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Correlation between biofilm formation and antibacterial resistance pattern among coagulase negative *Staphylococcus*.

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ABSTRACT

Identification of coagulase negative *Staphylococcus* species (CNS) involved in mastitis and providing information about pathogenicity and antimicrobial resistance are essential to developing efficient strategies to control mastitis caused by the CNS. A total of 100 raw milk samples from mastitic cows were collected aseptically from different private dairy cow farms at Alexandria Governorate. All samples were subjected to bacteriological isolation of the CNS. All isolates were identified biochemically and confirmed by Vitek compact system . The CNS strains were tested for their haemolytic activity. Biofilm formation and antimicrobial resistance were investigated. The haemolytic and biofilm forming isolates were further identified by 16S rRNA gene sequences. Antimicrobial resistance genes assayed by PCR. The obtained results revealed the isolation of 16 CNS isolates, with a percentage of 16%. Species identification of CNS isolates by Vitek compact system revealed *S. epidermidis*, *S. chromogenes*, *S. warneri*, *S. saprophyticus*, *S. simulans*, *S. haemolyticus* and *S. xylosus* (4, 3, 3, 2, 2, 1, and 1 isolate) respectively. All *S.warneri* and *S. haemolyticus* isolates showed haemolytic activity , while the other isolates were non-haemolytic. Four isolates that showed haemolytic activity were investigated for biofilm formation ability, which revealed that all isolates were biofilm formers to a different degrees. Identification on the basis of 16S rRNA of haemolytic and biofilm forming isolates revealed the presence of an amplicon gene of 412 bp. The results of in vitro antimicrobial susceptibility testing of haemolytic and biofilm forming CNS isolates against 11 antimicrobial agents showed high resistance against ampicillin, followed by amoxicillin-clavulanic acid , cefotaxime, gentamicin, deoxycycline HCL, and tertacycline. Meanwhile, they were highly sensitive to vancomycin, followed by ciprofloxacin, clindamycinand sulfamethoxazole-trimethoprim. The results of genotypic detection of *blaZ* and *mecA* resistance genes and the *icaD* biofilm coding gene using PCR showed that they were detected in 2 isolates (50%), 3 isolates (75%), and 3 isolates (75%) of the tested four haemolytic and biofilm forming isolates (3 *S.warneri* and 1 *S. haemolyticus*) respectively.

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INTRODUCTION

Bovine mastitis is a common condition in the dairy cattle industry around the world that is known to cause the most costly and greatest loss of dairy cattle worldwide. It is caused by either physical trauma or infection by microorganisms.

More than 140 different microorganisms have been isolated from dairy mastitic cow cases. Bacteria are the most common causative agents of mastitis, either contagious pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*, *Corynbacteriumbovis* and *Mycoplasma spp*., or environmental pathogens such as *Escherichia coli*, *Enterococcus faecalis*, *Streptococcus dysgalactiae*, *Streptococcus uberis,* and *Coagulase-negative Staphylococcus* **(Keane et al. 2013).**

Although CNS are less virulent as they do not produce a large number of toxins and toxic enzymes compared to coagulase-positive staphylococci **(Franca et al. 2021; Marek et al. 2021),** recent studies reported that the CNS has been increasingly recognized as an important cause of bovine mastitis around the world, with a noticeable increase in the prevalence of intramammary infections in bovines **(Melo et al. 2018; Kirwa et al. 2021).**

CNS mastitis is considered a serious hidden threat to dairy cows not only due to its horizontal transmission to lactating cows but also due to their vertical transmission to suckling calves **(Supre et al. 2011).**

CNS have different antimicrobial resistance genes such as *ermB, mecA, tetK, tetL, tetM*, and *dfrG*, which are recorded for erythromycin, methicillin, tetracycline, and trimethoprim resistance, respectively (Qu **et al. 2019).** The most important antibiotic-resistant gene in *Staphylococcus aureus* strains was the blaZ ne, which codes for -lactamase. **(Taponen and Pyoala, 2009).** Moreover, the high incidence of antimicrobial resistance among CNS could be attributed to their ability to form a biofilm, which facilitates persistent infections **(Cepas *et* al. 2019) and** also decreases their sensitivity to commonly used antimicrobial agents **(Tremblay et al. 2014).** CNS have the ability to form biofilms, which implies an important role in their pathogenicity **(Goetz et al. 2017).** Biofilms perform resistance actions to antimicrobials as it impairs their actions **(Gajewska and Chaj, 2020).**

The continuous surveillance and monitoring of the antimicrobial resistance genes of the CNS isolates will help in controlling mastitis by supplying a valuable database about the efficient antibiotics that could be used in the treatment of bovine mastitis.

