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COAGULASE-NEGATIVE STAPHYLOCOCCI COLLECTED FROM BOVINE MILK: SPECIES AND ANTIMICROBIAL GENE DIVERSITY

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ABSTRACT

The aim of this study was to examine the genetically mediated antimicrobial resistance in 94 coagulase-negative staphylococcal (CNS) milk isolates (buffalo, $n = 88$, and cow, $n = 6$), and to determine whether antimicrobial resistance profiles differed between bacterial species. Our analysis of 94 CNS isolates from milk confirmed the well-established multiresistant character of staphylococci in the dairy setting. Resistance against oxacillin, ciprofloxacin and cefoxitin was most frequently observed. Eleven CNS species isolated from buffalo's and cow's milk samples were 100% sensitive to gentamicin, erythromycin, clindamycin and ciprofloxacin. Resistance to oxacillin was attributed to the *mecA* gene in 44.7% of the oxacillin-resistant isolates. The *mecA* gene was detected in *Staphylococcus intermedius*, *epidermidis*, *hominis*, *hyicus*, *caprae*, *sciuri*, *lugdunensis* and *xylosus* while totally absent in *chromogenes*, *simulans* and *lentus*. Of the 11 CNS species, *S. epidermidis*, *S. lugdunensis*, *S. hominis*, *S. xylosus* and *S. intermedius* were the only species that exhibited multiple resistance.

PRACTICAL APPLICATIONS

Studying coagulase-negative staphylococci (CNS) at the species level can provide valuable information about species-specific differences that can be vital data to prevent the dissemination of antimicrobial-resistant genes and resistant pathogens to the community. CNS have long been regarded as pathogenic, but their important role as colonizers of the bovine udder has been recognized and studied in recent years. CNS have become increasingly resistant to multiple antibiotics. In recent years, increasing numbers of reports have shown that the *mecA* gene is present in CNS strains, including hospital-acquired infections, community-associated methicillin-resistant *Staphylococcus aureus* infections, animal epidermis, beaches and public transportation systems. Therefore, it is highly important to detect the *mecA* gene as a marker to investigate antibiotic resistance in milk that may represent a serious health and economic concern.

INTRODUCTION

Staphylococci are part of the normal microbiota of the skin and mucous membranes of mammals, but are also ubiquitously distributed in very different niches in nature, including soil, water, air and in a variety of foodstuffs, such as meat, cheese and raw milk (Irlinger 2008).

Currently, the *Staphylococcus* genus consists of 45 validated species and 24 subspecies resulting in more than 50 recognized systematic entities, of which the majority is coagulase negative (Euzeby 2014). They can be divided into two groups according to production of coagulase enzyme, which is capable of coagulating blood plasma. The synthesis of this enzyme is restricted to some species in the genus, among which *S. aureus*, *S. schleiferi* subsps. *coagulans*, *S. intermedius*, *S. hyicus* and *S. delphini* can be distinguished. The other staphylococci do not synthesize coagulase and make up the group known as coagulase-negative

staphylococci (CNS). CNS are commensal bacterial species and opportunistic pathogens that can cause infections in humans (most of the hospital-acquired infections, bacteremia related to indwelling devices, central nervous system shunt infections, native or prosthetic valve endocarditis, urinary tract infections and endophthalmitis) (Kloos and Bannerman 1994) and animals (Soares *et al*. 2012).

In the past few years, the interest in CNS species has significantly increased due to their impact on human health and disease (Oliveira and Cerca 2013). Several studies have reported the existence of CNS enterotoxin-producing strains (Veras *et al*. 2008; Zell *et al*. 2008; Oliver *et al*. 2009; Even *et al*. 2010; Hennekinne *et al*. 2010; Oliveira *et al*. 2011), and the wide dispersion of these enterotoxigenic CNS in the environment and in the foods analyzed indicates a risk to consumer health (de Mello *et al*. 2014).

There is concern that the antimicrobial-resistant elements of CNS in food animals can be disseminated to humans via food production chain (Irlinger 2008). CNS are considered a reservoir of various antimicrobial-resistant associated determinants (Bally *et al*. 2012). Previous studies indicated that staphylococcal resistance to penicillin is mediated by *bla*Z, the gene that encodes β-lactamase (Zapun *et al*. 2008). Four different tetracycline-resistant (*tet*) genes assigned to classes K, L, M and O of bacterial *tet* genes have been detected in staphylococci of animal origin (Schwarz *et al*. 1998). It has been determined that two mechanisms play a role in resistance to tetracyclines in staphylococci (Schnappinger and Hillen 1996). Active reflux that emerges with the acquisition of plasmid-based genes such as *tet*(K) and *tet*(L) and ribosomal protection mediated by transposon- or chromosome-located *tet*(M) and *tet*(O) determinants. It is well known that the majority of *tet*M-positive strains also contain *tet*K and that MRSA isolates possess the *tet*M or *tet*K genotype and that both drug efflux and ribosomal protection can be induced *in vitro* in *S. aureus* (Trzcinski *et al*. 2000; Fluit *et al*. 2001). The *tet*K gene, almost exclusively found on pT181 and related plasmids, encodes a tetracycline efflux pump. Far less frequently found in staphylococci is *tet*L, which encodes an efflux pump similar in structure to that of *tet*K. A third tetracycline-resistant gene, *tet*M, occurring in *S. aureus* is virtually identical to the *tet*M gene originally identified on the transposons Tn916 and Tn1545 in enterococci. Although pT181 plasmids are ubiquitous, the *tet*M determinant is probably more clinically significant in that it encodes resistance to all tetracyclines, including minocycline and doxycycline (Nesin *et al*. 1990). The resistance to methicillin is caused by the presence of the *mec*A gene, which encodes the 78 kDa penicillin-binding protein (PBP) 2a (or PBP2″) (Ender *et al*. 2004). The 2.1 kb *mec*A gene is located on a mobile genetic element, designated the staphylococcal cassette chromosome *mec* (SCC*mec*) (Deurenberg *et al*. 2007). *Staphylococcus* strains with *mec*A are resistant to lactam antibiotics and frequently code for multi-drug resistance, which may represent a serious health and economic concern (Cosgrove *et al*. 2003). Consequently, it is highly important to detect *mec*A, especially in *Staphylococcus* strains.

