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Evaluation of the effect of nanocopper oxide and nanochitosan on highly antibiotic resistant *Staphylococcus aureus* isolated from normal and mastitic bovine milk

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ABSTRACT:

The objective of study was to determine the rate of isolation of *Staphylococcus aureus* (*S. aureus*) in normal and mastitic bovine milk and their relationship with the milk composition also microbial, and chemical parameters changes in milk of normal apparently healthy bovine. Moreover, we study the effects of copper oxide Nano particles (Cuo- Nps) and chitosan Nano particles (Ch- Nps) on isolated antibiotic-resistant strains of *S. aureus*. One hundred milk samples (50 samples from apparently healthy lactating cows and buffaloes 25 for each) and (50 samples of mastitic milk 25 cows' milk and 25 buffaloes' milk from the neighboring animals in the same farms) were randomly collected from 10 privet farms (5 cows farm and 5 buffalo's farms) in Kafrelsheikh Governorate, Egypt.

Ten of 50 raw milk samples were positive for *S.aureus* (six raw cow's milk and 4 raw buffalo's milk samples) whereas 26 of 50 mastitic milk samples were positive for *S.aureus* (fifteen mastitic cow's milk samples and 11 mastitic buffalo's milk samples).

Our results revealed that direct relationship between isolation of *S. aureus* from normal milk samples and TBC, SCC, minerals (ash)g%, SNF g% and Total solid g% (significant increase) and inverse relationship with PH, Fat %, Lactose % and Water % (significant decrease), and no significant change with other parameters.

Susceptibility test of isolated *S. aureus* to antibiotics show that highly susceptible to Amoxicillin+Clavulonic acid (77.77%) but high resistant to Oxytetracycline (94.44%).

PCR analysis of *S. aureus* for general gene (23S rRNA) was 100%, while

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multiplex PCR for AB resistance genes represented by Aminoglycosides resistance gene Aac (6')/aph (2), Quinolones resistance gene (norA), and Chloramphenicol resistance gene (fexA) were 60%, 100% and 0% respectively.
On the other hand the zone of inhibition of Cuo- Nps and Ch- Nps against antibiotic resistant *S. aureus* isolated from milk was widest at 3200 µg/ml for each. The MIC of Cuo- Nps and Ch- Nps was 800 ug/ml, 200µg/ml respectively, while the MBC of Cuo- Nps and Ch- Nps was 1600 (µg/ml) for each and MBC/MIC ratio for Cuo- Nps and Ch- Nps 2, 8 respectively. The treated cells of *S. aureus* with Cuo- NPs and Ch- NPs were investigated using scanning electron microscope (SEM) which showed noticeable effect on treated *S. aureus* cells.
The study concluded that *S.aureus* isolated from mastitic bovine milk shown resistance to numerous antibiotics and it transmitted to the surrounding bovine causing subclinical mastitis. Cuo- Nps and Ch-Nps have antibacterial activity against the isolated antibiotic resistant *S. aureus*.

INTRODUCTION

Because milk has all the necessary nutrients protein, lactose , vitamins ,minerals and fat in a balanced ratio that other meals do not, it is regarded as a complete diet **(Hossain and Dev, 2013)**. In addition, milk has a number of active chemicals that are crucial for nutrition as well as health protection making it a source of both macro and micronutrients **(Ceballos et al. 2009)**.

Anywhere dairy animals are found in the world, mastitis is a common condition. There are two types of mastitis: clinical and subclinical. Subclinical mastitis does not result in obvious alterations to the milk or udder, in contrast to the evident changes associated with acute mastitis. Since most cases of mastitis are subclinical, maintaining a stress-free environment, and following proper milking techniques are the keyways to prevent mastitis in dairy herds **(Konwar et al. 2009)**.

S. aureus is a major food-borne pathogen linked to various diseases in humans and animals **(Fetsch and Johler, 2018)**, particularly mastitis, which is primarily transmitted between cows through contagious routes, causing both subclinical and clinical mastitis **(Veh et al. 2015).**

Mastitis affects the chemical composition of milk constituents depending on the onset of mastitis with *S. aureus* causing severe tissue damage and cell excretion into milk **(Petersson -Wolfe et al. 2010)**. BM poses significant public health risks due to the presence of toxin producers in milk **(kibebew, 2017)**.

Antibiotics are commonly used to treat bovine mastitis **(Kromker and leimbach 2017)**, but this approach has disadvantages, including increasing resistance to antibiotic residues in milk, which could potentially pose public health risks **(Tark et al. 2017)**.

Nanotechnology is being increasingly used in the design of new bovine mastitis therapies, as it aims to reduce antibiotic use. Nanoparticles (NPS) have been utilized as antimicrobial agents against drug-resistant bacterial strains **(Kumar et al. 2018)** due to their unique physical and chemical properties, including their small size, high reactivity, and large surface area, which enable easy penetration into microorganisms **(Morones et al. 2005)**.

Chitosan NPS (Ch-NP_S) exhibits antimicrobial activity against bovine mastitis pathogens without affecting cell viability. Its nanoderivates show a broad spectrum of antimicrobial activity **(Orellano et al. 2019**).

Copper NPS could be a potential alternative to traditional antibiotics, but they are more toxic for gram-positive bacteria, damaging their cell membranes **(Azam et al. 2012)**. The antimicrobial properties of copper oxide NPS (Cuo- NPs) have been evaluated in many bacterial species, including *S. aureus* **(Ahmed et al. 2014)** but have limited cytotoxicity against

mammalian cells. So, the purpose of this research is to isolate and identify the *S. aureus* from mastitic and normal un-mastitic milk, then evaluate the antimicrobial activity of Cuo-NPs and Ch-NP_S against the antibiotic resistant *S. aureus* bacteria isolates which pose potential public health hazards. In addition, to determine the bacteriological status and biochemical changes in milk samples of normal and sub -clinically infected milk for assessment of the bacteriological and compositional quality in relation to presence of *Staph aureus* bacteria.

