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Antibacterial Activities of Selenium Nanoparticles Against Multidrug Resistant *Staphylococcus aureus* and *Escherichia coli* Isolated from Mastitic Milk

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Abstract

Bovine mastitis is one of the most important diseases affecting dairy cows and subsequently milk production. There are several causes leading to the onset of mastitis in dairy cows, bacterial diseases are among the most important etiological agents. Therefore, this study was conducted to investigate the antibacterial activity of selenium nanoparticles against two major organisms causing mastitis in cows. Bacteriological examination of milk samples in the present study revealed that 25(40.32%) and, 27(43.54%) were positive for *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*), respectively. Among the examined samples 15(24.19%) had mixed *E. coli* and *S. aureus* infection. The antimicrobial susceptibility patterns showed that most isolates of *S. aureus* and *E. coli* were multidrug resistant, on the other hand they were sensitive to ciprofloxacin and gentamycin. Broth microdilution method was used for determination of the minimum inhibitory concentrations (MIC) of selenium nanoparticles against *S. aureus* and *E. coli* isolates. The MIC values against *S. aureus* isolates ranged from 15.62 to 31.25µg/ml, but in *E. coli* isolates ranged between 31.25 and 62.5 µg/ml. This *In vitro* result clearly indicates that the selenium nanoparticles have a good antibacterial activity against multidrug resistant bacteria causing mastitis.

KEYWORDS
Selenium nanoparticles, *Staphylococcus aureus*, *E. coli*, Bovine mastitis.

INTRODUCTION

Mastitis is one of the endemic diseases affecting dairy cows in many countries. It is considered one of the most important diseases to cause massive economic losses in the dairy industry (Du *et al*., 2022). According to Heikkilä *et al.* (2012), it has a significant impact on milk production, animal welfare, and food safety. Mastitis is a result of intramammary infection (IMI), which is mostly brought on by different bacterial species. For effective mastitis control and therapy, it is crucial to understand the bacterial etiology of IMI (Griffioen *et al*., 2016). The pathogens that cause IMI are distributed differently in different nations and herds. The major pathogens include *E. coli*, *S. aureus*, environmental streptococci, as well as the minor pathogens such as coagulase-negative staphylococci (CNS), are the most frequent causes of IMI (Riekerink *et al*., 2008; Levison *et al*., 2016). *Streptococcus* sp., *S. aureus*, *A. pyogenes*, *E. coli*, and Klebsiella sp. are the most common pathogens that harmfully affect elderly cows (Gröhn *et al*., 2004).

The somatic cells count (SCC) is a sign of udder health issues. The majority of somatic cells are white blood cells, such as macrophages and neutrophils, which enter inflamed mammary gland tissue from the blood (Akers and Nickerson, 2011). Increased somatic cell count is the initial indicator of mastitis in a herd. According to Gröhn *et al.* (2004), clinical mastitis is associated with significant yield losses in cows. In addition, Quinn *et al.* (2002) and Andrews *et al.* (2003) distinguish clinical mastitis from subclinical mastitis depending on the manifestations of the disease. When milk has chemical and physical abnormalities and seems abnormal, it has clinical mastitis. The morphology of the mammary gland may also change. The subclinical form is when the udder and milk both seem normal and show no obvious signs of irritation. In most dairy herds across the world, subclinical mastitis is more common than clinical mastitis and results in the highest overall losses (Eriskine, 2001).

Fever, animal depression, and appetite loss are among the most severe clinical indications of acute mastitis. Coliforms such *Escherichia coli*, Klebsiella, and *Streptococcus* species are among the most often found related organisms (Manasa *et al*., 2019). Subclinical mastitis is most associated with *S. aureus* and *Streptococcus* sp. (Zaatout *et al*., 2019).

Since they leave antibiotic residues in the milk and increase the likelihood that microbial resistance will spread to the environment in the case of mastitis, antibiotics have long been thought of as the first line of defense against bacterial infections in dairy cattle (Zduńczyk and Janowski, 2020). Food security concerns have been raised due to the development of multiple antibiotic-resistant (MAR) bacteria, which is a serious public health issue for both human and animal health (WHO 2015; Tang *et al*., 2017). It appears that cutting back on antibiotic use is a vital first step. The research on nanoparticles is currently of interest due

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to their potent antibacterial characteristics, which are preventing the emergence of more resistant forms of bacteria (Gong *et al*., 2007).

