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Efficacy of Green Silver Nanoparticles (GAbNPs) against Multidrug Resistance in *Staphylococcus aureus* from Mastitis-infected Cows

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ABSTRACT

Background: Antimicrobial resistance being the major emerging problem for management of mastitis in cow hence, the research work was undertaken in a geographical area around Shirwal town in Satara district of Western Maharashtra, India to investigate the occurrence of multidrug resistance in *Staphylococcus aureus* (MDRSA) isolated from mastitic cow milk samples. Further assessment was carried out for antimicrobial efficacy of formulation of Green Silver Nanoparticles (GAgNPs) synthesized from *Azadirechta indica* against MDRSA of mastitis origin.

Methods: A total of 250 milk samples from cows suspected of mastitis were screened by the California Mastitis Test (CMT). The CMT-positive samples were subjected to bacteriological investigation for the presence of *S. aureus* as an etiological agent. The isolates identified as *S. aureus* were further authenticated by the molecular method targeting 16s-rRNA and *nuc* gene. All isolates of *S. aureus* were subjected to an antimicrobial susceptibility test. The randomly selected MDRSA isolates were subjected to antimicrobial susceptibility testing with GAgNPs formulation were prepared from silver nitrate by reducing with *Azadirachta indica* leaf extract.

Result: Out of 250 cow milk samples suspected for having mastitis were screened with CMT, 170 tested positive. From these milk samples 145 isolates of *S. aureus* were isolated and bacteriologically identified. When processed for antibiotic sensitivity 60 were detected having multidrug resistance. Maximum resistance was shown against beta-lactam antibiotics. GAgNP synthesized and characterized with visual appearance, spectroscopy and TEM (Transmission electron microscopy) and tested for *in vitro* antimicrobial efficacy against MDRSA showed significant antimicrobial property.

Key words: AMR, *Azadirachta indica,* Green silver nanoparticles, Mastitis, MDR *S. aureus*.

INTRODUCTION

Mastitis in dairy animals is one of the most significant diseases costing the nation substantially in terms of economic output. Mastitis represents around 38% of the total direct costs of common industrial infections globally (Kossaibati and Esslemont, 1997). In India, mastitis-related economic losses have increased by roughly 115 times during the past five decades (Dua, 2001). Bacteria are more likely accountable and most frequently enter the teat canal through the teat opening, multiply quickly and then generate toxins and other enzymes, which trigger an inflammatory response. Staphylococcus is one of the main bacterial genera that cause mastitis in dairy cattle. *S. aureus* is one of the leading sources of intramammary infections in dairy cows (Dufour *et al*., 2012), which has a severe negative impact on the health of animals and the level of production as a whole. Antimicrobials have been used extensively and will continue to be utilized in veterinary and human medicine to treat bacterial ailments. However, drug-resistant bacteria are one of the emerging threats in the world today. Multidrug-resistant bacteria spreading into the community is a critical trend that is linked to higher morbidity, death, healthcare expenses and antibiotic use. To overcome the crisis of drug resistance, other alternatives are being researched and one of them is the use of nanoparticles. However, nanoparticles of metal origin come with certain risks to health and the environment. Hence, the quick, environmentally safe, nonpathogenic and economical protocol of using plants to produce green nanoparticles has gained interest in recent years, since it offers a single-step technique for the biosynthetic process. The simplest

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and least expensive method to make nanoparticles is to reduce and stabilize metal silver ions using a mixture of biomolecules that are already present in plant extracts, such as proteins, amino acids, polysaccharides, terpenes, alkaloids, phenolics, saponins and vitamins (Roy *et al*., 2017). Plant-assisted reduction is the primary mechanism for this process because of phytochemicals. The relatively high concentrations of the reducing agents (steroids, saponins, carbohydrates and flavonoids) and capping agents (phytoconstituents) ensure the stability of the green nanoparticles (Sondi and Salopek-Sondi, 2004).

MATERIALS AND METHODS

A total number of 250 HF-cross breed cows belonging to different unorganized farms located in and around Shirwal town, Dist. Satara, Maharashtra, India, were screened for the presence of mastitis by California Mastitis Test (CMT). CMT-positive samples were considered for further processing. Samples were brought to the laboratory on ice and processed immediately. Collected mastitic milk samples were subjected to the isolation of organisms as per the standard methods described in Bergey's Manual of Systematic Bacteriology, 1986. The samples were inoculated on media such as Blood agar and Nutrient agar and other selective and differential media such as Mannitol salt agar (MSA), MacConkey agar and Eosin methylene blue (EMB) agar. Inoculated plates were incubated aerobically at 37°C for 24 hours and examined for growth, pigmentation, hemolysis and colonial morphology. Yellow, round colonies presumptive of *S. aureus* were further confirmed by biochemical tests and PCR assay. Extraction and purification of DNA of *S. aureus* was carried out by snap chill method (Alwan and Talak, 2015). Briefly, pure colonies were selected from the Mannitol Salt agar and mixed with 150 µl of nutrient broth. Then this mixture was boiled for 10 min at 91°C and immediately kept in crushed ice for snap chilling for about 10 min. After chilling tubes were centrifuged at 10000 rpm for 10 min. The supernatant was used as template DNA. Purified DNA were processed for *S. aureus*-specific 16s-rRNA gene (Akindolire *et al*., 2015) as well as species-specific *nuc* gene PCR (Zhang *et al*., 2004). The primer sequences employed were 16s-rRNA F 5′GTAGGTG GCAAGCGTTACC3′ 16s-rRNA R 5'CGCACATCAGCG TCAG3′ and *nuc* F 5′GCGATTGATGGTGATACGGTT3′ *nuc* R 5′AGCCAAGCCTTGACGAACTAAAGC3′, respectively. The reaction was carried out in 25 µL volumes. Each PCR reaction mixture comprised 12.5 µL Himedia PCR mix, 1 µL each of forward and reverse primers, 3 µL of DNA and volume was made up to 25 µL using nuclease-free water. PCR amplification conditions for 16s-rRNA consisted of: Initial denaturation at 94°C for 05 min; followed by 30 amplification cycles of denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec and extension at 72°C for 1 min, final extension step at 72°C for 5 min, before cooling to 4°C. To carry out PCR amplification for *nuc* gene, the master mix was the same as described above, The PCR conditions were as follows: Initial denaturation at 94°C for 05 min; followed by 30 amplification cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min, final extension step at 72°C for 10 min, before cooling to 4°C (Table 1).

