Staphylococcus aureus in subclinical bovine mastitis: Prevalence and innovative treatment with *Moringa oleifera* and Selenium nanoparticles

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ABSTRACT

Background: Bovine subclinical mastitis is a significant disease that significantly impacts the health of dairy cows and subsequently affects milk production. Among the various causes of subclinical mastitis, bacterial infections, particularly *Staphylococcus aureus (S. aureus),* play a crucial role.

Aim: The aim of this investigation was to isolate and identify *S. aureus* strains from cows with subclinical mastitis, assess their antimicrobial susceptibility, virulence genes, and investigate the potential antibacterial activity of *Moringa oleifera* ethanolic extract and Selenium (Se) nanoparticles against both somatic cell count (SCC) and *S. aureus* count in subclinical mastitic cows.

Methods: We collected 110 milk samples from dairy cows with subclinical mastitis in the Kafrelsheikh governorate before treatment with intramammary antibiotics. Samples were identified using pH detection, SCC at ≥200,000 cells/ml, and California Mastitis Test.

Results: The effect of *M. oleifera* ethanolic extract, Se nanoparticles, and Ciprofloxacin antibiotic in reducing SCC and *S. aureus* counts was significant. Selenium nanoparticles and Ciprofloxacin antibiotics showed the highest reduction in the *S. aureus* count, comparable to *M. oleifera* ethanolic extract, while ciprofloxacin followed by *M. oleifera* ethanolic extract, and Selenium nanoparticles were effective against somatic cell count.

Conclusion: This study emphasizes the widespread occurrence of *S. aureus* in subclinical bovine mastitis and highlights the potential of *M. oleifera* ethanolic extract and Se nanoparticles as promising treatment options. Selenium nanoparticles demonstrated notable antibacterial effects against *S. aureus* and contributed to a reduction in somatic cell counts. Considering these findings, we propose additional studies on the potential of Selenium nanoparticles as antibacterial agents, as they demonstrate effectiveness in preventing and managing *S. aureus* subclinical mastitis.

Keywords: *mecA* gene, MRSA, *Moringa oleifera*, *Staph aureus*, Selenium nanoparticles, Subclinical mastitis.

Introduction

Mastitis, both in its clinical and subclinical forms, is a detrimental disease that significantly impacts the health of dairy animals, leading to reduced milk quality and quantity (Aamir *et al*., 2009). It is a complex condition influenced by three major factors: host resistance, causative agents, and environmental factors (Al Emon *et al*., 2024). The causative agents of mastitis typically include Gram-negative and Gram-positive bacteria, mycoplasmas, yeasts, and algae (Bhatt *et al*., 2012). *Staphylococcus aureus* is one of the main causes of contagious subclinical mastitis in cattle, causing severe economic losses (Farabi *et al*., 2024). It is also accountable for foodborne intoxication transmitted to humans via the ingestion of contaminated milk

(Ewida and Al-Hosary, 2020). The pathogenesis of *Staphylococcus aureus*, including intracellular parasitism, biofilm formation, and increasingly severe antimicrobial resistance, renders traditional treatment methods ineffective (Algharib *et al*., 2020). One type of *S. aureus* mastitis is methicillin-resistant *S. aureus* (MRSA), which was initially introduced as a hospital-acquired (*HA-MRSA*) infection in humans (Deurenberg *et al*., 2007). Recently, this pathogen emerged as livestock-acquired MRSA (LA-M*RSA*) (Ahangari *et al*., 2017), causing serious concern due to difficulties in therapeutic trials at both the human and livestock levels.

Milk samples from two Egyptian governorates—Assiut and El-Mansoura—contained *methicillin-*

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resistant S. aureus (Elshall, 2019). One of the most important problems associated with MRSA-induced mastitis is its controversial susceptibility to antibiotic therapy due to the cross-resistance of these isolates to most beta-lactams (Hoque *et al.*, 2023). Furthermore, some MRSA isolates are multidrug-resistant against many other antimicrobial families. In addition, it poses a potential threat to the selection and dissemination of resistance genes (Khazaie and Ahmadi, 2021).

The use of Selenium (Se) nanoparticles has become popular because of their potential applications as antioxidants, antimicrobials, and enzyme inhibitors (Ramamurthy *et al.*, 2013). Selenium helps heal udder epithelial cells inflamed by *S. aureus* by inhibiting the activity of nuclear factor kappa B, mitogen-activated protein kinase, and toll-like receptor 2 signaling pathways, while also promoting the expression of microRNA-146a (Wang *et al.*, 2018).

Moringa oleifera leaves have recently been recognized as a valuable source of nutrition with various beneficial activities. These leaves have promising antitumor, anti-inflammatory, antiulcer, antiatherosclerotic, and anticonvulsant properties (Dahiru *et al.*, 2006). Researchers have also explored the antimicrobial properties of plant-based compounds, including *M. oleifera*, as potential therapeutic alternatives (Bugno *et al.*, 2007).