Because they reduce the need for medicines or can be used to determine a specific bacteria's susceptibility to an antibiotic, nanoparticles can be a useful treatment for mastitis in dairy cows (Oliver and Murinda, 2012). Due to the production of reactive oxygen species (in the Fenton reaction), lipids and proteins being oxidized, and DNA being degraded, nanoparticles may potentially be hazardous to bacteria (Li *et al*., 2012).

One of the intriguing substances to combine with antibacterial drugs is selenium (Se), a dietary element that has a significant role in biological systems. Selenium is a vital trace element for growth and health maintenance in the diet (Devlin *et al*., 2017). Elemental Se is the least poisonous form (Housman *et al*., 2014), hence its nano form has garnered a lot of interest (Saini *et al*., 2010). Selenium nanoparticles (SeNPs) have been shown to possess anticancer (Feynman, 1992), antioxidant (Guisbiers 2010), antibacterial, and anti-biofilm effects in recent research. These nanoparticles have demonstrated exceptional antibacterial action so far against pathogenic bacteria, fungi, and yeasts (Jeevanandam, *et al*., 2018).

Here, the antibacterial efficacy of SeNPs was investigated against multidrug resistant *S. aureus* and *E. coli* that were isolated from mastitic milk.

MATERIALS AND METHODS

Samples

A total of 150 milk samples were taken with an aseptic technique (Hogan *et al*., 1999). Samples were collected from different farms in Sharkia Governorate, Egypt, over a period (August 2020– July 2021). Since all samples for the current study were taken during standard diagnostic exams, ethical approval was not necessary.

Determination of the Number of Somatic Cells in Milk

Somatic cells count (SCC) was determined as soon as possible at Animal Health Research Institute, Zagazig Lab using somatic cell counter (MT05, manufactured by PISOFT, SLOVAK REPUBLIC).

Isolation and Identification of the Involved Pathogens

After counting somatic cells, the milk sample of the high count was inoculated on a blood agar, MacConkey agar and tryptic soya agar then incubated for 24 h at 37°C. Further identification of specific bacterial isolates such as Staphylococci spp. and *E. coli* bacteria was done according to the manufacturer designated by the National Mastitis Council. Following the standard microbiological technique and procedures for the diagnosis of bovine mastitis infection, the bacteriological culture was formed based on Quinn *et al*. (2002).

Preparation of selenium nanoparticles (SeNPs)

SeNPs was prepared using a modified method of the Chen *et al*. (2011) approach. Simply, 100 mL of ethylene glycol (Merck Schuchardt - Hohenbrunn, Germany) and were mixed with 4 g of Na_2SeO_3 (LobaChemie, India; MW=173.01), 4 g of glucose (ADWIC, Egypt), and 100 mL of water. The beaker containing the reactants was then sealed and placed in an oven that had already been preheated to 80°C. The beaker was withdrawn and chilled after the reaction had continued for an hour.

Antimicrobial susceptibility testing

Using Mueller Hinton agar and commercial antibiotic discs, the Kirby-Bauer standard agar disc diffusion technique as described before (Bauer *et al*., 1966) was used to assess the *In vitro* susceptibility of *S. aureus* and *E. coli* isolates to routinely used antimicrobial medicines (Oxoid, Basingstoke, Hampshire, England, UK). The studied antibiotics were amoxicillin (AX), ampicillin (AM), erythromycin (E), vancomycin (VA), oxacillin (OX), doxycycline (Do), sulfamethoxazole/trimethoprim (SXT), gentamicin (CN), and ciprofloxacin (CIP). According to the breakpoints advised by the Clinical and Laboratory Standards Institute, the inhibition zones were tested twice and scored as sensitive, moderate, and resistant categories (CLSI, 2011).

Polymerase chain reaction procedure

DNA extraction from bacterial samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Primers used were supplied from Metabion (Germany) and listed in Table 1. According to the amplification reactions listed in Table 1, the amplification reaction was carried out in an applied biosystem 2720 thermal cycler. The PCR products were separated using gradients of 5V/cm electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) at room temperature. Each gel slot had 20 µl of the PCR product inserted for the gel analysis. The fragment sizes were measured using a Gelpilot 100 bp (Qiagen, Germany, GmbH). Using a gel documentation system (Alpha Innotech, Biometra), the gel was photographed, and the data were analyzed through computer software.