MATERIALS AND METHODS

1. Milk samples

One hundred milk samples (50 samples from apparently healthy lactating cows and buffaloes 25 for each) and (50 samples of mastitic milk; 25 cows' milk and 25 buffaloes' milk from the neighboring animals in the same farms) were randomly collected from 10 private farms, 10 samples from each farm (5 cows farm and 5 buffaloes farms) in Kafrelsheikh Governorate, Egypt. About 100 ml for each sample of fresh milk was directly collected from cows and buffaloes. The samples were used to isolate and identify *S. aureus*, determine the physicochemical properties of milk samples and changes in the biochemical parameters while the mastitic milk samples were used to isolate and identify *S. aureus*. All samples were randomly and aseptically collected after disinfection of teat orifice with 70% ethyl alcohol and discard of the first strain of milk. Collection of milk samples was done between Marchs to September 2022. The samples were immediately brought in ice-cooled containers to the lab where they were examined 12 hours later.

2. Biochemical parameter analyses of row milk

Chemical analyses (PH, Fat %, Protein %, Lactose %, minerals (ash) g%, SNF g% Total solid g%, Water %) of milk samples were performed using Lacto scan 90 milk analyzer (Foss). SCC and colony forming unit (CFU) were detected by Bacsomatic (Foss). Milk samples were mixed gently, then 5 ml of the sample were put in the sample holder with the analyzer in the recess position **(NMC 1999 and Catozzi et al. 2017)**.

3. Isolation and identification of *S. aureus*

The samples were immediately suspended in 10%NaCl tryptone soya broth (TSB, Oxoid) and then incubated at 37ºC for 24h.Following the streaking of Enrichment culture onto Baird -parker agar (BPA, Oxoid) which was supplemented with 5% egg yolk and tellurite the plates were incubated for 24 hours at 37ºc. The presumed isolates were (black colonies with clear zones ranging from 2-5 mm).The obtained colonies were further identified morphologically, microscopically and biochemically according to **MacFaddin, (2000)**.

4. Confirmation of *S. aureus* and detection of its antibiotic resistance genes by PCR assay.

Confirmation of the isolates as *S. aureus* was done by species–specific 23 Sr RNA gene, followed by screening by multiplex PCR assay for the presence of antibiotic resistance genes (Aac (6) aph, nor A and fex A). Aminoglycosides, Quinolones and chloramphenicol resistance genes, respectively.

4.a DNA extraction.

The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used to extract DNA from samples with certain changes made in accordance with the manufacturer's instructions.

4.b Primers of oligonucleotide.

The Primers utilized, which are mentioned in table (1) were provided by (Metabion, Germany).

PCR amplification.

The primers were used in a 50 ul reaction that included 5 ul of DNA template, 25 ul of Emerald Amp Max PCR Master Mix (Takara, Japan) , 1ul of each primer at a concentration of 20 pmol, and 14ul of water. The reation took place in a thermal cycler with an applied bio-system 2720.

4. c. Analysis of the PCR Products.

The PCR product were separated by electrophoresis on a 1.5% agarose gel(Applichem,

Germany, GmbH) . A gel documentation system (Alpha Innotech, Biometra) took pictures of the gel and computer software was used to analyse the data.

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

PCR type	Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles) Secondary denaturation	Annealing	Extension	Final extension	Reference
Uniplex	S. aureus 23S rRNA	ACGGAGTTACAAAGGACGAC AGCTCAGCCTTAACGAGTAC	1250	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 1.2 min.	72˚C 12 min.	Bhati et al., 2016
Multiplex	aac(6')aph (2")	GAAGTACGCAGAAGAGA ACATGGCAAGCTCTAGGA	491	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 1.2 min.	72˚C 12 min.	Duran et al., 2012
	norA	TTCACCAAGCCATCAAAAAG CTTGCCTTTCTCCAGCAATA	620						Pourmand et al., 2014
	fexA	GTA CTT GTA GGT GCA ATT ACG GCT GA CGC ATC TGA GTA GGA CAT AGC GTC	1272						Kehrenberg and Schwarz, 2013

5. Antibiotic Sensitivity testing:

Testing was performed using Muller Hinton agar in accordance with the worldwide criteria provided by the National Committee for Clinical Laboratory Standards **(NCCLS, 2002)**. The susceptibility of bacterial isolates to 7distinct antimicrobial discs was evaluated.These discs comprised Amoxicillin + Clavulonic acid (AMC) (20µg+ 10µg), Danofloxacin (DFX) (5 µg /disc), Gentamycin (CN) (10 µg /disc), Florphenicol (FFC) (30 µg /disc), Amoxicillin (AM) (10 µg /disc), Sulpha methoxazole + Trimethoprim (SXT) (25µg/disc) and Oxytetracycline (OT) (30 µg/disc).

6. Nano particles

Cuo-NPs and Ch-NPs suspensions were purchased from Nano Tech Egypt for Photo- Electronics, Al Giza, Egypt. The average particle size was measured using TEM (JEOL JEM-2100 high resolution transmission electron microscope) at an accelerating voltage of 200 KV. The average size was 40± 10 nm with quasi –spherical shapes and 20 ± 5 nm semi aggregated particulates for Cuo-NPs and Ch-NPs, respectively.

7. Determination of antimicrobial activity of Cuo-NPs and Ch-NPs by the well – diffusion method

Antimicrobial activities of Cuo-NPs and Ch-NPs against the isolated antibiotic resistant *S. aureus* were measured following a modified Kirby Bauer disc diffusion method **(Azam et al., 2012)**. In brief, 100 µL of the bacterial culture broth having 10^6CFU/ml of the tested organism was spreaded on nutrient agar plates. Wells (8 mm) were punched into the agar plates. Using a micropipette, 100 µL of different concentrations (100ug, 200ug, 400 µg, 800 µg, 1600 µg, and 3200 µg) were poured into each wells on the plates for each Cuo-NPs solution and Ch-NPs solution. The plates were incubated at 37^{o}C for 24h, the size of the inhibition zone was measured. A solvent blank and tetracycline antibiotic were run as negative and positive controls, respectively.