The investigation of alternative treatments, such as Se nanoparticles and *M. oleifera* extracts, shows great potential for combating subclinical mastitis and improving the overall health of dairy animals. Understanding the antimicrobial properties and effects of subclinical mastitis-causing microorganisms, including their susceptibility and virulence factors, can provide valuable insights for developing effective treatment strategies. Thus, this study aimed to estimate the prevalence of *S. aureus* subclinical mastitis among apparently healthy animals, identify the antibiotic resistance scheme of the isolated strains, and explore the *in-vivo* antibacterial efficacy of Se nanoparticles and *M. oleifera* extract on *S. aureus* subclinical mastitis, contributing to the expansion of novel beneficial methods for managing subclinical mastitis in dairy animals.

Material and Methods

This study was conducted according to research ethics approved by the committee on research of the College of Veterinary Medicine, Kafr-Elsheikh University, Kafr El-Shaikh, Egypt (KFS-IACUC/95/2019).

Clinical examination
Clinical examination of the cows' udders and regional lymph nodes was performed before sampling, as previously described (Constable *et al.*, 2016).

Milk samples
A total of 110 samples of milk from subclinical mastitic animals were collected after pH detection. Subclinical mastitis was detected using somatic cell count (SCC) and the California Mastitis Test (CMT). SCC was determined by automated counters with a threshold of ≥200,000 cells/ml to indicate subclinical cases, consistent with European guidelines. CMT was performed by mixing milk samples with a reagent on a paddle, with gel formation indicating elevated somatic cell counts in response to intramammary inflammation, following the method described by Ebrahimie *et al.* (2018). The samples were collected from dairy cow farms at the Kafrelsheikh governorate before the cows were treated with intramammary antibacterial agents. Before collecting milk samples, milkers thoroughly cleaned the udders, teat orifices, and hands with soap and water, followed by disinfection using 70% ethyl alcohol. The initial milk streams were disposed of, and approximately 20 ml of milk was kept in clean, sterile bottles, each labeled with a unique serial number. All samples were sent directly to the laboratory for bacteriological examination (Al Emon *et al.*, 2024; Farabi *et al.*, 2024).

Bacteriological examinations
A cow was considered to be subclinically infected after counting somatic cells, detecting pH, and indicating positivity for subclinical mastitis (SCM). Milk pH was measured at 37°C using a digital pH meter (modelAdwa1030, Romania) after calibration of the pH probe immediately after collecting milk samples, with beyond 6.8 indicating the prevalence of subclinical mastitis (Modh *et al.*, 2019). Furthermore, the SCC cut-off was employed to determine the frequency of SCM as having at least 200,000 cells/ml with or without positive isolation of the udder pathogen and when the cow was presented with no clinical signs (Ebrahimie *et al.*, 2018).

Isolation and identification of S. aureus
Milk samples were collected aseptically and centrifuged at 3,000 rpm for 20 min, after which the supernatant was discarded. A loopful of the sediment was then inoculated into Tryptic soy broth and aerobically incubated at 37°C for 18–24 hours. Subsequent subculturing was performed on Baird-Parker selective agar plates incubated aerobically at 37°C. Plates were examined after 24 hours and again at 48 hours for the presence of black, shiny, convex colonies with narrow white margins and surrounding clear zones (Baird-Parker, 1969). Mannitol salt agar (MSA) (DM160D) (Mast Diagnostics, Merseyside, United Kingdom) was also used to inoculate the samples (Wedley *et al.*, 2014) and incubated at 37°C for 24 hour. Clusters of *S. aureus* with a yellowish color on MSA plates were again examined for morphological and biochemical characteristics (Cheesbrough, 2006).

Antimicrobial susceptibility testing
according to the guidelines provided by CLSI, 2011, the susceptibility profiles of 10 isolated *S. aureus* strains to 9 commercially available antibiotic discs were determined using the disk diffusion technique. The following antibiotic discs were used in this

study: Amoxicillin clavulanic acid (AMC) 30 g, Sulfa trimethoprim (COT) 25 g, methicillin (ME) 5 g, cefotaxime 30 g, tetracycline (TE) 30 g, fosfomycin 200 g, tylosine 15 g, Ciprofloxacin (CIP) 5 g, and gentamicin 10 g. Measurement of millimeter-sized inhibitory zones that formed around the discs allowed us to ascertain the antibiotic sensitivity. A sensitivity, moderate, or resistant classification was given to the zone diameters.

Detection of S. aureus virulence genes by using polymerase chain reaction (PCR)

DNA was extracted from the samples using a QIAamp DNA Mini Kit (Qiagen, Germany, GmbH) with minor modifications. For 10 minutes, 200 µl of the sample suspension was treated with 20 µl of proteinase K and 200 µl of lysis buffer at 56°C. After incubation, 200 µl of 100% ethanol were added to the lysate. The samples were washed and centrifuged according to the manufacturer's instructions. Finally, 100 µl of the kit elution buffer was used to elute the nucleic acids. Table 1 lists Metabion (Germany) primers. The PCR reaction was conducted in a 25-µl volume with 12.5 µl EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer (20 pmol), 5.5 µl water, and 5 µl DNA template. The reaction was performed on an Applied Biosystems 2720 thermal cycler. The PCR products were electrophoresed on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x Tris-Borate-EDTA Buffer (TBE) buffer at room temperature with a 5 V/cm gradient. For analysis, 20 µl of uniplex PCR products and 30 µl of multiplex products were loaded into each gel slot. Fragment sizes were determined using a Fermentas 100-bp gene ruler ladder (Thermo, Germany). The gel data were analyzed by computer software from Alpha Innotech and the Biometra gel documentation system.