Antibiogram sensitivity patterns of target organism *S. aureus* isolates (n=170) from mastitis milk were interpreted using the Kirby Bauer disc diffusion method (Bauer, 1966) using commercially available antibiotic discs of M/s HiMedia Laboratories Pvt. Ltd., India, as per Clinical and Laboratory Institute (CLSI, 2018) guidelines (Table 2). Studies were aimed at the detection of AMR in *S. aureus* isolated from mastitis against multiple classes of antibiotics hence, analysis of multiple antibiotic resistance and the genes expressing it in *S. aureus* was performed by PCR with multiple oligonucleotide sequence primers (Table 3). AMR genes like *mec*A gene for methicillin, *tet*K, *tet*M for Tetracycline, *erm*A, *erm*C for Macrolide, Lincosamide, *aac*A for Aminoglycosides, *van*A, *van*B genes for Vancomycin resistance, *mrs*A, *mrs*B genes for Macrolide resistance were evaluated in three different sets of Multiplex PCR. In set I Multiplex PCR of *mec*A, *erm*A, *erm*C, *tet*K, *tet*M, *aac*A, While Set II Multiplex PCR was performed for *van*A and *van*B and Set III for *mrs*A and *mrs*B genes. For efficacy studies of GAgNP 10 isolates of exhibiting resistance to more than three antibiotics identified as MDRSA were randomly chosen having CFU much above permissible reference range (Mean range of 7.161±0.00866 to 9.083333±0.025783) for efficacy studies of GAgNPs.

For this study preparation of green silver nanoparticles were synthesized from Silver Nitrate reduced with *Azadirachta indica* leaf extract as per Roy *et al*. (2017) with slight modifications (Fig 1). Fresh green fully grown leaves were collected and thoroughly washed, twenty grams of it were finely chopped, added to 100 ml of double-distilled water and boiled for 10 min. The extract was cooled, filtered and stored in the refrigerator at 4°C-8°C until further use. Silver nitrate solution 0.1N (HiMedia Pvt. Ltd., India) was used for the green synthesis of GAgNPs. A set of 05 clean sterile test tubes were taken and labelled. Then 1 mL, 2 mL, 3 mL, 4 mL and 5 mL of neem extract respectively, were added to test tubes and 1ml of silver nitrate solution was added to each test tube. The test tubes were covered with silver foil and the entire set was incubated in the dark chamber to minimize the photo-activation of silver nitrate at room temperature for 48 hours. The color change from colorless to brown confirmed the reduction of silver ions. The optical density of the extract was measured to determine the concentration of GAgNPs formed by taking 50 µL of each in an ELISA plate and using an ELISA reader at different wavelengths of 405 nm, 420 nm and 450 nm, to record peak absorbance. The synthesis of GAgNP, their size and their antimicrobial activity against random ten isolates with MDRSA was evaluated with the Agar well diffusion method, MIC. Also Transmission Electron microscopy (TEM) images of GAgNP were taken by an Electron Microscope (JEOL JEM- 1011 100 kV model) (Fig 2).

To determine the effective GAgNP prepared from the suitable ratio of silver nitrate to neem leaf extract for implementation in the actual efficacy studies, it was first assessed for its efficacy by agar well diffusion test as per Roy *et al*. (2017) in five different ratios of neem extract to silver nitrate: N1 (1:1), N2 (2:1), N3(3:1), N4(4:1), N5(5:1).

The antimicrobial activity of the prepared GAgNP was assessed by performing the MIC using the method described by Loo *et al*. (2018) with slight modification. The MIC was performed in a 96-well flat-bottom micro-titer plate using the broth micro-dilution method. The bacterial inoculums were adjusted to the concentration of 10^5 CFU/mL samples as described in the manual of the American Society for Microbiology (2016). The 100 µL stock solution of GAgNPs were added in duplicate plates, one for RPMI and other for MBC. The drug was further diluted two-fold in 100 µL of NB starting from the highest concentration in column 12 to the lowest in column 03 while, column 01 served as the negative control (only medium) and column 02 served as the positive control (medium and bacterial inoculums). Each well of the microtiter plate was added with 100 µL of bacterial inoculums except negative control. Every well was added with 30 µL of the Resazurin solution and readings were taken after incubation at 37°C for 3 h, 6 h, 9 h, 12 h and 24 h, respectively. The mixture in every well was observed for visible change in color as per protocol of test, wherein, blue /purple color indicated no bacterial growth while the pink color or no change in color indicated bacterial growth in the well. Simultaneously, MBC (minimum bactericidal concentration) was checked by inoculating the mixtures of drug dilutions and bacterial inoculum mixtures in every wells of ELISA plate by inoculating it on agar plates and observing plates for any growth of MDRSA after incubation of 24 h. The lowest effective broth dilution of GAgNPs was considered as effective MBC value. The synthesized GAgNPs were also comparatively tested for antibacterial properties against commercially available nanoparticles procured from GreenVision Life Sciences Pvt. Ltd., Pune, India, against MDRSA.

