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Efficacy of green silver nano particles of *Azadirachta indica* against *Staphylococcus aureus* isolated from bovine mastitis

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Abstract

Background: *Staphylococcus aureus* being the major etiological agent of bovine mastitis causing huge economic loss of dairy sector in India, the research work was undertaken in and around Shirwal village of Satara district by screening of unorganized and organized farms for bovine mastitis to isolate, identify and to perform antibiogram studies of *Staphylococcus aureus*, furthermore green synthesis of Silver nanoparticles carried out using green leaves of *Azadirachta indica* and its characterization was performed by SEM, TEM and UV visible spectrophotometry. Efficacy of green silver nanoparticles against *Staphylococcus aureus* was investigated by Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) test.

Method: A total of 270 milk samples of cow were screened for mastitis by using California mastitis test (CMT). The CMT positive milk samples were processed for isolation of erization *Staphylococcus aureus* and its identification was carried out with different biochemical tests. The antibiogram studies were undertaken to know the antibiotic susceptibility of isolated *Staphylococcus aureus.* The Green synthesis of nanoparticles was undertaken using fresh green neem leaves extract in which 0.1 N $AgNO_3$ was added. Characterization was done by TEM, SEM and UV visible spectrophotometry. The *In-vitro* efficacy studies of GAgNP were carried out against *Staphylococcus aureus* isolated from bovine mastitis by microdilution MIC test for which the bacterial count in bacterial culture was maintained at 10^5 CFU/ml by serial dilution method. The combination with lowest MIC was used for MBC test.

Result: Out of total 270 milk samples screened by California mastitis test (CMT) 200 samples were CMT positive in which prevalence of clinical, sub-clinical mastitis and chronic mastitis was 18%,71.5% and 10.5% respectively. 90 Staphylococcal isolates were obtained from CMT positive milk samples. Antibiogram studies of *Staphylococcus aureus* isolates revealed that all 90 isolates were methicillin resistant (MRSA) with highest sensitivity for Gentamicin. Green synthesis of Siver nanoparticles using green leaves of *Azadirachta indica* was confirmed with characterization by Transmissible Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), Spectrophotometry. The *in-vitro* efficacy of green silver nanoparticles against *Staphylococcus aureus* using tests like Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) revealed the antimicrobial effect of Green Silver nanoparticles of *Azadirachta indica* against *Staphylococcus aureus*.

Keywords: Bovine mastitis, MRSA, *S. aureus,* Antibiotic resistance, Green silver nanoparticles

Introduction

Indian economy is based on agriculture as its backbone, especially in rural India. Dairying, which was previously known as secondary to agriculture, now has emerged as the sole bread earner for many native farmer families and replaced agriculture as the main business. The most extensive inventory of cattle in the world is in India in 2023, followed by Brazil and China. India's cattle inventory was reported at 307.5 million head in 2023, accounting for roughly 33% of the world's inventory. Milk Production during 2021-22 was 221.06 million tonnes. The availability of milk per capita in India was around 444 grams/day in 2021-22 (Annual report AHD GOI, 2022-23). The leading cause of mastitis in bovines is *Staphylococcus aureus.* Inspite of known methods of understanding the disease pathogenesis, control of infections in mastitis has many limitations. *Staphylococcus aureus* mastitis remains a significant disease burden. *Staphylococcus aureus* is highly pathogenic and most frequently causes severe clinical and sub-clinical mastitis in bovines. In the wake of evolution, *Staphylococcus aureus* has acquired many factors that actively contribute to antibiotic resistance.

Mastitis is one of the most common reasons for antimicrobial use to control or prevent bacterial infections of the udder, especially Staphylococcal infections, being found in the majority of dairy cattle. Unfortunately, the selective pressure from antimicrobial agents significantly contributes to the dissemination of resistant strains, which greatly attenuates the therapeutic effectiveness of antimicrobial therapy (Ahmed *et al*., 2020) [5]. Initially, penicillin was considered efficacious against many staphylococcal infections, but in the mid-1940s, the strains of *Staphylococcus aureus* started showing resistance to penicillin. Due to its increased resistance towards penicillin, methicillin drugs were used, which were semi-synthetic beta-lactam antibiotics and resistant to β-lactamase enzyme and were considered efficacious against penicillin-resistant *Staphylococcus aureus.* However, the acquisition of the mecA gene caused methicillin resistance with a decreased affinity for β-lactam antibiotics.

The process of rendering green nanoparticles environmentally friendly by the use of different plants with potent bactericidal properties, like neem, was supposed to deliver the added advantage of synergistic antibacterial effect to the biosynthesized nanoparticles. This type of nanoparticle was used successfully for the delivery of therapeutic agents against chronic diseases. Green synthesis of nanoparticles has been considered as one of the promising methods because of their biocompatibility, low toxicity, and eco-friendly nature. Green nanoparticle synthesis is a simple, convenient, economical, and environmentally friendly method as the toxic nature of metals is reduced. Recently, new nature-friendly processes have been adopted, making use of organic reducing and stabilizing agents.