Therefore, screening for these elements is important for public health reasons and despite the importance of such a screen, limited data are available for CNS at the species level of strains isolated from buffalo and cow milk. Thus, the focus of the current study was to compare pheno and genotypic indicators of antimicrobial-resistant profiles in CNS at the species level isolated from buffalo and cow milk to antibiotics of clinical relevance in dairy practice with emphasis on penicillin, tetracycline and oxacillin resistance. As to our knowledge, this is the first report on the *mec*A and *tet* (KLMO) elements in buffalo and cow milk CNS isolates in Egypt.

MATERIALS AND METHODS

Sampling

A total of 386 milk samples were collected from buffalo ($n = 338$) and cow ($n = 48$), with visually normal milk and mammary gland from private dairy farms around Cairo. The animals had not been treated with an antibiotic for at least 30 days prior to collection. A composite milk sample (all four quarters in one collection vial to represent one udder) was taken after gentle stirring under aseptic conditions for bacteriological examinations. A subsample of 15 mL of milk, taken from the composite milk sample, was collected in sterile universal bottles. The milk samples were quickly transported to the laboratory under chilled conditions and stored at 4C until bacteriologically analyzed.

Isolation of Bacterial Strains and Growth Conditions

Ninety-four CNS were isolated from milk samples collected in duplicate from mammary quarters. Milk samples were plated on plates containing Columbia agar base with 5% defibrinated sheep blood (Oxoid Ltd., Hampshire, United Kingdom). Test plates were incubated for 24–48 h at 37 ± 1C. All isolates were presumptively identified as CNS based on colony morphology, Gram staining (positive), coagulase plasma test, catalase test (positive), hemolysis and KOH test. Additional tests included a API staph kit (BioMerieux, Honeydew, South Africa) as described by Petzer *et al*. (2013). Strains identified as CNS were subcultured to obtain single colonies in pure culture. Colonies were then transferred to Todd Hewitt broth (Becton Dickinson Diagnostic Systems, Sparks, MD), cultured at 37C for 18 h and stored in 20% glycerol solution at −80C until use.

Phenotypic Antimicrobial-Resistant Tests

Antimicrobials were selected for testing based on the licensing for mastitis treatment in cattle, use in human medicine and potential resistant determinant phenotypes (FAO/WHO/OIE 2008; WHO 2009). Susceptibility of the isolates was determined against 10 antimicrobial agents commonly used for treatment of bovine mastitis in Egypt or considered as important agents for humans as follows: antimicrobials used for treatment of bovine mastitis included in this study were ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), penicillin (10 U) and tetracycline (30 μg). Antimicrobials not used for treatment of bovine mastitis but important for humans were clindamycin (2 μg), oxacillin (1 μg), rifampicin (5 μg) and vancomycin (30 μg). Confirmed CNS isolates were inoculated into Mueller–Hinton broth (Oxoid) and incubated overnight at 37C. The turbidity of the suspensions was adjusted to a 0.5 McFarland standard and streaked onto Mueller–Hinton agar (Oxoid) plates. Antimicrobial disks were added on the plates and they were incubated aerobically at 35C for 16–18 h. The results were recorded as susceptible, intermediate or resistant by measurement of the inhibition zone diameter. Resistance was determined by measurement of inhibition of growth around the antimicrobial disk according to the zone diameter interpretative standards of CLSI (2012) or according to the antimicrobials manufacturer's instructions. The reference strain *S. aureus* ATCC 25923 was used as the quality control organism and included with each batch of isolates tested.

Polymerase Chain Reaction Screening of the Genetic Determinants of Antibiotic Resistance

DNA Isolation for Polymerase Chain Reaction. Prior to DNA extraction, the bacterial strains were cultivated on blood agar base (Oxoid) containing 5% defibrinated sheep blood for 24 h at 37C. A total of 5–10 *S. aureus* colonies were suspended with 200 μL TE buffer (1% Triton X-100, 0.5% Tween-20, 10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid [EDTA] [pH 8]) in 2 mL microfuge tubes. The suspensions were incubated for 30 min at 56C, and then for 10 min at 95C before being spun at 6,000× g for 2 min. After centrifugation, 5 μL of the supernatant was used as template in a 50 μL polymerase chain reaction (PCR). DNA was stored at −20C.

***nuc* Gene Detection.** The PCR assay for the detection of the *nuc* (encoding for the *S. aureus*-specific thermonuclease) gene, in the 94 CNS isolates, was performed based on published primers and methods. PCR amplification was performed with a PT-100 thermal cycler (PT-100 thermocycler, MJ Research, Watertown, USA) in a volume of 50 μL, consisting of the following components: 1.5 mM $MgCl_2$; 200 μM each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP); 2 μM of each primer; 0.1 μg template DNA; and 1.25 U *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). A *nuc* gene was amplified using *nuc* gene primers as described by Brakstad *et al.* (1992). Forward GCGATTGATGGTGATACGGTT, reverse AGCCAAGCCTTGACGAACTAAAGC giving a PCR product of 270 bp. All primers were supplied by Sigma Genosys (Sigma-Genosys, Houston, TX, USA). The extracted DNA was amplified for 30 cycles consisting of 60 s at 94C for denaturation, 30 s at 50C for annealing and 90 s at 72C for primer extension. Twenty microliters of the PCR product was then analyzed by agarose gel electrophoresis (2% agarose prepared in TAE [1 mM EDTA/40 mM Tris acetate, pH 8] buffer). Gels were stained with ethidium bromide.

