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Original Research Article

THE ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF SILVER NANOPARTICLES ON STAPHYLOCOCCI ISOLATES FROM COW MILK

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Abstract: Biofilm-producing ability has been identified as a serious virulence factor in staphylococci and increases their antimicrobial resistance. This study aimed to investigate the biofilm forming ability of staphylococci isolated from cow milk samples. Moreover, we assessed the antibiofilm activity of silver nanoparticles (AgNPs) against methicillin resistant (MRSA) and biofilm forming staphylococci. The results revealed that 82.14% (23/28) and 91.66% (11/12) of the coagulase positive staphylococci (CPS) and coagulase negative Staphylococci (CNS) isolates, respectively produced biofilm on Congo red agar (CRA). In the case of the microtiter plates (MTP) method, all CPS and CNS isolates produced biofilm at different levels. These results revealed a substantial agreement between CRA and MTP results according to the kappa coefficient test (kappa value = 0.773). *Staphylococcus aureus* species specific *nuc* gene and the determinant of methicillin resistance (*mecA*) gene were amplified from *S. aureus* isolates (n=10). The intercellular adhesion gene A (*icaA*), and intercellular adhesion gene D (*icaD*) were amplified from all the CPS and CNS isolates, but none of the isolates were positive for the biofilm associated protein (*bap*) gene. Antibiotic susceptibility testing showed that all isolates were oxacillin resistant. AgNPs (100 µg/mL) inhibited the growth of Staphylococci isolates (inhibition zone diameters ranged from 22 to 28 mm). AgNPs decreased biofilm formation in the biofilm forming Staphylococci isolates with percent ranged from 67.05 to 98.02% using MTP assay. In conclusion, AgNPs have antistaphylococcal activity and inhibit biofilm formation.

Key words: biofilm; antibiofilm; Staphylococci; milk; AgNPs

Introduction

Staphylococcus species are recognized worldwide as significant pathogens causing acute and chronic contagious bovine mastitis (1), and it is one of the major pathogens causing food poisoning (2). Staphylococci associated with mastitis are surrounded by a layer of slime and an exopolysaccharide called biofilm (3). Biofilm production is a common virulence factor of staphylococci that facilitates their colonization and adherence to the mammary gland epithelium. In addition to, leading to the evasion of the immunological defenses, the difficulty of pathogen eradication (4), and resistance to some antibiotics (5), leading to recurrent or persistent infections. Factors that contribute to biofilm-mediated antimicrobial resistance include inefficient diffusion or sequestering of the agent within the biofilm matrix (6), the slow growth rate of biofilm cells (7), the presence of persister cells or antibiotic-resistant small-colony variants (8) and other unknown phenotypic differences. Polysaccharide intercellular adhesion (PIA) is composed of β -1,6 *N*- acetyl glucosamine which is necessary for biofilm formation and responsible for cell to cell adhesion (9). The locus of chromosomal intercellular adhesion (*ica*) encoding PIA

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consists of the *icaADBC* structural and *icaR* regulatory genes. The intercellular adhesion gene A (*icaA*) and intercellular adhesion gene D (*icaD*) genes play a central role in biofilm production (10). Recently, a surface protein, *bap* (biofilm associated protein) has been identified to aid in bovine *S. aureus* biofilm formation (11). There are three methods for the detection of biofilm production in bacteria: the tube method (TM), qualitative Congo red agar (CRA) (12), and quantitative microtiter plates (MTP) (13). In addition, molecular techniques such as PCR have been used recently to understand the molecular mechanisms of biofilm formation (14). Phenotypic methods in combination with genotypic methods must be carried out for confirmation of biofilm formation (15).

Silver nanoparticles (AgNPs) are one of the most extensively utilized antibacterial nanomaterials(16). Studies have shown that AgNPs not only has an antibacterial effect on planktonic bacteria (17) but also has an antibiofilm effect (18). The antibacterial effect of AgNPs is justified by the interaction of AgNPs with bacterial membrane proteins, phosphate residues in DNA, intracellular proteins, and interference with cell division, leading to cell death (19). Therefore, the present study aimed to investigate the phenotypically and genotypically biofilm forming ability of Staphylococci isolated from raw cow milk samples and evaluate the anti-biofilm activity of AgNPs on methicillin resistant and biofilm forming Staphylococci isolates.