RESULTS AND DISCUSSION

Bacterial isolation and identification

From the suspected cases of symptomatically mastitis positive 250 cows screened, 170 were found CMT-positive out of which 30 cows had clinical mastitis with +++ reaction and the remaining 140 showed a reduction in average milk production with samples being ++ and + CMT reaction. The gram-positive organisms isolated were *S. aureus* 145/170 (85.29%), *Bacillus* spp. 40/170 (23.52%), coagulase-negative *Staphylococci* spp. 25/170 (14.70%), unidentified gram-positive bacillus 10/170 (5.88%) and *Streptococcus* spp. 02/170 (1.17%). Major gram-negative organisms isolated from mastitis milk samples of cows were *E. coli* 100/170 (58.82%). *S. aureus* being the target organism of the present investigation, all 145 isolates identified phenotypically and biochemically were reconfirmed by conventional with 16S-rRNA gene PCR showing 228 bp product (Fig 3A) on similar lines with Andrade *et al*. (2021) and species-specific 279 bp product of *nuc* gene (Fig 3B).

Antimicrobial susceptibility of *S. aureus* organisms isolated from cow mastitis

In the present research only MDRSA were considered for the antimicrobial efficacy studies against GAgNP hence,

Table 1: Oligonucleotide sequences of primers used in the studies of AMR genes of *S. aureus*.

Gene	Primer	Oligonucleotide sequence(3′-5′)	Amplicon size (bp)
*mec*A	*mec*A-F	AAAATCGATGGTAAAGGTTGGC	532
	*mec*A-R	AGTTCTGCAGTACCGGATTTGC	
*erm*A	*erm*A-F	AAGCGGTAAACCCCTCTGA	190
	*erm*A-R	TTCGCAAATCCCTTCTCAAC	
*erm*C	*erm*C-F	AATCGTCAATTCCTGCATGT	299
	*erm*C-R	TAATCGTGGAATACGGGTTTG	
*tet*K	*tet*K-F	GTAGCGACAATAGGTAATAGT	360
	*tet*K-R	GTAGTGACAATAAACCTCCTA	
*tet*M	*tet*M-F	AGTGGAGCGATTACAGAA	158
	*tet*M-R	CATATGTCCTGGCGTGTCTA	
*aac*A-D F	aacA-D	TAATCCAAGAGCAATAAGGGC	227
*aac*A-D R	aacA-D	GCCACACTATCATAACCACTA	
*mrs*A	*mrs*A-F	GGCACAATAAGAGTGTTTAAAGG	940
	*mrs*A-R	AAGTTATATCATGAATAGATTGTCCTGTT	
*mrs*B	*mrs*B-F	TATGATATCCATAATAATTATCCAATC	595
	*mrs*B-R	AAGTTATATCATGAATAGATTGTCCTGTT	
*Van*A	*Van*A-F	GTAGGCTGCGATATTCAAAGC	231
	*Van*A-R	CGATTCAATTGCGTAGTCCAA	
*Van*B	*Van*B-F	GTAGGCTGCGATATTCAAAGC	330
	*Van*B-R	GCCGACAATCAAATCATCCTC	

the results of 60 MDRSA isolates detected showing resistance against different classes of antibiotics were analyzed. Out of 145 isolates of *S. aureus*, 60 isolates were found to have AMR by ABST as shown in Table 2. Further, 60 MDRSA isolates demonstrating multi-drug resistance were subjected to genotypic characterization of resistance genes (Fig 4.). As shown in Table 4, overall, *erm*A gene was found in the highest number of samples, followed by *aaca*-D, *tet*K, *erm*C, *mec*A, *van*B and *mrs*B. The genes *tet*A, *mrs*A and *van*A were not present in any of the isolates tested. It was thus interpreted that in MDRSA highest resistance was displayed to ß lactam group of antibiotics followed by the macrolide group of antibiotics. This could be due to ß lactam group of antibiotics being commonly used to treat mastitis and subclinical mastitis and macrolide antibiotics are preferred to treat penicillin-resistant infections in the area. Significant resistance was noted for the tetracycline group could be because of the indiscriminate use of this antibiotic against mastitis prophylaxis along with other non-specific infections, parasitic/haemo-protozoan infections in ruminants in the study area in animals. Because glycopeptides are not frequently utilized in veterinary services, there are very few reports on the prevalence of Vancomycin-Resistant *S. aureus* (VRSA) in animals. Although, the possibility of VRSA contamination of the surrounding environment or pastureland and subsequent colonization of domestic animals cannot be completely ruled out (Bhattacharyya *et al*., 2016) which could be the reason for the considerable resistance to vancomycin found in the current study. Overall moderate resistance was recorded against aminoglycosides and quinolones although streptomycin and norfloxacin showed higher degree of resistance. The least resistance was observed for the phenicol group of antibiotics as the use of chloramphenicol is not very common in the treatment of animals in study area (Fig 5). Similar findings were noted by Sharma *et al*. (2015), with 100% Penicillin resistance in *S. aureus* isolated from mastitis-affected cows whereas, Ramasamy *et al*. (2021) and Shrestha *et al*. (2021) had noted 100% resistance to Ampicillin in *S. aureus* isolated from mastitic cows. Chandrasekaran *et al*. (2014) had detected 100% Methicillin resistance in *S. aureus* isolated from mastitic cows, which was higher, while resistance to penicillin detected by them was lower than the current findings. In concurrence to our study Ramasamy *et al*. (2021) and Ali *et al*. (2021) reported complete to very high resistance to