Identification of the *bla*Z, *mec*A and the *tet*KLMO by d- and m-PCR. The 94 CNS isolates were investigated to detect the presence of genes associated with the screened antibiotic resistances, namely, the β-lactamase gene *bla*Z (PEN resistance) was detected by primers designed by Vesterholm-Nielsen *et al.* (1999); *mec*A (OXA resistance) (Zhang *et al.* 2005); and the *tet*K, *tet*L, *tet*M and *tet*O (TET resistance) that confer resistance to tetracycline by two different mechanisms: efflux (*tet*[K] and *tet*[L]) and ribosomal protection (*tet*[M] and *tet*[O]) (Ng *et al.* 2001). Primer pairs targeting four different *tet* (*tet*[K], *tet*[L], *tet*[M] and *tet*[O]) genes were used in multiplex, in addition to two other different genes that were used in duplex and the target pairs were *bla*Z gene (a determinant of β-lactamase production) and *mec*A gene (a determinant of methicillin resistance) (Table 1).

Reactions for Amplification of *bla*Z and *mec*A Resistance Genes. PCR conditions included a 4 min initial denaturation at 94C followed by 35 cycles of 94C for 1 min, 55C for 1 min and 72C for 1 min and a final extension for 10 min at 72C.

Reactions for Amplification of Tetracycline-Resistant Genes. For PCR amplification reactions, a final volume of 50 μL contained 5 μL DNA template (target DNA); 1× PCR buffer II (PerkinElmer) (Perkin-Elmer Applied Biosystems, Foster City, CA, USA); 3 mM $MgCl_2$; 300 μM each dATP, dCTP, dGTP and dTTP; 200 μM each deoxynucleoside triphosphate; and 2.5 U of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA). The primer concentrations were optimized for

TABLE 1. PCR-SPECIFIC OLIGONUCLEOTIDE PRIMERS, AMPLICON SIZE AND CONDITIONS FOR SIX GENES SPECIFIC TO THREE ANTIBIOTIC-RESISTANT STRAINS

Gene	Oligonucleotide sequences (5′-3′)	Annealing	PCR product size (bp)	Reference
blaZ	F-AAG AGA TTT GCC TAT GCT TC R-GCT TGA CCA CTT TTA TCA GC	55C	517	Vesterholm-Nielsen *et al*. (1999)
*mec*A	F-GTG AAG ATA TAC CAA GTG ATT R-ATG CGC TAT AGA TTG AAA GGA T	55C	147	Zhang *et al*. (2005)
tet(K)	F-TCG ATA GGA ACA GCA GTA R-CAG CAG ATC CTA CTC CTT	48C	169	Ng *et al*. (2001)
tet(L)	F- TCG TTA GCG TGC TGT CAT TC R- GTA TCC CAC CAA TGT AGC CG	48C	267	
tet(M)	F-GTG GAC AAA GGT ACA ACG AG R- CGG TAA AGT TCG TCA CAC AC	48C	406	
tet(O)	F-AAC TTA GGC ATT CTG GCT CAC R-TCC CAC TGT TCC ATA TCG TCA	48C	515	

bp, base pair; PCR, polymerase chain reaction.

each multiplexed primer as follows: *tet*(K) 1.25 μM, *tet*(L) 1.0 μM, *tet*(M) 0.5 μM and *tet*(O) 1.25 μM. An initial denaturation hot start of 7 min at 95C was followed by 40 cycles consisting of 30 s ofdenaturation at 95C, 30 s of annealing at 48C and 1 min of extension at 72C. The last cycle was followed by a 10 min extension at 72C. *S. aureus* ATCC43300 as *mec*A+ and *blaZ*+ control, *S. epidermidis* DSM 20044 as a negative control for gene *nuc* and *S. aureus* ATCC 29213 as a positive control for targeting *nuc* gene and negative for gene *mec*A. Isolates putatively containing genes encoding for tetracycline resistance were identified by comparison with positive controls (Ng *et al*. 2001). All PCR assay runs incorporated a reagent control (without template DNA), and the PCR amplicons were simultaneously visualized and resolved using a UV light box after electrophoresis on a 2% agarose gel containing 0.5 μg/mL ethidium bromide.

RESULTS

Distribution of Resistance to Individual Antimicrobial Agents

The *in vitro* sensitivity of 94 CNS isolates (88 isolates were recovered from buffalo milk and six isolates were recovered from cow's milk) was tested against 10 antimicrobial drugs. The 94 CNS isolates were resistant to oxacillin, ampicillin, penicillin, tetracycline, vancomycin and rifampicin in an incidence of 48.9% (46/94), 42.6% (40/94), 32.0% (30/94), 25.5% (24/94), 8.5% (8/94) and 4.3% (4/94), respectively.

