Prevalence, Detection Of Marker And Virulence Genes Of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolated Form Milk And Dairy Products And Their Antimicrobial Susceptibility

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ABSTRACT

The present work was undertaken to study the prevalence, genetic profile and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) in raw milk and dairy products in Mansoura City, Egypt. MRSA was detected in 53% (106/200) among all milk and dairy product samples with prevalence rates of 75% (30/40), 65% (26/40), 40% (16/40), 50% (20/40), and 35% (14/40) in raw milk, Damietta cheese, Kareish cheese, ice cream, and yoghurt samples, respectively. The mean *S. aureus* counts were 3.49, 3.71, 2.93, 3.40 and 3.23 $\log_{10}$ cfu/g in the tested raw milk, Damietta cheese, Kareish cheese, ice cream and yoghurt, respectively, with an overall count of 3.41 $\log_{10}$ cfu/g. Unexpectedly, all of the *S. aureus* isolates (n=414) detected were genetically verified as MRSA strains. PCR analyses verified the existence of *nuc*, *coa*, and *mecA* genes in all of the 414 isolates. All of the 414 MRSA isolates were also positive for *hla* gene. The antimicrobial susceptibility pattern for the 414 MRSA strains against 13 tested antimicrobials indicated that the least effective drugs were penicillin G, cloxacillin, tetracycline, and amoxicillin with bacterial resistance percentages of 87.9%, 75.9%, 65.2% and 55.6% respectively, while the most effective antimicrobials against MRSA isolates were vancomycin, sulphmethazole/ trimethoprim, ciprofloxacin, netilmicin, and gentamicin, which revealed bacterial sensitivity percentages of 76.3%, 75.45 70.1%, 69.1%, and 63.3%, respectively. Of the 414 MRSA strains tested, 348 (84.1%) were multidrug resistance. This study presents the first genetic characterization of MRSA isolated from milk and dairy products in Egypt.

INTRODUCTION

Staphylococcus aureus has become one of the most important pathogens in microbiological safety and quality of food *(1)*. *S. aureus* is ubiquitous in nature. It is highly vulnerable to destruction by heat treatment and nearly all sanitizing agents. Thus, the presence of this bacterium or its enterotoxins in processed foods or in food processing equipment is generally an indication of poor sanitation *(2)*. *S. aureus* counts should reach 6 $\log_{10}$ cfu/g of food to produce sufficient amounts of enterotoxins to cause illness *(3)*.

Foods of animal origin, specially milk and dairy products, are associated with foodborne diseases *(4, 5)*. Milk is a good substrate for *S. aureus* growth, and the dairy products are a well-known source of intoxication. The enterotoxigenic *S. aureus* have been reported to cause contamination of raw milk *(6, 7)*, cheeses *(8, 9)*; ice cream *(10)*; and yoghurt *(11)*.

S. aureus may be contaminating milk and dairy products from the udder of dairy animal, from human beings, and from inadequately cleaned equipment. It is also a major causative pathogen for clinical or subclinical mastitis of dairy domestic ruminants *(12)*.

The enumeration of Staphylococci was routinely conducted on dairy products as a good indicator of quality of sanitation during its

production and distribution and to establish the occurrence of post-processing contamination *(13)*.

Enterotoxigenic *S. aureus* is the major bacterial pathogen that develops multidrug resistance to antibiotics *(14)*. Methicillin-resistant *S. aureus* (MRSA) is often present in various kinds of food and causes foodborne intoxication *(15)*. *Kluytmans et al. (16)* reported the first foodborne outbreak of MRSA that caused the death of 5 out of 21 patients. The *mecA* gene considered as a useful molecular marker of putative methicillin resistance in coagulase-negative staphylococci (CoNS) and *S. aureus (17)*.

Kareish cheese (acid curd skim milk cheese) is a kind of homemade soft cheese manufactured in Egyptian villages and can be considered the main protein supplement consumed by most Egyptian farmers, while Damietta cheese is a famous soft, white pickled cheese industrially processed and distributed in Egypt under different market names. Unfortunately, there is uncontrolled use of antimicrobial agents for treatment in Egypt which make the screening for antibacterial agent of *S. aureus* organisms is very important. The present study was planned to throw light on the Prevalence, genetic profile and antimicrobial susceptibility of MRSA in raw milk, Damietta cheese, Kareish cheese, ice cream, and yoghurt marketed in Mansoura City, Egypt.

MATERIAL AND METHODS

Milk and dairy products samples

A total of 200 samples (40 each of raw milk, Damietta cheese, Kareish cheese, ice cream and yoghurt) were collected from retail outlets, different shops and supermarkets in Mansoura City, Egypt. All samples were transported in a refrigerated box (4 to 8 °C) to Food Hygiene and Control Department Laboratory, Mansoura University, Egypt, where the conventional bacteriological analysis was done immediately.