Table 2: The result of antibiotic sensitivity and resistance pattern of MDRSA isolates (n=60) of bovine mastitis.

Group of antibiotic	Antibiotics (mcg conc.)	Sensitivity (%)	Intermediate sensitive (%)	Resistance (%)	X^2
ß lactam	Penicillin G (02)	0 (0)	0 (0)	60 (100)	35.694*
	Cloxacillin (01)	0 (0)	0 (0)	60 (100)	
	Methicillin (30)	7 (11.67)	0 (0)	53 (88.33)	
	Ampicillin (10)	0 (0)	0 (0)	60 (100)	
	Amoxycillin- clavulanic Acid (30/15)	0 (0)	0 (0)	60 (100)	
	Amoxycillin-Sulabactum (30/15)	0 (0)	0 (0)	60 (100)	
Tetracycline	Oxytetracycline (30)	3 (5)	9 (15)	48 (80)	
Aminoglycoside	Streptomycin (10)	18 (30)	0 (0)	42 (70)	43.278*
	Gentamicin (50)	50 (83.33)	3 (5)	7 (11.67)	
	Amikacin (30)	32 (53.33)	3 (5)	25 (41.67)	
Quinolones	Ciprofloxacin (10)	39 (65)	0 (0)	21 (35)	18.701*
	Enrofloxacin (10)	43 (71.67)	0 (0)	17 (28.33)	
	Norfloxacin (10)	22 (36.67)	0 (0)	38 (63)	
Phenicol	Chloramphenicol (30)	52 (86.67)	0 (0)	8 (13.33)	
Glycopeptide	Vancomycin (10)	18 (30)	0 (0)	42 (71.67)	
Macrolide-Lincosamide	Azithromycin (30)	3 (5)	0 (0)	57 (95)	

*Significant at P≤05.

Table 3: PCR conditions for the amplification of AMR genes.

Cycling conditions		Temperature	Time	Temperature	Time	Temperature	Time
		SET - I		SET - II		SET - III	
Initial denaturation (1 cycle)		94°C	4 min	94°C	2 min	94°C	10 min
35 cycles of	Denaturation	94°C	30 sec	94°C	1 min	94°C	1 min
	Annealing	55°C	1 min	54°C	1 min	54°C	1 min
	Extension	72°C	1 min	72°C	1 min	72°C	2 min
Final extension (1 cycle)		72°C	5 min	72°C	10 min	72°C	10 min

oxytetracycline. Similar to our findings Siddiki *et al*. (2019) and Pu *et al*. (2014), had found 70% resistance to Streptomycin and 11.17% resistance to Gentamicin in *S. aureus* isolated from bovine milk. In the ABST pattern for Gentamicin, higher resistance rates than those seen in the current study were reported by Brahma *et al*. (2022). In correlation with the results of our study Mushtaq *et al*. (2019) had reported 100% sensitivity to Enrofloxacin. The sensitivity to Chloramphenicol for *S. aureus* reported by Nelli *et al*. (2022) was closely similar to the present study. As compared

