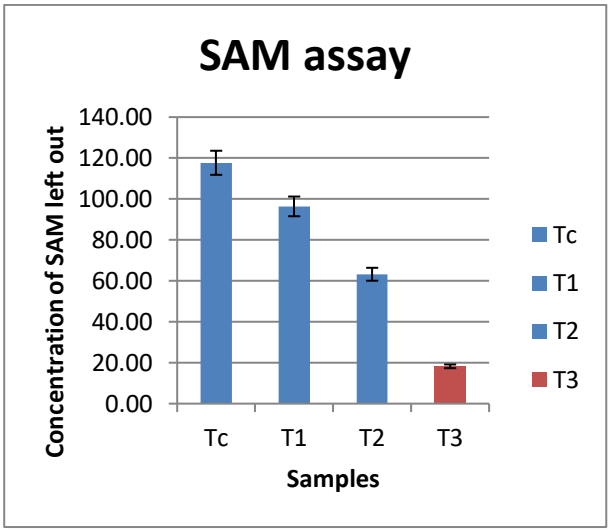

Figure 6: Graph showing the S.adenosyl methionine assay with the values of SAM left out after the treatment. All the values are average of triplicates. Values are expressed as concentration ± s.e (standard error).

Discussion:

Biofilms represent an earnest concern in medicine, as they contribute to chronic infections, complicate wound healing, contaminate medical devices, and exhibit resistance to conventional antimicrobials.

In this study, we confirmed that AgNPs is effective in eliminating planktonic populations of bacterial growth and biofilm formation of UPEC strains(18). In the presence of the treatment, UPEC strains were found to be susceptible and the inhibition in growth and biofilm was dose dependent , Figure (2). As the concentration increases, the growth also got reduced, Figure (1). The study primarily focused on how the treatment affects the gene expression especially the members which aid in the biofilm formation. The present result is in accordance with the previous report showing the viability of E. coli AB1157 cells in biofilms was considerably reduced by AgNPs concentrations above 100 −150 μg/ml. E. coli strains with mutations in genes responsible for the repair of DNA containing oxidative lesions (mutY, mutS, mutM, mutT, nth) were less resistant to AgNPs than wild type strains. This suggests that these genes might be involved in the repair of DNA damage caused by AgNPs (19). Motility B (motB) was found to be under expressed along with the AI2 repression system. This under expression explains clearly the major role of motB and AI-2 genes in the formation of biofilm and antibacterial resistance.

Additionally, the present study is very informative in finding out the AI-2-controlled genes (motB and AI-2 kinases) and serves to elucidate the significant effect of silver nanoparticles in regard to AI-2 signaling.

Conclusion:

Our results indicate that the biofilm forming genes are under the control of the AI2 repression system and as such both play a coordinating role in forming biofilms and pathogenesis. From the study we conclude that silver nanoparticles are having a significant negative effect on the growth of the UPEC strains. As noted from the previous reports UPEC strains are gaining antibacterial resistance to many of the antibiotics. As such the diseases incidence has been growing to vast extent. This leads to the development of pathogenesis and antibacterial strains as such it is of mandate to design and hunt for novel drug targets and antimicrobials which can reduce the invasiveness of the pathogenic strains especially UPEC.

Author's declaration:

- Conflicts of Interest: None.
- I hereby confirm that all the Figures and Tables in the manuscript are mine. Besides, the Figures and images, which are not mine, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Technology.

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الجسيمات النانوية الفضية ودورها في التعبير الجيني للجينات الحركية *motB* وقمع الجينات التي تسيطر عليها بواسطة AI-2

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الخلاصة :
مقاومة المضادات الحيوية هي قدرة السلالات على المقاومة أو الحماية من تأثيرات المضاد الحيوي. هذه المقاومة نحو مضادات الميكروبات الحالية أدت إلى البحث عن مضادات الميكروبات الجديدة.حيث استخدم النانوتكنولوجي بالعديد من فروع العلوم المختلفة ، وأحدها هو استخدام الجسيمات النانوية كعوامل مضادة للبكتيريا. يبدو أن القناة الهضمية هي المستودع الأساسي لـ UPEC عند البشر. تحوي سلالات E.coli Uropathogenic UTI وتسبب التهاب المسالك البولية الخطير للبشر . حيث قامت بتطوير المقاومة وزيادة الضراوة من خلال تشكيل الأغشية الحيوية. كما أنها تظهر حركة حركية ملحوظة بمساعدة الجينات التي تسيطر على(AI-2) autoinducer.
الدراسة الحالية هي لمعرفة مستويات التعبير للجينات المضبوطة AI-2 والجينات الحركية في وجود المواد النانوية. تم التضخيم RT PCR جنبا إلى جنب مع دراسات مضادة للجراثيم والحركة لمقارنة تأثير كبير على الممرض. تم إجراء اختبار S-adenosylmethionine أيضا لتأكيد دور العلاج على قمع الجينات AI-2.
وجد أن جسيمات الفضة النانوية أظهرت تأثيرًا كبيرًا على دراسات الحركة. تم التعبير عن التعبير النسبي لجينات motB مع قمع بروتين AI-2 kinase. هذا يؤكد الدور المحتمل للجسيمات النانوية تجاه الممرض UPEC. كان هناك أيضا تثبيط على تشكيل بيوفيلم تحت تأثير العلاج جسيمات متناهية الصغر. وخلصت الدراسة إلى أن الجسيمات النانوية الفضة يمكن استخدامها كعامل مضاد للبكتيريا جديد ضد سلالات UPEC وبالتالي توقف المقاومة المضادة للبكتيريا.

الكلمات المفتاحية : محفز تلقائي 2 , جينات الحركة , اجسام نانوية , فحص S-أدينوزيل مثيونين ,الايكولاي المسببة لالتهاب المسالك البولية.