Material and Methods

Screening of cows and collection of samples: Total 270 cows from different organized and unorganized farms in and around Shirwal were screened for mastitis using California Mastitis Test (CMT). The milk samples positive for CMT were carried to the laboratory in box with ice packs and without any contamination. Milk samples were preserved in a refrigerator at 4 °C until further processing.

Isolation of organisms from milk samples: 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. A loopful of milk sample was inoculated in a test tube containing 3 ml of sterile nutrient broth. It was incubated at 37 ºC for 12 h. After 12 h, the test tubes were checked for turbidity/sedimentation/ pellet formation. The mastitic milk samples collected were inoculated on media such as Blood agar and Nutrient agar using a sterile loop around the Bunsen burner. Other selective and differential media such as Mannitol Salt Agar (MSA), Baird Parker Agar (BPA) were also used for the cultivation of organisms. Colonies grown on different types of media were stained by Gram's method and then examined microscopically for Gram staining reaction (positive staining purple or negative staining pink), size (small, medium, or large), and shape (rods, cocci, or coccobacilli). Further confirmation of the organisms was done biochemically. Different biochemical tests like Coagulase test, catalase test, DNase test, Methyl Red, Voges Proskauer test, Oxidase test were performed for identification of *Staphylococcus aureus* isolated from bovine mastitis.

Antibiogram Studies of Staphylococcus aureus by disc diffusion method

Antibiogram sensitivity and resistance pattern of *Staphylococcus aureus* isolated from mastitis milk was interpreted as per (Bauer, 1966) [10]. Antibiotic discs were obtained from M/s HiMedia Laboratories Pvt. Ltd. Mumbai and results were recorded following the guidelines given by the Clinical and Laboratory Institute. Overuse of antibiotics not only hampers the health of animal but also compromise the health of human and environment so that "one health" concept is getting attention worldwide. This study was undertaken to evaluate the antibiotic sensitivity in study area. Total 11 antibiotic discs were used for antibiogram studies.

Synthesis, characterization and efficacy studies of Green Silver Nanoparticles against *Staphylococcus aureus* isolated from bovine mastitis.

Fresh green fully grown neem leaves were collected from the area around Shirwal town, Dist. Satara, Maharashtra. Leaves were thoroughly washed in running water to remove the dirt and dust on the surface. Twenty grams of finely chopped Neem leaves were added to 100 ml of double-distilled water and boiled for 10 min. The extract was cooled, filtered, and stored in the refrigerator at 4ºC-8ºC until further use. This solution was used for the green synthesis of Silver Nanoparticles (GAgNPs). Silver Nitrate solution 0.1N (HiMedia, India) was used for the green synthesis of silver nanoparticles. A set of 05 clean, sterile test tubes were taken labeled 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml, respectively. Then 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml of Neem silver foil, and the entire setup was incubated in the dark chamber to minimize the photo-activation of Silver Nitrate at room temperature. The color change from colorless to brown confirms the reduction of silver ions. Optical density was also used to measure the concentration of formed GAgNPs. 50 µl of the sample was used to calculate the Optical Density (OD) at 48 h with the help of an Enzyme-Linked Immunosorbent Assay (ELISA) reader at different wavelengths, such as 405 nm, 420 nm, and 450 nm, to record peak absorbance. The characterization of synthesized nanoparticles was done for assessment of its size and shape by visual inspection, Transmissible Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), and Uv- visible spectrophotometry using ELISA reader.

Assessment of antimicrobial efficacy of Green Silver Nanoparticles against Staphylococcus aureus

The antimicrobial activity of the prepared GAgNP was assessed by performing the minimum inhibitory concentration (MIC) within the micro-titer plates using the micro-dilution method described by Roy *et al*. (2018) and Ramteke *et al*. (2024) with slight modification. For this purpose, stock solutions of GAgNP and Nutrient broth were prepared.

The MIC test was performed in a 96-well flat-bottom microtiter plate using standard broth microdilution methods. The *Staphylococcus aureus* inoculums were adjusted to the concentration of 10^5 CFU/ml. For dilution of samples 50 µl NB is added in each of the well, then 50 µl Green Nanoparticle sample from test tube N1, N2, N3, N4, N5 added to the 1st well of each row which is named as 1ML, 2ML, 3ML, 4ML, 5mL and in two separate well Gentamicin and Ceftriaxone/Tazobactam was added as they so show highest and lowest sensitivity against *Staphylococcus aureus* respectively. After this perform two fold dilution up to 10th well and discard 50µl from last well. Then add the 50 µl bacterial inoculum in each well and incubate the plate at 37 °c for 24 hrs. The 11th well is considered as negative control and 12th well is considered as positive control. Each well of the microtiter plate was added with 30 µl of the Resazurin solution and incubated at 37 °C for 3 h, 6 h, 9 h, 12 h, and 24 h. Any color changes were observed. Blue/purple color indicated no bacterial growth while pink/colorless indicated concentration of an antimicrobial drug that is bactericidal. MBC was determined as per described by Sykes and Rankin (2013) [28].