Moringa oleifera ethanolic extract

Moringa oleifera leaves were shade-dried and ground into a powder. The hydroethanolic extract was prepared by dissolving 500 g of this powder in 2 L of 80% ethanol at room temperature with occasional shaking. After three days, the mixture was filtered using sterile filter paper (No. 1. The ethanolic extract was concentrated and evaporated below 40°C to form a semisolid mass. This extract was reconstituted in sterile phosphate-buffered saline (PBS, pH 7.4) at a concentration of 1-g extract per 10 ml of PBS. The reconstituted extract was filtered through a 0.22 µm membrane (Millipore, India) and stored in a sterile vial under refrigeration for intramammary infusion. The dosage was determined based on prior research findings (Sharma and Sharma, 2022).

Pharmaceutical compounds in Moringa oleifera ethanolic extract

Assessing the total flavonoid concentration

Following Marinova *et al*. 2005, a modified AlCl₃ colorimetric method was used to measure flavonoid content. In a 10-ml volumetric flask, 1 ml of the extract

was dissolved in 2 ml of methanol. Solutions of 5% NaNO₃, 5% NaOH, and 7% AlCl₃ were prepared in a 25 ml flask. Then, 200 µl of the extract was placed in a sealed glass vial, mixed with 75 µl of 5% NaNO₃, and left for 5 minutes at room temperature. Subsequently, 1.25 ml of AlCl₃ and 0.5 ml of NaOH were added to each vial, followed by sonication and incubation for 5 minutes. With the use of a quercetin standard calibration curve, the flavonoid content was quantified as micrograms of quercetin equivalent per gram of dry extract. The absorbance of each solution was measured at 510 nm.

Total phenolic content

By employing a colorimetric technique with the Folin-Ciocalteu reagent, the total phenolic content of the extracts was determined. One milliliter of the extract was dissolved in two milliliters of methanol. Subsequently, 2.5 ml of 75 g/l sodium carbonate and 500 µl of Folin-Ciocalteu reagent diluted 10 times were mixed with 2.5 ml of this solution. Before allowing the mixture to sit for 2 hours at 25°C, it was vortexed for 10 seconds. The absorbance was measured at 765 nm compared with a blank reagent after incubation. Gallic acid equivalent milligrams per gram of dry extract was used as the unit of expression for the results Marinova *et al*. 2005.

Total alkaloid content

Following (Shamsa *et al*., 2008), to prepare the bromocresol green (BCG) solution, 69.8 mg of BCG was dissolved in 3 ml of 2 N NaOH and 5 ml of distilled water, and then brought to a volume of 1,000 ml with distilled water. A phosphate buffer (pH 4.7) was created by adjusting the pH of a 2M sodium phosphate solution with citric acid. The atropine standard solution was prepared by dissolving 10 mg of atropine in 10 ml of distilled water. Aliquots (5–60 µg/ml) of this solution were mixed with 5 ml of phosphate buffer and 5 ml of BCG solution, then shaken with chloroform in increments. The extracted solutions were collected and diluted with chloroform, and their absorbance was recorded at 470 nm. For plant extraction, 5 g of the plant material was finely ground and extracted with methanol at 26°C over 24 hours, followed by concentration under vacuum at 45°C. A portion of the residue was dissolved in 2N HCl, filtered, neutralized with NaOH, and then combined with 5 ml of BCG and 5 ml of buffer. The solution was shaken and extracted with chloroform, and its absorbance was measured at 470 nm.

Determination of total saponins content (TSC)

Following (Le *et al*. (2018), a standard curve for aescin was prepared by dissolving 150 mg of aescin in 10 ml of methanol (15 mg/ml stock) and performing serial dilutions in triplicate using methanol as the blank. For extraction, 1 g of powder was mixed with 30 ml of ethanol, left for 30 minutes at room temperature, cooled in an ice bath, and filtered through a 0.45 µm membrane. This clear extract was used to determine

Table 1. Primer sequences, target genes, amplicon sizes, and cycling conditions.

| Target gene | Primers sequences | The amplified segment (bp) | Primary denaturation | Amplification (35 cycles) | | | Final extension | Reference |
				Secondary denaturation	Annealing	Extension		
MecA	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	310 bp	94°C 5 minutes	94°C 30 seconds	50°C 30 seconds	72°C 30 seconds	72°C 7 minutes	McClure *et al.,* 2006
Coa	ATA GAG ATG CTG GTA CAG G GCT, TCC; GAT, TGT, TCG; ATG, C	Four different types of bands can be detected: 350 bp 430 bp 570 bp 630 bp	94°C 5 minutes	94°C 30 seconds	55°C 40 seconds	72°C 45 seconds	72°C 10 minutes	Iyer and Kumosani, 2011
nuc	ATATGTAT-GGCAATC-GTTTCAAT GTAAATG-CACTTGCTTCAG-GAC	395 bp	94°C 5 minutes	94°C 30 seconds	55°C 40 seconds	72°C 40 seconds	72°C 10 minutes	Gao *et al.,* 2011

TSC. Samples were heated at 65°C to evaporate methanol, treated with 0.5 ml of 4% vanillin and 2.5 ml of 72% H_2SO_4, incubated at 60°C for 15 minutes, and cooled. The absorbance at 560 nm was measured, and the values were plotted for the standard curve. TSC was calculated as: \text{Total saponin content} = \frac{\text{weight of saponins in extract (mg)}}{\text{weight of powder (g)} \times (1 - \frac{\text{moisture %}}{100})} and expressed as (mg AE/g).