The aim of this study was to investigate the prevalence of CNS and determine their potential pathogenicity by monitoring the relationship between antimicrobial resistance and biofilm formation among CNS isolates recovered from bovine mastitis in Egypt.

MATERIALS and METHODS

1-Collection of samples:

One hundred milk samples were collected from different private dairy bovine farms in Alexandria Governorate , Egypt. All samples were collected in sterile containers and then transferred as soon as possible in an ice box to the laboratory of the Animal Health Research Institute, Alexandria branch, Egypt, for isolation and identification of CNS

2- Isolation of CNS (Waller et al. 2011) :

All mastitic milk samples were centrifuged at 3,000 rpm for 15 minutes, then the supernatant and cream layer were discarded. The sediment was inoculated into tryptone soy broth (Oxoid) and incubated aerobically at 37 °C for 18–24 hrs. A loopful of inoculated broth was streaked onto sheep blood agar 7%, Baird-Parker, and mannitol salt agar (Oxoid) and incubated at 37 °C for 18–24 hrs aerobically.

3-Identification of CNS isolates:

All plates were examined for their bacterial growth and cultural characteristics. Isolated purified strains were identified microscopically for the presence of Gram positive cocci occurring in clusters and biochemically for confirmation **(Collee et al. 1996) and (Quinn et al. 2011).** The Vitek compact system (BioMérieux); used for Gram positive cocci identification, was applied on pure cultures for

complete identification of CNS isolates. For further confirmation of haemolytic and biofilm forming CNS isolates , the polymerase chain reaction **(Hwang et al. 2011)** was applied to pure cultures for complete identification of the most virluent CNS isolates. Identification of the the most virluent CNS isolates on species level was based on 16S rRNA gene sequencing by PCR.

4- Gene sequencing of CNS isolates:

***DNA extraction.**

DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the isolate suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

***Oligonucleotide Primer.** Primers used were supplied from Metabion (Germany) are listed in **table (1).**

***PCR amplification.**

PCR. Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmolconcentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

***Analysis of the PCR Products.**

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. Agene-ruler 100 bp ladder (Fermentas, thermofisher, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

*** Phylogenetic analysis:**

PCR products were purified using QI-Aquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit(Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) (**Altschul et al. 1990**) was initially performed to establish sequence identity to GenBank accessions. The phylogenetic tree was created by the MegAlign module of LasergeneDNAStar version 12.1 **(Thompson et al. 1994)** and Phylogenetic analyses was done using maximum likelihood, neighbour joining and maximum parsimony in MEGA6 (**Tamura et al. 2013).**

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

Target gene	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)					Final extension	Reference
			Primary Denaturation	Secondary denaturation	Annealing	Extension			
16S rRNA	GCCAGTTGAGGACGTATTCT CCATTTCAGTACCTTCTGGTAA	412	94˚C 5 min.	94˚C 30 sec.	56˚C 40 sec.	72˚C 45 sec		72˚C 10 min.	Hwang et al., 2011

Biofilm Formation Assay (Stepanovic et al. 2007):

The tested strains were refreshed by cultivation on trypticase soya agar (TSA) plates and incubated for 24h at 37°C aerobically. Then 3-4 typical colonies from fresh cultivated TSA plates are suspended in 5ml TSB and incubated for 18h at 37°C, after incubation vortex each tube and diluted 1:100 using TSB 1% glucose. The diluted bacterial broth was vortexed and inculate (200µl / well) in 96-well flat -bottom microtiter plates, this done in triplicate for each strain and one well as a negative control (200 µl TSB1%glucose). The plates were incubated aerobically at 37°C for 48 hr. After incubation the content of the wells were discarded into a discard container and each well washed three times with 300µl of sterile phosphate buffered saline; then wells allowed to drain in an inverted position. After that, 150 µl of methanol was added to each well for 15 minutes, then drain and wells allowed to dry in an inverted position at room temperature. For staining of the adhered cells, 150 µl of 0.1% crystal violet solution was added for 15 minutes then the stain should be aspirated by the automated pipette and the well washed twice with sterile water to remove the excess stain, then allow plate to dry. Finally, 150 µl glacial acetic acid 33% added gently for each well to solubilize the bounded dye then cover the plate with lid to avoid evaporation. The optical density of each well was measured at 570 nm (OD570) using ELISA reader. The interpretation of the biofilm formation results was made according to the following rule: (OD> 4×ODC) as strong, (2×ODC<OD ≤ 4×ODC) moderate positive, (ODC<OD ≤ 2×ODC) weak positive (OD ≤ ODC) as negative.