Result and Discussion

Prevalence of mastitis: Total 270 cows were screened for mastitis by California Mastitis Test out of which 200 cows were CMT positive. Depends on types of mastitis prevalence of clinical mastitis was 18% (36/200), sub-clinical mastitis 71.5% (143/200), and of chronic mastitis 10.5% (21/200). The prevalence of bovine mastitis is largely influenced by many factors which may be extrinsic and extrinsic.

A) **Influence of age of animals on the occurrence of mastitis:** The incidence of mastitis in Cows of 3yrs age was (8/200; 4%), 4yrs age (16/200; 8%), 5yrs age (27/200; 13.5%), 6yrs (53/200; 26.5%), 7yrs (60/200; 30%), 8yrs (20/200; 10%), 9yrs (10/200; 5%) and of 10yrs age was (6/200; (3%). The cows within the age group of 5 to 7 years were mostly found to be mastitis-positive by the CMT test. As the cow gets older, there are changes in the immune system, and the physiology of the udder changes as the teat sinus remains wide open for more time in older cows, so they become more prone to mastitis.

B) **Influence of lactation no of animals on the occurrence of mastitis:** mastitis was most common in cows during 4th lactation 68/200 (34%), followed by cows in 3rd lactation 57/200 (28.5%), 2nd lactation 41/100 (20.5%), 5th lactation 14/100 (7%), 6th lactation 9/200 (4.5%), 1st lactation 8/200 (4%) and least during 7th lactation 3/100 (1.5%). The higher incidence of mastitis was because of the stress of higher milk production in these lactations. At 4th -6th lactation, the milk production is at its peak, and in high milker cows, stress is more; they need more nutrition and are more susceptible to mastitis.

C) **Influence of seasonal factor on the occurrence of mastitis in cows:** The highest number 59.5% (119/200) of mastitis-positive cases occurred in the monsoon season during the month of July-Sept, followed by the pre-monsoon period during the month of May-June 26% (52/200), and post-monsoon during months Oct-Dec 14.5% (29/200). The environmental conditions of the monsoon season, like humidity, wet floor, and change in temperature, are suitable for the growth of *Staphylococcus aureus* bacteria in the environment, animal body surface, and surroundings.

Isolation and identification of Staphylococcus aureus: All milk samples were processed for bacterial isolation by streaking on Nutrient agar. The bacterial colonies, which are gram-positive and suspected as *Staphylococcus aureus*, were streaked on specific media Mannitol Salt Agar (MSA). The growth of mannitol fermenting yellow-colored colonies on MSA confirms the presence of Staphylococcus aureus in mastitic milk. Some of the samples were streaked on Baird Parker Agar (BPA) and obtained the growth of colonies with grey to jet black color and smooth to shiny appearance having 1-3 mm diameter. The pure colonies grown on NA were processed with Gram staining, which revealed the round, grapes-like clusters of colonies retaining the violet color indicative of *Staphylococcus aureus* in mastitis-positive milk samples. *Staphylococcus aureus* were processed for a battery of biochemical tests to identify them, including Catalase, DNAse, Methyl red, Voges- Proskauer, and Coagulase tests. All 90 isolates were positive for these biochemical tests but negative for the oxidase test.

Antibiogram studies of Staphylococcus aureus isolated from bovine mastitis: In the case of bovine, due to overuse of a higher generation of antibiotics, mastitis has become incurable. *Staphylococcus aureus* is one of the leading causes of bovine mastitis. Increasing AMR over a period of time has become a big trouble for the dairy industry. Many cases are becoming chronic, and reoccurrence is common due to AMR in *Staphylococcus aureus*. In the present investigation, an antibiotic sensitivity test was performed using Kirby's disc diffusion method with 11 different antibiotics. The aim of the study was to investigate the efficacy of an alternative candidate, Green Silver nanoparticle, against *Staphylococcus aureus.*

As per the observations in Table no 4.6, all 90 (100%) isolates of *Staphylococcus aureus* tested resistant to methicillin. The Methicillin-resistant *Staphylococcus aureus* (MRSA) primarily involves the production of an altered penicillin-binding protein (PBP). The main mechanism of MRSA is the acquisition of the mecA gene, which codes for an alternative penicillin-binding protein called PBP2a. This protein has a low affinity for β-lactam antibiotics like methicillin. 44 (48.88%) *Staphylococcus aureus* isolates were found sensitive to Ampicillin-sulbactam, intermediate sensitive to 21(23.33%), and resistant to 25 (27.77%). Chandrasekaran *et al.* (2014) reported similar results in 100% methicillin-resistant *Staphylococcus aureus* isolates (MRSA). Out of 90 isolates, 17 (18.8%) were sensitive, 27 (30%) were intermediate sensitive, and 46 (51.11%) were resistant to oxytetracycline. Specifically, in the present research, MRSA showed resistance against oxytetracycline. Generally, oxytetracycline is widely used in the management of haemo protozoal infection in our area, which may be the reason for its increasing resistance. Abdeen *et al*. (2021) [2] and Faramaway *et al*. (2019) found resistance in *Staphylococcus aureus* against oxytetracycline in the range of 50% - 60%. Out of a total of 90 isolates of *Staphylococcus aureus*, 82 (91.11%) were sensitive, 3 (3.33%) were intermediate sensitive, 5 (5.55%) were resistant to gentamicin and in case of amikacin 73 (81.11) isolates are sensitive, 10 (11.11%) isolates were intermediate sensitive and only 7 (7.77) were resistant. Such a remarkable sensitivity of gentamicin observed by Abera *et al.* (2013) against *Staphylococcus aureus.* The treatment of mastitis concerned gentamicin is commonly used as intramammary infusion instead of systemic injection, therefore to overcome the systemic symptoms like fever, pain and to check the secondary complications the broad spectrum antibiotic frequently are used in combination with gentamicin. The quinolone antibiotics ciprofloxacin and enrofloxacin were tested against Staphylococcus aureus, and results revealed 65% and 70% sensitivity, respectively. Enrofloxacin and Ciprofloxacin are broad spectrum antibiotics and are found quite effective against *Staphylococcus aureus* from bovine mastitis.