Total tannins assessment
Tannin content was measured using tannic acid as a reference. For this, 400 µL of extract was mixed with 3 ml of 4% vanillin solution in methanol and 1.5 ml of concentrated hydrochloric acid. After 15 min of incubation, absorbance was recorded at 500 nm (Broadhurst and Jones, 1978).

Preparation of Selenium nanoparticles
One gram of sodium selenite was dissolved in 100 ml of double-distilled water until a clear solution was obtained. Then, 0.5 ml of hydrazine hydrochloride was added as a reducing reagent. The mixture solution was left to react by stirring for 1 hour, then heated for 1 hour, and a red precipitate formed. The Se nanoparticles were centrifuged, washed, and dried. The characterization of Se nanoparticles was performed using various techniques, such as X-Ray diffraction (XRD), scanning

electron microscopy, and Zeta potential (Chudobova *et al*., 2014; Fig. 1).

Experimental design
The experimental design was completely randomized using three intramammary treatments. Ten subclinically dairy cows selected based on CMT, SCC, and *S. aureus* examination were involved as three groups (Group 1: *n* = 3 for *M. oleifera* ethanolic extract; Group 2: *n* = 3 Se nanoparticles and Group 3: *n* = 4 for Ciprofloxacin antibiotic treatment according to in vitro sensitivity test).

Concerning Group 1, an ethanolic extract of *M. oleifera (*1 g/10 ml of PBS) was prepared according to Sharma and Sharma (2022) and was infused in the intramammary once daily for 3 days; the Nano-Se-treated group cows (Group 2) were infused in the intramammary with nano-Se at a concentration of (500 µg/ml) according to Hassan *et al*. (2019). Furthermore, the third group (antibiotic) commercial CIP (Ciprolac® 100 mg intramammary antimicrobial syringe; Ourofino Animal Health) was chosen for this study after passing an in vitro sensitivity test using the gold standard—the disc diffusion method since 100 mg intramammary was infused once daily for 3 days.

Milk samples were collected before treatment as a control group and on the 1st, 2nd, and 3rd days after
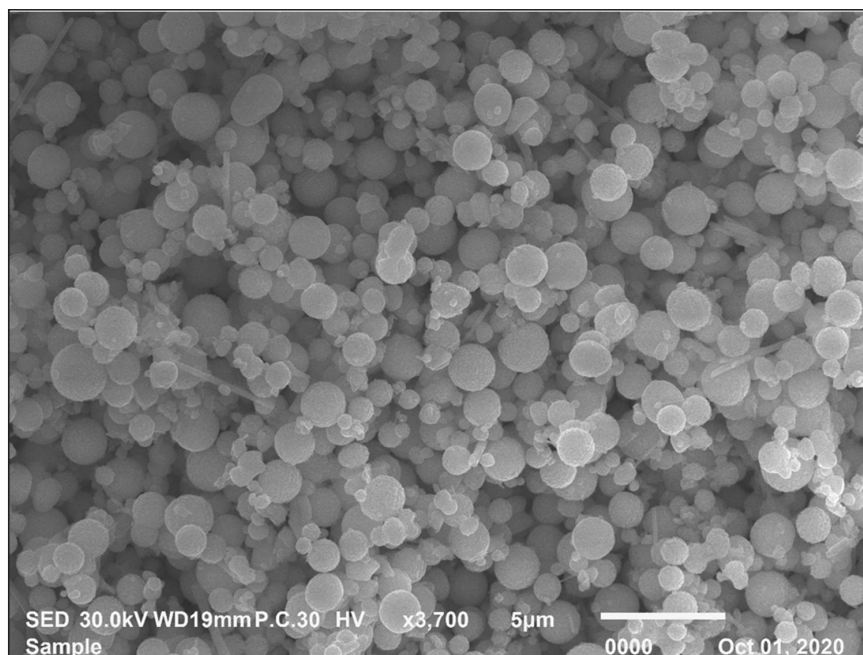
Fig. 1. Shows the Se nanoparticles' scanning electron microscopy (SEM). The size and morphology of Selenium (Se) nanoparticles were studied by placing a small sample on a holder and measuring the average particle size of the synthesized Se nanorods. The high-resolution SEM images of the fabricated Se nanoparticles were acquired at different magnifications to perform the measurements. The Se nanostructure appeared nanospherical in the images, with an almost uniform morphology and width of approximately 30 nm.

therapy with *M. oleifera* extract, Se nanoparticles, and Ciprofloxacin. Bacteriological examination of milk samples before and after treatment was performed for the control and three treatment groups by counting the number of *S. aureus* colonies. A colony counter was used to count the SCC, and the values were expressed as Colony-forming units (CFU)/ml.