6- Antimicrobial Susceptibility Testing:

According to Clinical and Laboratory Standards Institute guidelines **(CLSI, 2018).** The antimicrobial susceptibility of all CNS isolates was determined by disc diffusion method using Muller-Hinton agar against the most 11 different antimicrobials used in the field. Antimicrobial discs included ampicillin (10µg), amoxicillin-clavulanic A (30µg), cefotaxime (30µg), vancomycin (30µg), clindamycin (2µg), gentamicin (10µg), doxycycline HCl (30µg), ciprofloxacin (5µg), amikacin (30µg), colistin (10µg), and sulfamethoxazole-trimethoprim (25µg) (Oxoid, Basing Stoke, UK). Multidrug resistance (MDR) was defined as an acquired resistance to at least one agent in three or more antimicrobial classes (**Magiorakos et al. 2012**).

7- Detection of biofilm-associated genes and antimicrobial resistance genes by polymerase chain reaction:

DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the isolate suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer. Primers used were supplied from **Metabion (Germany)** are listed in **table (2)**.

PCR amplification:

PCR. Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmolconcentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. Agene-ruler 100 bp ladder (Fermentas, thermofisher, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 2. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
16S rRNA	GCCAGTTGAGGACGTATTCT CCATTTCAGTACCTTCTGGTAA	412	94˚C 5 min.	94˚C 30 sec.	56˚C 40 sec.	72˚C 45 sec	72˚C 10 min.	Hwanget al., 2011
icaD	AAA CGTAAG AGA GGT GG GGC AAT ATG ATC AA-GATA	381	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 40 sec	72˚C 10 min.	Ciftci*et al*., 2009
mecA	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	310	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec	72˚C 7 min.	McClure *et al*., 2006
blaZ	TACAACTGTAATATCG-GAGGG CATTACACTCTT-GGCGGTTTC	833	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 50 sec	72˚C 10 min.	Bagcigil et al. 2012

RESULTS

Prevalence of different CNS among the examined mastitic cow's milk samples:

Out of 100 mastitic cow milk samples,16 CNS were recovered with a percentage of 16%. Vitek compact system of CNS isolates revealed presence of S.*epidermidis*, *S.chromogens, S.warnei, S.saprophyticus, S.simulants, S.haemolyticus* and *S.xylosus* (4,3,3,2,2,1 and 1 isolate) respectively.

Haemolytic activity of CNS:

All *S.warneri* and *S.haemolyticus* isolates (3 and 1 respectively) showed haemolytic activity , while the other isolates showed no haemolytic activity .

Results of biofilm formation assay by microtiter plate:

All CNS isolates which showed haemolytic activity were tested for production of biofilm . Biofilm binding activity and optical density were calculated for detection of biofilm as shown in the following **table (3)** revealed that all tested isolates have the ability for biofilm production with a percentage of 100% but with a different degree. Where Out of 4 isolates,1 *S.haemolyticus* and 2 *S.warneri* isolates showed strong activity while, 1 *S.warneri* isolate showed weak activity.

Table 3. Calculation of optical density for biofilm strength detection:

Cut-off value calculation (ODc) a	Average OD value	Biofilm production strength	Tested strains
More than 4 cut off OD value	OD> 0.43	Strong	*S.haemolyticus* (1) & *S.warneri*(2)
More than 2 cut off OD up to 4 cut off OD value	0.21<OD ≤0.43	Moderate	-
1 cut off OD up to 2 cut off OD value	0.109<OD ≤0.21	Weak	*S.warneri* (1)
Equal or lower than 1 cut off OD	OD ≤ 0.109	Negative	-

a: Optical density cut-off = average OD of -ve control + 3 x (SD) of -ve control
Cut off -ve control OD= (0.079 + 3× 0.010) = 0.109

Results of antimicrobial susceptibility test of CNS isolates:

All CNS isolates (n=4) which showed the ability for haemolytic activity and biofilm production were montiored to detect their antimicrobial susceptability pattern against 11 antimicrobial agents as shown in **Table (4)**. Results indicated that tested CNS isolates were mostly resistant to ampicillin (100%), followed by amoxicillin-clavulanic, cefotaxime, gentamicin, deoxycyclin HCL and tertacyclin (75% for each), and finally Trimethoprim-Sulphamethoxazole (50%). Meanwhile, they were highly sensitive to vancomycin (100%), followedby ciprofloxacin, clindamycin and sulfamethoxazole-trimethoprim (50% for each), and finally each of amoxicillin-clavulanic, cefotaxime and gentamycin (25% for each)**.**