Similar results were recorded by Pavulraj *et al*. (2013) [23] with 70% sensitivity to enrofloxacin. Also, similar results were observed by Awandkar *et al*. (2013) [6], who showed more than 50% sensitivity to enrofloxacin and ciprofloxacin. Pati and Mukherjee (2022) found 72% sensitivity of enrofloxacin against *Staphylococcus aureus*. The data and information collected from farmers during the screening of cows for mastitis in the research area showed that the most frequently used antibiotic for mastitis treatment was Ceftriaxone + Tazobactam. Out of 90 isolates of *Staphylococcus aureus*, 65/90 (72.22%) were resistant, 19/90 (21.11%) isolates were intermediate sensitive, and only 6/90 (6.66%) isolates were sensitive to Ceftriaxone + Tazobactam. Use of long-acting Ceftriaxone + Tazobactam without necessity may have created resistance to this antibiotic combination. Like many other potent antibiotics, ceftriaxone + Tazobactam also loses its efficacy against *staphylococcus aureus*. Awandkar and Kulkarni (2021) found more than 50% resistance of Ceftriaxone + Tazobactam for *Staphylococcus aureus*. Crossbreeds cows in the research area with a previous history of affection of mastitis suffer from reoccurring mastitis, and such relapsing mastitis cases are resistant to treatment with Ceftriaxone +Tazobactam.

Synthesis and Characterization of Green Silver Nanoparticles of *Azadirachta indica*: The Green Silver nanoparticle synthesis was carried out with reference to Roy *et al*. (2017) [25] and Ramteke *et al*. (2024) with slight modifications. The synthesized silver nanoparticles were set up in a dark room for 24-48 hours as direct light exposure hampers the synthesis of nanoparticles. For the synthesis of green silver nanoparticles, the neem extract was added to an aqueous silver nitrate solution. The phytochemicals terpenoids and flavonoids present in the neem leaf extract act as reducing agents, and the synthesis of green silver takes place Ramteke *et al.* (2024). Besides this, terpenoids and flavonoids act as capping and stabilizing agents, which enhance the antimicrobial effect of green silver nanoparticles. After incubation in a dark room, the pale yellow colour is changed into a dark brown colour, which is visible to the naked eye and is the primary indication of the biosynthesis of green silver nanoparticles. The dark brown color also signifies that the concentration of Silver nanoparticles increases with time. This happens due to the Plasmon resonance (SPR) phenomenon (Plate 4.7).

Biosynthesis and analysis of Green silver nanoparticles by Optical Density: The change in colour previously discussed was considered the endpoint of Green silver Nanoparticles biosynthesis. Further evaluation involved measuring the optical density (OD) values of each sample treated with varying concentrations of Neem extract using ELISA reader set at different wavelengths (420 nm,405 nm,445 nm) to record peak absorbance. The results observed in Table no. 4.7 revealed that the absorbance at 405 nm for sample N1, N2, N3, N4, N5 have the OD values 0.3421, 0.4325, 0.5111, 0.3225, 0.3371 respectively. At 420 nm, the OD values for Sample N1, N2, N3, N4, and N5 are 0.6785, 1.234, 1.564, 0.8343, 0.5864, respectively. Similarly, at 490 nm, the OD values for samples N1, N2, N3, N4, and N5 are 0.0882, 0.1308, 0.1332, 0.1237, 0.1175, respectively. The absorbance at different wavelengths and their OD values indicate that sample no N5 has the highest OD values for all wavelengths as it contains 5 ml neem extract, which, ultimately, provides more capping and reducing agents, which results in an increased concentration of Nanoparticles in it. For this study, a wavelength between 390-520 nm range was preferred, as we got higher absorbance at 420 nm. The OD values and absorbance at a preferable range of wavelength were primary indications of the formation of Green silver nanoparticles from neem leaf extract. Ahmed *et al.* (2016) [11] reported similar findings from their study. They concluded that as the concentration of neem extract increased, the absorbance also increased, which resulted in an increase in OD values. Asif *et al*. (2022) synthesized Green Silver nanoparticles using neem leaf extract. After UV-visible spectrophotometry, he found peak absorbance around 419 nm wavelength.