Antibacterial activity test of SeNPs

Agar Well diffusion assay

The initial evaluation of SeNPs' antibacterial effectiveness against isolates of *S. aureus* and *E. coli* isolates in comparison to gentamycin was conducted using the agar well diffusion method. The examined isolates were added to 10 mL of sterile nutrient

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

Bacteria	Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles) Secondary denaturation	Annealing	Extension	Final extension	Reference
S. aureus	*23S rRNA*	ACGGAGTTACAAAGGACGAC AGCTCAGCCTTAACGAGTAC	1250 bp	94˚C 5 min	94˚C 30 sec	55˚C 1 min	72˚C 1.2 min	72˚C 12 min	Bhati *et al*. (2016)
E. coli	*16S rRNA*	GACCTCGGTTTAGTTCACAGA CACACGCTGACGCTGACCA	585 bp	94˚C 5 min	94˚C 45 sec	58˚C 45 sec	72˚C 60 sec	72˚C 3 min	Hassan *et al*. (2014)

broth and allowed to grow there for 8 hours at 37°C. Using a sterile cotton swab, the cultures were swabbed onto the sterile nutrient agar plates. With the aid of a 10 mm diameter sterilized cork borer, agar wells were made (NCCLS, 2002). 100 µl of SeNPs in a variety of concentrations were added to the plate's wells using a micropipette. The plates were incubated for 24 h at 37°C while standing upright. Inhibition zones' diameters were measured in millimeters, and the data were recorded. It was reported that the inhibitory zones with a diameter of less than 12 mm lacked antibacterial effects (Durairaj *et al*., 2009).

Microdilution method

By using the conventional microdilution method, SeNPs was tested for the minimum inhibitory concentration (MIC) that would prevent bacterial growth (CLSI, 2013). In a broth dilution susceptibility test, the MIC is the lowest dosage of an antimicrobial agent that inhibits a microbe from growing visibly. The MIC was calculated using a 96-well microtiter plate dilution. In a nutshell, each well containing 50 µL of Muller-Hinton soup received 50 µL of SeNPs solution at twice the desired final concentration. The direct colony suspension method was used to create the inoculums. Isolated colonies were chosen from a 24 h agar plate and placed in a direct saline suspension. The solution was then adjusted to reach a turbidity that was equal to the 0.5 McFarland standard. Total of 10 µL of each tested strain's activated culture, containing 5 x10^6 CFU per mL, was introduced. Bacterial growth inhibition was assessed after 20 hours of incubation at 37 °C by measuring the optical density at 630 nm using an ELX 808 microplate reader (Biotek Instruments, Winooski VT, USA). The bacterial growth was visible in the absorbance at 630 nm. The lowest concentration of SeNPs solution that inhibited observable microbial growth was recorded as the MIC value. The control was a blank control without SeNPs solution.

RESULTS AND DISCUSSION

Mastitis is a major problem worldwide that affects dairy industry and leads to massive economic losses due to condemnation of huge quantities of milk either because of apparent defects in the sensory characteristics of milk, or due to unsafety of the milk because of bacterial contaminations (Sharma *et al*., 2012). In the present study, a total of 150 milk samples were examined for determination of somatic cell counts, a marker of mastitis. The obtained results revealed that 62 samples (41.33%) had higher SCC (more than 200000 cells/mL of each milk sample), which confirmed the occurrence of mastitis. Likely, mastitis was confirmed at a prevalence rate of 42.92% in dairy herds in Beni Suef, Egypt (Elbably *et al*., 2013). Mastitis cases were also recorded at a higher rate (85.33%) in Sokoto, Northwest Nigeria (Adamu *et al*., 2020). Milk samples (62 samples) with SCC higher than 200000 cells/mL were examined bacteriologically for isolation and identification of Staphylococcus spp., and *E. coli*, two major bacterial causes of mastitis (Ombarak *et al*., 2019; Algammal *et al*., 2020). Microorganisms were typed based on their cultural characteristics, biochemical tests, staining characteristics, and molecular confirmation of their specific genes (Fig. 1). Results showed that 32 (51.61%), 27 (43.54%) and 15 (24.19%) samples had Staphylococci, *E. coli* and mixed Staphylococci and *E. coli* contamination, respectively. *S. aureus* was confirmed at 25/62 (40.32%) of the examined Staphylococcus isolates. Similarly, *E. coli* was frequently recognized as the most important Gram's negative bacteria linked to mastitis cases in Egypt (Ombarak *et al*., 2019). *S. aureus* was also among the most significant Gram's positive bacteria associated with the onset of bovine mastitis in Egypt (Ahmed *et al*., 2020; Algammal *et al*., 2020).