Material and methods

Samples collection and bacteriological analysis

This study was carried out on 100 milk samples from individually raised dairy cows, including 65 apparently healthy and 35 cows suffering from clinical mastitis in Sharkia Governorate, Egypt. The samples were collected aseptically during the period from April 2020 to March 2021. Isolation and identification of Staphylococci were carried out according to Bergey and Holt (20). Biochemical identification of Staphylococci isolates was done by performing catalase, coagulase, growth at 10% NaCl, mannitol fermentation tests, and hemolysis on 5% sheep blood agar (21).

Staphylococcus aureus counts in milk samples

Milk samples were serially diluted up to 10^{-6} in normal saline and 1mL sample suspension was transferred aseptically from each dilution to Baird Parker agar (Oxoid-UK). The suspected colonies of *S. aureus* (black, shiny appearance and surrounded by a clear zone) were counted. According to Egyptian Standards (ES, 154|1|2005), the Permissible limit of *S. aureus* in milk should not exceed 100 CFU/mL) (22).

Phenotypic characterization of biofilm formation

Congo Red Agar method**:**

All staphylococci isolates were cultivated on CRA plates which were prepared as mentioned by Mathur et al. (13). The inoculated CRA plates were incubated at 37°C in aerobic conditions for 24 h. The colonies formed over the medium were categorized as strong black, and black according to the strength of biofilm production. Non-biofilm producers are typically found in red colonies (12).

Microtiter Plate (MTP) method:

The Microtiter plate test was performed according to Stepanović et al. (23). The optical density (OD) value of stained adherent biofilm was obtained with a microELISA auto-reader (BioTek, USA) at wavelength 570 nm. The OD value of < 0.120 was defined as none or weak biofilm producer, 0.120 to 0.240 as moderate biofilm producer, and > 0.240 as strong biofilm producer (2).

Antimicrobial susceptibility testing

Antimicrobial susceptibility test was done according to Kirby Bauer disc diffusion method (24) using the following antibiotic discs, ampicillin (10 µg), ciprofloxacin (5µg), oxacillin (1µg), vancomycin (30 µg), amikacin (30 µg), amoxicillin (10 µg), clindamycin (2 µg) and gentamycin (10 µg) (Oxoid Ltd., Basingstoke, Hampshire, UK). Interpretation of the results was done according to Clinical and Laboratory Standards institute (CLSI) guidelines (25). Multiple antibiotic resistance (MAR) index was determined as previously described (26).

Molecular identification of Staphylococci isolates and biofilm-encoding genes

The identification of the recovered staphylococci isolates was confirmed by PCR amplification of the staphylococcus genus-specific 16S rRNA gene. Furthermore, CPS isolates were tested for the *S. aureus* species-specific *nuc* gene and methicillin resistance gene (*mecA*) (27-29) (Table 1).

Table 1: Oligonucleotide primers used for identification of staphylococci and biofilm genes

Target Gene	Primer sequence (5'-3')	Amplicon size (bp)*	Primary denaturation	Amplification (30 cycles)			Final extension	Reference
				Denaturation	Annealing	Extension		
Staphyloccocci identification *16S rRNA*	CCTATAAGACTGG-GATAACTTCGGG CTTTGAGTTTCAAC-CTTGCGGTCG	791	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	72°C 10 min.	(27)
S. aureus identification *nuc*	GCGATTGATGGTGA-TACGGTT AGCCAAGCCTTGA-CGAACTAAAGC	270	94°C 5 min.	94°C 1 min	50°C 1 min	72°C 2 min.	72°C 10 min	(28)
mecA	TAG AAA TGA CTG AAC GTC CG TTG CGA TCA ATG TTA CCG TAG	154	94°C 3 min.	94°C 1 min	58°C 1 min.	72°C 1 min.	72°C 5 min.	(29)
Biofilm genes *icaA*	CCT AAC TAA CGA AAG GTA G AAG ATA TAG CGA-TAA GTG C	1315	94°C 5 min.	94°C 30 sec.	49°C 1 min.	72°C 1 min.	72°C 12 min.	(30)
icaD	AAA CGTAAG AGA GGT GG GGC AAT ATG ATC AAGATA	381	94°C 5 min.	94°C 30 sec.	49°C 30 sec	72°C 30 sec.	72°C 7 min.	
bap	CCC TAT ATC GAA GGT GTA GAA TTG GCT GTT GAA GTT AAT ACT GTA CCT GC	971	94°C 5 min.	94°C 30 sec.	62°C 40 sec	72°C 50 sec.	72°C 10 min.	(11)