Characterization of GAgNPs by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

SEM revealed the size, shape, and morphology of GAgNP. The nanoparticles were clustered and varied in size and shape. The characterization of GAgNP by SEM confirms the formation of nanoparticles (Plate 4.7). Sengupta and Sarkar (2022) [27] carried out the characterization of nanoparticles by ESEM and concluded that generated nanoparticles were spherical in shape. The TEM revealed the size of GAgNPs was 10-5 nm. The nanoparticles were scattered and less dense. Ramteke *et al*. (2023) [24] carried out a characterization of GAgNP and revealed the size of the nanoparticle was 10-70 nm with height 15-25 nm. Regmi *et al*. (2021) investigate the nanoparticles with size 2-14 nm under TEM. (Plate 4.7).

Antimicrobial effect of Green Silver Nanoparticles against *Staphylococcus aureus*

A) MIC of GAgNPs against *Staphylococcus aureus* isolated from bovine mastitis

The efficacy of Green Silver Nanoparticles was estimated by the Minimum inhibitory concentration test. The sample from test tube N3 had 3 ml neem extract and 1 ml 0.1N silver nitrate, which inhibited the bacterial growth up to the 9th well. In 1st well, a 50 µl sample was added, and by two-fold dilution in the 9th well, the concentration was 0.1953125 µl, which was taken as the MIC value for sample N3. Similarly, for N1, complete inhibition up to the 7th well was observed, so MIC was 0.78125 µl; for N2, inhibition was up to the 8th well, so MIC was 0.39625 µl; for N4, inhibition was up to the 7th well, so MIC was 0.78125 µl, and for N5 inhibition was up to 5th well MIC was 3.125 µl. Along with Green Silver Nanoparticles, the MIC of Gentamicin and Ceftriaxone/Tazobactam was also estimated, which showed that Gentamicin inhibits bacterial growth up to 9th well, having MIC similar to sample N3 and the efficacy of Ceftriaxone + Tazobactam is poor as it inhibited the growth up to 3rd well with MIC 12.5 µl. As we got the effective OD values of Green silver nanoparticles, we concluded that the absorbance of UV light for each wavelength we used was higher for sample no 3. Hence, the higher OD values for the N3 sample were indicative of the fact that the concentration of nanoparticles achieved at this dilution was higher for all isolates of *Staphylococcus aureus*; thus, MIC for the N3 sample was considered promising and selected for further evaluation. The antimicrobial efficacy of GAgNP was estimated by Roy *et al.* (2017) [25] and Ramteke *et al*. (2023) [24]. The highest dilution of GAgNP, which inhibits the growth of *Staphylococcus aureus*, was further processed for MBC. The sample with the same dilution was poured on NA and incubated overnight. No visible growth of *Staphylococcus aureus* was observed on the plate, which indicates the antimicrobial activity of GAgNP against *Staphylococcus aureus.*

Table 1: Prevalence of different types of mastitis

Sr. No.	Mastitis	Total no of cases	Prevalence%
1	Clinical mastitis	36	18%
2	Sub-clinical mastitis	143	71.5%
3	Chronic mastitis	21	10.5%

Table 2: Influence of age of animals on the occurrence of mastitis

Sr. No.	Age of cow (Years)	No of cases	Prevalence%
1	3	8	4%
2	4	16	8%
3	5	27	13.5%
4	6	53	26.5%
5	7	60	30%
6	8	20	10%
7	9	10	5%
8	10	6	3%
	Total	200	

Table 3: Influence of lactation number of animals on the occurrence of mastitis (n=200)

Sr. no.	Lactation no	No of Mastitis positive cases	Prevalence%
1	1st	8	4%
2	2nd	41	20.5%
3	3rd	57	28.5%
4	4th	68	34%
5	5th	14	7%
6	6th	9	4.5%
7	7th	3	1.5%
8	Total	200	

Table 4: Influence of seasonal factor on the occurrence of mastitis cases in cows (n=200)

Season	Sub-clinical mastitis	Clinical mastitis	Chronic Mastitis	Total	%
Pre-monsoon	39	6	7	52	26
Monsoon	87	19	13	119	59.5
Post-monsoon	17	11	1	29	14.5

Table 5: Results of Antibiogram studies of Staphylococcus aureus isolated from bovine mastitis

Group of Antibiotics	Antibiotics	S		I/S or M		R	
		Total	%	Total	%	Total	%
B- Lactam	Ampicillin-Sulbactam AMS (30/15 mcg)	44	48.88	17	18.88	29	32.22
	Methicillin MET (30 mcg)	0	0	0	0	90	100
Tetracycline	Oxytetracycline 0 (30 mcg)	17	18.88	27	30	46	51.11
Aminoglycosides	Amikacin C (30 mcg)	73	81.11	10	11.11	7	7.77
	Gentamicin GEN (30 mcg)	82	91.11	3	3.33	5	5.55
Quinolones	Ciprofloxacin CIP (5 mcg)	65	72.22	5	5.55	20	22.22
	Enrofloxacin EX (5 mcg)	70	77.77	5	5.55	15	16.66
Glycopeptide	Vancomycin VA (10 mcg)	35	38.88	0	0	55	61.11
Macrolide	Azithromycin AZM (15mcg)	25	27.77	5	5.55	60	66.66
Cephalosporins	Ceftriaxone/Tazobactam CIT (30/10 mcg)	6	6.66	19	21.11	65	72.22
	Cefotaxime CTX (30 mcg)	18	20	42	46.66	30	33.33

Table 6: MIC of GAgNPs against *Staphylococcus aureus* isolated from bovine mastitis.