The abuse of antimicrobials in dairy farms for the prevention and control of bacterial diseases had led to the development of the antimicrobial resistance among several bacterial pathogens (Alsayeqh *et al*., 2021). Antimicrobial resistance is a challenging concern worldwide. Therefore, the susceptibility of the recovered *S. aureus*, and *E. coli* isolates was screened against the most widely used antimicrobials as presented in (Fig. 2). *S. aureus* isolates showed a higher degree of antimicrobial resistance against ampicillin (100%), followed by amoxicillin (96%), and oxacillin (96%). On the other hand, *S. aureus* isolates showed higher sensitivity to vancomycin (100%), followed by ciprofloxacin (96%) and gentamicin (92%). Meanwhile all *E. coli* isolates were found to be 100% susceptible to gentamicin and ciprofloxacin followed by sulphamethoxazole-trimthoprim (74%) and doxycycline (55.5%). While *E. coli* isolates were highly resistant to ampicillin (92.5%), followed by amoxicillin (88.8%), and erythromycin (70.3%). Out of the examined isolates of *S. aureus* and *E. coli*, 15/25 (60%) and 18/27 (66.66%), respectively were recorded as multidrug resistant (MDR), which were resistant to at least one antibiotic in three or more antimicrobial classes. Likely, multidrug resistant *E. coli* and *S. aureus* were isolated from mastitic milk in Egypt as recorded in several studies (Ombarak *et al*., 2019; Ahmed *et al*., 2020; Algammal *et al*., 2020).

Selenium is a vital trace element for growth and health maintenance, but depending on the dose and chemical form, it can be poisonous and cause considerable adverse effects. Due to their decreased toxicity and capacity to release selenium gradu-

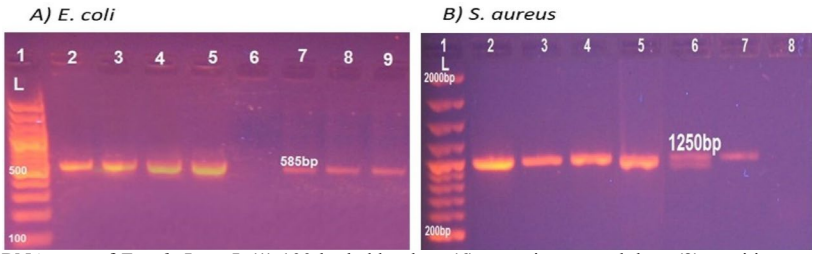

Fig. 1. A) PCR amplicons of 16S rRNA gene of *E. coli*. Lane L (1):100-bp ladder; lane (6): negative control; lane (2): positive control; lanes 3, 4, 5, 7, 8, 9: positive *E. coli* isolates at 585 bp. B) PCR amplicons of 23S rRNA gene of *S. aureus* Lane L (1):200-bp ladder; lane (8): negative control; lane (2): positive control; lanes 3, 4, 5, 6, 7: positive *S. aureus* isolates at 1250 bp.

Table 2. Antibacterial activity of SeNPs against *S. aureus* and *E. coli* isolates.

Isolates	Inhibition zone dimeter (mm) Range			
	SeNPs			Gentamycin
	500 µg/ml	250 µg/ml	125 µg/ml	
S. aureus (n.= 25)	28-32	25-23	18-21	15-22
E. coli (n.= 27)	25-29	22-24	17-20	17-23

Table 3. Minimum inhibitory concentrations (MICs) of SeNPs against *S. aureus* and *E. coli* isolates.

Isolates	MIC values of SeNPs (µg/ml)						
	500	250	125	62.5	31.25	15.62	7.81
S. aureus (n.= 25)	0	2	3	4	6	10	0
E. coli (n.= 27)	1	1	4	10	8	3	0