8. Determination of minimum inhibitory concentration (MIC).

MIC technique of Cuo-NPs and Ch-NPs was performed using successive serial 2-fold dilution technique **(CLSI 2018)**. A dilution series with 10

ml nutrient broth medium containing different concentrations of Cuo-NPs and Ch-NPs (100-3200ug/ml) for each was prepared. Each set was inoculated aseptically with 100 µL of isolated *S. aureus* suspension (approximately 10^6CFU/ml). The MIC was determined after incubation for 24h at 37°C as the lowest concentration without visible growth.

9. Determination of minimum bactericidal concentration (MBC).

The contents of all positive MIC tubes were subcultured on TSA plates and incubating them for 24 houre at 37°C . The MBC was determined as the lowest concentration of Cuo-NPs and Ch-NPs suspension that killed 100% of bacteria.

10. Scanning Electron Microscope (SEM) "jump up"(Tahmasebi et al. 2015)

The morphological changes in the *S. aureus* bacterial cells treated with sub MIC of Cuo-NPs and Ch-NPs were examined by SEM.

RESULTS

Table 2. Rate of isolation of *S. aureus* from 50 samples of normal cow and buffalo milk (25 for each) and from 50 samples of mastitic cow and buffalo milk (25 for each).

	samples	+ve No of *S.aureus* isolation	total No.
Cow	normal milk	6	25
	mastitic milk	15	25
buffalo	normal milk	4	25
	mastitic milk	11	25
total		36	100

Table 2 shows the rate of isolation of *S. aureus* in both normal and mastitic milk .Fifty normal milk samples, 10 were positive for *S. aureus* (6 normal cow's milk samples and 4 normal buffalo's milk samples). While, 26 of 50 mastitic milk samples were positive for *S. aureus* (15 mastitic cow's milk samples and 11 mastitic buffalo's milk samples).

Table 3. Total bacterial count (TBC) and Somatic cell count (SCC) based on presence or absence of *S. aureus* in the examined normal Cow milk samples.

variable	Normal values	*S. aureus* isolation	No	AB No.	AB%	Min	Max	Mean ± SE
TBC(cfu)	Up to10^4	-ve	19	0	0	2.30 x10^3	9.80 x10^3	5.837 x10^3 ± 3.36 x10^3
		+ve	6	6	100	26.50 x10^4	73.1x10^4	40.9x10^4 ± 12.3 x 10^4 (↑)
SCC/ml	Up to 750×10^3	-ve	19	0	0	254 x10^3	554 x10^3	371 x10^3 ± 184 x10^3
		+ve	6	6	100	780 x10^3	920 x10^3	858x10^3± 199 x10^3 (↑)

Data show Number (No), Abnormal (AB), Minimum (Min), Maximum (Max), mean ± Standard Error (SE)
Somatic cell count according to Sharma, et al., (2011). Normal values of
Normal values of colony forming unit according to Nicla Marri, et al., (2020).
Somatic cell count (SCC) and colony forming unit (CFU) for the examined row Cow milk samples in table **3** show positive or negative *S. aureus* isolation in which there is increase in SCC and CFU over the normal in cow milk in the samples with positive *S. aureus* isolation (6 isolates) with direct relationship between isolation of *S. aureus* with CFU and SCC (significant increase).

Table 4. Total bacterial count (TBC) and Somatic cell count (SCC) based on presence or absence of *S. aureus* in the examined normal buffalo milk samples

variable	Normal values	*S. aureus* isolation	No	AB	AB%	Min	Max	Mean ± SE
TBC(cfu)	Up to 10^4	-ve	21	0	0	2.80×10^3	9.80×10^3	$5.819 \times 10^3 \pm 3.81 \times 10^3$
		+ve	4	4	100	37.8×10^4	45.20×10^4	$40.7 \times 10^4 \pm 6.2 \times 10^4$(↑)
SCC/ml	Up to 750×10^3	-ve	21	0	0	230×10^3	523×10^3	$312 \times 10^3 \pm 149 \times 10^3$
		+ve	4	4	100	790×10^3	920×10^3	$852 \times 10^3 \pm 119 \times 10^3$ (↑)

Data show Number (No), Abnormal (AB), Minimum (Min), Maximum (Max), mean ± Standard Error (SE)
Normal values of somatic cell count according to Patil et al., (2015).
Normal values of colony forming unit according to Han, et al., (2007).
Somatic cell count (SCC) and colony forming unit (CFU) for the examined row buffalo milk samples in table **4** show positive or negative *S. aureus* isolation in which there is increase in SCC and CFU over the normal in buffalo milk in the samples with positive *S. aureus* isolation (4 isolates) with direct relationship between isolation of *S. aureus* with CFU and SCC (significant increase).

Table 5. Biochemical changes (Mean± SE) based on presence or absence of *S. aureus* in the examined normal cow milk samples

variable	Normal values	*S. aureus* isolation	No	AB	AB%	Min	Max	Mean ± SE
PH	6.26 –6.58	-ve	19	0	0	6.28	6.52	6.38 ± 0.069
		+ve	6	6	100	5.8	6.1	5.93 ± 0.113 (↓)
Fat %	2.5 – 6.0	-ve	19	0	0	2.89	5.77	3.91 ± 0.684
		+ve	6	1	16.7	2.18	4.27	3.48 ± 0.703 (↓)
Protein %	2.9 – 5.0	-ve	19	1	5.3	3.32	4.76	3.95 ± 0.41
		+ve	6	6	100	3.75	3.9	3.82 ± 0.057
Lactose %	3.6 – 5.5	-ve	19	0	0	3.67	5.22	4.37 ± 0.502
		+ve	6	6	100	3.33	3.43	3.4 ± 0.037 (↓)
Minerals (ash) g%	0.6 – 0.8	-ve	19	0	0	0.63	0.79	0.71 ± 0.045
		+ve	6	6	100	0.81	0.89	0.83 ± 0.03 (↑)
SNF g%	8.30 –8.49	-ve	19	2	10.5	8.14	8.49	8.38 ± 0.091
		+ve	6	6	100	8.54	9.52	8.75 ± 0.379 (↑)
Total solid g%	10.5 – 14.5	-ve	19	0	0	11.22	14.2	12.73 ± 0.832
		+ve	6	6	100	15.47	16.9	16.13 ± 0.517 (↑)
Water %	85.5 – 89.5	-ve	19	0	0	85.8	88.78	87.28 ± 0.83
		+ve	6	6	100	83.1	84.53	83.87 ± 0.517 (↓)