*bp: base pair

Staphylococci isolates that exhibited methicillin resistance and strong biofilm producers on MTP were examined using specific primers targeting biofilm genes including *icaA*, *icaD*, and *bap* genes (30, 11) (Table 1). DNA extraction was performed using the QIAamp DNA mini kit (Qiagen, Germany, GmbH). DNA amplification was performed in a reaction volume of 25 µL containing 12.5 µL of Emerald Amp GT PCR master mix (2x premix), 1 µL of 20 pmol of each primer (Biobasic, Canada), 5 µL of DNA template, and 5.5 µL of DNase/RNase-free water. The T3 thermal cycler **(**Biometra**)** was used to carry out the cycling conditions. PCR products were analyzed by electrophoresis and visualized under a UV transilluminator (Spectroline, USA).

Characteristics of silver nanoparticles (AgNPs)

AgNPs (Sigma-Aldrich, St. Louis, MO, USA) were of a spherical shape, size (20-40 nm) and 99.97% purity. They were soluble in water (100µg /mL) yielding a clear, colourless solution.

Determination of the antibacterial activity of AgNPs

Agar well diffusion test was used to screen the antistaphylococcal activity of AgNPs as previously described (31). Staphylococci suspensions were prepared in physiological saline and adjusted approximately to reach the density of $1.5x10^8$ CFU/mL. Wells of 6-mm diameters were made on Mueller Hinton agar (MHA) (Oxoid, UK) plates using a sterile cork borer. The prepared bacterial suspensions were swabbed uniformly on the individual plates by using sterile cotton swabs. Different concentrations of AgNPs (6.25, 12.5, 25, 50, and 100 µg/mL) were poured into wells (32). The plates were left for 30 min at room temperature to allow the diffusion of the suspension and then incubated at 37°C for 24 h. The inhibition zone diameters were measured and recorded.

Testing antibiofilm activity of AgNPs against biofilm-producing Staphylococci isolates

Briefly, 100 µL of AgNPs were added to the selected wells of a 96-well flat-bottom microtiter plate (Cleanway, Egypt) seeded with 100 µL of trypticase soy broth (TSB) (Oxoid, UK) that contained 10^8cells/mL and incubated for 24 h at 37°C to allow the biofilm formation. The contents of the wells were removed and washed three times using sterile phosphate-buffered saline (PBS) (Al-Gomhorya Company, Egypt). The extent of biofilm formation was detected by using the crystal violet staining assay. The negative control (silver nanoparticles free wells) and the positive controls (wells containing biofilms) were included. Experiments were carried out in triplicate. The percentage of biofilm inhibition was calculated by the formula: percentage of biofilm inhibition = 1- (Test OD570 nm/ Control OD570 nm) x 100 (33).

Data analysis

Statistical analysis was done by IBM SPSS Statistics 23 (IBM Corp., Armonk, NY). The significance of differences in CPS and CNS recovery rates from raw and mastitic milk samples was assessed by Mann-Whitney test, and the Binomial test was used to compare between the frequency

of CPS and CNS in each of raw milk and mastitic milk. Differences were considered significant when $P < 0.05$.The agreement between the CRA and MTP test results was determined by the kappa coefficient test (34) and their specificity and sensitivity were calculated according to Ilstrup (35).

Results

Occurrence of staphylococci in the examined cow milk samples

Based on bacteriological examination, staphylococci were isolated from 40% of milk samples (26.15% from raw milk and 65.7% from mastitic milk). There is a non-significant difference ($P > 0.05$) in the occurrence rate of Staphylococci in milk samples. Twenty-eight isolates yielded the characteristic golden yellow pigments onto mannitol salt agar (MSA) medium, produced β-hemolysis onto blood agar, black shiny colonies surrounded by a clear zone onto baired parker agar medium, exhibited agglutination of rabbit plasma in coagulase test, and strong bubbles in catalase test were identified as CPS. The recovery rate of CPS was 20 (57.14%) from mastitic milk samples that was significantly higher than raw milk samples 8 (12.30%) (Table 2). On the other hand, the other 12 (12%) isolates produced red pigmented colonies on MSA, α or δ hemolysis on blood agar, and no agglutination of rabbit plasma. They were identified as CNS. Of note, there is a non- significant difference ($P = 1.00$) between the frequency of CNS and CPS in raw milk samples.