Table 4. Antimicrobial susceptibility pattern of haemolytic CNS isolates:

Chemotherapeutic Agents	Disk Concentrations	CNS tested isolates (n= 4) S		I		R	
		No.	%*	No.	%*	No.	%*
Amoxicillin-Clavulanic acid	30 µg	1	25	0	0	3	75
Ampicillin	10 µg	0	0	0	0	4	100
Cefotaxime	30 µg	1	25	0	0	3	75
Ciprofloxacin	15µg	2	50	1	25	1	0
Clindamycin	2 µg	2	50	1	25	1	25
Gentamicin	30 µg	1	25	0	0	3	75
Vancomycin	30 µg	4	100	0	0	0	0
Deoxycyclin HCL	30 µg	0	0	1	25	3	75
Tetracyclin	30 µg	0	0	1	25	3	75
Trimethoprim-Sulphamethoxazole	25 µg	2	50	0	0	2	50

* Percentage according to total number of examined CNS isolates (n=4).
S: Sensitive, I: Intermediated sensitive, R: Resistant.

Detection of biofilm-associated gene and antimicrobial resistance genes by polymerase chain reaction:

The PCR results are shown in **Table (5)** and **Figure (1)** represented that the presence of biofilm-associated genes *ica*D was detected in 3 isolates. Genes encoding resistance to β-lactams; *bla*Z and *mec*A were detected in 2 and 3 isolates, respectively.

Table 5. Prevalence of biofilm-associated genes and antimicrobial resistance genes among haemolytic CNS isolates

CNS examined Isolates	Antimicrobial resistance genes		biofilm-associated gene (*icaD*)
	blaZ	*mecA*	
S.warneri	-	-	+
S.warneri	-	+	+
S.warneri	+	+	-
S.haemolyticus	+	+	+
Total percentage (%)	50	75	75

Figure (1): Prevalence of biofilm-associated genes and antimicrobial resistance genes among haemolytic CNS isolates:

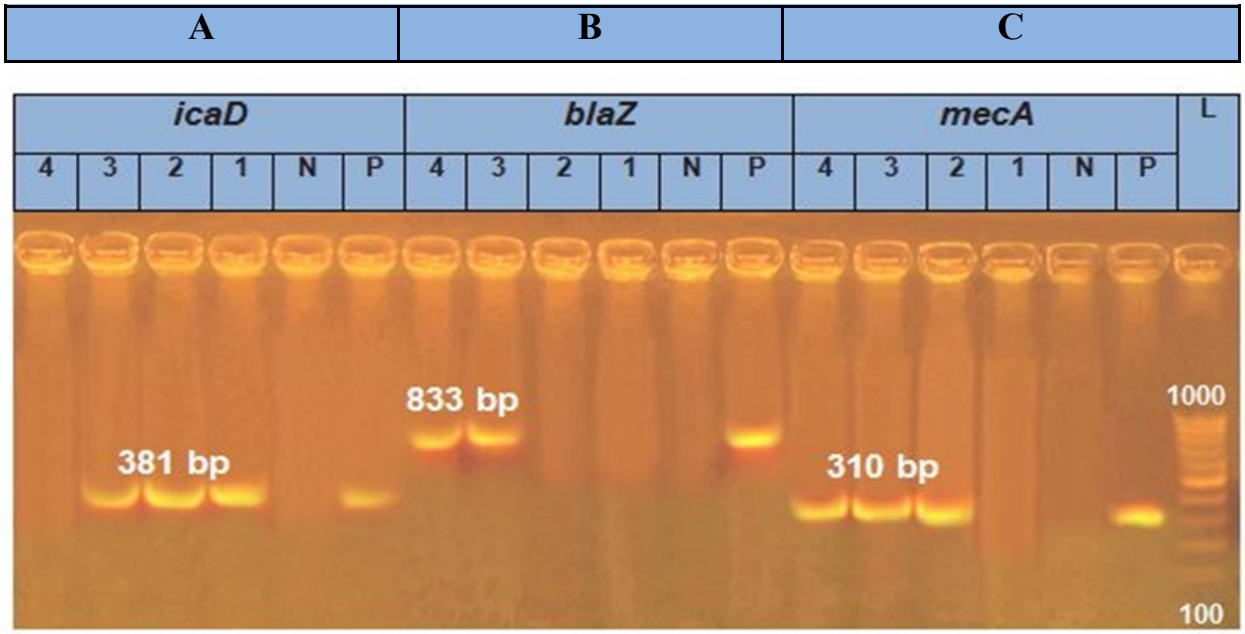


Fig. 1. (A) Agarose gel electrophoresis of PCR products of CNS found in examined samples by PCR. *icaD* (381 bp), lanes (1, 2, 4) are *S.warneri*isolates, Lane (3) *S.haemolyticus* isolate and lane (L) is DNA ladder 100 bp molecular weight marker, lane (N) is negative control, and lane (P) is positive control. **(B)** Agarose gel electrophoresis of PCR products of CNS found in examined samples by PCR.*blaZ*(833bp), lanes (1, 2, 4) are *S.warneri* isolates, Lane (3) *S.haemolyticus* isolate and lane (L) is DNA ladder 100 bp molecular weight marker, lane (N) is negative control, and lane (P) is positive control **(C)** Agarose gel electrophoresis of PCR products of CNS found in examined samples by PCR. *mecA* (310 bp), lanes (1,2,4) are *S.warneri*isolates, Lane (3) *S.haemolyticus* isolate and lane (L) is DNA ladder 100 bp molecular weight marker, lane (N) is negative control, and lane (P) is positive control.

Sequencing of 16S rRNA gene and phylogenetic analysis for haemolytic CNS isolates identification:

All haemolytic and biofilm forming activity CNS isolates were identified by biochemical tests and Vitek compact system were further characterized by 16S rRNA gene PCR and sequencing of a 412 bp region (Fig. 2). Species identification of CNS by gene sequences revealed *S.wareni, and S.haemolyticus* (3 and 1) isolates respectively. The sequences of the most virluent isolates which were 3 *S. warneri* and 1 *S.haemolyticus* were submitted to the GenBank database and were given the accession numbers as BankIt2712089 Seq1 OR119739, BankIt2712089 Seq2 OR119740, BankIt2712089 Seq3 OR119741 and BankIt2712093 Staphylococcus OR119742 for isolates WISH1, WISH2, WISH4 as *S.warneri*, and WISH3 as *S.haemolyticus* respectively.

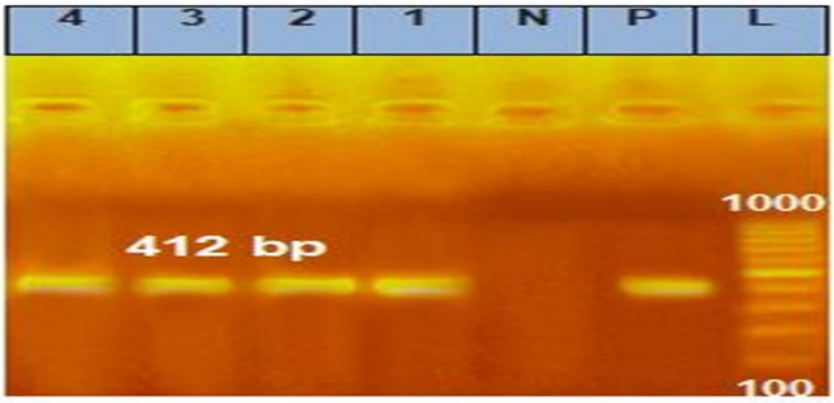
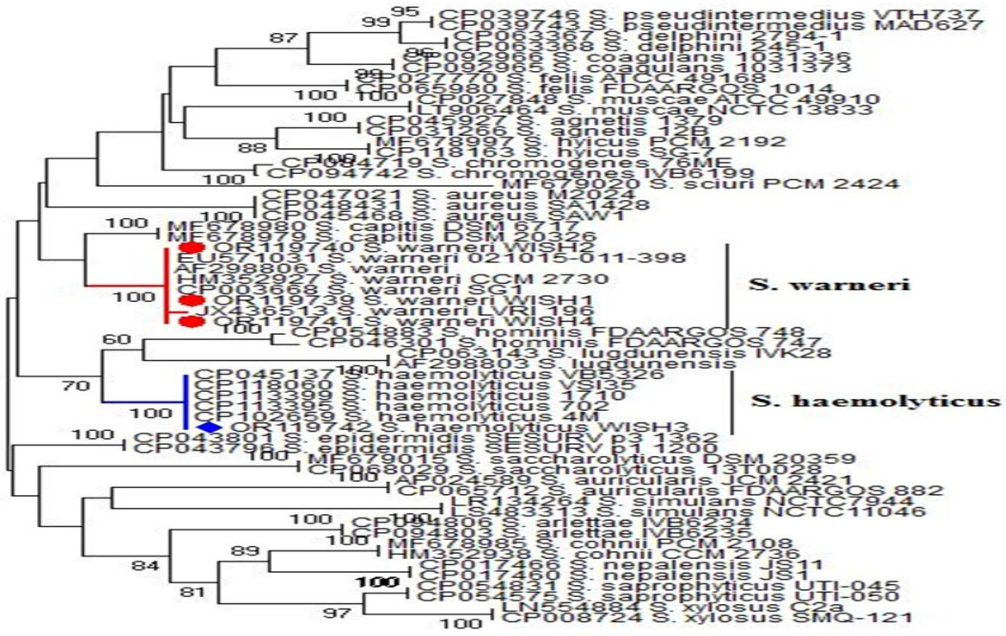