Normal values according to Hamad and Baiomy (2010).
Changes of composition of row cow milk samples in relation to *S. aureus* isolation (6 isolates) in table 5 show direct relationship with Minerals (ash) g%, SNF g% and Total solid g% (significant increase) and inverse relationship with PH, Fat %, Lactose % and Water % (significant decrease) and no significant change with other parameters.

Table 6. Biochemical changes (Mean± SE) based on presence or absence of *S. aureus* in the examined normal buffalo milk samples.

variable	Normal values	*S. aureus* isolation	No	AB	AB%	Min	Max	Mean ± SE
PH	6.13 –6.44	-ve	21	0	0	6.15	6.42	6.26 ± 0.017
		+ve	4	4	100	5.89	6.10	5.97 ± 0.049 (↓)
Fat %	5.5 – 9.0	-ve	21	0	0	5.54	8.90	6.85 ± 0.197
		+ve	4	1	25	5.00	6.65	6.07 ± 0.368 (↓)
Protein %	3.2 – 5.3	-ve	21	0	0	3.21	4.87	3.92 ± 0.097
		+ve	4	0	0	3.42	3.87	3.63 ± 0.094
Lactose %	4.1 – 6.2	-ve	21	0	0	4.18	5.82	4.87 ± 0.102
		+ve	4	4	100	3.41	3.91	3.66 ± 0.111 (↓)
Minerals (ash) g%	0.69 – 0.89	-ve	21	0	0	0.70	.86	0.78 ± 0.011
		+ve	4	4	100	0.94	.98	0.96 ± 0.009 (↑)
SNF g%	8.50 –9.40	-ve	21	3	14.3	8.38	9.99	9.05 ± 0.092
		+ve	4	4	100	9.62	9.90	9.73 ± 0.063 (↑)
Total solid g%	14.5 – 16.5	-ve	21	5	23.8	14.69	16.85	15.77 ± 0.156
		+ve	4	4	100	9.62	9.90	17.01 ± 0.137 (↑)
Water%	83.5 – 85.5	-ve	21	4	19	83.15	85.31	84.27 ± 0.147
		+ve	4	4	100	82.65	83.23	83 ± 0.137 (↓)

Normal values according to Hamad and Baiomy (2010).
Changes of composition of row buffalo milk samples in relation to *S. aureus* isolation (4 isolates) in table **6** show direct relationship with Minerals (ash) g%, SNF g% and Total solid g% (significant increase) and inverse relationship with PH, Fat %, Lactose % and Water % (significant decrease) and no significant change with other parameters.

Table 7. Susceptibility of isolated *S. aureus* to antibiotics (36 isolates).

Antibiotic	36 isolates			
	Sensitive		Resistant	
	No.	%	No.	%
Amoxicillin + Clavulonic acid (20µg+ 10µg)	28	77.77	8	22.23
Danofloxacin (5 µg /disc)	24	66.67	12	33.33
Gentamycin (10 µg /disc)	21	58.34	15	41.66
Florphenicol (30 µg /disc)	19	52.78	17	47.22
Amoxicillin (10 µg /disc)	11	30.56	25	69.44
Sulpha methoxazole + Trimethoprim (25µg)	7	19.45	29	80.55
Oxytetracycline (30 µg)	2	5.56	34	94.44

The antibiotic susceptibility of 36 *S. aureus* isolates were shown in table 7.The isolates showed sensitivity to ,amoxicillin+clavulonic acid, Danofloxacin , , gentamycin and florphenicol with 77.77%,66.67%,58.34% and 52.78% respectively, however, the isolates showed resistance to oxytetracycline , sulphamethoxazole+trimethoprim, amoxicillin with 94.44%,80.55%,69.44% respectively.

Table 8. Results of PCR for general gene (23S rRNA) at1250bp, and multiplex PCR for AB resistance genes represented by Aminoglycosides resistance gene Aac (6')/aph (2) at 491bp, Quinolones resistance gene (norA) at 620bp, and Chloramphenicol resistance gene (fexA) at 1272bp for *S. aureus*

Source	Sample	*23S rRNA*	*aac(6')aph (2'')*	*norA*	*fexA*
normal cow milk	1	+	-	+	-
	2	+	-	+	-
normal buffalo milk	3	+	-	+	-
	4	+	-	+	-
Mastitic cow milk	5	+	+	+	-
	6	+	+	+	-
	7	+	+	+	-
Mastitic buffalo milk	8	+	+	+	-
	9	+	+	+	-
	10	+	+	+	-

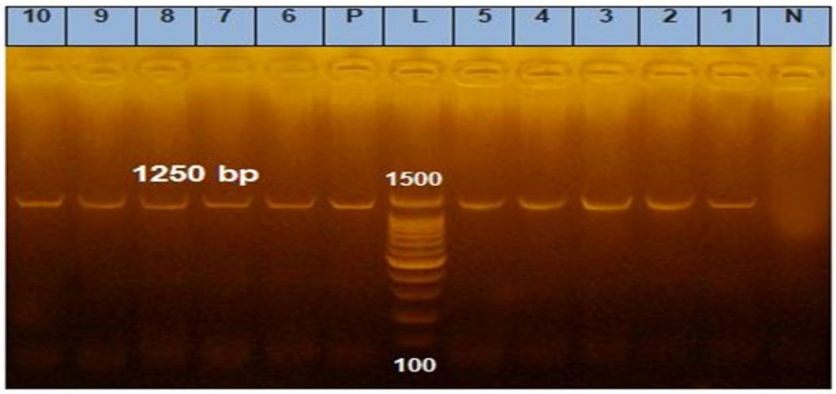

Photo 1. Agarose gel electrophoresis of PCR for 23s rRNA General gene at 1250bp for *S. aureus*.
Photo details
Lane L: 100 bp ladder as molecular size DNA marker.
Lane P: Control positive genes.
Lane N: Control negative.
Lanes 1 to 10: Positive for 23s rRNA gene