Statistical analysis

Two-way repeated measures analysis of variance was conducted to evaluate the effects of different treatment groups across various examination days on somatic cell counts and CFUs per ml. The statistical analysis was performed using IBM SPSS Statistics 23 (IBM, Armonk, NY).

Results

Prevalence of S. aureus *in Bovine subclinical mastitis samples*

A total of 110 milk samples were obtained after investigation of subclinical mastitis cases using somatic cell count ($\geq$200 000 cells/ml), pH ($\geq$ 6.8), and positive CMT, yielding 10 *S. aureus*-positive isolation samples**.** Antibiotic susceptibility analyses of the *S. aureus* isolates showed that the most susceptible to Ciprofloxacin CIP and COT (100%) were followed by tylosin and gentamicin (80%) (Table 2). Only three out of ten isolates were identified as MRSA based on the methicillin-resistant disk diffusion method and

confirmed by the presence of *mecA* gene, although the remaining seven isolates were negative for methicillin-resistant disk diffusion but carried *mecA* gene as presented in Tables 2 and 3 and Fig. 2. All of the ten isolates were carrying *nuc* and *coa* genes as presented in Table 3 and Figs. 2, 3, and 4.

The effect of *M. oleifera* ethanolic extract, Se nanoparticles, and Ciprofloxacin antibiotics on reducing somatic cell count cells/ml, as shown in Table 5, the highest significant decrease in somatic cell counting at $p < 0.05$ was achieved in the CIP antibiotic-treated group. The other two groups did not show a significant difference at $p < 0.05$. Furthermore, there was a substantial difference at $p < 0.05$ in the somatic cell counts between days of treatment, where the highest number of cells was in control a and the lowest was in the 3rd day of treatment in all 3 treatments. The effects of *M. oleifera* ethanolic extract, Se nanoparticles, and Ciprofloxacin antibiotics on *S. aureus* counting are shown in Tables 5 and 6, which showed that the highest significant decrease in *S. aureus* counting at $p < 0.05$ was achieved in the Se nanoparticle-treated group and Ciprofloxacin antibiotic-treated group than in the moringa-treated group. However, there was a significant difference between day 0 and the other 3 days of treatment in the Ciprofloxacin, *moringa* extract, and Se nanoparticle groups. The findings presented in Table 6 demonstrate

Table 2. Antimicrobial susceptibility test for *S. aureus* isolates (no = 10).

Antimicrobial agent	Sensitive (N0.)		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Ciprofloxacin (CIP)	10	100	-	-	-	-
Sulfa trimethoprim (COT)	10	100	-	-	-	-
Tylosine (TL)	8	80	2	20	-	-
Gentamicin (CN)	8	80	-	-	2	20
Methicillin (ME)	7	70			3	30
Tetracycline (TE)	6	60	2	20	2	20
Amoxicillin clavulanic acid (AMC)	6	60	-	-	4	40
Cefotaxime (CTX)	5	50	5	50	-	-
Fosfomycin (FO)	5	50	2	20	3	30

Table 3. Prevalence of *mecA*, *nuc*, and *coa* viral genes in *S. aureus* isolates from subclinical mastitis cases.

Isolates	*mecA*	*nuc*	*coa*
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
Total %	10 (100%)	10 (100%)	10 (100%)

the antibacterial effects of *M. oleifera* ethanolic extract, Se nanoparticles, and Ciprofloxacin against *S. aureus* counts (CFU/ml) over the treatment period. In the control group, the initial *S. aureus* counts were 3×10^3 CFU/ml, 2.8×10^5 CFU/ml, and 6.82×10^5 CFU/ml for the Ciprofloxacin, *M. oleifera,* and Se nanoparticle groups, respectively, highlighting the severity of infection. CIP showed the most consistent reduction in bacterial counts, decreasing significantly to 3.15×10^2 CFU/ml by the third day. Selenium nanoparticles demonstrated comparable efficacy, reducing bacterial counts to 3.2×10^2 CFU/ml by the third day, suggesting that they are an effective alternative to Ciprofloxacin. *Moringa oleifera* extract also significantly reduced *S. aureus* counts, but the reduction (to 1.19×10^3 CFU/ml by the third day) was less pronounced compared with the other treatments. The statistical analysis revealed significant differences in bacterial reduction between treatments ($p < 0.05$) and across treatment days ($p < 0.05$), emphasizing the superior effectiveness of Ciprofloxacin and Se nanoparticles. These results

suggest that Se nanoparticles represent a promising alternative therapy for managing *S. aureus* infections in subclinical mastitis.

Discussion

Subclinical mastitis is responsible for substantial monetary losses in dairy farming, and *S. aureus* is considered the most frequent bacterial species responsible for both clinical and subclinical types of mastitis in cattle: clinical and subclinical (Neelam *et al*., 2022). Moreover, MRSA is a type of *S. aureus* infection and is considered an emerging problem in dairy farms worldwide, including Egypt (Ewida, 2009). MRSA is resistant to commonly used antimicrobials, and it possesses virulent factors, such as enterotoxins and hemolysins, which have public health consequences. This highlights the importance of continuous surveillance for estimating the extent of the problem in dairy farms, controlling antimicrobial resistance, and prescribing appropriate antimicrobial agents in both veterinary holdings and public health hospitals.