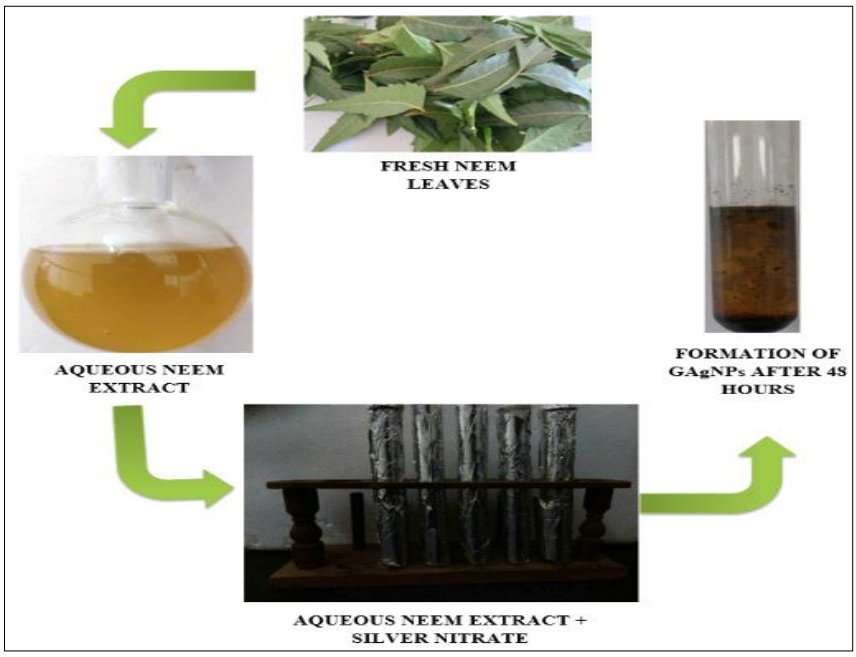
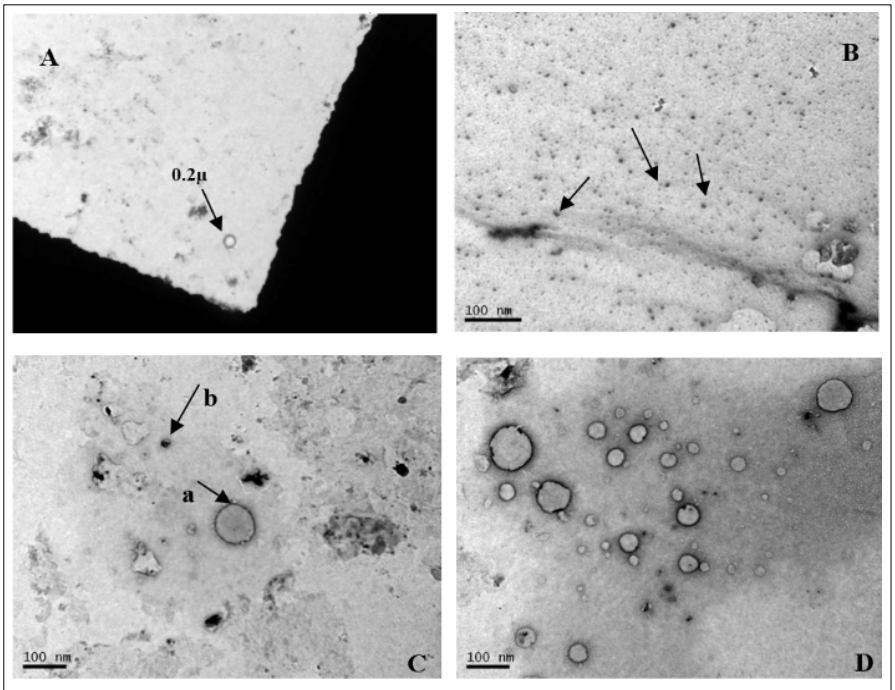


Fig 1: Synthesis of GAgNPs from aqueous neem extract (*Azadirachta indica*) leaves extract.



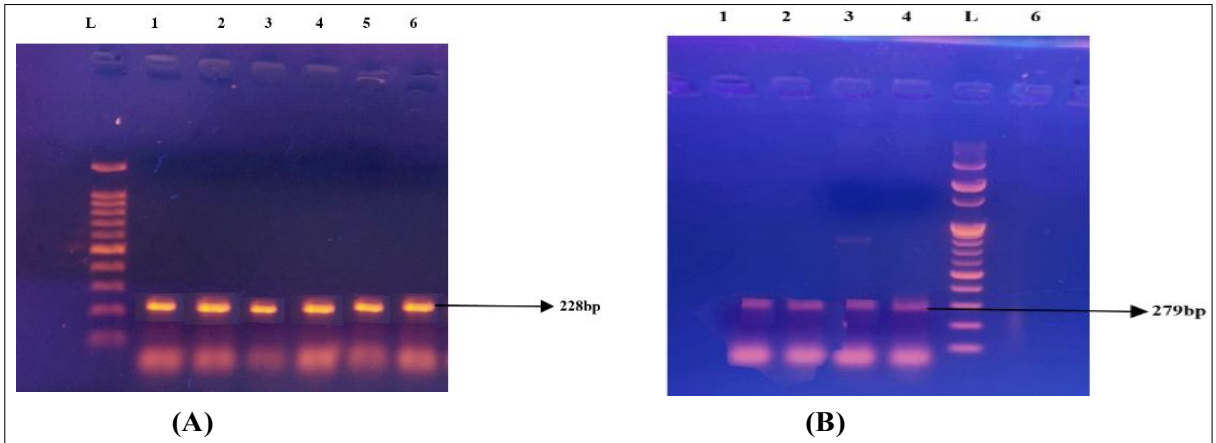
(A) *S. aureus* size-0.2 ì, (B) Scattered GAgNPs size ranging from 10-70 nm, (C) arrow a. attachment of GAgNPs to *S. aureus* cell, whereas arrow b. Agglomerates of GAgNPs and (D) showing lysis of *S. aureus* cells by GAgNPs.

Fig 2: Transmission electron microscopy (TEM) images of green silver nanoparticles (GAgNPs) prepared from aqueous neem extract.

to results of our studies Sharma *et al*. (2014) had documented higher resistance while Singh *et al*. (2016) had noted slightly less resistance to Vancomycin earlier.