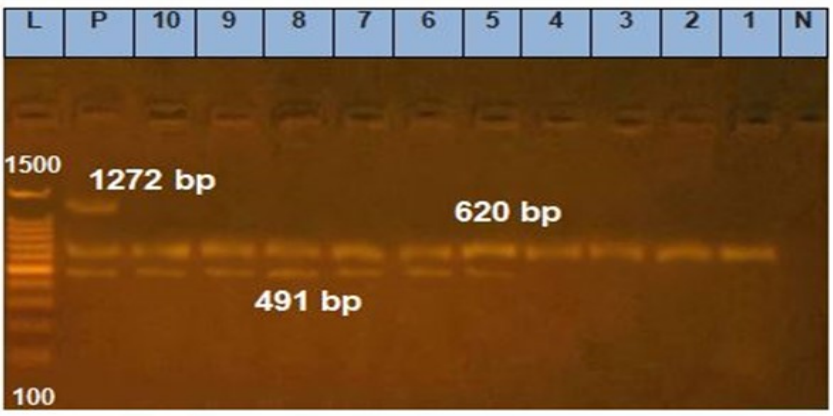

Photo 2. Agarose gel electrophoresis of multiplex PCR for antibiotic resistant genes, gene Aac (6')/aph (2) at 491bp, gene (norA) at 620bp, and gene (fexA) at 1272bp for *Staphylococcus aureus*.
Photo details
Lane L: 100 bp ladder as molecular size DNA marker.
Lane P: Control positive genes.
Lane N: Control negative.
Lanes 1 to 4 were Negative for Aac (6')/aph (2) gene and Lanes 5 to 10 were positive for this gene at 491bp.
Lanes 1 to 10 were positive for (norA) gene at 620bp.
Lanes 1 to 10 were Negative for (fexA) gene at 1272bp

Table 9. Values of zones of inhibitions (mm) of Cuo- Nps and Ch- Nps on antibiotic resistant *S. aureus* isolated from milk samples.

Conc	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml	1600 µg/ml	3200 µg/ml
Cuo Nps	no	no	8	11	16	20
Ch Nps	no	12	15	16	18	21

The results in table 9 and fig1 showed the antibacterial activity of Cuo-NPs and Ch-NPs against the isolated antibiotic resistance strain of *S. aureus* using the agar well diffusion method . Cuo-NPs don't have antibacterial activity against *S.aureus* at a concentration of 100 ug/ml and 200 ug/ml whereas show antibacterial effect against *S.aureus* at a concentration of 400 ug/ml, 800 ug/ml, 1600 ug/ml and 3200 ug/ml with inhibition zone of 8 mm, 11 mm,16 mm,18 mm and 20 mm respectively .For Ch-NPs don't have antibacterial activity against *S.aureus* at a concentration of 100 ug/ml .However the inhibition zones were about 12 mm,15 mm,16 mm,18 mm and 21mm for the concentration of 200 ug/ml , 400 ug/ml , 800 ug/ml, 1600 ug/ml and 3200ug/ml respectively.

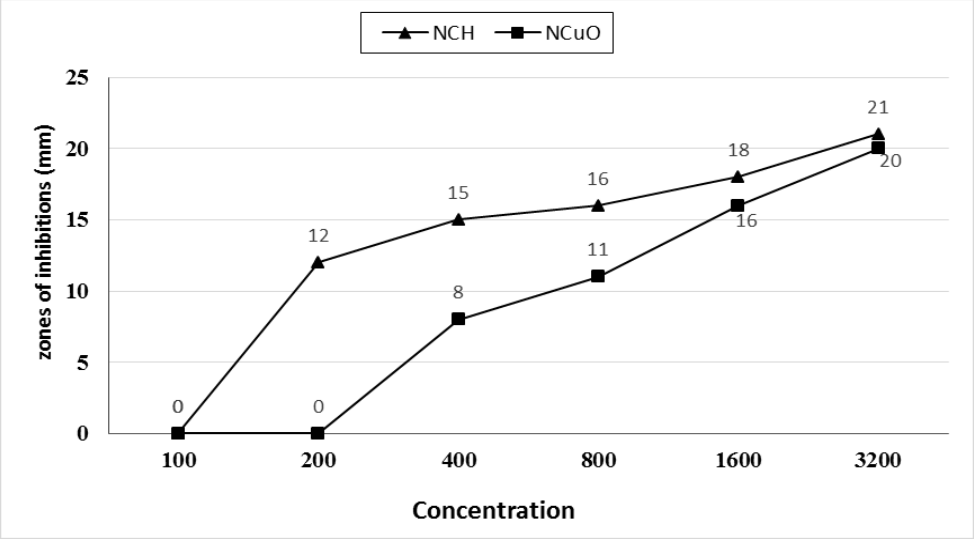

Fig 1. Show values of zones of inhibitions (mm) of CuoNps and CHNps on antibiotic resistant *Staphylococcus aureus* isolated from milk.

Table 10. Values of minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of copper oxide Nano particles (Cuo- Nps) and chitosan Nano particles (Ch- Nps) on antibiotic-resistant *Staphylococcus aureus* isolated from milk and MBC/MIC ratio.

	Cuo NPs	Ch- Nps
MIC µg/ml	800	200
MBC µg/ml	1600	1600
MBC/MIC ratio	2	8

As showed in table 10 and fig 2 the MIC , MBC and ratio MBC/MIC values of Cuo-NPs against *S. aureus* were at concentration of 800 ug/ml, 1600 ug/ml and 2 respectively .Whereas for Ch-NPs were found at concentration 200ug/ml, 1600ug/ml and 8 respectively.