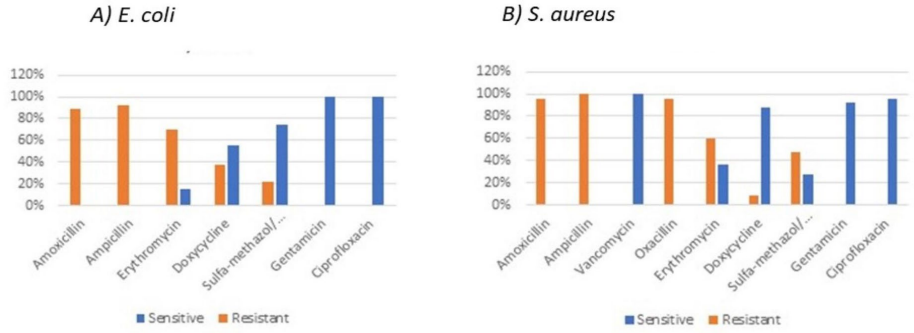

Fig. 2. *In vitro* susceptibility results of A) *E. coli*, and B) *S. aureus* to commercially used antimicrobials.

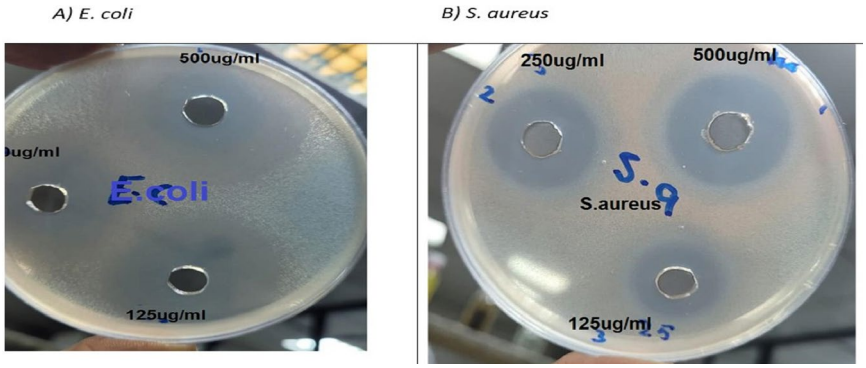

Fig. 3. Antimicrobial activities of different concentrations of selenium nanoparticles against A) *E. coli,* and B) *S. aureus* using the disk diffusion method.

ally after ingestion, SeNPs represent what we think to be a fresh option for nutritional supplementation (Skalickova *et al*., 2017). Moreover, SeNPs were reported to have several medicinal applications such as acting as anticancer candidates, immunostimulants and as antimicrobials (Khurana *et al*., 2019). In this regard, SeNPs exhibited antibacterial activities at concentrations of 125, 250 and 500 µg/mL against both *S. aureus* and *E. coli* isolates in a dose-dependent manner (Fig. 3). The inhibition zone diameters for SeNPs at concentrations of 125 µg/mL ranged from 18-21 mm, and 17-20 mm, for *S. aureus* and *E. coli* isolates, respectively. Such values reached 28-32 mm against *S. aureus*, and 25-29 mm against *E. coli* at 500 µg/mL (Table 2). Calculation of the minimum inhibitory concentrations (MICs) of SeNPs revealed lower MICs against *S. aureus* (15.62-31.25 µg/mL), compared with that against *E. coli* (31.25- 62.5 µg/mL) (Table 3). Similarly, the growth of *S. aureus* was significantly reduced after incubation for 3, 4, and 5 hours at 7.8, 15.5, and 31 g/mL of selenium nanoparticles. Moreover, in the presence of SeNPs, the proportion of living *S. aureus* also fell (Tran and Webster, 2011). Furthermore, SeNPs was successfully synthesized by laser ablation in water. The minimum concentration necessary of the resultant SeNPs to achieve 50% suppression of either *E. coli* or *S. aureus* after 24 h *In vitro* is found to be at least 50 ppm. While at 107.12 and 79.4 ppm, respectively, total inhibition of *E. coli* and *S. aureus* is anticipated to take place. Also, our findings somewhat comparable to that of Vahdati and Moghadam (2020), who reported that gram-negative bacteria displayed remarkable resistance to SeNPs over a broad concentration range. The highest amount of growth inhibition for *E. coli* was reported at 660 µg. mL−1 of SeNPs, whereas in the case of *S. aureus* the inhibitory concentration was 82 µg. mL^{-1}.

An overall finding of the current investigation demonstrated *In vitro* evidence to use SeNPs for the prevention and control of *S. aureus* and *E. coli*-related mastitis.

CONCLUSION

This work therefore recommends that selenium nanoparticles should be further investigated for their application as antimicrobials since they may be utilized to successfully prevent and treat *S. aureus* and *E. coli* infections.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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