Table 2 records the results of antimicrobial sensitivity tests on 94 isolates (11 species) of CNS isolated from buffalo and cow's milk. A 100% resistance in *S. lentus* (2/2), *S. lugdunensis* (2/2) and *S. sciuri* (2/2) was found to ampicillin and penicillin while in *S. hominis* (8/12) and *S. hyicus* (4/8) resistance reached to 75 and 50%, respectively. The lowest resistance to penicillin was observed in *S. xylosus* (10/28, 35.7%), *S. epidermidis* (2/10, 20%) and *S. intermedius* (4/30, 13.3%), whereas their resistance to ampicillin was 50% (14/28), 40% (4/10) and 20% (6/30), respectively. *S. caprae* (2/2), *S. intermedius* (18/30) and *S. xylosus* (16/28) were resistant to oxacillin in an incidence of 100, 60 and 57.1%, respectively, and 50% in *S. hyicus* (4/8), *S. lentus* (2/4) and *S. lugdunensis* (2/4). Resistance to tetracycline was in an incidence of 100, 75, 40, 21.4 and 20% in *S. lugdunensis* (2/2), *S. hominis* (8/12), *S. epidermidis* (4/10), *S. xylosus* (6/28) and *S. intermedius* (6/30), respectively. The 11 CNS species isolated from buffalo's and cow's milk samples were 100% sensitive to gentamicin, erythromycin, clindamycin and ciprofloxacin.

The antimicrobials to which the CNS isolates were resistant fell into eight different antimicrobial-resistant classes of which penicillin was common to all 11 CNS species. On the other hand, six of the CNS (*S. chromogenes*, *S. hyicus*, *S. simulans*, *S. lentus*, *S. caprae* and *S. sciuri*) were resistant to only one class of the antibiotics (penicillin). The CNS species (*S. epidermidis* and *S. lugdunensis*) were resistant to two classes of antibiotics (penicillin and tetracycline) while *S. hominis* was resistant to three of the antibiotic classes (penicillin, vancomycin and tetracycline). Resistance to four of the antibiotic classes (penicillin, vancomycin, rifampicin and tetracycline) was evident in *S. xylosus* and *S. intermedius*. Of the 11 CNS species *S. epidermidis*, *S. lugdunensis*, *S. hominis*, *S. xylosus* and *S. intermedius* were the only species that indicated multiple resistance (two or more antimicrobial classes).

Results of Duplex PCR for Amplification of 147 and 517 bp Fragment for *mec*A and *blaZ* Genes Performed with Their Specific Primer

The results observed in Table 2 revealed that the *mec*A gene was detected in 58 CNS isolates in an incidence of 61.7% and positive amplification for the *blaZ* gene in 46 CNS iso-

TABLE 2. DISTRIBUTION OF ANTIBIOTIC-RESISTANT GENE COMBINATIONS IN 11 DIFFERENT CNS SPECIES CONSTITUTING THE 94 CNS ISOLATES FROM THE BUFFALO AND COW MILK

Staphylococcus species identified by API	Antibiotics										Antibiotic-resistant genes					
	P	AM	CN	CIP	T	VA	E	DA	OX	RF	*mec*A	*bla*Z	*tet*K	*tet*L	*tet*O	*tet*M
Buffalo																
Staphylococcus intermedius	**R**	**R**	S	S	S	**R**	S	S	**R**	S	+	+	–	–	–	–
S. intermedius	S	S	S	I	S	S	I	I	**R**	S	+	–	–	–	–	–
S. intermedius	S	**R**	S	S	S	S	S	I	**R**	S	+	+	–	–	–	–
S. intermedius	S	S	S	S	S	S	S	S	S	S	–	–	–	–	–	–
S. intermedius	S	S	S	S	S	S	S	I	**R**	S	+	–	–	–	–	–
S. intermedius	S	S	S	S	**R**	S	I	I	S	S	–	–	+	–	–	–
S. intermedius	S	S	S	S	**R**	S	S	S	**R**	S	+	+	+	–	–	–
S. intermedius	S	S	S	S	S	S	S	S	S	S	+	+	–	–	–	–
S. intermedius	S	S	S	S	I	S	I	S	S	S	+	–	–	–	–	–
S. intermedius	S	S	S	S	S	S	S	I	S	S	–	–	+	–	–	–
S. intermedius	**R**	**R**	S	I	I	S	I	I	**R**	**R**	+	+	+	–	–	–
S. intermedius	S	S	S	S	S	S	I	I	**R**	S	+	–	–	–	–	–
S. intermedius	S	S	S	S	**R**	S	I	I	**R**	S	+	–	+	–	–	–
S. intermedius	S	S	S	S	S	S	S	S	**R**	S	+	–	–	–	–	–
S. intermedius	**R**	**R**	S	S	S	**R**	S	S	**R**	S	+	+	–	–	–	–
S. intermedius	S	S	S	I	S	S	I	I	**R**	S	+	–	–	–	–	–
S. intermedius	S	**R**	S	S	S	S	S	I	**R**	S	+	+	–	–	–	–
S. intermedius	S	S	S	S	S	S	S	S	S	S	–	–	–	–	–	–
S. intermedius	S	S	S	S	S	S	S	I	**R**	S	+	–	–	–	–	–
S. intermedius	S	S	S	S	**R**	S	I	I	S	S	–	–	+	–	–	–
S. intermedius	S	S	S	S	**R**	S	S	S	**R**	S	+	+	+	–	–	–
S. intermedius	S	S	S	S	S	S	S	S	S	S	+	+	–	–	–	–
S. intermedius	S	S	S	S	I	S	I	S	S	S	+	–	–	–	–	–
S. intermedius	S	S	S	S	S	S	S	I	S	S	–	–	+	–	–	–
S. intermedius	**R**	**R**	S	I	I	S	I	I	**R**	**R**	+	+	+	–	–	–
S. intermedius	S	S	S	S	S	S	I	I	**R**	S	+	–	–	–	–	–
S. intermedius	S	S	S	S	**R**	S	I	I	**R**	S	+	–	+	–	–	–
S. intermedius	S	S	S	S	S	S	S	S	**R**	S	+	–	–	–	–	–
S. xylosus	S	**R**	S	S	S	S	S	I	**R**	S	+	–	–	–	–	–
S. xylosus	**R**	**R**	S	S	S	S	S	S	**R**	S	+	+	–	–	–	–
S. xylosus	S	S	S	S	S	S	S	S	**R**	S	–	–	–	–	–	–
S. xylosus	S	S	S	S	S	S	I	S	S	S	–	–	–	–	–	–
S. xylosus	S	S	S	S	S	S	I	S	S	S	–	–	–	–	–	–
S. xylosus	**R**	**R**	I	I	**R**	**R**	I	I	**R**	**R**	+	+	+	–	–	–
S. xylosus	S	S	S	S	S	S	I	I	S	S	–	–	–	–	–	–
S. xylosus	S	**R**	S	S	**R**	S	S	S	**R**	S	+	+	+	–	–	–
S. xylosus	**R**	**R**	S	S	S	S	I	S	S	S	+	+	–	–	–	–
S. xylosus	**R**	**R**	S	I	**R**	S	S	I	**R**	S	+	+	+	–	–	–
S. xylosus	**R**	**R**	S	S	S	S	S	I	**R**	S	+	+	–	–	–	–
S. xylosus	S	S	S	I	S	R	I	I	**R**	S	+	–	–	–	–	–
S. xylosus	S	S	S	S	S	S	S	I	S	S	–	–	–	–	–	–
S. xylosus	S	**R**	S	S	S	S	S	I	**R**	S	+	–	–	–	–	–
S. xylosus	**R**	**R**	S	S	S	S	S	S	**R**	S	+	+	–	–	–	–
S. xylosus	S	S	S	S	S	S	S	S	**R**	S	–	–	–	–	–	–
S. xylosus	S	S	S	S	S	S	I	S	S	S	–	–	–	–	–	–
S. xylosus	S	S	S	S	S	S	I	S	S	S	–	–	–	–	–	–
S. xylosus	**R**	**R**	I	I	**R**	**R**	I	I	**R**	**R**	+	+	+	–	–	–
S. xylosus	S	S	S	S	S	S	I	I	S	S	–	–	–	–	–	–
S. xylosus	S	**R**	S	S	**R**	S	S	S	**R**	S	+	+	+	–	–	–
S. xylosus	**R**	**R**	S	S	S	S	I	S	S	S	+	+	–	–	–	–
S. xylosus	**R**	**R**	S	I	**R**	S	S	I	**R**	S	+	+	+	–	–	–
S. xylosus	**R**	**R**	S	S	S	S	S	I	**R**	S	+	+	–	–	–	–
S. xylosus	S	S	S	I	S	**R**	I	I	**R**	S	+	–	–	–	–	–