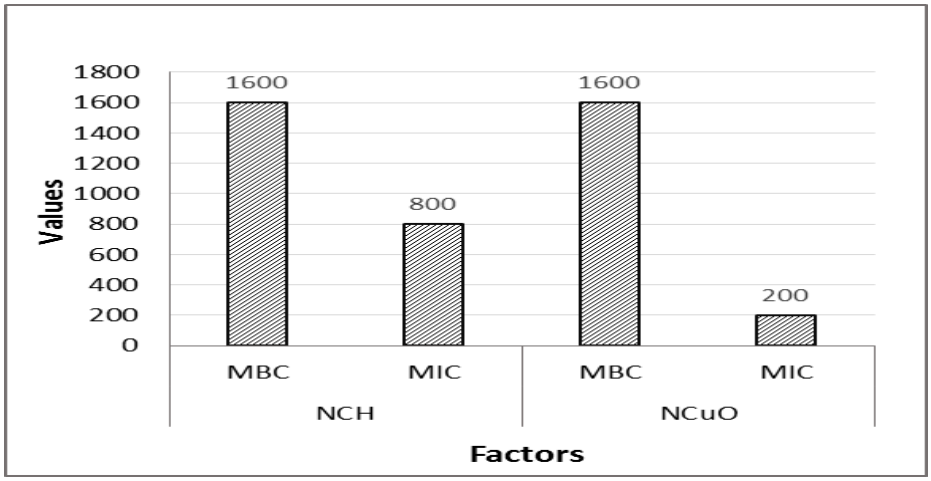

Fig 2. Show values of minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of copper oxide Nano particles (Cuo- Nps) and chitosan Nano particles (Ch- Nps) on antibiotic-resistant *Staphylococcus aureus* isolated from milk.

Result of scanning electron microscope (SEM):

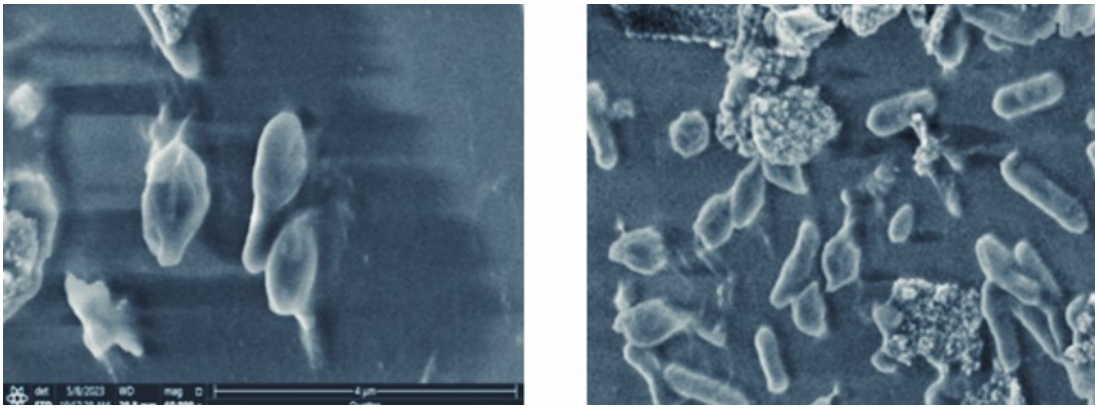

Photo 3. SEM analysis of *S. aureus* cells treated with Cuo NPs.

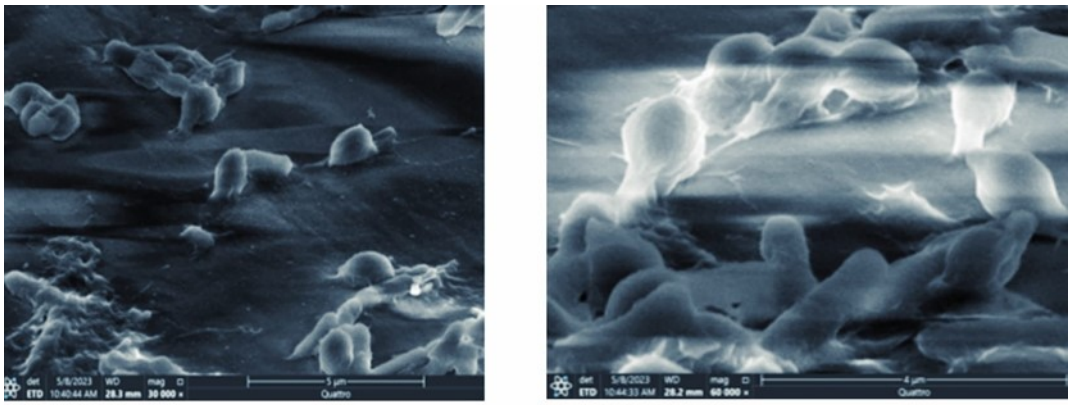

Photo 4. SEM analysis of *S. aureus* cells treated with ChNPs.

The treated cells of *S. aureus* with Cuo NPs (Photo 3) and Ch NPs (Photo 4) were investigated using SEM. The ultra–structure of the treated cells showed the effect of Ch NPs and Cuo NPs causing abnormal cell shapes and sizes, complete cytoplasmic lysis and leakage out of the cell membrane and damage of the cell, while the other intact cell walls attached with noticeable amounts of Cuo NPs.as seen in Photo 3.

DISCUSSION

S. aureus is one of the main pathogens responsible for contagious bovine mastitis. Our research revealed that 26 of 50 mastitic milk samples (52%) were positive for S. aureus (Fifteen mastitic cow' milk samples and 11 mastitic buffalo's milk samples) Table 2. These result nearly similar to **Pavlak et al. (2008)** they stated that the incidence of *S. aureus* was 58%. While our results were lower than previous report by **Klibi et al. (2018)** isolated *S. aureus* from mastitic milk by (5%). n the other hand, as shown in Table 2,10 of 50 raw milk samples (20%) were positive for *S. aureus* (Six raw cow's milk and4 raw buffalo's milk samples). These findings such as isolated by **Santana et al. (2010)** 18.80%, higher incidence was reported by **Olivera et al. (2022)** 53% while lower prevalence was mentioned by **Kumar and Prasad (2010)** who found 6.6% prevalence. The differences in frequencies of *S. aureus* in milk could be due to poor farm management, and improper or suboptimal hygiene.