Fig. 2. Agarose gel electrophoresis of PCR products for 16S rRNA , lane (L) is DNA ladder 100 bp molecular weight marker, lane (N) is negative control, and lane (P) is positive control and Lanes 1-4: CNS isolates from cows mastitis showing 412 bp amplicon of 16S rDNA gene

The phylogenetic analysis of 16S rRNA sequences of the most virluent four CNS isolates of the identified species (3 *S.wareni* and 1*S.haemolyticus* collection identified the three CNS isolates WISH1,WISH 2and WISH4 as *S. warneri*, and WISH3 as *S. haemolyticus*(Fig. 3).

Fig (3) Phylogenetic analysis of 16S rRNA sequences of haemolytic CNS isolates



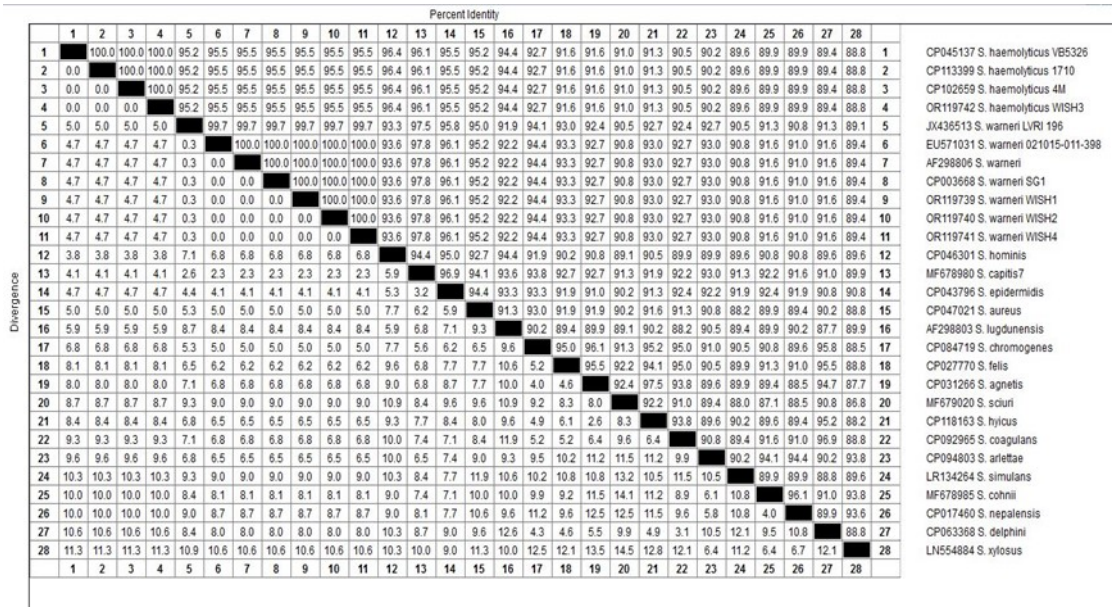


Fig 4. Sequence distances of haemolytic CNS isolates

DISCUSSION

Bovine mastitis is one of the most economically important diseases affecting dairy farms and causing economic losses to farmers in Egypt. There are 50 staphylococci or more that have been reported as a cause of staphylococcal mastitis; among them, CNS species are considered the major pathogens causing mastitis throughout the world, and CNS were described as emerging mastitis pathogens **(El-Jakee et al. 2013; Taponen et al. 2006).** CNS is becoming a significant mastitis-causing pathogen in several nations, including developing countries **(Petzer et al. 2009; Sender et al. 2017).**