TABLE 2. (*continued*)

Staphylococcus species identified by API	Antibiotics										Antibiotic-resistant genes					
	P	AM	CN	CIP	T	VA	E	DA	OX	RF	*mec*A	*bla*Z	*tet*K	*tet*L	*tet*O	*tet*M
S. xylosus	S	S	S	S	S	S	S	I	S	S	–	–	–	–	–	–
S. epidermidis	S	S	S	S	**R**	S	S	S	S	S	+	+	+	–	–	–
S. epidermidis	S	S	S	S	**R**	S	S	S	**R**	S	+	–	+	–	–	–
S. epidermidis	S	**R**	S	I	S	S	I	I	**R**	S	+	+	–	–	–	–
S. epidermidis	S	S	S	S	S	S	S	I	S	S	–	–	–	–	–	–
S. epidermidis	S	S	S	S	**R**	S	S	S	S	S	+	+	+	–	–	–
S. epidermidis	S	S	S	S	**R**	S	S	S	**R**	S	+	–	+	–	–	–
S. epidermidis	S	**R**	S	I	S	S	I	I	**R**	S	+	+	–	–	–	–
S. epidermidis	S	S	S	S	S	S	S	I	S	S	–	–	–	–	–	–
S. hominis	**R**	**R**	S	I	**R**	S	S	I	**R**	S	+	+	+	–	–	–
S. hominis	S	S	S	S	S	S	S	S	S	S	–	–	–	–	–	–
S. hominis	**R**	**R**	S	S	**R**	S	S	S	S	S	–	+	+	–	–	–
S. hominis	**R**	**R**	S	S	**R**	**R**	I	S	S	S	–	+	+	–	–	–
S. hominis	**R**	**R**	S	I	**R**	S	S	I	**R**	S	+	+	+	–	–	–
S. hominis	S	S	S	S	S	S	S	S	S	S	–	–	–	–	–	–
S. hominis	**R**	**R**	S	S	**R**	S	S	S	S	S	–	+	+	–	–	–
S. hominis	**R**	**R**	S	S	**R**	**R**	I	S	S	S	–	+	+	–	–	–
S. hyicus	S	S	S	S	S	S	S	S	S	S	–	+	–	–	–	–
S. hyicus	**R**	**R**	S	S	S	S	S	S	**R**	S	+	+	–	–	–	–
S. hyicus	S	S	S	S	S	S	S	S	S	S	–	+	–	–	–	–
S. hyicus	**R**	**R**	S	S	S	S	S	S	**R**	S	+	+	–	–	–	–
S. chromogenes	S	S	S	S	S	S	S	I	S	S	–	–	–	–	–	–
S. chromogenes	S	S	S	S	S	S	S	S	S	S	–	–	–	–	–	–
S. chromogenes	S	S	S	S	S	S	S	I	S	S	–	–	–	–	–	–
S. chromogenes	S	S	S	S	S	S	S	S	S	S	–	–	–	–	–	–
S. caprae	S	S	S	S	S	S	S	S	**R**	S	–	–	–	–	–	–
S. caprae	S	S	S	S	S	S	S	S	**R**	S	–	–	–	–	–	–
S. simulans	S	S	S	S	S	S	S	S	S	S	–	–	–	–	–	–
S. simulans	S	S	S	S	S	S	S	S	S	S	–	–	–	–	–	–
S. lentus	**R**	**R**	S	S	S	S	S	I	S	S	–	+	–	–	–	–
S. lentus	**R**	**R**	S	S	S	S	S	I	S	S	–	+	–	–	–	–
S. lugdunensis	**R**	**R**	S	S	**R**	S	I	S	**R**	S	+	+	+	–	–	–
S. lugdunensis	**R**	**R**	S	S	**R**	S	I	S	**R**	S	+	+	+	–	–	–
S. sciuri	**R**	**R**	S	S	S	S	S	S	S	S	+	+	–	–	–	–
S. sciuri	**R**	**R**	S	S	S	S	S	S	S	S	+	+	–	–	–	–
Cow																
S. intermedius	S	**R**	S	S	S	S	S	I	S	S	+	–	–	–	–	–
S. intermedius	S	**R**	S	S	S	S	S	I	S	S	+	–	–	–	–	–
S. xylosus	S	S	S	S	S	S	I	I	S	S	+	–	–	–	–	–
S. xylosus	S	S	S	S	S	S	I	I	S	S	+	–	–	–	–	–
S. epidermidis	**R**	**R**	S	S	S	S	S	S	S	S	–	+	–	–	–	–
S. epidermidis	**R**	**R**	S	S	S	S	S	S	S	S	–	+	–	–	–	–

