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Research Article

Antibiotic Resistance of Staphylococci Concerning Strains Included in Food Industry in Egypt

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ABSTRACT

Antibiotic resistance increased in humans and in veterinary medicine which may occur from direct way like improper treatment with antibiotic or addition of sub therapeutic doses of antibiotic in ration but there is also an in direct way which is food product as it may contaminated with multiple antibiotic resistance bacteria or bacteria carried the antibiotic resistance genes even if not expressed as we detect in our study so the objective of this study is to isolate and identify *staphylococci* from raw cow milk, pasteurized milk, pasteurized yogurt, beef burger and minced beef meat and to examine the isolated staphylococci phenotypically against seven 7 anti-microbial agents considered the most important agents: ciprofloxacin (5 µg), erythromycin(15 µg), tetracycline (30 µg), methicillin (5 µg), rifampicin (5 µg), penicillin (10 IU) and vancomycin (30 µg). We investigated the presence of antibiotic resistance genes by PCR; the β-lactamase gene *blaZ*(PEN resistance),*mecA* (methicillin resistance) and the *tetK* (TET resistance) that confer resistance to tetracycline. In the present study we record *S. aureus* and coagulase negative staphylococci (CNS) with multiple antibiotic resistance attributes. Also we found isolates of *S. aureus* and CNS carried *blaZ* gene and *mecA* gene but not expressed when they examined by disc diffusion test which let us highlight on these isolates as it could be a source of transmission of resistant genes to human and other bacteria. We also for the first time record *S. aureus* with 100% resistance to tetracycline recovered from food product phenotypically and genotypically while resistance to tetracycline was 33.3% in *S. epidermidis.*

Keywords: *Staphylococcus aureus*, CNS, methicillin resistance, PCR, antibiotic resistant genes, *blaZ*, milk, *mecA, tetK*.

INTRODUCTION

Anti-microbial agent are broadly spread and seriously utilized as a part of the food industry[1]. With numerous old antibiotics no more powerful and no new ones going onto the market to supplant them, the therapeutic group is presently shortening abuse of anti-microbial to keep the last few lifesaving drugs viable for no less than a couple of more years.

The antibiotic time had quite recently started when specialists coincidentally found that the expansion to sustain of low, or subtherapeutic, measurements of antibiotic made domesticated animals become speedier, conceivably by stifling microorganisms in the gut. Albeit, antibiotic use in food animals has been covered from general society scene yet, antibiotic application in meat delivering animals have affected on general wellbeing and has ruled out disarray in the wake of being experimentally archived[2].

Presently, the Staphylococcus variety comprises of 45 approved species and 24 subspecies bringing about more than 50 perceived efficient substances, of which the lion's share is coagulase negative[3]. They can be classified into two gatherings as indicated by creation of coagulase enzyme, which is fit for coagulating blood plasma. The combination of this protein is confined to a 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 don't blend coagulase and make up the gathering known as coagulase-negative staphylococci (CNS).

Staphylococcus aureus is a danger in view of its impeding consequences for animal health and its ability for transmission from animal to people and the other way around[4]. The segregation comprehensiveness of multidrug-resistant staphylococci from meat tests has been seen by a few specialists[5,6,7,8]. This ought to be considered important, particularly when the finding of Waters[9] records that, near 50 percent of meat samples examined from U.S. supermarkets are debased with the microorganisms *S. aureus* and resistant to no less than three classes of anti-microbial agents. Methicillin-resistant strains of *S. aureus* (MRSA), which is connected to an extensive variety of human ailments[10,11,12,13], is famous for its relatednous to food contamination and its probability for its transmission to people.

Methicillin-resistant *Staphylococcus aureus* (MRSA) have been found in different animal species all through the world. In Egypt, there was a study on hamburger meat observed that Twenty seven disconnects involving five species (*S.hyicus*, *S.aureus*, *S.schleiferisubsp.coagulans*, *S.intermedius*, and *S.lentus*) were portrayed for their anti-microbial resistance phenotypic profile and anti-microbial resistance genes[14].

CNS are commensal bacterial species and opportunistic pathogens that can bring about diseases in people (the vast

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majority of the doctor's facility obtained contaminations, bacteremia identified with indwelling gadgets, central nervous system shunt contaminations, local or prosthetic valve endocarditis, urinary tract diseases and endophthalmitis)[15] and animals[16]. Additionally, its capacity of biofilm arrangement appears to assume a crucial part in the destructiveness of coagulase-negative staphylococci[17,18].