Although lactating animals produce normal milk, the routine testing can reveal subclinical mastitis affecting milk quality and quantity. Pathogenic bacteria coexisting in dairy animals threaten human health, necessitating herd health improvement interventions **(Saleem et al. 2021)**.

Therefore, one hundred milk samples were taken from apparently healthy lactating cows and buffalos from many different private farms. Milk samples were used to isolate and identify *S. aureus,* determine the physicochemical properties of milk samples and changes in biochemical parameters.

In table 3 and 4 show somatic cell count (SCC) and colony forming unit (CFU) for the examined row Cow and buffalo milk samples respectively in positive or negative *S. aureus* isolation in which there is increase in SCC and CFU over the normal in cow and buffalo milk in the samples with positive *S. aureus* isolation (6 isolates from cow milk and 4 isolates from buffalo milk) with direct relationship between isolation of *S. aureus* with CFU and SCC (significant increase)

Higher SCCs indicates tissue alterations resulting from long-lasting infections with pathogens or even with subclinical mastitis in which milk production decrease with no clinical signs **(Abed et al. 2021),** and our results agree with **Catozzi et al. (2017)** in which subclinical mastitis increase SCC and rate of isolation of *S. aureus* from buffalo's milk compared to healthy status.

The CFU is crucial for dairy farmers and processors as it indicates herd health, sanitation efficacy, and proper handling of milk, and storage temperature **(Schalk et al. 2002)**. Determining milk SCCs is a recommended method to diagnose pathogenic subclinical mastitis, as it elicits different immune responses in the mammary gland **(Saleem et al. 2021)**.

In table 5 **a**nd 6 show changes of composition of row cow and buffalo milk samples respectively in relation to *S. aureus* isolation (6 isolates from cow milk and 4 isolates from buffalo milk) with direct relationship with Minerals (ash) g%, SNF g% and Total solid g% (significant increase) and inverse relationship with PH, Fat %, Lactose % and Water % (significant decrease) and no significant change with other parameters. Results of previous studies on the prevalence of health status of the udder in dairy animals were useful for monitoring the structural alterations in milk composition **(Saleem et al. 2021)**. Milk pH increased positively depending on the severity of the inflammatory process **(Qayyum et al. 2016)**. **(Romero et al. 2018)** hypothesized that milk pH testing could be used as an accurate, low-cost, and practical on-farm method to diagnose subclinical mastitis.

Subclinical mastitis is considered as one of the most common intra-mammary infections, which results in a significant change in milk components lower the milk quality **(Tuaillon et al. 2017)**. Our results of biochemical parameters analysis of cow's milk agree with results showed by **Saleem et al. (2021)** and our results of biochemical parameters analysis of buffalo's milk agree with results showed by **Catozzi et al. (2017)**.

Antibiotic resistance correlates to several factors, including increased use of antibiotics, background antibiotic resistance levels, and spread of resistance **(Crofts et al. 2017)**.The results of the antimicrobial susceptibility tests of *S. aureus* in this study (table7) showed that the bacteria had the greatest resistance to Oxytetracyclin (94.44%), Sulphamethoxazole + Trimethoprim (80.55%) and Amoxicillin (69.44%) which is in line with the previous studies **(Alekish et al. 2013; Wang et al. 2015 and Ismail 2017)** who reported that *S. aureus* was resistance to Sulphamethoxazole + Trimethoprimits (its resistance was from 90 to 100%) and Oxytetracycline (its resistance was from 72 to85%) also **(Jain et al. 2022)** who found that Oxytetracyclin (98.18%) and Amoxicillin(89.09%) were resistant to *S. aureus*. However highest sensitivity (77.77%), (66.67%) and (58.34%) were exhibited against Amoxicillin+Clavulonic acid, Danofloxacin and Gentamycin. These results in harmony with results of **Sekkin et al. (2010)** who reported that Amoxicillin+Clavulonic acid and Danofloxacin (93.6% each) was the most proper antibiotics and **Kaczorektukowska et al. (2022)** who revealed that Amoxicillin+Clavulonic acid and Gentamycin (100% each) were sensitive to *Staph aureus* .

S. aureus resistance to Oxytetracyclin might be due to its repeated use, while the high sensitivity to Cefoperazone and Cefotaxime was probably because of less use of these antibiotic for the treatment of bovine mastitis. Based on the high resistance percentage, these antibiotics should be used with caution for treatment of mastitis caused by *S. aureus.*

The current study approved that the 10 tested *S. aureus* strains recovered from the examined normal and mastitic milk samples contained 16s r RNA gene. Table (8) and photo (1).These isolates were characterized by phenotypic and confirmed by PCR that quick, sensitive and accurate than routine diagnosis of bacterial identification **(Atea and Jabber 2021)**. Antibiotic resistance poses a significant global health challenge, with microbes showing varying levels of resistance to most antimicrobials (**Mekonnen et al. 2018)**. Similar findings have been observed in the current study as well. The results represented in the table (8) and photo (2) revealed that the aminoglycosides resistance gene aac (6) aph (2) detected in 60% of *S. aureus* isolates. These findings nearly agree with **Liu et al. (2022)** who reported the presence of aac(6) aph(2) gene in 50% of *S. aureus* isolates. While **Liu et al. (2022)** reported lower presence of aac (6) aph (2) (33.3%) compared to the present study. On the other hand quinolones resistance gene norA present in all isolates (100%) while multiplex PCR results were negative for chloramphenicol resistance gene FexA in all *S. aureus* isolates. These data were similar to that obtained by **Patel et al. (2021)** who detected nor A gene in 99.2 % of the *S. aureus* isolates and reported that FexA gene were negative for *S. aureus* isolates. The complex mechanisms of resistance to antibacterial drugs make it difficult to determine if an isolate is resistant or sensitive to the corresponding antimicrobial agent **(Gow et al. 2008)**. Antibiotic resistance among bacteria is causing numerous research to discover new, more potent antimicrobial agents (**Zu et al. 2014)**. The field of nanotechnology and its nanoparticle-based applications is expanding due to its significant biological effectiveness.

