



Official Journal Issued by
Faculty of
Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: <https://bvmj.journals.ekb.eg/>



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Original Paper

Antibiogram pattern and antimicrobial effect of Cao Nanoparticles on *Staphylococcus aureus* isolated from different sources

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ARTICLE INFO

Keywords

Cao Nanoparticles,
Staphylococcus aureus,
High-resolution
transmission electron
Microscopy,
milk,
semen,
dairy workers

Received 16/09/2023

Accepted 26/09/2023

Available On-Line

31/12/2023

ABSTRACT

The emergence and increasing of multidrug resistant bacteria have become an urgent threat requiring the development of a new antimicrobial class. This study was planned to investigate the effects of calcium oxide nanoparticles (CaO-Nps) on *S. aureus*. So, 140 samples from different sources, such as raw milk, milk by-products, intestinal contents of poultry, animal semen, and hand swabs of milkers and workers were collected. The findings illustrated *S. aureus* isolated from 30 samples (21.42%) using bacterial isolation which confirmed using PCR technique. Their Antibiogram pattern showed high resistance to cefoxitin, oxacillin, tetracycline, and gentamicin while were sensitive to lenzolid and clindamycin. The concentration of Cao-NPs in the prepared solution was determined by atomic absorption apparatus, and the other characters were detected by high-resolution transmission electron microscopy (HRTEM). The HRTEM showed that the size of Cao-NPs ranged from 31 to 120 nm, with a mean of 67.62 NM. The crystalized Cao-NPs show of 11.291/nm, 9.431/nm, and 6.181/diameter. The results also revealed an average zeta potential (ZP) of the prepared CaO NPs of -18.00 mV in 418 mJ. This proved that CaO NPs have a high degree of stability. Its antimicrobial activity against *S. aureus* was studied by a well diffusion method. The results showed that the inhibition zone diameter varied from 8 mm to 16 mm starting from Cao NPs concentration 100 NPs/ml to 600NP/ml, while 50 NPs/ml didn't cause inhibition.

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacteria arranged in grape-like clusters that vary in size from 0.5-1.5 μm . Its colony color varies from white, Grey to grey, or Grey-White with shades ranging from yellow to orange. Depending on the growth conditions, typical hemolysis may be produced on blood agar (Muruhan et al., 2012). Due to its widespread distribution, it is considered as a zoonotic pathogen of concern to public health, high rate of contamination, and rapid transmission. It results in a range of clinical manifestations, including life-threatening invasive illnesses and superficial, mild-local cutaneous lesions (Turner et al., 2019). *S. aureus* infection symptoms and severity are influenced by virulence factors (Xing et al., 2016). *S. aureus* is the cause of opportunistic, nosocomial, opportunistic, and population-related illnesses worldwide (Paterson et al., 2013). *Staphylococcus aureus* has been determined to cause mastitis in animals and food poisoning due to the consumption of contaminated dairy products, particularly yoghurt and cheese (Feyissa et al., 2022). Raw milk and milk by-products, especially kareish cheese which is not exposed to any type of thermal treatment, serve as a method for the transmission of many types of microorganisms, such as *S. aureus*. Also, a deficiency of hygienic handling of food products, especially those of animal origins like raw milk, milk byproducts, and poultry meat, during and after preparation facilitates microbial contamination (Akabanda et al., 2017). *Staphylococcus*

aureus became highly resistant to antibiotics. in addition to, their ability to overcome the immune system (Liu et al., 2017). Multi-drug-resistant (MDR) *S. aureus* has evolved from a single-drug-resistant, which overburdens the problem of drug susceptibility (Gomes and Henriques, 2015). Nanoparticle-based treatment offers a very promising strategy to overcome the resistance built up by bacteria. As a result, using nanoparticles in conjunction with antibiotics may increase their ability to limit growth and reduce the chance that bacteria will develop resistance (Sobhani et al., 2017).

A nanoparticle is a small particle that has at least one dimension (1-100 nm) in the range of nanometer scale (Edmundson et al., 2013). Many factors affect the mechanism of action of NPs, including size, shape, surface coating, surface charge nature, dosage, protein binding, and animal species of the particles. NPs are now coming to be seen as a successful alternative to antibiotics, (Cao et al., 2019). Also, Nanoparticles have a stronger ability to penetrate cells and tissues due to their small particle size and strong antimicrobial effect (Mohammadi et al., 2011). CaO NPs have strong antibacterial properties, due to increased exposure to active oxygen species and alkalinity. By hydrating CaO with water and superoxide, pH is increased built up on its surface. Also, CaO NPs have adequate antimicrobial activity due to large surface area to volume ratios, where the small size of the particles can easily enter the bacterial cells leading to destruction and distortion of the

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cell membrane, then bacterial cell death (Ramola et al., 2019).