Enumeration and Isolation of *S. aureus*

The count of *S. aureus* was determined according to *(13)* by using surface plate technique onto Baird Parker agar (BP) with Egg Yolk Tellurite Emulsion (Baird Parker, Oxoid, CM0257; Egg Yolk Tellurite Emulsion, Oxoid, SR0054) followed by incubation at 37 °C for 48 h. Suspected colonies (1-12) were selected and picked up onto a slope agar, purified and identified biochemically according to Bennett and Lancette *(2)*.

Molecular characterization of MRSA

Molecular identification and characterization of the isolated S. aureus strains was carried out in Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Hokkaido, Japan. Genomic DNA of *S. aureus* isolates was prepared according to the method described by *Sallam et al. (18)* with the use of Maxwell 16 cell DNA purification kit (Promega Corporation, Madison, WI, USA). Genomic DNA from E. coli K12DH5α strain was similarly prepared and used as a negative control template for PCR analyses.

PCR was conducted for the presence of the marker genes *nuc*, *coa*, and *mec*A, as well as *hla* virulence gene. Primer sets used for PCR amplification of the target genes are listed in Table (1). The oligonucleotide primers were synthesized by Hokkaido System Science Co. Ltd. (Hokkaido, Sapporo, Japan).

GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) was used for detection of the various target genes. PCR was carried out in a 20-µl reaction mixture containing 1.6 µl *S. aureus* genomic DNA template, 1 µl (6 pmol) for each of forward and reverse primers, 4 µl dNTPs (2 mM), 0.4 µl KOD FX Neo Polymerase enzyme (1.0U/µl), 10 µl of 2×PCR Buffer for KOD FX Neo (Toyobo Co., Ltd., Japan) and 2 µl PCR

grade water. After an initial denaturation at 94 °C for 2 min, 35 cycles PCR amplification cycles consisting of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, and extension at 68 °C for 1 min/kbp, were performed followed by a final extension at 68 °C for 7 min. Amplified genes of each PCR reaction mixture were separated by subjecting 3 µl aliquots to agarose (1.2%) gel electrophoresis for 30 min at 100 V followed by a 20-min staining in ethidium bromide solution. The separated PCR products were then visualized under UV light and photographed.

Table 1.Primer sets for PCR amplification of molecular identification of *Staphylococcus aureus*

Gene	Oligonucleotide primer sequences	Amplified DNA size	References
nuc	F: 5´-GTGCTGGCATATGTATGGCAATTG-3´ R: 5´-CTGAATCAGCGTTGTCTTCGCTCCAA-3´	660 bp	*(18)*
coa	F: 5´-TAGGCGCATTAGCAGTTGCATC-3´ R: 5´-CCAGCCGTAGTTTTAACCTCTTG-3´	1000 bp	*(18)*
mecA	F:5´-GATTGGGATCATAGCGTCA-3´ R:5´-CAGTATTTCACCTTGTCCG-3´	1200 bp	*(18)*
hla	F: 5´-CCGGTACTACAGATATTGGAAGC-3´ R: 5´-GGTAATCATCACGAACTCGTTCG-3´	744 bp	*(18)*

For confirmation of the amplified genes, DNA sequencing was completed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems) following the manufacturer's instructions on an ABI Prism 3100 automated sequencer (Applied Biosystems). Primers used for PCR for sequencing were the same used for DNA amplification. Nucleotide sequence data were then analyzed by the GENETYXMAC software, version 12 (GENETYX Corp., Tokyo, Japan).

Antimicrobial susceptibility testing

The Kirby-Bauer test for antimicrobial susceptibility, which is called the disc diffusion test, was applied according to Jorgensen and Turnidge *(5)*. *S. aureus* strains were swabbed onto Muller-Hinton medium (Oxoid) and the antimicrobial discs (Oxoid) were placed on the top of the medium. The plates were then incubated at 35 C for 24 h. The presence or absence of an inhibitory area around the disc identifies the bacterial sensitivity to the drug. The zone sizes were looked up on a standardized chart to give a result of sensitive, resistant or intermediate.

RESULTS AND DISCUSSION

Prevalence of MRSA in milk and dairy products

In the present study, *S. aureus* (MRSA) was detected in 53% (106/200) among all milk and dairy product samples tested with prevalence rates of 75% (30/40), 65% (26/40), 40% (16/40), 50% (20/40), and 35% (14/40) in raw milk, Damietta cheese, Kareish cheese, ice cream, and yoghurt samples, respectively (Fig. 1). Unexpectedly, all of the *S. aureus* isolates detected were genetically verified as MRSA strains.