For example, *S. simulans*, *S. chromogens*, *S. hyicus*, and *S. epidermis* represent an emerging mastitis pathogen that has been isolated in many countries (**Taponen and Pyorala. 2009).** Many studies propose that infections of the CNS may cause more serious harm than previously thought (**Schukken et al. 2009).** In the present study, the prevalence of CNS among 100 clinically mastitic dairy cow milk samples was investigated. The present data revealed the recovery of 16 CNS isolates out of 100 collected mastitic cows milk samples, with a percentage of 16%. Many studies in different regions all over the world have investigated the prevalence of CNS-caused mastitis. **EL-Diasty et al. (2019**) recoreded that the incidence of CNS in clinical mastitic cows was 25.7%. **Amer et al. (2018)** showed that examination of clinical mastitic cow milk samples revealed the isolation of the CNS by 20.4%. **Gao et al. (2017)** isolated 11.3% CNS isolates from cow's milk samples with clinical mastitis. The need for differentiating and identifying CNS at the species level has become increasingly important, but it is currently limited by the availability of suitable, cost-effective diagnostic tests. Identification based on biochemical test profiles, VITEK-2, the API-STAPH system, MALDI-TOF-MS, and 16S rRNA gene sequencing are used to identify and differentiate the staphylococcal species. A number of PCR amplicon sequencing-based methods for the identification of CNS have been reported **(Capurro et al. 2009; Sampimon et al. 2009; and Schukken et al. 2009).**The identified 16 CNS isolates by Vitek compact system revealed presence of *S.epidermidis*, *S.chromogenes, S.warenri, S.saprophyticus, S.simulans, S.haemolyticus, S.haemolyticus* and *S.xylosus* (4, 3, 3, 2, 2, 1, and 1 isolate), respectively.

In this study, haemolytic activity was investigated in all CNS isolates. Haemolytic activity was recorded at 100% among *S.warenri* and *S. haemolyticus* isolates, while the other

CNS isolates showed no haemolytic activity.

We chose 16S rRNA sequencing as the means of identification of the most virluent CNS isolates because it is extremely specific—the identity can be clearly defined by the number of base differences between the isolate and the existing species. Further confirmation for identification of the four haemolytic CNS isolates by 16S rRNA were revealed *S.warenri and S.haemolyticus* (3 and 1) isolates respectively. Hosseinzadeh and Dastmalchisaei (2014**)** identified nine different CNS species from bovine mastitis: 44 *S. haemolyticus* (40.7%), 17 *S. chromogenes* (15.7%), 11 *S. epidermidis*, *S. warneri,* and *S. cohnii* each (10.2%), 6 *S. simulans* (5.5%), 4 *S. hominis* (3.7%), 3 *S. capitis* (2.7%), and 1 *S. xylosus* (0.9%). *S. haemolyticus*, *S. chromogenes,* and *S. warneri* were the only species identified from clinical mastitis. Walid (2021) recorded that most CNS isolates from bovine mastitis in Egypt were *S. epidermidis* (48.4%), *S. saprophyticus* (32.3%), and *S. haemolyticus* (19.4%).Production of slime and the capability of surfaces' attachment to assist the formation of biofilm are essential properties related to the pathogenicity of *Staphylococcus* species and their intramammary survival **(El-Seedy et al., 2017).** The biofilm of the CNS is composed of a layer of extracellular polymeric substance called polysaccharide intercellular adhesion (PIA) matrix, which is encoded by the *ica* operon (*icaADBC* genes) **(Mack et al. 1999).** Beneath the biofilm matrix, bacteria are protected from physical, chemical, and biological stresses imposed by the antibiotics and the host immune cells **(Von-Eiff et al. 2002).** Through biofilms, the CNS can adhere to and colonise biotic as well as abiotic surfaces. **(Von-Eiff et al. 2002; Ziebuhr et al. 2006).** Indeed, biofilms reduce AMS, impairing antimicrobial therapy **(Tremblay et al. 2013).** In particular, several CNS, such as *S. epidemidis*, *S. chromogenes*, and *S. xylosus*, showed higher pathogenicity by forming biofilms for bacterial aggregation for better growth and resistance to adverse conditions **(Maity and Ambatipudi, 2021).** In the current study, biofilm formation ability was investigated in all tested CNS isolates that showed hemolytic activity, revealing that all CNS isolates have the ability for biofilm production with a percentage of 100% but to a different degree. Where Out of 4 isolates, 1 *S. haemolyticus* and 2 *S. warneri* isolates showed strong activity, while 1 *S. warneri* isolate showed weak activity. **Abed et al. (2022)** concluded that 62.5 percent of CNS isolates were phenotypically biofilm formers. Of them, 4 (50%) were strong biofilm formers, while only one isolate (12.5%) was intermediate. Meanwhile, three isolates (37.5%) were negative. **Abed et al. (2021)** showed that biofilm formation occurred in 46.8% of *Staphylococcus* isolates, of which 33.8% were strong and 13% were intermediate. **Sarita et al. (2021)** demonstrated strong and moderate biofilm producers in 35 (16.4%) and 55 (25.7%) CNS isolates, respectively. Among all CNS species, *S. epidermidis* was the most frequent species to produce biofilm in all phenotypic methods. Antibiotic resistance is a major livestock health problem. The present result demonstrated that all haemolytic CNS isolates tested were resistant to ampicillin (100%). However, the majority of the isolates were resistant to amoxicillin-clavulanic, cefotaxime, gentamicin, deoxycyclin HCL, and tertacyclin (75% for each), followed by trimethoprim-sulfamethoxazole (50%). However, the study also demonstrated that the majority of isolates were highly susceptible to vancomycin (100%), followed by ciprofloxacin, clindamycin, clindamycin and sulfamethoxazole-trimethoprim (50% for each), and finally each of amoxicillin-clavulanic, cefotaxime, and gentamycin (25% for each)**. Abed et al. (2022**) recorded that CNS isolates were highly resistant to penicillins: ampicillin, amoxicillin-clavulanic acid, cefoxitin, and cefotaxime sodium. On the other hand, high susceptibilities were recorded against ciprofloxacin, levofloxacin, florophenicol, vancomycin, doxycycline HCl, clindamycin, gentamicin, and sulfamethoxazole-trimethoprim. Fowoyo and Ogunbanwo (2017) said that CNS isolates showed high resistance against ampicillin (86.7%), followed by sulfuramethoxazole or trimethoprim (74.9%), amoxicillin-clavulanic acid (52.5%), and oxacillin (35.7%). Mohammed **et al. (2019)** noticed that the highest number of CNS isolates were resistant against Ampicillin and oxycillin