So, the current research was aimed to isolate *S. aureus* from various sources and determine of antibiogram pattern of the isolates. Then study the antimicrobial effect of (CaO-Nps) on *S. aureus*.

2. MATERIAL AND METHODS

2.1. Sampling:

A total of 140 samples were randomly collected from variety of sources, including raw milk, Pasteurized milk, Yoghurt, kareish cheese, soft cheese, Ice cream, intestinal content of chicken, Semen of dogs and hand swabs from factory workers (Batta bros Dairy product factory and Abu Shaqra factory for meat product) and dairy farm in Giza Governorate (ethical number: BUFVTM04-06-23 using sterile containers and cotton swabs under aseptic conditions in an ice box and then transferred to the lab for a bacteriological examination.

2.2. Isolation and identification of *S. aureus*:

2.2.1. Isolation

The inoculated transport media (peptone water) incubated at 37°C for 24 hr. then cultivated on sheep blood agar (5%) and mannitol salt agar (MSA) then incubated for 24 hr at 37°C (Saab et al., 2017).

2.2.2. Morphological identification

The purified colonies were picked up and stained with Gram's stain for microscopical demonstration of *S. aureus* morphological characters (Tong et al., 2015).

2.2.3. Biochemical tests

S. aureus was identified using the coagulase and catalase tests (Abdelhamid and Wu, 2018).

2.3. Confirmation of *S. aureus* by PCR assay

DNA extraction: According to the manufacturing kit (Qiagen, Germany, GmbH)

Oligonucleotide Primer: Primer sequence specific to 23S *rRNA* gene provided by Metabion (Germany) Table (1).

PCR amplification: A PCR reaction mix of (25- µl) was prepared from (12.5 µl) of Emerald Amp Max PCR Master Mix (Takara, Japan), (1 µl) of each primer of 20 pmol concentration, (4.5µl) of water, and (6 µl) template of DNA. A specific biosystem 2720 thermal cycler was used to perform the reaction.

Analysis of the (PCR) Products: The PCR products were separated by electrophoresis on 1.5% agarose gel (AppliChem, Germany, GmbH) in 1x TBE buffer at room temperature Using ingredients of 5V/cm. Gel pilot (100 bp) plus DNA ladder (GmbH, Qiagen, Germany) and a gene ruler (100 bp) (Germany, Fermentas) were used to determine the fragment sizes. Using a gel documentation device, the gel was photographed by (Biometra Alpha Innotech) and computer software was used to analyze the data.

Table (1) Primers sequences, amplicon sizes, cycling conditions and Target genes.

Target gene	Primers sequences	Amplified segment (bp.)	Prim. (Den.)	Amplification (35 cycles)			Final extension	Reference
				Sec.(den.)	(Ann.)	(Ext.)		
23S <i>rRNA</i>	AC GGAGTTACAAAGGACGAC	1250	94°C	94°C	55°C	72°C	72°C	(Bhati et al., 2016)
	AGCTCAGCCTTAACGAGTAC		(5 min).	(30 sec)	(40 sec).	(1.2 min).	(12 min)	

2.4. Antimicrobial sensitivity testing

The In-vitro antimicrobial susceptibility was determined against antimicrobial types on Mueller-Hinton agar (Oxoid) plates using the technique of Kirby-Bauer disc diffusion (CLSI, 2018).

2.5. Calcium oxide nanoparticles preparation and characterization:

CaO NPs were synthesized from hen eggshell powder (by sol-gel) derived technique (Habte et al., 2019).

Briefly, the eggshells were washed and then dried in the oven at 120°C for 1.5–2 hours. Then it was ground into powder using a mortar and pestle. CaCl₂ solution was prepared by dissolving 12.5 gm of eggshell powder in 250 mL of 1M HCl with a magnetic stirrer (moderate stirring) for 30 minutes. During stirring, 250 mL of 1 M NaOH solution were dripped slowly, drop-by-drop. The slow addition of NaOH leads to the subsequent formation of CaOH₂ in the form of a highly crystalline inorganic gel network within the NaCl liquid, The prepared Ca (OH)₂ gel-containing solution was kept for one night at room temperature and then filtered. The filtrate was washed several times with distilled water to remove any impurities.