AM, ampicillin; CIP, ciprofloxacine; CN, gentamicin; DA, clindamycin; E, erythromycin; OX, oxacillin; P, penicillin; RF, rifampicin; T, tetracyclin; VA, vancomycin; I, intermediate; R, resistant; S, susceptible.

lates in an incidence of 48.9%. The *mec*A gene was detected in *S. sciuri*, *S. lugdunensis* and *S. caprae* in an incidence of 100% but decreased in *S. intermedius*, *S. xylosus*, *S. epidermidis*, *S. hyicus* and *S. hominis* to reach 73.3, 71.4, 60.0, 50.0 and 25.0%, respectively. On the other hand, the *bla*Z gene was evident in *S. lentus*, *S. sciuri*, *S. lugdunensis* and *S. hyicus* in an incidence of 100%, but its presence decreased in *S. hominis*, *S. epidermidis*, *S. xylosus* and *S. intermedius* to reach 75.0, 60.0, 50.0 and 33.3%, respectively.

Results of Multiplex PCR for the Detection of *tet*KMOL Genes of 94 CNS Species

The results observed in Table 2 revealed that the *tet*K gene was detected in 28 CNS isolates in an incidence of 29.8%,

whereas the *tet*LMO genes were absent from the 94 CNS isolates in an incidence of 100%. At the species level, the *tet*K gene was carried by the *S. lugdunensis*, *S. hominis*, *S. epidermidis*, *S. xylosus* and *S. intermedius* in an incidence of 100.0, 75.0, 40.0, 28.6 and 26.7%, respectively.

Association of Antimicrobial-Resistant Phenotype with Resistance-Associated Genes

Analysis of the presence of the *mec*A, *bla*Z and *tet*KLMO genes in the 94 CNS isolates with various antimicrobial resistance patterns was conducted as shown in Table 2. A detailed analysis displayed associations of resistance/susceptibility phenotypes with potential resistance genes. We evaluated the *tet*K, *tet*L, *tet*M and *tet*O to determine the TET resistance genotypic distribution and found that none of the 94 tested isolates were positive for *tet*L, *tet*M or *tet*O. The intermediate resistance phenotype (18/39, 46.2%) harbored the *tet*K determinant.

DISCUSSION

Emerging antimicrobial resistance among CNS is a concern in veterinary and human medicine. Archer and Climo (1994) reported an escalation of resistance for almost all antimicrobial classes excluding glycopeptides: β-lactams aminoglycosides, trimethoprim, rifampin, fluoroquinolones, macrolides and tetracyclines. Humans and dairy cattle may share CNS strains, implying that bovine multidrug resistant staphylococci might be zoonotic pathogens (Soares *et al.* 2012).

The widespread use of antibiotics on dairy farms and other food-producing animals could lead to the selection and emergence of antibiotic-resistant bacterial strains (Oliver *et al.* 2011) to become a serious public health problem because of the possibility of dissemination of the antimicrobial-resistant bacteria to humans via food. In Egypt, very little is known about the use of antibiotics on small dairy farms as is the case in lower/middle-income countries. Redding *et al.* (2014) found that the farmers' knowledge of antibiotics was significantly associated with the use of antibiotics for preventative reasons, the purchase of antibiotics from feed stores, the experience of complications in animals after having administered antibiotics, the number of workers on the farm, the educational level of the farmer and its infrequent use, because therapeutic interventions were sought only when the animal had reached an advanced stage of clinical disease. Also, because of their inability to define an antibiotic and in contrast to Redding *et al.* (2014), many farmers do not understand that the use of antibiotics carried inherent risks to their animals and potentially to the consumers of dairy products from treated animals.