CNS in the surrounding and in the food investigated shows a danger to customer wellbeing[19]. There is worry that the anti-microbial-resistant components of CNS in food animals can be scattered to people by means of food production chain[20]. CNS are viewed as a store of different anti- microbial-resistant related determinants[21]. The investigation of 94 CNS strains from milk affirmed the entrenched multiresistant character of staphylococci in the dairy setting. Resistance to oxacillin was ascribed to the *mecA* quality in 44.7% of the oxacillin-resistant strains. The *mecA* quality was recognized in *Staphylococcus intermedius*, *epidermidis*, *hominis*, *hyicus*, *caprae*, *sciuri*, *lugdunensis* and *xylosus* while thoroughly truant in *chromogenes*, *simulans* and *lentus*[22].

As a result of the likelihood of scattering of the anti-microbial-resistant microorganisms to people through the food processing chains[23], screening the anti- microbial resistance of microscopic organisms in the food industry ought to be performed further. The resistance genes may in a few examples exchange from staphylococci of animal inception to staphylococci that cause disease in people, in this manner bargaining anti-microbial treatment[20].

MATERIALS AND METHODS

Sample Collection

During the year 2016, 150 samples were collected 30 samples from each food product as fresh normal cow milk, pasteurized milk, pasteurized yogurt, beef burger and minced beef meat collected from several sources in the Great Cairo zone. Each sample was aseptically collected in ice boxes and immediately conveyed to the laboratory to be microbiologically examined.

Isolation and Identification

Under complete aseptic condition 1ml of milk, 1 gm of yogurt and 1 gm of beef burger and minced beef meat were taken to be added to 10 ml peptone water and vigorously shaken for 2min and then incubated for 8 to 12 h at 37 °C. Ten microliters of each sample was inoculated on mannitol salt agar plates were incubated at 37 °C for 18 plus 24 h. All isolates were identified as staphylococci based on colony morphology, Gram staining, catalase reaction, oxidase test and oxidative–fermentative testing. After confirmation of the genus Staphylococcus, the enzyme coagulase was characterized among all isolates in tubes using both the slide and tube methods. Coagulase-negative isolates resistant to oxacillin and/or cefoxitin were subjected to identification to the species level using the API Staph commercial identification system (API Staph ID32 test; bioMérieux, Marcy l'Etoile, France)[24,25]. Strains identified were subcultured 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 Anti-microbial-Resistant Tests

Anti-microbials were selected for testing based on the WHO's critically important anti-microbials list (2012) were selected for testing according to their importance to human and animal health[26,27]. Susceptibility of the isolates was determined against 7 anti-microbial agents considered as important agents included in this study were ciprofloxacin (5 µg), erythromycin (15 µg), tetracycline (30 µg), methicillin (5 µg), rifampicin (5 µg), penicillin (10 IU) and vancomycin (30 µg). One loopful of colony material was subcultured in 5 ml Mueller-Hinton broth for an overnight at 37°C, vortexed well and adjusted to obtain a turbidity comparable to that of 0.5 McFarland opacity standard (bioMe´rieux, Marcy 1'Etoile, France). The bacterial suspensions were streaked on MHA plates with a cotton swab and with an antibiotic disc dispenser, the discs were placed on the agar surface, plates were incubated at 37°C, the standard temperature used for anti-microbial susceptibility testing, for 24 h, and the diameter of the inhibition zones was measured. The isolates were classified as sensitive, intermediate, and resistant based on the diameter of the clearing zone according to CLSI[28] guidelines. 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 extraction

Bacterial DNA from the 10 staphylococci were isolated from an overnight cultures using the boiling protocol[29]. DNA precipitates were resuspended in an appropriate volume of Tris-EDTA buffer solution. DNA was stored at -20°c.

Identification of the blaZ, mecA and the tet K genes

The isolated staphylococci were investigated to detect the presence of genes associated with the screened antibiotic resistances, namely, the β-lactamase gene *blaZ* (PEN resistance) was detected by primers designed by Vesterholm-Nielsen[30]; *mecA* (methicillin resistance)[31]; and the *tet K* (TET resistance) that confer resistance to tetracycline, *tet K* gene was detected by single PCR and two other different genes that were used in duplex and the target pairs were *blaZ* gene (a determinant of β-lactamase production) and *mecA* gene (a determinant of methicillin resistance) (Table .1)

Reactions for Amplification of blaZ and mecA 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 tet K Resistance Gene.