(85% of each), followed by Cefoxitin, sulfamethoxazole/trimethoprim (75% of each), cefotaxime (70% of each), tetracycline (65%), ampicillin/sulbactam, erythromycin (60% of each), chloramphenicol, gentamicin, ofloxacin (45% of each), Ciprofloxacin (40%), and vancomycin (5%).According to **Waters et al. (2011),** a single strain that exhibits multi-drug resistance (MDR) is resistant to three or more antimicrobial classes. The CNS isolates in the current investigation were resistant to a variety of antibiotics. These findings may be explained by the widespread usage of these medications to treat mastitis, a condition in which beta-lactam antibiotics like penicillin and cephalosporin play a significant role. The extremely seldom usage of preparations containing vancomycin is thought to be the cause of the high rate of sensitivity to the drug **(Lüthje and Schwarz, 2006).**Genotypic methods to detect antimicrobial resistance genes have the advantage of being more rapid than conventional susceptibility tests that depend on culturing bacteria **(Asfour and Darwish, 2011)**. In the current study, the *blaZ* and *mec*A resistance genes were investigated using PCR among 4 haemolytic and biofilm forming CNS isolates, and both genes were found in 50% and 75% of the tested isolates, respectively. These results are nearly similar to those reported by **Abed et al. (2022),** who pointed out that the *blaZ*and *mec*A resistance genes were detected in 60% and 80% of the examined CNS isolates, respectively. **Abed et al. (2018)** showed that *blaZ* and *mec*A were detected in 55% and 70% of isolates, respectively. Regarding the biofilm-coding gene, *icaD, which* was investigated using PCR in the same four CNS isolates, was detected in 75% of the tested isolates. These results ran parallel to the incidence obtained by **Osman et al. (2015), 77%, and Abed et al. (2021),** 77.8%. Meanwhile, our results are slightly lower **than Abed et al. (2022)** who recorded the *icaD* genes in 60% of the CNS-tested isolates.

CONCLUSION

One of the most frequent causes of clinical cow mastitis in different countries is CNS. The identification of CNS isolates is improved by the use of genotypic approaches because it has been demonstrated that they have higher specificity and sensitivity than other methods for differentiating between species. The ability of biofilm to spread resistance genes and possess a phenotypic tolerance to antibiotics force it to be considered as being related to antibiotic resistance. Farmers and veterinarians are under pressure to select effective antimicrobial therapies or prophylaxes due to the high prevalence of antimicrobial resistance, the presence of resistance and virulence genes, as well as the risks to public health and the risk of lateral transfer of resistance-associated genes between human and animal pathogens

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