Sr. No.	Sample No	MIC OF GAgNPs against Staphylococcus aureus
1	N1	0.78125 µL
2	N2	0.39625 µL
3	N3	0.193125µL
4	N4	0.78125 µL
5	N5	3.125 µL
6	Gentamycin	0.193125µL
7	Ceftriaxone+ Tazobactum	12.5 µl

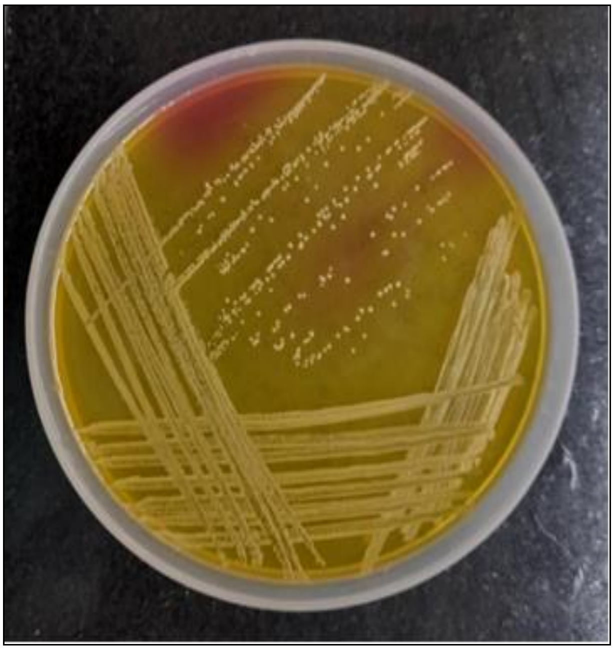


Fig 1: *Staphylococcus aureus* on MSA plate

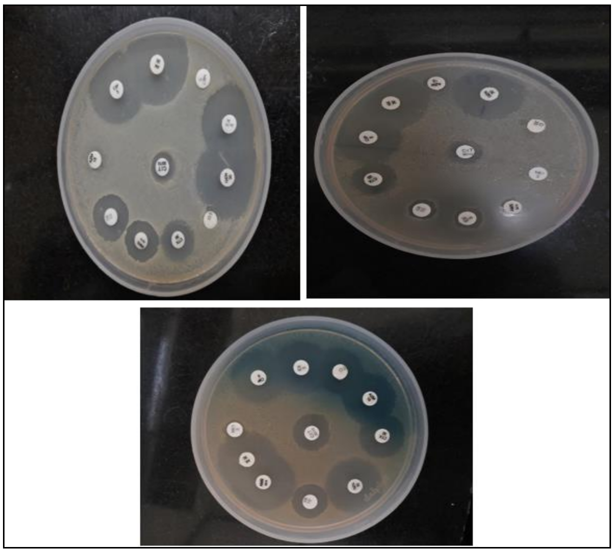
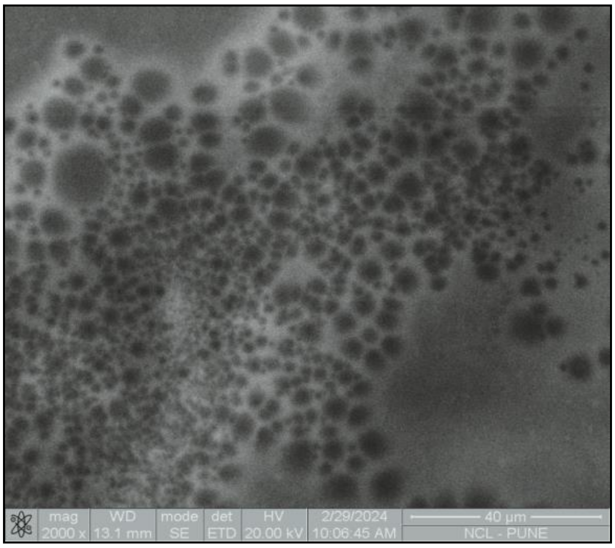
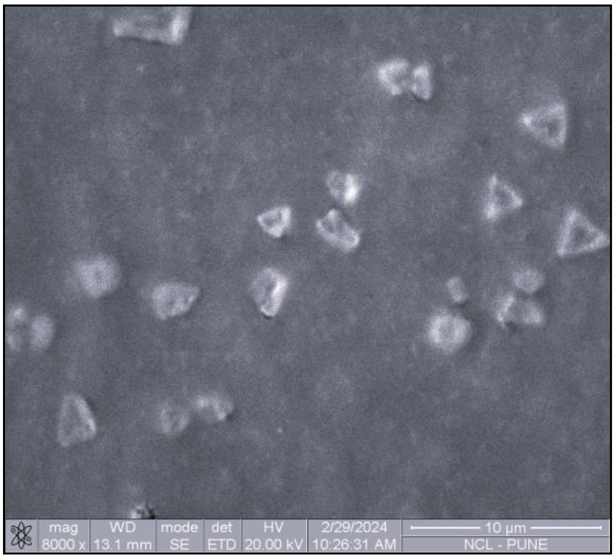