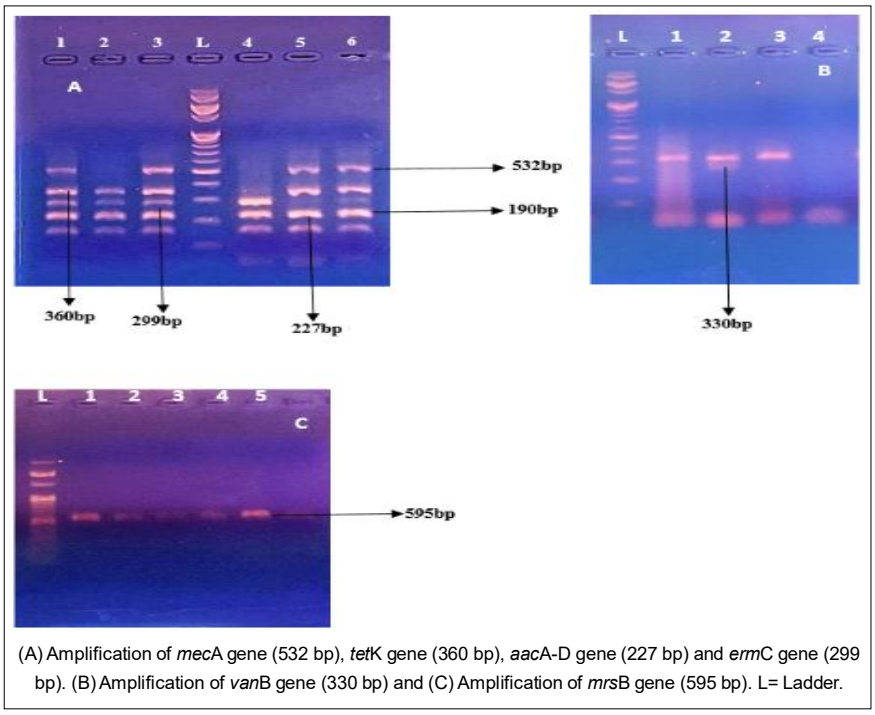
Biosynthesis and characterization of GAgNPs

Green silver nanoparticles were synthesized successfully for the present research from Silver Nitrate reduced with *Azadirachta indica* leaf extract on the similar lines with Roy *et al*. (2017). Characterization studies of GAgNP were done by assessment of OD values of every sample prepared and the results of absorbance obtained of N1, N2, N3, N4 and N5 at 405 nm wavelength were 0.533, 0.84, 1.146, 1.444 and 1.531, respectively. At 420 nm the absorbance recorded was 0.192, 0.273, 0.357, 0.517 and 0.604. And at 450 nm the absorbance was found to be 0.147, 0.187, 0.233, 0.279 and 0.477. Thus, the maximum absorbance values were recorded at 405 nm. Characterization of GAgNP for their shape size and appearance was carried out with Transmission Electron microscopy (TEM) which revealed general spherical shape with the images showing agglomerates of microscopic granules and a few dispersed particles while their size



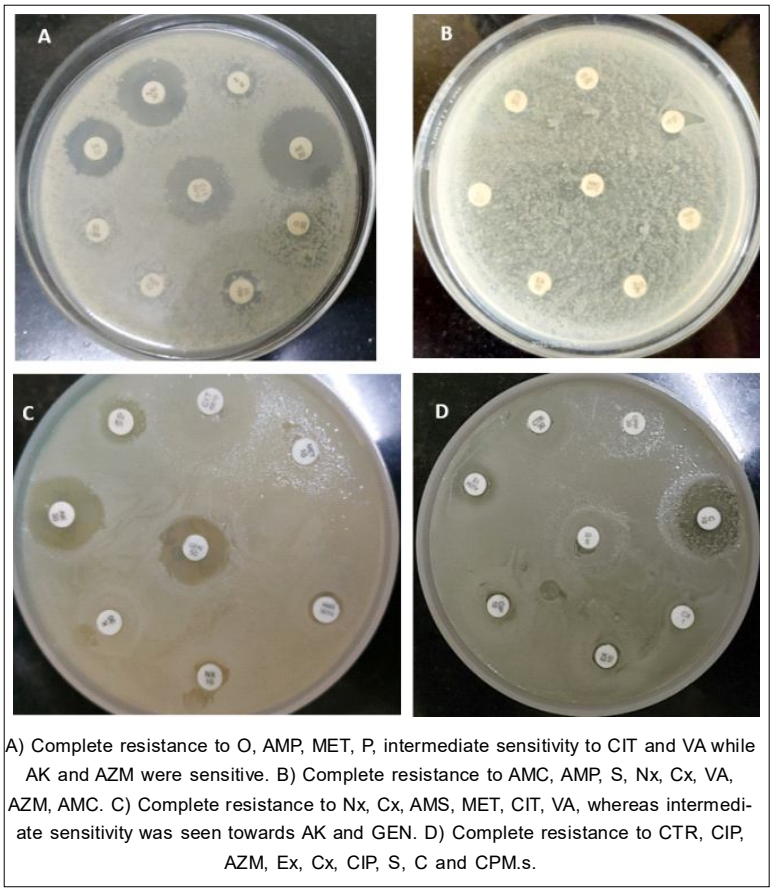
(A) 228 bp product of 16S-rRNA gene and (B) 279 bp product of *nuc* gene (L= Ladder).

Fig 3: Amplified PCR products of *S. aureus*.



(A) Amplification of *mec*A gene (532 bp), *tet*K gene (360 bp), *aac*A-D gene (227 bp) and *erm*C gene (299 bp). (B) Amplification of *van*B gene (330 bp) and (C) Amplification of *mrs*B gene (595 bp). L= Ladder.

Fig 4: MDRSA isolates from mastitis positive cow milk showing AMR genes in multiplex PCR.



A) Complete resistance to O, AMP, MET, P, intermediate sensitivity to CIT and VA while AK and AZM were sensitive. B) Complete resistance to AMC, AMP, S, Nx, Cx, VA, AZM, AMC. C) Complete resistance to Nx, Cx, AMS, MET, CIT, VA, whereas intermediate sensitivity was seen towards AK and GEN. D) Complete resistance to CTR, CIP, AZM, Ex, Cx, CIP, S, C and CPM.s.