PCR conditions included a 5 min initial denaturation at 94C followed by 35 cycles of 94C for 30 sec, 54C for 30 sec and 72C for 30 sec and a final extension for 10 min at 72C.

All primers included in this study were supplied by Sigma Genosys (Sigma). The reaction with these primers were

carried out using a total volume of 25 µl reaction mixtures contained 5 µl of DNA as template, 20 pmol of each primer and 1 X of PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science). The amplification cycles were carried out in a PT- 100 Thermocycler (MJ Research, USA). The PCR products were stored in the thermal cycler at 4°C until they were used. PCR was performed, with a quality control for the test using a positive control DNA of standard *Staphylococcus aureus* (ATCC 33591) and *Escherichia coli* DNA (NCIMB 50034) which served as negative control. 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

Isolation and biochemical identification

The colonies were described for their morphological characters and appearance. Film prepared from pure culture of organisms were stained with Gram's stain to detect Gram positive cocci occurring singly, in pairs, in short chain or in irregular clusters like bunch of grapes when examined under light microscope.

The results of traditional biochemical tests indicated that all suspected isolates are Staphylococci spp. A total of 10 staphylococci strains were isolated with percentage 6.66% (10/150).

Investigation of Coagulase Production (coagulase test)

From total 10 staphylococcal isolates, 7 isolates were coagulase positive *staphylococci* in a percentage of 70% and 3 isolated were coagulase negative *staphylococci*) in a percentage of 30%.

Identification of Staphylococci isolates

The identified10 staphylococcal isolates by using the API Staph commercial identification system (API Staph ID32 test; bioMérieux, Marcy l'Etoile, France) were belonging to four different staphylococcal species. These included *S. aureus* (n = 7, 70%), *S. epidermidis*(*n* = 1, 10 %), *S. xylosus*(*n* = 1,10 %),and *S. sciuri*(*n* = 1, 10 %).We found that 3 isolates of *S. aureus* were isolated from raw cow milk 30%, 3 isolates of *S. aureus* were isolated from minced beef meat 30%,1 isolate of *S. aureus* were isolated from beef burger 10% and 30% of isolates were isolated from fresh raw milk as CNS (*S. epidermidis, S. xylosus* , *S. sciuri*)as 10% of each.

Results of antibiotic resistance among the isolated staphylococci

Distribution of resistance to individual anti-microbial agents

The *in vitro* sensitivity of 10 Staphylococcus isolates (7*S.aureus* and 3 CNS) recovered from different food products were examined against 7 anti-microbial drugs.

The 7 staphylococcus aureus isolates were resistant to penicillin and methicillin in an incidence of 57.14% (4/7)and the resistance to tetracycline was in an incidence of 100% while the 7 staphylococcus aureus isolates were sensitive to vancomycin , rifampicin, ciprofloxacin and erythromycin in an incidence of 100% (7/7).while the CNS were resistant to penicillin , methicillin and tetracycline in an incidence of 33.33% (1/3) as recorded in table (2) the resistance to penicillin was found in *S. sciuri* and the resistance to methicillin and tetracycline were found in *S. epidermidis.*

Genotypic detection of antibiotic resistance genes

From screening of antibiotic resistance genes we found the duplex PCR that target pairs were *blaZ* gene (a determinant of β-lactamase production) and *mecA* gene (a determinant of methicillin resistance) in all *staphylococcus aureus* were resistant to *blaZ* gene in an incidence of 100% (7/7) while the *mecA* gene resistance was in an incidence of 85.7%(6/7). The PCR for detection of the *tet K* (TET resistance) that confer resistance to tetracycline was in an incidence of 100% (7/7) in *staphylococcus aureus.*

The resistance to *blaZ* gene and *mecA* gene in CNS were in an incidence of 33.3% (1/3) and 66.6% (2/3) respectively. While the resistance to *tet K* was in an incidence of 33.3% (1/3).

The resistance to *blaZ* gene and mecA gene was found in *S. sciuri* in an incidence of 33.3% (1/3) for each gene. While the resistance to *tet K* gene and *mecA* gene was found in *S. epidermidis* in an incidence of 33.3% (1/3) for each gene on the other hand *S. xylosus* was sensitive to *blaZ, mecA* and *tet K* genes.