Enumeration of S. aureus in milk samples

As listed in Figure (1), the mean count of *S. aureus* reached 4.25×10^4 and 2.6×10^5 in raw and mastitic milk samples, respectively. In addition, 3.07% and 48.57% of raw and mastitic milk samples, respectively, exceed the permissible limits of *S. aureus* count in milk.

Phenotypic characterization of biofilm formation using CRA and MTP methods

About 34/40 (85%) of the examined CPS and CNS isolates were positive for biofilm production with varying degrees on CRA (Figure 2A); but by MTP method, all isolates were biofilm producers with different adherence levels (Figure 2B) (Table 3). The CRA method revealed that out of 28 CPS isolates, seven (25%) yielded strong black colonies and only five (17.85%) demonstrated red colonies. In the examined 12 CNS isolates, three (25%) and one (8.33%) were strong black and red colonies, respectively. MTP results showed 3 adherence levels; strong, moderate, and weak or none-biofilm producers. CRA and MTP results showed a substantial agreement according to the kappa coefficient test (kappa value = 0.773), but MTP exhibited higher sensitivity (94.4%) in detecting the positive strains and higher specificity (100%) in screening the negative isolates than CRA.

Antibiotic susceptibility testing of strong biofilm producers

The susceptibility testing of 20 CPS and 8 CNS isolates that exhibited strong biofilm production on MTP was conducted against the most widely commercial antibiotics. The higher degrees of antibiotic resistance were against oxacillin (100%), clindamycin (67.85%), amoxicillin (64.28%) and ampicillin (60.71%). On the other hand, the higher sensitivity of the tested isolates was recorded against ciprofloxacin (85.71%), amikacin (78.57%), and vancomycin (71.425%) (Table 4). Out of 28 examined isolates, 10 (35.71%) were recorded as multidrug resistant (MDR), which were resistant to at least one antibiotic in three or more antimicrobial classes. Oxacillin showed a higher MAR index (0.125) but ciprofloxacin recorded a lower MAR index (0.004).

PCR characterization of Staphylococci and detection of biofilm-encoding genes

All the examined 20 CPS and 8 CNS isolates successfully amplified the 16S rRNA gene with amplicon sizes of 791 bp. On the other hand, 10 isolates were positive for *nuc* gene and *mec*A genes with amplicon sizes of 270 bp and 154 bp, respectively. Ten examined methicillin-resistant and strong biofilm producer isolates successfully amplified the *icaA* and *icaD* with amplicon sizes of 1315 bp and 381 bp, respectively, and were negative for the *bap* gene.

Antibacterial and antibiofilm activities of AgNPs

Our results showed antibacterial activity of AgNPs at concentrations of 50 and 100 µg/mL against 10 staphylococci isolates (7 CPS and 3 CNS) (Figure 3). The inhibition zone diameters with AgNPs at concentrations of 100 µg/ml and 50 µg/mL ranged from 22-28 mm and 16-20 mm, respectively. Using the MTP method, AgNPs at a concentration of 50 µg /mL inhibited biofilm formation by Staphylococci isolates by 67.05-98.02% (Table 5).

Table 2: Recovery rate of staphylococci from the examined milk samples

Milk samples	No. of CPS (%)	No. of CNS (%)	Total (%)	Binomial test *P*-value
Raw milk (n = 65)	8 (12.30)	9 (13.84)	17 (26.1)	1.000
Mastitic milk (n = 35)	20 (57.14)	3 (8.57)	23 (65.7)	0.000 [c]
Total (100)	28 (28)	12 (12)	40 (40)	0.017
	Mann-Whitney test			
P-value	0.000 [a]	0.44	0.00 [b]	

CPS; coagulase positive Staphylococci, CNS; coagulase negative Staphylococci.
[a, b, c] *P*-values (Mann-Whitney_test and Binomial test) < 0.05 were considered statistically significant

Table 3: Results of CRA and MTP methods for detection of biofilm production in CPS and CNS isolates