Fig 5: Antibiotic sensitivity pattern of MDRSA isolates from cow mastitis showing multi drug resistance pattern.

Table 4: Prevalence of antimicrobial resistance genes in (n=60) isolates of MDRSA from cows having mastitis.

Gene	Number of positive isolates	Per cent pisolates
mecA	31	51.67
ermA	47	78.33
ermC	32	53.33
tetK	37	61.67
tetM	00	00
aaca-D	43	72.00
vanA	00	00
vanB	23	38.00
mrsA	00	00
mrsB	15	25.00

measured ranging between 10 to 70 nm in diameter (Fig 2). The TEM images obtained of the broths used during MIC studies reveled the nanoparticles adhered over the surface of MDRSA cells resulting in the disintegration of cell wall and lysis of bacterial cells.

Antimicrobial efficacy of GAgNP against MDRSA

As a prerequisite for determining the optimum effective antimicrobial concentration of nanoparticle drug for further antibiotic sensitivity assay studies against MDRSA, 50 µl of the green nanoparticles prepared and labeled as N1, N2, N3, N4 and N5 were added in five wells prepared in agar plates inoculated with MDRSA in well diffusion technique. The plates were incubated overnight and the diameters of zones of inhibition were measured. The biggest zone of inhibition (23 nm) was observed around the well containing N5 followed by N4 (21 nm), N3 (18 nm) and N2 (13 nm) while negligible inhibition was noted around N1. Hence, for further studies of MIC, drug N5 was selected on line with the observations made by Roy *et al*. (2017).

Result of MIC of GAgNP against MDRSA *in vitro*

The drug concentrations of N5 used in MIC were 4, 3, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03 and 0.015 µl/Ml, respectively. The appreciable antibacterial efficacy against MDRSA was noted for all these concentrations used. When compared with the results of the efficacy of commercially available nanoparticles (NW: 50) against MDRSA antibacterial efficacy comparable results were observed for all these drug concentrations used. The confirmation of the efficacy of drugs was also carried out by doing MBC of all the wells added with MDRSA and treated with different drug dilution mixtures, wherein visible growth was not observed even in the lowest concentration of GAgNPs of 0.015 µl/mL, thus, confirming the potential candidature of GAgNPs for its

antibacterial activity against MDRSA present in mastitis samples of cows.

CONCLUSION

It was concluded that *S. aureus* was the predominant bacteria responsible for mastitis in dairy cows in the study area. These isolates showed the highest resistance to ß lactamase group of antibiotics followed by macrolide group of antibiotics. However Green silver nanoparticles GAgNPs synthesized by reducing with *Azaderichta indica* leaf extract provided significant antibacterial properties against MDRSA indicating its potential for future applications against MDRSA.

Conflict of interest

All authors declare that they have no conflicts of interest.

REFERENCES

Akindolire, M.A., Babalola, O.O., Ateba, C.N. (2015). Detection of antibiotic-resistant *Staphylococcus aureus* from milk: A public health implication. International Journal of Environmental Research and Public Health. 12(9): 10254-10275. https://doi.org/10.3390/ijerph120910254.

Ali, S.S., Moawad, M.S., Hussein, M.A., Azab, M., Abdelkarim, E.A., Badr, A., Sun, J., Khalil, M. (2021). Efficacy of metal oxide nanoparticles as novel antimicrobial agents against multi-drug and multi-virulent *Staphylococcus aureus* isolates from retail raw chicken meat and giblets. International Journal of Food Microbiology. 344: 109116. doi: 10.1016/j.ijfoodmicro.2021.109116.

Alwan, A.H. and Talak, M.A. (2015). Isolation and characterization of *Staphylococcus aureus* in spoiled food samples. International Journal of Current Microbiology and Applied Science. 4(3): 645-651.

American Society for Microbiology, (2016). Clinical Microbiology Procedures Handbook, Fourth Edition. eISBN: 9781555818814, Published 05/15/2016, Editor: Amy L. Leber.

Andrade, N.C., Laranjo, M., Costa, M.M., Queiroga, M.C. (2021). Virulence factors in Staphylococcus associated with small ruminant mastitis: Biofilm production and antimicrobial resistance genes. Antibiotics. 10(6): 633. https://doi.org/10.3390/antibiotics10060633.

Bauer, A.W. (1966). Antibiotic sensitivity testing by a standardized disc diffusion method. American Journal of Clinical Pathology. 45(4): 149-158.

Bergey, D. (1986). Manual of Systemic Bacteriology, 3rd ed. Springer Publication. 392-420.

Bhattacharyya, D., Banerjee, J., Bandyopadhyay, S., Mondal, B., Nanda, P.K., Samanta, I., Mahanti, A., Das, A.K., Das, G., Dandapat, P., Bandyopadhyay, S. (2016). First report on Vancomycin-resistant *Staphylococcus aureus* in bovine and caprine milk. Microbial Drug Resistance. 22(8): 675-681.

Brahma, U., Suresh, A., Murthy, S., Bhandari, V., Sharma, P. (2022). Antibiotic resistance and molecular profiling of the clinical isolates of *Staphylococcus aureus* causing bovine mastitis from India. Microorganisms. 10(4): 833. doi: 10.3390/microorganisms10040833.