In the existing study, upon conducting a bacteriological examination of the subclinical milk samples, we found that *S. aureus* is responsible for almost 10% of SCM in dairy farms in the study area. In contrast, a study by (Ewida and Al-Hosary, 2020) reported higher results, with *S. aureus* detected in 46 out of 48 samples (95.83%) that were positive for CMT. Moreover, our findings demonstrated a lower prevalence than previous research in Benha Province, which found *S. aureus* in 90.4% of milk samples, and in Dakahlia Province, it was found in 81.3% (Adwan and Isayed, 2018). The high prevalence of *S. aureus* in these studies may be ascribed to inadequate hygiene before and after milking and the prevailing environmental conditions.

Antimicrobial resistance poses a significant global challenge. Therefore, the susceptibility of the recovered *S. aureus* isolates to commonly used antimicrobials was examined. Table 2 shows that the isolates were vulnerable to the antimicrobials tested. The isolates
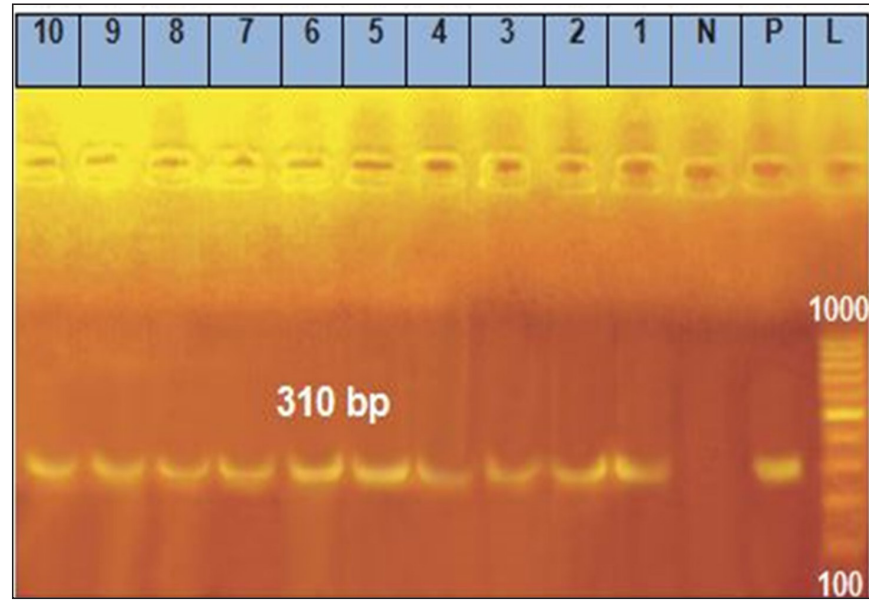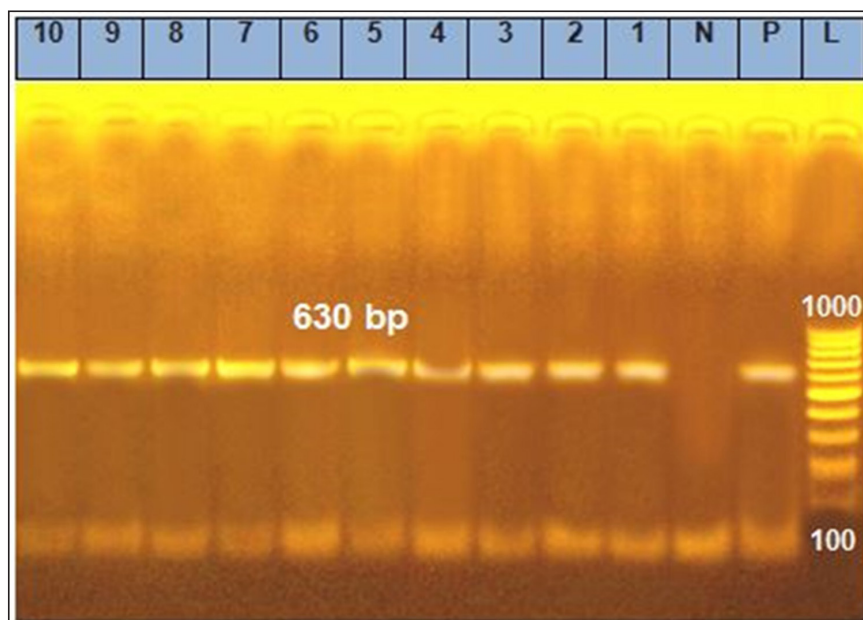
Fig. 2. Agarose gel electrophoresis of the polymerase chain reaction result for *mecA* virulence. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10: Positive for *mecA* gene with 310 bp band.
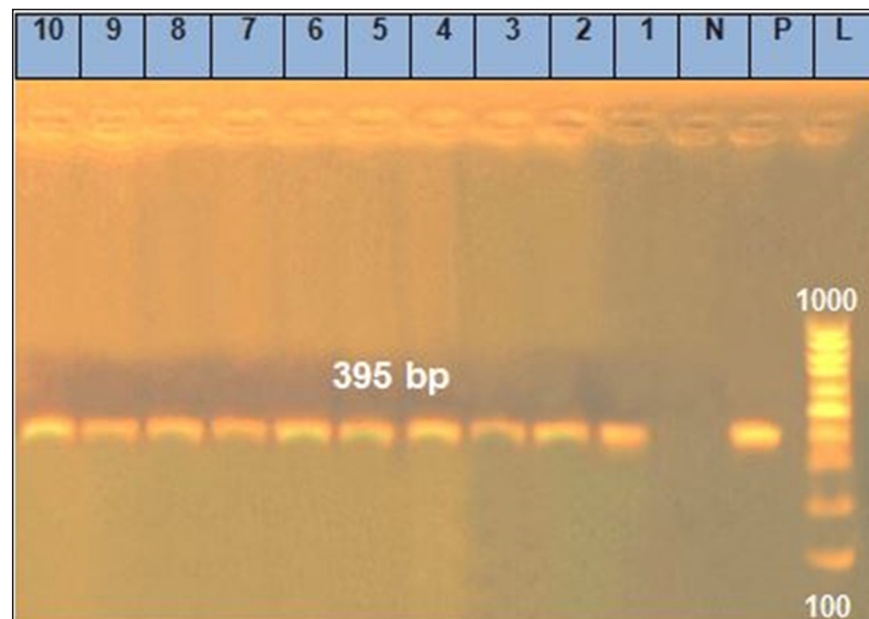
Fig. 3. Agarose gel electrophoresis of the polymerase chain reaction result for *nuc* virulence. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10: Positive for *nuc* gene with 395 bp band.