Accurate detection of oxacillin/methicillin resistance can be difficult due to the possible coexistence of two subpopulations (susceptible and resistant) within a culture termed heteroresistance (Bannerman 2003). It is a problem for clinical laboratory personnel because cells expressing resistance may grow more slowly than the susceptible population. This heterogeneity can lead to failure of treatment due to false perception of susceptibility. Despite the sensitivity of the MIC, it is a very laborious and subjective assay and this has induced Klement *et al.* (2005) to suggest the adoption of disk diffusion test by microbiology diagnostic laboratories for the provision of accurate antimicrobial susceptibility results. This was further recommended by the veterinary CLSI (2012) to adopt the agar screen and disk diffusion as standard tests for oxacillin evaluation in animal samples.

Using the disc diffusion method, Kaliwal *et al.* (2011) found that the highest numbers of CNS were susceptible to ciprofloxacin (73.52%), erythromycin (70.05%), gentamicin (42.94%) and the lowest susceptibility was shown toward ampicillin (29.41%) and penicillin (23.23%). On the contrary, the present investigation found that susceptibility to gentamicin, ciprofloxacin, penicillin and ampicillin reached higher levels (97.9, 85.1, 68.1 and 57.4%, respectively), and the lowest susceptibility was shown toward oxacillin (51.1%). Noble and Allaker (1992) indicated that tetracycline resistance was most frequently observed in isolates of various CNS species that attained up to 70.2% tetracycline resistance in our investigations. The results could be explained by the prophylactic use of these compounds on the respective farms. Not only were the differences found between bacterial species for antimicrobial susceptibility, there were also differences among the CNS species and regions (Myllyls *et al.* 1998; Pitkala *et al.* 2004; MARAN 2007; Sampimon *et al.* 2009; Buttner *et al.* 2011; Szymanska *et al.* 2011) and that 91.5% of our CNS isolates were multidrug resistant to two to four antibiotic classes of phenotype found in previous CNS isolates from bovine mastitis that showed resistance to two or more antimicrobial agents (Soares *et al.* 2012). In the present work, the highest resistance rate was observed against penicillin, ampicillin and oxacillin. The emergence of high levels of penicillin resistance followed by the development and spread of strains resistant to the semisynthetic penicillin (oxacillin), macrolides, tetracyclines and aminoglycosides has made the therapy of staphylococcal disease a global challenge (Soares *et al.* 2012). The penicillin, ampicillin, oxacillin and tetracycline resistance detected can be attributed to the large use of these antibiotics in mastitis treatment and in the water of the herd as a prophylactic measure aimed at reducing infections (Booth and McDonald 1992).

In the present work, the highest resistance rate was observed against penicillin, ampicillin and tetracycline. Penicillin and ampicillin resistance in *Staphylococcus* spp. is a worldwide phenomenon, with increasing prevalence, as a result of the selective pressure caused by the improper use of β-lactams in mastitis treatment (Bonna *et al*. 2007). The emergence of high levels of penicillin resistance followed by the development and spread of strains resistant to semisynthetic penicillins (methicillin, oxacillin and nafcillin), macrolides, tetracyclines and aminoglycosides has made the therapy of staphylococcal disease a global challenge (Soares *et al*. 2012). Resistance to β-lactam antimicrobials (including penicillin and cephalosporins), aminoglycosides (gentamicin and neomycin) and macrolides (spiramycin), which are commonly used to treat mastitis, has been increasingly reported in CNS associated with bovine mastitis (Frey *et al*. 2013).

Very few studies have investigated differences in antimicrobial resistance among CNS species (Sampimon *et al*. 2009). Identification to species level would be important if it has impact on management and treatment decisions (Soares *et al*. 2012). Myllyls *et al*. (1998) reported that much of the differences in resistance were due to the strains' capabilities of producing β-lactamase, which inhibits some β-lactam antimicrobials. Capurro *et al*. (2009) reported 18% of bovine milk samples contained CNS with resistance to antimicrobials and that four species produced β-lactamase (*S. chromogenes*, *S. epidermidis*, *S. haemolyticus* and *S. xylosus*).

Three species of our CNS isolates (*S. chromogenes*, *S. simulans* and *S. caprae*) were susceptible to antimicrobials commonly used in mastitis treatment. Four isolates of *S. intermedius* (4/30) and two isolates of each of *hominis* (2/8), *hyicus*, (2/4) *chromogenes* (2/4) and *simulans* (2/2) were totally susceptible to all of the tested antibiotics. To the best of our knowledge, this is the first study that showed the presence of classical staphylococcal-resistant genes in various species of CNS isolates from buffalo milk. Differences between Egypt and other countries in antimicrobial usage could be contributed to differences in resistance gene profiles of CNS isolates originating from the same host species in different countries (Pitkala *et al*. 2004; MARAN 2007; Sampimon *et al*. 2009; Buttner *et al*. 2011; Frey *et al*. 2013). In the case of mastitis-causing CNS, it is important to detect resistant strains because such strains can serve as a reservoir of resistance genes that can be transferred to other bacteria posing additional difficulties to the control and cure of mastitis (Irlinger 2008) and that could potentially pose a human health hazard (Soares *et al*. 2012). They are amplified through antibiotic selection that occurs when antibiotics administered to animals achieve low concentrations on the animals' body surface especially with regard to the following species, namely, *S. xylosus*, *S. caprae* and *S. sciuri* (Perreten *et al*. 1997; Irlinger 2008) contaminating milk or meat and be subsequently isolated in fermented foods made from raw materials (Irlinger 2008). Some studies have shown that staphylococci from cheese, mostly identified as *S. xylosus* strains, harbored resistance to chloramphenicol, tetracycline, erythromycin and, in lower proportions, to gentamicin, penicillin, lincomycin and kanamycin (Perreten *et al*. 1997; Irlinger 2008). The tetracycline-resistant genes, localized in most *S. xylosus* strains, were identified as tetracycline efflux resistance gene (*tet*K) (Perreten *et al*. 1997; Irlinger 2008). CNS, and particularly *S. xylosus*, *S. sciuri* and *S. caprae*, could represent a natural reservoir of antibiotic-resistant genes (Irlinger 2008).