Chandrasekaran, D., Venkatesan, P., Tirumurugaan, K.G., Nambi, A.P., Thirunavukkarasu, P.S., Kumanan, K., Vairamuthu, S., Ramesh, S. (2014). Pattern of antibiotic resistant mastitis in dairy cows. Veterinary World. 7(6): 389-394.

CLSI, (2018). Performance standards for antimicrobial susceptibility testing: Twenty-eighth information supplement. M 100-S28. 28th Ed. Clinical Laboratory Standards Institute.

Dua, K. (2001). Incidence, etiology and estimated economic losses due to mastitis in Punjab and India update. Indian Dairyman. 53(10): 41-52.

Dufour, S., Dohoo, I.R., Barkema, H.W., DesCôteaux, L., Devries, T.J., Reyher, K.K., Roy, J.P., Scholl, D.T. (2012). Manageable risk factors associated with the lactational incidence, elimination and prevalence of *Staphylococcus aureus* intramammary infections in dairy cows. Journal of Dairy Science. 95(3): 1283-1300.

Kossaibati, M.A. and Esslemont, R.J. (1997). The costs of production diseases in dairy herds in England. The Veterinary Journal. 154(1): 41-51.

Loo, Y.Y., Rukayadi, Y., Nor-Khaizura, M.A.R., Kuan, C.H., Chieng, B.W., Nishibuchi, M., Radu, S. (2018). *In vitro* antimicrobial activity of green synthesized silver nanoparticles against selected gram-negative foodborne pathogens. Frontiers in Microbiology. 9: 1555. https://doi.org/10.3389/fmicb.2018.01555.

Mushtaq, M., Agrawal, R., Bhat, M.A., Singh, R., Pande, N. (2019). Antibiotic resistance gene typing in *Staphylococcus aureus* isolated from bovine mastitis. Indian Journal of Animal Science. 89: 1188-1191.

Nelli, A., Voidarou, C., Venardou, B., Fotou, K., Tsinas, A., Bonos, E., Fthenakis, G.C., Skoufos, I., Tzora, A. (2022). Antimicrobial and methicillin resistance pattern of potential mastitis-inducing *Staphylococcus aureus* and coagulase negative *Staphylococci* isolates from the mammary secretion of dairy goats. Biology. 11(11): 1591. doi: 10.3390/biology11111591.

Pu, W., Su, Y., Li, J., Li, C., Yang, Z., Deng, H., Ni, C. (2014). High incidence of oxacillin-susceptible mecA-positive *Staphylococcus aureus* (OS-MRSA) associated with bovine mastitis in China. Plos One. 9(2): e88134. https://doi.org/10.1371/journal.pone.0088134.

Ramasamy, T., Keerthana, S., Srinivasan, M.R., Chandrasekar, D., Porteen, K., Borthakur, A., Elamaran, P., Sriram. (2021). Molecular characterization of antibiotic resistance gene pattern of *Staphylococcus aureus* and *Escherichia coli* in mastitis affected dairy cows. Indian Journal of Animal Research. 55(4): 463-468. doi: 10.18805/ijar.B-3972.

Roy, P., Das, B., Mohanty, A., Mohapatra, S. (2017). Green synthesis of silver nanoparticles using *Azadirachta indica* leaf extract and its antimicrobial study. Applied Nanoscience. 7(8): 843-850. https://doi.org/10.1007/s13204-017-0621-8.

Sharma, I., Brinty, A., Sondi, P., Kumar, A. (2014). Isolation and identification of *Staphylococcus aureus* from bovine mastitis milk and their drug resistance patterns in Silchar town dairy farms, NE India. Online International Interdisciplinary Research Journal. 4: 256-260.

Sharma, L., Verma, A., Kumar, A., Rahal, A., Nigam, N., Nigam, R. (2015). Incidence and pattern of antibiotic resistance of *Staphylococcus aureus* isolated from clinical and subclinical mastitis in cattle and buffaloes. Asian Journal of Animal Sciences. 9: 100-109.

Shreshtha, A., Bhattarai, R.K., Luitel, H., Karki, S., Basnet, H.B. (2021). Prevalence of methicillin-resistant *Staphylococcus aureus* and pattern of antimicrobial resistance in mastitis milk of cattle in Chitwan, Nepal. BMC Veterinary Research. 17: 1-7.

Siddiki, S.H.M.F., Samad, M., Saha, S., Badiuzzaman, M., Islam, M. (2019). Comparison of bacterial pathogens associated with different types of bovine mastitis and their antibiotic resistance status in Bangladesh. Journal of Veterinary Medical and One Health Research. 1(1): 17-27.

Singh, N., Singh, P., Patel, R.K. (2016). Isolation and identification of bacterial organisms from mastitic milk. Journal of Livestock Science. 7: 46-48.

Sondi, I. and Salopek-Sondi, B., (2004). Silver nanoparticles as antimicrobial agent: A case study on *E. coli* as a model for Gram-negative bacteria. Journal of Colloid and Interface Science. 275(1): 177-182.

Zhang, K., Sparling, J., Chow, B.L., Elsayed, S., Hussain, Z., Church, D.L., Gregson D.B., Louie T, Conly, J.M. (2004). New quadruplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of *Staphylococcus aureus* from coagulase-negative *Staphylococci*. Journal of Clinical Microbiology. 42(11): 4947-4955. https://doi.org/10.1128/JCM.42.11.4947-4955.2004.