displayed 100% sensitivity to Ciprofloxacin and COT. Moreover, the strains exhibited 80% sensitivity to tylosin and gentamicin, 40% were resistant to Amoxicillin clavulanic acid (AMC), and 30% were resistant to Methicillin (ME). They also found similar results (El-Azzouny *et al*., 2022), who observed that

S. aureus isolates showed sensitivity to Ciprofloxacin (96%) and gentamicin (92%), and by (Saidi *et al*., 2019), who found that *S. aureus* isolates exhibited sensitivity to Tetracycline (TE) (65.71%) and Amoxicillin clavulanic acid (AMC) (51.42%). This study identified a high rate of MRSA discrepancies,

Fig. 4. Agarose gel electrophoresis of the polymerase chain reaction result for *coa* virulence gene. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10: Positive for *coa* gene with 630 bp band.

Table 4. Determination of total phenolic, flavonoid, and alkaloid content, total saponin content, total alkaloid content, and total tannin content of *Moringa oleifera* (ethanolic extract) (mean ± SE).

Moringa oleifera (ethanolic extract)	Total Phenolic con. (mg gal/ml)	Flavonoids con. (mg QUE/ml)	Total saponin conc. (μg AE/ml)	Total Alkaloid (μg /ml)	Total Tannins con. (μg TanE/ml)
	2.001 ± 0.00	1.807 ± 0.00	708.667 ± 1.00	609.467 ± 1.59	29.967 ± 0.95

Table 5. Effects of *Moringa oleifera*, Selenium nanoparticles, and Ciprofloxacin on the mean Somatic cell count cells ml.

Day	Ciprofloxacin	Moringa group	Selenium nanoparticles
Control	$407.5 \times 10^{3a*}$	$496.66 \times 10^{3a**}$	$483.33 \times 10^{3a**}$
1st	$352.5 \times 10^{3b*}$	$396.66 \times 10^{3b**}$	$343.33 \times 10^{3b**}$
2nd	$290 \times 10^{3c*}$	$350 \times 10^{3c**}$	$283.33 \times 10^{3c**}$
3rd	$240 \times 10^{3d*}$	$340 \times 10^{3d**}$	$300 \times 10^{3d**}$

Cells in the same row with different (*) expression levels are significantly different at $p < 0.05$. Cells in the same column with different letters are significantly different $p < 0.05$.

with phenotypically MRSA-negative isolates testing positive for *mecA* by PCR. These findings suggest that reliance solely on *mecA* expression alone is insufficient for accurately distinguishing MRSA or MSSA. Another gene, similar to *mecA*, also contributes to methicillin resistance (Paterson *et al.*, 2014). Another study reported that a group of genes located on the staphylococcal chromosomal cassette *mec*, which enhances antibiotic resistance, can lead to inaccurate results in disc diffusion tests (Aqib *et al.*, 2018). The occurrence of MRSA in dairy farms is likely due to the widespread use of antimicrobials, which has driven the development of antimicrobial resistance in bacterial pathogens. Antibiotic administration is

Table 6. Effects of *Moringa oleifera*, Selenium nanoparticles, and Ciprofloxacin antibiotics on *S. aureus* count: CFUs per ml.