Later on, the gel was dried at 60°C for 24 hours. CaO nanoparticles were formed by the calcination of the dried gel in the oven at 900°C for 1 hour.

Characterization of CaO-NPs:

The concentration of Cao-NPs in the prepared solution was determined by atomic absorption apparatus. The size and shape of CaO particles were confirmed by high-resolution transmission electron microscopy (HRTEM). It displayed the size and shape distribution of CaO nanoparticles in the form of histograms. The zeta potential of CaO NPs was measured by a Malvern Zeta sizer-nano instrument (Liang et al., 2014).

2.6. Antibacterial activity of CaO NPs

Using agar-well diffusion approach, the CaO NPs antibacterial efficacy was evaluated against isolated *S. aureus*. Different CaO NP concentrations (50, 100, 200, 300, 350, 400, 450, 500, 550, and 600 NPs/ml) had been used after cultivating the organisms in the bored wells. CaO NPs and the tested organisms were incubated at 37 °C for 24 hours. After that, the average diameter of the bacterial

inhibition zones produced by the CaO NPs concentrations was measured and recorded (Kadhim *et al.*,2019).

3. RESULTS

3.1. Identification and isolation of S. aureus

Out of 140 samples,30 *S. aureus* isolates (21.42%) depending on culture and biochemical properties. All isolates found white to yellow tint, creamy, opaque, and convex colonies surrounded by β-hemolysis on sheep blood agar (5%). While on MSA, they fermented mannitol and grew as small yellow colonies. Gram-stained smears showed

clusters of Gram-positive cocci. Biochemically, these isolates showed positive for both the test coagulase (curd-like clotting) and catalase (bubble formation), confirming that the isolates were *S. aureus*.

3.2. Incidence rate of S. aureus isolated from different samples:

Staphylococcus aureus was found in 30 samples from tested samples. with an incidence rate of (21.42%). The incidence of isolated *S. aureus* among examined samples showed the highest percent from Yoghurt and kareish cheese (50%), while the lowest was from intestinal content of chicken (8%) Table (2).

Table (2) Incidence of *S. aureus* among different samples.

Sample type	Sample No	positive		Negative	
		No.	%	No.	%
Intestinal content of chicken	25	2	8%	23	92%
Semen of dogs	10	1	10%	9	90%
Pasteurized milk	10	1	10%	9	90%
Ice cream	10	1	10%	9	90%
Hand swabs of farmers milkers	20	5	25%	15	75%
Hand swabs of factory workers	20	3	15%	17	85%
Yoghurt	10	5	50%	5	50%
Soft cheese	10	3	30%	7	70%
Raw milk	15	4	26%	13	74%
Kareish cheese	10	5	50%	5	50%

3.2. Confirmation of S. aureus by PCR

The isolates were determined as *S. aureus* by PCR depending upon(23S rRNA gene), which resulted in product of 1250 bp (Figure 1).

3.3. Anti-microbial sensitivity of isolated S. aureus

The high degree of resistance was detected by isolated *S. aureus* against Cefoxitin (96.7%), followed by Oxacillin (93.3%), Tetracycline and Gentamicin (60%), while they were highly sensitive to Lenzolid and clindamycin (93.3%) (Table 3).

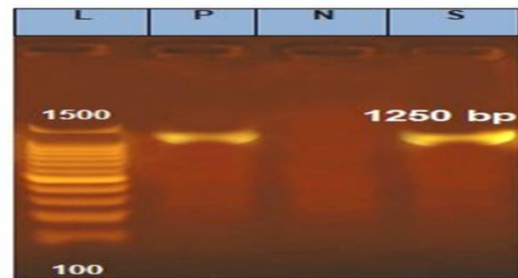


Fig (1): Electrophoretic gel imaging of PCR for *S. aureus* 23S rRNA gene (1250 pb) Lane (L): 100-1500 bp DNA Ladder, Lane (P): Positive control, Lane (N): Negative control