The CNS harbor the antimicrobial-resistant element, SCC*mec*, that encodes alternative PBP2a and confers resistance to all β-lactam antimicrobials and other antibacterial drugs. This element exists only in *Staphylococcus* strains (Enright *et al*. 2002) and can easily be transferred to all methicillin-resistant CNS strains (Koksal *et al*. 2009). In this study, *mec*A was detected from only one out of 15 isolates showing the oxacillin-resistant phenotype. Among CNS species found less frequently in samples from mastitis is *S. sciuri*, a species that has gained recent attention as it carries a *mec*A homologue from which the *mec*A gene of methicillin-resistant staphylococci may have developed (Fuda *et al*. 2007). Interestingly, two *mec*A-positive oxacillin susceptible isolates were recovered from the buffalo milk. In a French study (Martin and Maris 1995), their *S. xylosus* isolates recorded elevated MIC values for erythromycin while our isolates did not indicate any resistance. Also, considerable numbers of *S. epidermidis* isolates exhibiting resistance to tetracycline, ampicillin and even oxacillin were reported by Martin and Maris (1995). This was recorded in our results in the case of *S. lentus*, *S. intermedius*, *S. lugdunensis*, *S. sciuri*, *S. hominis* and *S. hyicus* while our *S. epidermidis* isolate did not exhibit any resistance to gentamicin. Antimicrobial resistance of *S. epidermidis* may be important to those who consume raw milk because this organism is isolated frequently from bulk tank milk. In a U.S. study (Todhunter *et al*. 1993), *S. chromogenes* showed resistance rates of approximately 30% for penicillin/ampicillin. In contrast, our *S. chromogenes* and *S. simulans* isolates did not show any resistance against the examined antimicrobial agents.

The antimicrobial resistance of importance found in bovine-associated CNS in this investigation was for oxacillin and tetracycline. The former is mediated by the *mec*A gene, whereas the latter is conferred by *tet* genes. In recent years, increasing numbers of reports have shown that the *mec*A gene is present in CNS strains, including hospital-acquired infections, community-associated methicillin-resistant *S. aureus* infections (Hisata *et al*. 2011), animal epidermis

(Schnellmann *et al*. 2006), beaches (Soge *et al*. 2009) and public transportation systems (Zhou and Wang 2013). According to recommendations of the CLSI, oxacillin-resistant *Staphylococcus* isolates shall be reported as resistant to other β-lactam antibiotics (Aarestrup and Schwarz 2006).

In the present investigation, resistance to oxacillin could be attributed to the *mec*A gene in 44.7% of the oxacillin-resistant isolates. The *mec*A gene was detected in *S. sciuri*, *S. lugdunensis* and *S. caprae* with an incidence of 100% and a lower incidence in the case of *S. intermedius*, *S. xylosus*, *S. epidermidis*, *S. hyicus* and *S. hominis* (73.3, 71.4, 60.0, 50.0 and 25.0%, respectively). In addition, resistance to tetracycline was attributed to the presence of the *tet*K gene in 24.5% of the tetracycline-resistant isolates. In this study, the tetracycline-resistant gene (*tet*K) was detected with an incidence of 100% in *S. lugdunensis* but decreased in the case of *S. hominis*, *S. epidermidis*, *S. xylosus* and *S. intermedius* with an incidence of 75.0, 40.0, 28.6 and 26.7%, respectively. On the other hand, while penicillin resistance was attributed to the presence of *bla*Z gene in 31.9% of the penicillin-resistant isolates. Positive amplification of the *bla*Z gene was recorded in 100% of the *S. lentus*, *S. sciuri*, *S. lugdunensis* and *S. hyicus* isolates. This incidence decreased in the *S. hominis*, *S. epidermidis*, *S. xylosus* and *S. intermedius* (75.0, 60.0, 50.0 and 33.3%, respectively). All of the *S. sciuri* and *S. lugdunensis* isolates were *bla*Z and *mec*A+ and 60% of *S. epidermidis* were also *bla*Z and *mec*A+. Soares *et al*. (2012) found the *mec*A gene in 4% of the CNS isolates, all represented by the *S. xylosus* species. The *bla*Z gene was detected in 16% of the isolates and all *bla*Z isolates were *mec*A+ (Soares *et al*. 2012).

In the oxacillin-resistant, tetracycline-resistant and/or penicillin-resistant isolates, the *mec*A, *tet* and *bla*Z were present, either alone or in different combinations. *S. epidermidis* was the predominant CNS species among those that contained multiple antimicrobial-resistant genes. Multiple resistant genes were also found in *S. sciuri* and *S. chromogenes*, and these genes were frequently associated with the presence of the *mec*A gene. In total, 29.8% (28/94) of the isolates studied were resistant to more than two antimicrobials and some strains were virtually resistant to all antimicrobials authorized for the treatment of mastitis.

CONCLUSIONS

Because of the possibility of dissemination of the antimicrobial-resistant bacteria to humans via the food processing chains, screening the antimicrobial resistance of bacteria in the dairy industry should be performed further. The resistance genes might in some instances transfer from staphylococci of animal origin to staphylococci that cause infections in humans, thereby compromising antimicrobial treatment (Irlinger 2008). CNS colonizing the udder of buffaloes and cows may represent a reservoir of different antibiotic-resistant genes and SCC*mec* elements. Could this genetic background be a reservoir for interspecies gene transfer among CNS and *S. aureus* in the udder as it was previously suggested in the intestinal tract (Vitali *et al*. 2014)?

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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