Species (No.)	CRA	No. (%)	MTP	No. (%)
CPS (28)	Strong black	7(25)	Strong	20 (71.14)
	Black	16 (57.14)	Moderate	6 (21.42)
	Red	5 (17.85)	Weak /none	2 (7.14)
CNS (12)	Strong black	3 (25)	Strong	8 (66.66)
	Black	8 (66.66)	Moderate	2 (16.66)
	Red	1 (8.33)	Weak /none	2 (16.66)

CPS; coagulase positive Staphylococci, CNS; coagulase negative Staphylococci, CRA; congo red agar, MTP; microtiter plate method

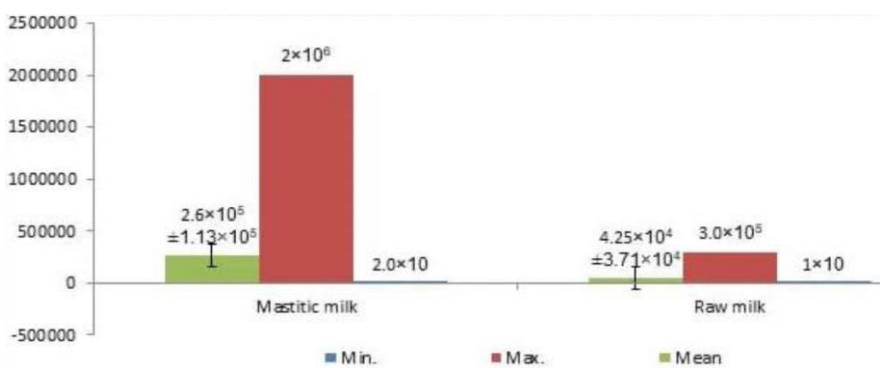

Figure 1: The colony count (CFU/mL) of coagulase positive Staphylococci in the examined milk samples

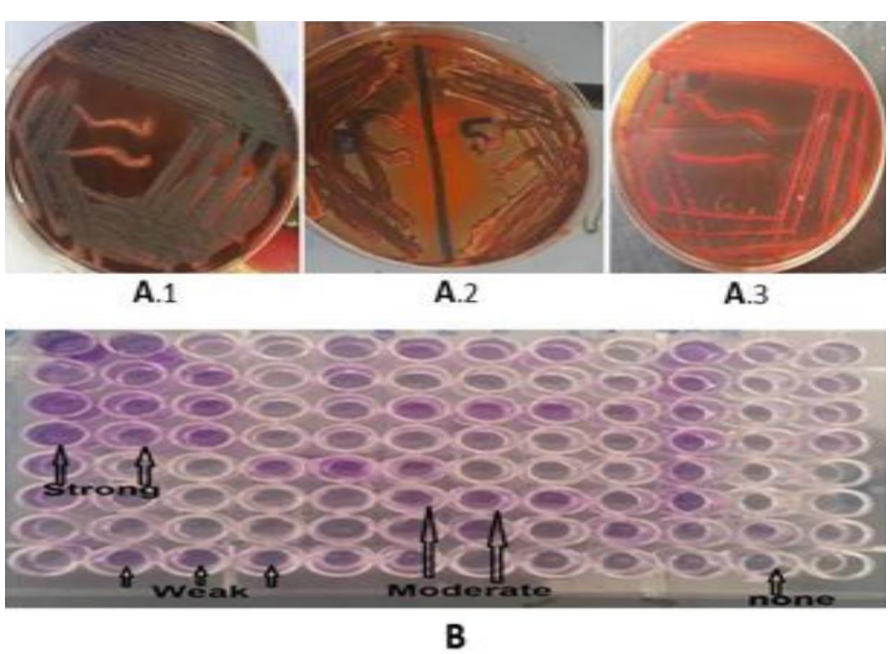

Figure 2: A. Screening of biofilm forming staphylococci on congo red agar plate (A.1) strong black colonies; (A.2) black colonies; (A.3) red colonies.B. Microtiter plate method reveals that among Staphylococci isolates, none, strong, moderate, and weak biofilm producers exist

Table 4: Susceptibility profile of 28 strong biofilm-producers Staphylococci isolates