Table (3): Antibiogram Pattern of isolated *S. aureus*

Antibiotic	Antibiotic Code	Concentration/ µg	Sensitive		Inter mediate		Resistant		AA
			NO.	%	NO.	%	NO.	%	
Tetracycline	TET	30 µg	2	6.7	10	33.3	18	60	R
Amikacin	AK	30 µg	19	63.3	7	23.3	4	13.3	S
Lenzolid	LZ	30 µg	28	93.3	1	3.3	1	3.3	S
Gentamicin	GEN	10 µg	10	33.3	2	6.7	18	60	R
Levofloxacin	Lev	5 µg	18	60	0	0	12	40	S
Streptomycin	S	10 µg	13	43.3	12	30	5	16.7	S
Cefoxitin	Cx	30 µg	1	3.3	0	0	29	96.7	R
Azithromycin	AZM	15 µg	2	6.7	16	53.3	12	40	I
Mecillinam	MET	5 µg	20	66.7	9	30	1	3.3	S
Ciprofloxacin	CIP	10 µg	17	56.7	4	13.3	9	30	S
Chloramphenicol	C	30 µg	24	80	2	6.7	4	13.3	S
clindamycin	DA	2 µg	28	93.3	0	0	2	6.7	S
vancomycin	VA	30 µg	1	3.3	17	56.7	12	40	I
Oxacillin	OXI	1 µg	0	0	2	6.7	28	93.3	R

AA: Antibiogram activity. No=30. %: percentage in relation to the whole number of isolates

R: Resistance. S: Sensitive. I: Intermediate.

3.5. Nanoparticle synthesis and characterization

The calcium oxide NPs were prepared by the thermal decomposition of eggshell at 900 °C. Its examination by a transmission electron microscope showed spherical particles of size ranging from (31-120) nm with a mean 67.62 NM as

diagramed by the histogram (Figure, 2, the crystalized CaO-NPs showed diameters of 11.291/nm, 9.431/nm, and 6.181/nm, (Figure, 3). The zeta potential of CaO NPs was - 18.00 mV in 418Mj

3.6. Antimicrobial Activity of Cao NPs against the *S. aureus*

According to the findings, the inhibition zone diameter ranged from 8 mm to 16 mm starting from Cao NPs

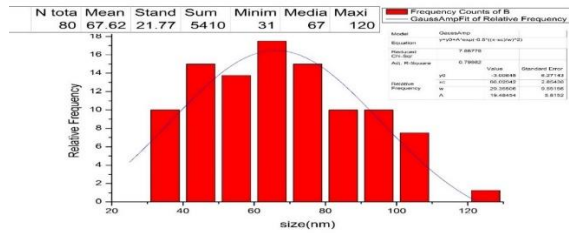


Fig (2) Histogram of the CaONPs size distribution by HRTEM

concentration of 100 NPs/ml to 600NP/ml, while 50 NPs/ml didn't cause inhibition (Table 4).

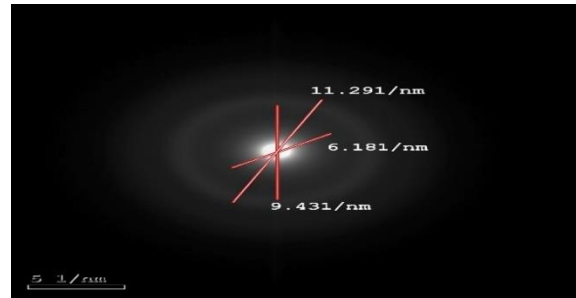


Fig (3) Diameters of crystallized CaO NPs by a transmission electron microscope

Table (4): DIZ of CaO nanoparticles by using different concentrations.

Concentration NPs/ml	50 NPs/ml	100 NPs/ml	200 NPs/ml	300 NPs/ml	350 NPs/ml	400 NPs/ml	450 NPs/ml	500 NPs/ml	550 NPs/ml	600 NPs/ml
DIZ	0	12mm	8 mm	12mm	8mm	8mm	10mm	12mm	10mm	16mm

The diameters of the inhibition zone (DIZ).