Fig 2: Antibiotic Sensitivity Test of Staphylococcus aureus isolated from bovine mastitis

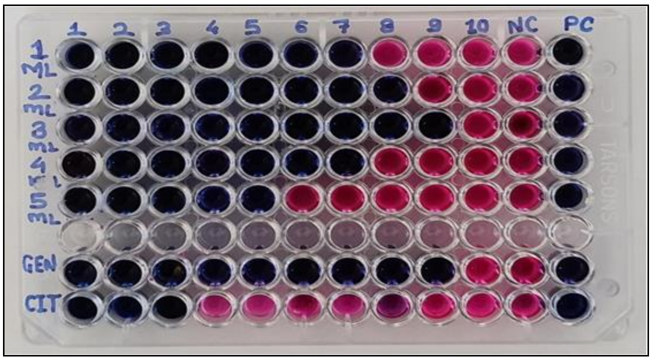


A) Scanning electron microscopy (SEM)



B) Transmission Electron Microscopy (TEM)

Fig 3: Characterization of Green silver Nanoparticles



The MIC of Sample N1, N2, N3, N4, N5 was carried out against *Staphylococcus aureus*

Sample N3 shows complete inhibition of bacteria up to 9^{th} well where dilution of $GAgNO_3$ is 0.193125µl which is MIC of that sample.

Column 11 and 12 are negative and positive control respectively

The MIC of Gentamycin and Ceftriaxone+ Tazobactam was estimated which is 0.193125µl and 12.5 µl. respectively.

Fig 4: Minimum Inhibitory Concentration (MIC) Test of Green Silver Nanoparticles of *Azadirachta indica* against *Staphylococcus aureus*

Conclusion

Exotic cow breeds were more susceptible to mastitis than native breeds, and extrinsic and intrinsic factors like age, lactation number, and environment played an important role in the incidence of mastitis. The prevalence of Sub-clinical mastitis was more which remains undetected and causes huge economic losses of dairy industry. In the study area, *Staphylococcus aureus* was predominantly isolated from bovine mastitis cases. *Staphylococcus aureus* showed complete resistance against methicillin, indicated the threat of complete resistance for cephalosporins like Ceftriaxone+ Tazobactam and β-lactam antibiotics. Green synthesis of Silver Nanoparticles was easy, economical and quick method of preparing the alternative drugs for therapy against MRSA.