Antibiotics	Sensitive (%)	Intermediate (%)	Resistant (%)	MAR index
Ampicillin	6 (21.42)	5 (17.85)	17 (60.71)	0.075
Ciprofloxacin	24 (85.71)	3 (10.71)	1 (3.57)	0.004
Oxacillin	0 (0)	0 (0)	28 (100)	0.125
Vancomycin	20 (71.42)	3 (10.71)	5 (17.85)	0.022
Amikacin	22 (78.57)	2 (7.14)	4 (14.28)	0.017
Amoxicillin	4 (14.28)	6 (21.42)	18 (64.28)	0.080
Clindamycin	4 (14.28)	5 (17.85)	19 (67.85)	0.084
Gentamycin	15 (53.57)	2 (7.14)	11 (39.28)	0.049

MAR; multiple antibiotic resistance index

Table 5: Resistance patterns of staphylococci isolates and antibiofilm effect of AgNPs

Isolates Code No.	Resistance pattern*	Biofilm formation (OD values)**		Inhibition % of biofilm formation§
		None treated	treated with 50 µg/mL AgNPs	
CPS				
1	OX,AM, DA, AX,VA	Strong (0.522)	Weak /none (0.031)	94.04
2	OX,VA, DA, CN, AX	Strong (0.284)	Weak /none (0.014)	95.07
3	OX, AM, DA,VA, AX	Strong (0.735)	Weak /none (0.024)	96.73
4	OX, AM, AX, DA,CIP	Strong (0.280)	Weak /none (0.021)	92.50
5	OX, AM, DA, CN, AK	Strong (0.255)	Weak /none (0.084)	67.05
6	OX, AM, CN, AX, AK	Strong (0.260)	Weak /none (0.035)	86.53
7	OX, AM, DA, AX,VA	Strong (0.522)	Weak /none(0.020)	96.16
CNS				
8	OX, AM,CN, AX, AK	Strong (0.384)	Weak /none (0.023)	94.01
9	OX, AX, DA, CN, AM	Strong (0.620)	Weak /none(0.030)	95.16
10	OX, AX, DA, CN, VA	Strong (0.760)	Weak /none (0.015)	98.02

CPS; coagulase positive Staphylococci, CNS; coagulase negative Staphylococci, *DA; clindamycin, CIP; ciprofloxacin,OX; oxacillin, AK; amikacin, Ax; amoxicillin, AM; ampicillin , CN; gentamycin, and VA; vancomycin. ** Optical density values at 570 nm by microtitre plate method. §Inhibition % of biofilm formation was calculated using this equation 1- (Test OD570 nm/ Control OD570 nm) x 100

A B

Figure 3: Agar well diffusion test showing inhibition of Staphylococci isolates (code No. 1: A and No.10: B) by AgNPs at different concentrations

Discussion

Out of the 100 analyzed cow milk samples, 40 (40%) were contaminated with Staphylococci. Based on bacteriological identification, 28 (28%) isolates were identified as CPS and 12 (12%) were characterized as CNS. This result was contrary to an earlier study in which CNS accounted for

68.35%, while *S. aureus* accounted for 3.2% (36). Mahmoud et al. recorded a higher CPS isolation percentage (48.2%) and a lower isolation rate of CNS (8.9%) (37). In this study, the occurrence rate of staphylococci isolates in mastitic milk samples was 65.71% (23/35). This occurrence rate was higher than that recorded in another work (71.5%) (36). 57.14% (20/35) of isolates from mastitic milk were CPS which is higher than that reported in another study (32.8%) (38). In accordance with a previous study that showed 6.8 to 23.3% of mastitis in cattle was caused by CNS, 8.57% (3/35) of isolates were CNS (39). The differences in these isolation rates may be associated with several causes, such as conditions during milking, sample sources, and differences in locality. The mean of *S. aureus* count in raw milk samples obtained from apparently healthy cows was $4.25x10^4$. Another research recorded a lower count ($2.5x10^4$) of *S. aureus* in milk (40). In case of mastitic milk samples, *S. aureus* count was $2.6x10^5$, which was lower than the previously mentioned ($1.1x10^6$) (41). The results showed that 3.07% and 48.57% of milk samples obtained from the apparently healthy cows and those with clinical mastitis, respectively, had *S. aureus* with a count ranging from 10^3 to10^6 CFU/mL. These results exceed the permissible limits set by Egyptian Standards (ES, 154|1|2005) (22) but are lower than the results of another study, which found that the frequency distribution of *S. aureus* in raw milk was 80% within the range of $10^4 \leq 10^6$ CFU/mL (42). Consequently, we need strict hygienic measures for milk production processes (43).