Day	Ciprofloxacin	Moringa group	Selenium nanoparticles
Control	$3 \times 10^{3a*}$	$2.8 \times 10^{5a*}$	$6.82 \times 10^{5a*}$
1st	$1.06 \times 10^{3b*}$	$2.5 \times 10^{4b**}$	$6.95 \times 10^{2b*}$
2nd	$4.2 \times 10^{2b*}$	$6.61 \times 10^{3b*}$	$5.3 \times 10^{2b*}$
3rd	$3.15 \times 10^{2b*}$	$1.19 \times 10^{3b**}$	$3.2 \times 10^{2b*}$

Cells in the same row with different (*) numbers are significantly different at $p < 0.05$. Cells in the same column with different letters are significantly different $p < 0.05$.

strongly associated with the emergence and persistence of resistance traits in pathogenic strains. These traits are encoded by specific genes located on the bacterial chromosome, plasmids, transposons, or gene cassettes within integrons (Daka *et al*., 2012). Consequently, isolates can easily transfer these genes (Hassan *et al*., 2016).

Molecular identification and detection of virulence genes were performed on the isolates. The screening revealed that all 10 *S. aureus* isolates carried *mecA, nuc*, and *coa*. In contrast, (Ewida and Al-Hosary, 2020) reported lower results, with the *nuc* virulence gene Confirmed in 33 (71.73%) of the milk samples examined, whereas *mecA* was not found in this study. These results are consistent with findings from a previous study conducted by (Abd El-Hamid and Bendary, 2013) in Egypt, which confirmed the presence of the *nuc* gene in all *S. aureus* strains and differed from the findings of (Al-Ashmawy *et al*., 2016), who detected only the *mecA* gene in 53% of the scanned samples, and (El-Tawab *et al*., 2017), who noticed that *S. aureus* bacteria taken from mastitic cows tested positive for the *coa* gene 50% of the time.

Moringa plants contain a number of compounds. There are multiple possible medical uses for each plant. The values of total phenolics, flavonoid components, total saponin, total alkaloid, and total tannins in the ethanolic extract of *M. oleifera* are presented in Table 4. Similar results were reported by (Abadallah and Ali, 2019), who revealed that the leaves' aqueous and ethanol extracts included alkaloids, saponins, flavonoids, reducing sugar, glycosides, quinones, tannin, and terpenoids. A vast class of nitrogenous chemicals known as alkaloids has been extensively applied as chemotherapeutic agents against cancer, as well as anesthetics and CNS stimulants (Madziga *et al*., 2010). Alkaloids regulate the growth and metabolism of living things (Edeoga *et al*., 2006). A study by (Salah *et al*., 1995) validated the antioxidant and antibacterial capabilities of flavonoids. Flavonoids have the potential to prevent tumor invasion and kill cancer cells through the processes they induce (Benavente-García *et al*., 1997). Also, (Arabski *et al*., 2012) noted that saponins possess noteworthy biological properties, including cytotoxic, hemolytic, molluscicidal, anti-inflammatory, antifungal, anti-yeast, antibacterial, and antiviral activities.

In our study, Tables 5 and 6, we observed that using Ciprofloxacin followed by each Se nanoparticle and *M. oleifera* ethanolic extract decreased somatic cell counts. In contrast, Se nanoparticles and Ciprofloxacin antibiotics were used before the ethanolic extract of *M. oleifera* to decrease the *S. aureus* count. The difference in the results between the somatic cell count and *S. aureus* count may be attributed to the fact that the components of the somatic cell count are bacterial cells responsible for mastitis, such as *S. aureus*, as well as the immune cells responsible for defense (Ewida and Al-Hosary, 2020). In a laboratory setting, extracts of *M. oleifera* leaves in aqueous, ethanol, and methanol solutions were found to slow down the expansion of certain bacteria (Singh and Tafida, 2014). Different findings were informed by (Hassan *et al*., 2019), who reported that the ethanol and methanol extracts showed higher antibacterial activity than the antibiotic extract, with the ethanolic extract showing the strongest antimicrobial action against the bacteria tested. This suggests that plants could potentially be used to source new antibiotic compounds**.**

Studies have shown that Se nanoparticles can interact with the surface of bacterial cell membranes, disrupting their permeability and respiratory function. They can also penetrate bacteria or fungi, making them a potential alternative to antibiotics and aiding in overcoming antibiotic resistance (Hassan *et al*., 2019). Proof of the potent suppressive effect of Se nanoparticles on *S. aureus* in vitro growth, with a declining percentage of viable bacteria detected in the presence of Se nanoparticles at different doses and over time (Tran and Webster, 2011).

Conclusion

In conclusion, *Staph aureus* is a significant causative agent of subclinical bovine mastitis. Antimicrobial susceptibility testing revealed that Ciprofloxacin and COT are effective against *S. aureus*. Furthermore, all 10 isolates carried the virulence genes *mecA*, *nuc*, and *coa*, while three isolates were methicillin-resistant using the disk diffusion method, which has implications for public health. MRSA and the potential pollution of

milk with this bacterium pose a severe risk to public health, necessitating sustained efforts to control the infection. Finally, Se nanoparticles and Ciprofloxacin had a better effect on *S. aureus* counting than *M. oleifera* ethanolic extracts. In contrast, Ciprofloxacin, followed by *M. oleifera* ethanolic extracts and Se nanoparticles, effectively reduced somatic cell counts. Based on these findings, we recommend further investigation into the use of Se nanoparticles as antibacterial because they show promise in preventing and treating *S. aureus* subclinical mastitis.

Acknowledgments

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