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References

1. Aarestrup FM, Wegener HC, Rosdahl VT. Evaluation of phenotypic and genotypic methods for epidemiological typing of *Staphylococcus aureus* isolates from bovine mastitis in Denmark. Vet Microbiol. 1995;45(2-3):139-50.
2. Abdeen EE, Mousa WS, Abdel-Tawab AA, El-Faramawy R, Abo-Shama UH. Phenotypic, genotypic and antibiogram among *Staphylococcus aureus* isolated from bovine subclinical mastitis. Pak Vet J. 2021;41(2):289-93.
3. Abebe R, Hatiya H, Abera M, Megersa B, Asmare K. Bovine mastitis: prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. BMC Vet Res. 2016;12:1-11.
4. Abera M, Demie B, Aragaw K, Regassa F, Regassa A. Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. J Vet Med Anim Health. 2010;2(3):29-34.
5. Ahmad S, Ahmed S, Sabir MS, Khan H, Rehman M, Niaz Z. Frequency and comparison among antibiotic resistant *Staphylococcus aureus* strains in selected hospitals of Peshawar, Pakistan. J Pak Med Assoc. 2020;70(7):1199-202.
6. Awandkar SP, Bhikane AU, Kulkarni MB. Antibiotic resistance trends in subclinical bovine mastitis. Biolife. 2013;1(3):139-43.
7. Baghel A, Chhabra D, Sharda R, Shukla S, Audarya S, Sikrodia R, Gangil R. Isolation of *Staphylococcus* from bovine mastitis and their antibiotic sensitivity pattern. Indian J Vet Sci Biotech. 2018;13(4):49-52.
8. Barber DA, Miller GY, McNamara PE. Models of antimicrobial resistance and foodborne illness: examining assumptions and practical applications. J Food Prot. 2003;66(4):700-9.
9. Baştan A, Kaçar C, Acar DB, Şahin M, Cengiz M. Investigation of the incidence and diagnosis of subclinical mastitis in early lactation period cows. Turk J Vet Anim Sci. 2008;32(2):119-21.
10. Bauer AW. Antibiotic sensitivity testing by a standardized disc diffusion method. Am J Clin Pathol. 1966;45(4):149-58.
11. Ahmed S, Ahmad M, Swami BL, Ikram S. Green synthesis of silver nanoparticles using *Azadirachta indica* aqueous leaf extract. J Radiat Res Appl Sci. 2016;9(1):1-7.
12. Chandrasekaran DP, Nambil AP, Thirunavukkarasu PS, Venkatesan P, Tirumurugaan KG, Vairamuthu S. Incidence of resistant mastitis in dairy cows in Tamil Nadu, India. J Appl Nat Sci. 2015;7(1):304-8.
13. Cheesbrough M. District Laboratory Practices in Tropical Countries. Cambridge: Cambridge University Press; c2006.
14. Cruickshank R, Duguid JP, Marmion BP, Swain RH. Medical Microbiology: The Practice of Medical Microbiology. New York: Longman Group Ltd.; c1975.
15. Cui L, Ma X, Sato K, Okuma K, Tenover FC, Mamizuka EM, Hiramatsu K. Cell wall thickening is a common feature of vancomycin resistance in *Staphylococcus aureus*. J Clin Microbiol. 2003;41(1):5-14.
16. Abdeen EE, Walid M, Hussien H, Roshdy S. PCR for detection of virulence and antibiotic resistance genes of coagulase positive *Staphylococcus aureus* from clinical mastitis in Egypt. Int J Basic Appl Sci. 2015;4(3):315-9.
17. Fesseha H, Mathewos M, Aliye S, Wolde A. Study on prevalence of bovine mastitis and associated risk factors in dairy farms of Modjo town and suburbs, central Oromia, Ethiopia. Vet Med Res Rep. c2021; p.271-83.
18. Feyissa N, Alemu T, Jirata Birri D, Desalegn A, Sombo M, Abera S. Isolation and determination of antibacterial sensitivity characteristics of *Staphylococcus aureus* from lactating cows in West Shewa Zone, Ethiopia. Vet Med Int.; c2023: [Article number].
19. Jagadeesh DS, Puttamallappa RK, Keregallokoppalu HG, Lakshminarasimhiah M. Prevalence of sub-clinical mastitis in cattle and effect on milk quality. Adv Anim Vet Sci. 2016;4(5):237-40.
20. Krishnamoorthy P, Goudar AL, Suresh KP, Roy P. Global and countrywide prevalence of subclinical and clinical mastitis in dairy cattle and buffaloes by systematic review and meta-analysis. Res Vet Sci. 2021;136:561-86.
21. Kumari SA, Patlolla AK, Madhusudhanachary P. Biosynthesis of silver nanoparticles using *Azadirachta indica* and their antioxidant and anticancer effects in cell lines. Micromachines. 2022;13(9):1416.
22. Kurjogi MM, Kaliwal BB. Epidemiology of bovine mastitis in cows of Dharwad district. Int Sch Res Notices; c2014: [Article number].
23. Pavulraj S, Amsaveni S, Kalaiselvi L, Ramesh S. Screening of *Staphylococcus aureus* isolates from mastitis for antibacterial susceptibility pattern and beta-lactamase production. Tamilnadu J Vet Anim Sci. 2013;9(4):300-7.
24. Ramteke MD, Mhase PP, Muglikar DM, Budhe MS, Tumlam UM, Kolhe RP, Jadhav SN, Pawar PD, Singla LD. Efficacy of green silver nanoparticles (GAbNPs) against multidrug resistance in *Staphylococcus aureus* from mastitis-infected cows. Agric Res Commun Cent ARCC/B-5207; c2023.
25. Roy P, Das B, Mohanty A, Mohapatra S. Green synthesis of silver nanoparticles using *Azadirachta indica* leaf extract and its antimicrobial study. Appl Nanoscience. 2017;7(8):843-50. doi: 10.1007/s13204-017-0726-7.
26. Sadashiv SO, Kaliwal BB. Prevalence of bovine mastitis in north Karnataka, India. Int J Pharma Health Care Res. 2013;1(4):169-77.
27. Sengupta A, Sarkar A. Synthesis and characterization of nanoparticles from neem leaves and banana peels: a green prospect for dye degradation in wastewater. Ecotoxicol. 2022;31(4):537-48.
28. Sykes JE, Rankin SC. Isolation and identification of aerobic and anaerobic bacteria. In: Canine and Feline Infectious Diseases. St. Louis: Elsevier Inc; c2013. p. 17-28.
29. Tesfaheywet Zeryehun TZ, Gerema Abera GA. Prevalence and bacterial isolates of mastitis in dairy farms in selected districts of Eastern Harrarghe Zone, Eastern Ethiopia. [Publication details missing].

30. Yadav MM. Prevalence of *Staphylococcus aureus* in lactating cows with subclinical mastitis and their antibiogram in organized dairy farm, Maharashtra, India. Int J Curr Microbiol Appl Sci. 2018;7(3):3674-80.
31. Yang F, Shi W, Meng N, Zhao Y, Ding X, Li Q. Antimicrobial resistance and virulence profiles of staphylococci isolated from clinical bovine mastitis. Front Microbiol. 2023;14:1190790.

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