Evaluation of biofilm production by staphylococci isolates using CRA and MTP methods showed about 85% of the examined isolates were positive for biofilm production with a varying degree on CRA, but on MTP, all isolates were biofilm producers with different adherence levels. These findings were higher than those of Darwish and Asfour (10) who stated that 70.4% of the isolates were positive for biofilm production with a varying degree on CRA and 96.3% of the isolates were biofilm producers by using the MTP method. The percentage of positive biofilm forming isolates was higher than stated in other research (44).

According to Darwish and Asfour (10), Mathur et al. (13), the CRA method alone cannot be used to screen for Staphylococci biofilm formation. The difference in results obtained from both CRA and MTP methods may be attributed to the fact that phenotypic expression of biofilm formation is highly sensitive to *in vitro* conditions and can be detected variably by different methods. Also, both tests measure the same phenomenon but in different ways. Our findings illustrate that the MTP method has higher sensitivity (94.4%) for detection of positive strains and higher specificity (100%) in screening the negatve isolates than CRA. So, our study and a previous study recommended using the MTP assay as a routine method for biofilm analysis (15).

The susceptibility test results showed that all isolates were resistant to oxacillin. The higher degrees of antibiotic resistance were against clindamycin (67.85%), amoxicillin (64.28%) and ampicillin (60.71%). These results agreed with previous studies (45) and were dissimilar to other work that found low resistant rates in examined isolates (46). Moon et al. (47) stated that 60.2% of CNS were resistant to penicillin. Taponen and Pyörälä (48) mentioned that the high prevalence of resistance mechanisms in staphylococci is due to β- lactamase production that results in penicillin resistance. The higher sensitivity of the tested isolates was recorded against ciprofloxacin (85.71%), amikacin (78.57%) and vancomycin (71.42%). Dhakal (49) recorded a similar sensitivity rate of staphylococci against the same antibiotics. This high increase in antimicrobial resistance is attributed to the overuse of antibiotics in animal production and agriculture, as well as the very easy purchase of antibiotics without a prescription (50-52).

Amplification of the *mec*A gene confirmed that 100% of the tested staphylococci isolates were methicillin resistant, which is in accordance with the previous study (53). The prevalence rate of *ica*A and *icaD* genes was 100% (10/10), which was higher than previously reported (15% and 62.5% for *ica*A and *Ica*D, respectively in *S. aureus*) (10) and agreed with another findings that all the examined 35 isolates possessed *icaA* and *icaD* genes (54). The absence of the *bap* gene in all isolates has been previously stated (55). All isolates that produced strong biofilm on MTP and CRA were amplified *ica*A*D* genes (*ica*A and *icaD* that agreed with previous research (56). Vasudevan et

al. (54) declared that screening of *ica* genes is a better methodology for biofilm detection, in addition to MTP or CRA methods, to avoid missing the strains genotypically positive and negative by phenotypic methods.

Our results showed that AgNPs at a concentration of 50 and 100 µg/mL had antistaphylococcal activity. The biofilm formation ability of the examined staphylococci isolates was highly inhibited (67.05 to 98.02%) post exposure to 50 µg/mL AgNPs, implying its efficiency as an antibiofilm agent. This result is in agreement with Mohanty et al. (57) who showed that AgNPs decreased biofilm formation by 88%. In our study, AgNPs were used with a small size (mean diameter < 40 nm) which was one of the contributing factors to their antibacterial and antibiofilm activities. Similarly, Kalishwaralal et al. (58) found that AgNPs (mean diameter 50 nm) completely inhibited *S. epidermidis* biofilms by blocking the initial step of biofilm formation (bacterial adhesion to the surface).

Conclusion

This work demonstrates that MTP has good sensitivity and specificity in the screening of biofilm-forming Staphylococci isolates. Genetic detection of biofilm encoding genes is an alternative rapid method for confirmation of biofilm activity. AgNPs have efficient antibacterial and antibiofilm activity against MRSA. The presence of MDR *S. aureus* in raw milk above the permissible limit contributes a potential risk to public health. Hence, it is very important to apply strict hygienic measures during milking and milk packaging. Further studies focusing on the *in vivo* efficacy and safety application of AgNPs as an antibacterial and antibiofilm agent are recommended. Also, further studies are recommended on the ability of incorporation of AgNPs into milk packaging materials.

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Conflict of interest. The authors declare no conflicts of interest.

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