DISCUSSION

This study aims to isolate and recognize *S. aureus* and CNS from raw milk, pasteurized milk, pasteurized yogurt, beef burger and minced beef meat. Foodborne pathogens, including *S. aureus*, have been secluded from milk and dairy items overall[33,34,35,36,37,38].

People are at danger of being contaminated with food borne microscopic organisms by means of the food chain[39,40] from repository animals[41,42,43,44]. A past exploration[9] has recommended that, when purchasing meat at any butcher shop, general store or nearby basic supply shop, is a similarly likely risk of it being tainted with drug-resistant bacteria. The WHO has uncovered a report on the worldwide genuine risk of anti-microbial resistant strains of bacterial pathogens happening right now in each region of the world without any impediments and to influence everyone, at any age and in any nation[45]. In animals, examination of resistance rates to anti-microbial can topographically fluctuate among universally and broadly as indicated by the anti- microbial utilization administration[46,47]. Improper food handling and unhygienic practices among food handlers amid generation, preparation and dispersion, have added to food harming episodes[48]. The investigation of Sasidharan[37] was directed to isolate *S. aureus* and decided the predominance of anti-microbial resistance among the confines from the nearby dairy products *S. aureus* shows some tricky elements which are not found in other applicable microbes. This bacterium is equipped for communicating virulence components and in this way is considered therapeutically pertinent when experienced in dairy products. *S. aureus* keeps on showing the capacity to create and extend resistance to an expansive exhibit of anti- microbial classes[49]. It is a noticeable pathogen in both the healing

Table 1: PCR-specific oligonucleotide primers, amplicon size and conditions for genes specific to three antibiotic-resistant strains.

Gene	Oligonucleotide sequences **('5-'3)**	Annealing	PCR product size **(**bp**)**	Reference
*bla*Z	F-AAG AGA TTT GCC TAT GCT TC R-GCT TGA CCA CTT TTA TCA GC	55°C	517	30
*mec*A	F-GTG AAG ATA TAC CAA GTG ATT R-ATG CGC TAT AGA TTG AAA GGA T	55°C	147	31
tet(K)	F-GTAGCGACAATAGGTAATAGT R-GTAGTGACAATAAACCTCCTA	54°C	360	32

bp, base pair; PCR, polymerase chain reaction.

Table 2: Distribution of antibiotic-resistant gene combinations in *staphylococcus aureus* and 3 different CN.

Staphlococcal species identified by API	Source	Antibiotic							Antibiotic–resistant genes		
		P	E	CIP	T	VA	M	RF	*bla*Z	*mec* A	*tet* K
S. aureus	Fresh milk	R	S	S	R	S	R	S	+	+	+
S. aureus	Fresh milk	S	S	S	R	S	R	S	+	+	+
S. aureus	Fresh milk	R	S	S	R	S	S	S	+	+	+
S. aureus	Beef burger	S	S	S	R	S	S	S	+	-	+
S. aureus	Minced beef meat	S	S	S	R	S	R	S	+	+	+
S. aureus	Minced beef meat	R	S	S	R	S	I	S	+	+	+
S. aureus	Minced beef meat	R	S	S	R	S	R	S	+	+	+
S. epidermidis	Fresh milk	S	S	S	R	S	R	S	-	+	+
S. xylosus	Fresh milk	S	I	S	S	S	S	S	-	-	-
S. sciuri	Fresh milk	R	S	S	S	S	S	S	+	+	-

center and the group settings. Sasidharan[37] examined 5 dairy items, it demonstrated that contamination happens in fresh cow milk and sanitized milk. Milking operations, including capacity, taking care of and transportation, are considered as basic focuses that sully milk items.

Susceptible populations of microorganisms may get to be resistant to anti- microbial agents through mutation and determination or by the obtaining of new genetic material from other resistant living beings through change, transduction and conjugation. One disconnect of *S. aureus* was resistant to numerous classes of anti-microbial agents (methicillin and vancomycin), which can bring about genuine wellbeing problems[50]. The way that resistance is high in ecological disconnects is chiefly in light of the fact that anti-microbials are oftentimes endorsed by veterinarians as treatment for gram-negative bacterial contaminations on farms[51]. Subsequently, the unpredictable utilization of those anti- microbial agents may account, in any event to some extent, for such a high resistance[51]. In our study, we examined 30 samples from each food product as fresh normal cow milk, pasteurized milk, pasteurized yogurt, beef burger and minced beef meat we found that 10 staphylococci strains were isolated with percentage 6.66% (10/150) and differentiated as follow 3 isolates of *S. aureus* were isolated from raw cow milk 30%, 3 isolates of *S. aureus* were isolated from minced beef meat 30% ,one isolate of *S. aureus* were isolated from beef burger 10% and 30% of isolates were isolated from fresh raw milk as CNS (*S. epidermidis*, *S. xylosus* , *S. sciuri*) as 10% of each.