The antibacterial activity of Cuo-Nps and Ch-Nps against the isolated antibiotic resistance strain of *S. aureus* was evaluated. The agar well diffusion method and the broth dilution method were two common techniques used for this purpose **(Azam et al. 2012)**. The results in the table (9) and fig (1) showed that both types of nanoparticles don't have antibacterial activity against *S. aureus* at a concentration of 100 ug /ml. Cuo-Nps show antibacterial effect against *Staph .aureus* at a concentration of 400ug/ml, 800ug/ml, 1600ug/ml and 3200ug/ml with inhibition zone of 8mm, 11mm, 16mm, and 20mm, respectively. For Ch-Nps, the inhibition zone was about 12 mm, 15 mm, 16 mm, 18 mm, and 21 mm for the concentration of 200ug/ml, 400ug/ml, 800ug/ml, 1600ug/ml and 3200ug/ml, respectively. The 3200ug/ml concentration exhibited a maximum zone of inhibition against the tested organism. This data revealed that higher concentration of Cuo-Nps is required to inhibit the growth of *S. aureus.* On the other hand, the

results revealed that the antibacterial activity of both nanoparticles against *S. aureus* increased with their concentration. This is in agreement with **Raheem et al. (2019)** who reported increased diameter of inhibition zone proportional to the rise in the nanoparticle's concentration. The maximum zone of inhibition was exhibited by Ch- Nps followed by Cuo- Nps. The presence of an inhibition zone clearly indicated the antibacterial effect of Cuo -Nps and Ch-Nps. This agrees with **Wardani et al. (2018)** proved a good demonstration of the Ch-NPS ability against wide range of bacteria. The present results regarding the inhibition zones (mm) of Cuo-Nps and Ch-Nps on antibiotic resistant *S. aureus* isolated from milk revealed no zone at 100 and 200 µg/ml and the widest zone (20mm) was at 3200 µg/ml for Cuo- Nps, and for Ch- Nps no zone at 100 µg/ml and the widest zone (21mm) was at 3200 µg/mm which agree with previous results **(Hussain et al. 2015 and Orellano et al. 2021)**.

The antimicrobial property of Ch-NPS was attributed to their small size, strong curvature of the surface, high surface area, and charge density that enable them to interact with negatively charged bacteria cell surface causing leakage of intracellular substances and cell death **(Yien et al. 2012)**. While Cuo-Nps suddenly decrease the bacterial cell membrane integrity, and release reactive oxygen species (Ros) where superoxide species is generated and contributed in the degradation of biomolecules **(Jadhav et al. 2011)**.as showmen in table (10) and fig (2) The MIC, MBC and ratio MBC/MIC values of Cuo- Nps against *S. aureus* were at concentration of 800ug/ml, 1600ug/ml and 2, respectively. Whereas for Ch- Nps were found at concentration 200ug/ml, 1600ug/ml and 8, respectively. The above results were in agreement with **Ren et al. (2009)** who reported a high concentration of Cuo- Nps were required to achieve a bactericidal action with MBC of 5000ug/ml. Also agree with (**Hussain et al. (2015)** who recorded that the MIC and MBC of Cuo- Nps against MRSA was 800ug/ml and 1600ug/ml, respectively. For the obtained MIC values of Ch- NPs the data agree with **Orellano et al. (2021)** who found that MIC was 200ug/ml for *S. aureus*. The difference in MIC and MBC values might be attributed to the nanoparticles' shape and size, the methods of preparation, and the genetic variation of the isolated organisms. The MBC /MIC value indicates the bacterial efficiency of Ch- Nps and Cuo- Nps. Antibacterial agents are often classified as bactericidal if the MBC /MIC ratio is greater than 4 and bacteriostatic if it is less than 4 **(Sader et al. 2006)**. The obtained results showed that the MBC / MIC ratio of Cuo- Nps and Ch- Nps were 2 and 8 indicating bactericidal activity and bacteriostatic effect, respectively.

The treated cells of *S. aureus* with Cuo-NPs and Ch- NPs were investigated using SEM to show the effects of the NPs on the ultrastructure of the treated cells. The used NPS induced abnormal cell shapes and sizes, damage of the cell, complete cytoplasmic lysis and leakage out of the cell membrane. The morphological changes and disruption of the bacterial cell membrane of Cuo- Nps treated *S. aureus* bacteria as seen in **photo 3** agrees with the previous studies **(Syame et al. 2017)**. However, in case of Ch- NPs treated *S. aureus* bacteria as seen in **photo 4** similar pattern of results were observed by **Hipalaswins et al. (2016)**.

CONCLUSION

In conclusion, changes in biochemical parameters in cow's and buffaloe's milk with subclinical mastitis were related to the increased SCC and TBC, which could be used as an indicator to diagnose subclinical mastitis in cows. This suggests that biochemical parameters can be used as potential biomarkers for the early detection of mastitis. As well as milk biochemical changes with isolation of *S. aureus* from normal row milk may predict sub clinical mastitis in bovine and differentiate between healthy and sub clinically mastitic cattle. Also concluded that *S. aureus* isolated from mastitic bovine milk was resistant to many antibiotics which have public health interests and it may transmitted to the surrounding bovine causing subclinical mastitis resulting in change in normal milk composition so application of hygienic practices is necessary to control the spread of pathogen on the other hand it was observed that Cuo- Nps and Ch- Nps have an-

tibacterial activity against the isolated antibiotic resistant *S. aureus*.

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