4. DISCUSSION

Staphylococcus aureus is an opportunistic microbe that causes soft-tissue infections, skin infections, and other diseases (Corrado et al., 2016). *Staphylococcus aureus* is an essential global cause of food-borne illness. According to Hennekinne et al., (2012), In the present study, from 140 collected samples, *S. aureus* was isolated from 30 samples, with a prevalence rate of 21.42%. The incidence of *S. aureus* from different clinical samples was represented by 5 positive samples (50%) from Kareish cheese samples, followed by yoghurt (5/50%), then Soft cheese (3/30%); raw milk (4/26%); hand swabs of farmers milkers (5/25%); hand swabs of factory workers (3/15%); (1/10%) from pasteurized milk; ice cream (1/10%); semen of dogs (1/10%) and intestinal content of chicken (2/8%), (Table 2) This was similar to what was seen by Reta et al., (2016), who reported an incidence rate of 24.2% from dairy products. While in India, a lower incidence of 10.16% was reported by Patel et al., (2018) from raw milk. However, a greater prevalence rate was reported in raw milk by Abbas et al. (2018) and Jorgensen et al. (2005) who found 45.6% and 96.2%, respectively. These variations in the prevalence of *S. aureus* between different studies could be due to the differences in geographic location, management methods, hygienic standards, and sample size used in farms and milk collection sites. Globally, antibiotic resistance is a serious issue. It has been discovered to be more prevalent among pathogenic bacteria. The misuse and overuse of these drugs, as well as the pharmaceutical industry's absence of new drug research because of high costs and heavy restrictions from regulators, may be responsible for the problem of antibiotic resistance (Gould and Bal 2013).

In the present study, *S. aureus*' antibiogram showed high resistance to cefoxitin (96.7%) and Oxacillin (93.3%), followed by tetracycline (60%) and gentamicin (60%) (Table 3). The current investigations were in harmony with Ahmad et al. (2013), who showed a 68.6% tetracycline resistance rate. However, both Ahmed et al. (2014) and Gitau et al. (2018) reported higher resistance rates (90.3%) and lower resistance rates (33%), respectively. Marais et al. (2009) found a high resistance of *S. aureus* to gentamycin (65.7%). *S. aureus* isolated from the current research had azithromycin resistance (40%). This matched the 37.2% resistance indicated by Dweba et al., (2019). *S. aureus*

isolates showed low resistance against ciprofloxacin and chloramphenicol (30% and 13.3%, respectively). The findings agreed with those found by Wu et al., (2018) when the resistance rates were 23.3%. The resistance rate to ciprofloxacin was recorded at 22.8% by Kumari et al. (2008), other than a greater resistance rate (75.75%) found by Marais et al., (2009). Ahmed et al. (2014) found a high resistance to chloramphenicol of 61.3%. In this investigation, *S. aureus* isolates showed a low resistance to clindamycin. This result was the same as that of Gitau et al. (2018) who found a 14% resistance rate. However, Akanbi et al., (2017) reported a higher resistance rate (76.7%)

Characterization of prepared CaO NPs cleared that the zeta potential of CaO NPs was -18.00 mV in 418 m J. It has been known that higher negative zeta potential value confirms repulsion between the particles which prevents aggregation and agglomeration due to highly charged particles, which confirms the high degree of stability to the nanoparticles (Clogston & Patri, 2011). Where the positively charged surface of metallic nanoparticles aids in their adhesion to the negatively charged surface of bacterial cell walls, increasing the bactericidal action. In addition to calcium oxide's histocompatibility and its ability to resist microbes (Mohammadi and Dummer, 2011)

The results of the used concentrations of Cao NPs (100,200,300,350,400,450,500,550 and 600) NPs/ml showed the diameters of inhibition zone (DIZ) from 8 mm to 16 mm as (12mm,8mm,12mm,8mm,10mm,12mm,10mm, and 16mm) respectively, where the highest mean diameter of inhibition appeared at the highest concentration (Table 4). These results were consistent with Gedda et al. (2015) who determined the diameters of the inhibition zone 17±2 mm against *S. aureus* against different concentrations (5, 10, 25, 50 µg /ml) of CaO NPs. Roy et al., (2013) found that the MIC and MBC of Cao NPs against *S. epidermidis* were 2 mM and 4 mM, respectively.

5. CONCLUSION

The present study concluded that the recovery of *S. aureus* was the highest from Yoghurt and kareish cheese (50%). Most of the isolates showed multiple antimicrobial resistances against Cefoxitin (96.7%), followed by Oxacillin (93.3%), Tetracycline. Even though CaO-NPs demonstrated

inhibitory activity against *S. aureus* at concentrations ranged from 100-600 NPs/ml against *S. aureus*, which provide a promising antibacterial agents alternative.

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