Sasidharan[37] found that from 50 samples inspected, 5 (10%) were tainted with *S. aureus*. Along these lines, the 5 strains were subjected to anti- microbial resistance design utilizing five antibiotic discs (methicillin, vancomycin, kanamycin, chloramphenicol and tetracycline). One strain indicated resistance to methicillin and vancomycin.

Osman[14] gathered examples amid 2013 from hamburger meat at retail. Twenty seven strains containing S. aureus and non S. aureus were described for their anti-microbial resistance phenotypic profile and anti-microbial resistance genes (*mecA*, *cfr*, *gyrA*, *gyrB* and *grlA*). Out of the 27 Staphylococcus strains one and only separate was resistant to the 12 anti-microbials speaking to nine classes. Crude hamburger meat sold over the Great Cairo zone, contains 66.7% of methicillin resistant staphylococci, with most noteworthy commonness was accounted for in S. aureus (66.7%), while the methicillin resistant non-*S. aureus* strains constituted 66.7% from which *S. hyicus* (60%), *S. intermedius* (33.3%), *S. schleiferisubsp. coagulans* (100%) and *S. lentus* (100%) were methicillin resistant staphylococci and 11/27 (40.7%) conveyed the *mecA* gene. In this study the *in vitro* sensitivity of 10 Staphylococcus isolates (7*S.aureus* and 3 CNS) were examined against 7 anti-microbial drugs. The 7 staphylococcus aureus isolates were resistant to penicillin and methicillin in an incidence of 57.14% (4/7) and they carried *blaZ* gene in an incidence of 100% (7/7) while the *mecA* gene in an incidence of 85.7%(6/7) while *staphylococcus aureus* were resistant to tetracycline in an incidence of 100% (7/7) and carried *tet K* gene in an incidence of 100% (7/7) while the 7 staphylococcus aureus isolates were sensitive to vancomycin , rifampicin, ciprofloxacin and erythromycin in an incidence of100% (7/7). In CNS The resistance to penicillin, methicillin and tetracycline were in incidence of

33.3%(1/3) while *S. sciuri* was resist to penicillin and carried the *mecA* gene.

Huber[5] mention that in minced meat samples, MR-CNS were detected in 32.4% of minced meat. MR-CNS from these sample were *S. leurettii* accounting for 76.2% of all analyzed strains, followed by *S. sciuri* comprising 15.5% of strains.

Akindolire[52] recorded that *S. aureus* isolated from milk got from various inspecting destinations tried to assess their susceptibilities against a board of 11 anti-microbial agents. An extensive extent (60%–100%) of the *S. aureus* strains were resistant to penicillin G, ampicillin, oxacillin, streptomycin, vancomycin, and erythromycin. Also, a comparably substantial extent of strains from different destinations were resistant to penicillin G and oxacillin. A reason for concern is the way that numerous antibiotic resistant strains that are likewise resistant to oxacillin were identified in his study. These strains could serve as supplies for the transmission and spread of antibiotic resistant determinants inside a populace.

CONCLUSIONS

In the present study, *S. aureus* and CNS were successfully isolated from milk samples, minced beef meat and beef burger and a large proportion of *S. aureus* isolates exhibited resistance towards the most important antibiotics tested (penicillin, methicillin, tetracycline). Thus, it is evident that most of these isolates possessed multiple antibiotic resistance attributes. Though the development of antibiotic resistant determinants in *S. aureus* is associated with the uncontrolled usage of antibiotics in human and veterinary medicine, the incidence of drug-resistant *S. aureus* in raw milk samples warrants closer monitoring. Resistance to tetracycline was recorded in high percentage also CNS were multiple antibiotic resistance, Also we found isolates of *S. aureus* and CNS carried *blaZ* gene and *mecA* gene but not expressed when they examined by disc diffusion test which let us highlight on these isolates as it could be a source of transmission of resistant genes.

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