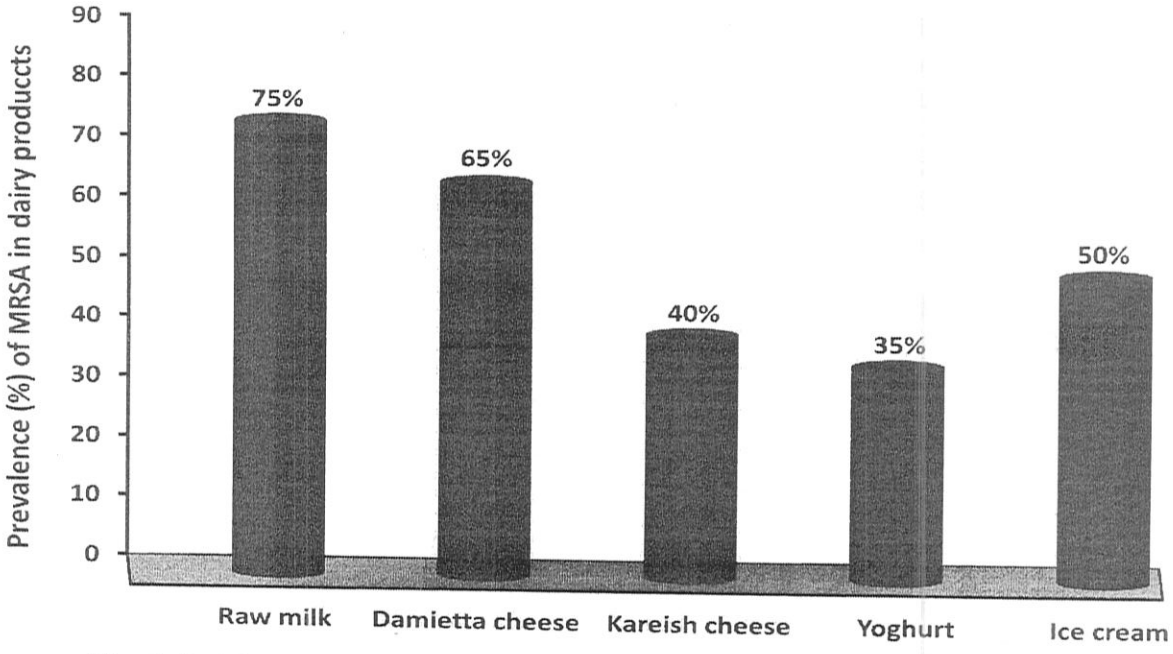

Fig. 1. Incidence of contamination of milk and dairy product samples with MRSA

Several studies were conducted in different countries to screen milk and dairy products for the presence of MRSA. A study in USA has identified MRSA in 21.8% (29/133) of bovine milk samples *(19)*. Very low contamination rates for MRSA in milk and dairy products were recorded, however in different countries. ***Gücükoğlu et al. (10)*** revealed that 2.8% (1/35) of ice cream samples tested in Turkey were positive for MRSA, while *Normanno et al. (20)* in Italy indicated that 3.75% (6/160) of dairy products were positive for MRSA. In Korea, *Lee (21)* revealed the occurrence of MRSA in 1.34% (12/894) of bovine milk samples. Another study in Iran revealed that 4% (2/50) of both pasteurized milk and traditional soft cheese samples were positive for MRSA while it could not be detected in raw milk *(22)*. In Turkey, 7.5% (3/40) of tested isolated *S. aureus* from Urfa cheese samples were MRSA *(23)*. MRSA was not detected among any of the *S. aureus* positive samples associated with foodborne illness in Alberta, Canada *(24)*. It has been indicated that the prevalence of MRSA varied dramatically around the world and reflects inappropriate antimicrobial administration and prevention practices *(25, 26)*.

In this study, the mean *S. aureus* counts were 3.49, 3.71, 2.93, 3.40 and 3.23 $\log_{10}$ cfu/g in the tested raw milk, Damietta cheese, Kareish cheese, ice cream and yoghurt, respectively, with an overall count of 3.41 $\log_{10}$ cfu/g (Table 2). The frequency of *S. aureus* counts in examined milk and dairy product samples is shown in table (3). It has been indicated that 75% (30/40) of raw milk samples exceeded the maximal limits of 100 cfu/ml set by both Egyptian standards *(27)* and European regulations *(28)*, while 65% (26/40), 40% (16/40), 40% (16/40) and 30% (12/40) of Damietta cheese, Kareish cheese, ice cream and of yoghurt samples, respectively (Table 2) exceeded the maximal limit of 100 cfu/g set for *S. aureus* by European Economic Communities food legislation for soft and fresh cheeses, frozen milk-based products and fermented dairy products *(28)*. The Egyptian standards specified that soft cheeses (including Damietta and Kareish cheeses), ice cream and Yoghurt must be free from pathogenic organisms and their toxins. All cheese, ice cream, yoghurt samples in this study exceeded the permissible limits of *S. aureus* counts reported in Egyptian Standards.

Table 2. ***Staphylococcus aureus*** **counts (log_{10} cfu/g or ml) on Baird parker Agar (BP) from milk and dairy products**

Product	No. of samples tested	Minimum	Maximum	Mean	Samples exceeded the maximal limit†
Raw milk	40	2.0	4.45	3.49*	30 (75%)
Damietta cheese	40	2.3	5.04	3.71*	26 (65%)
Kareish cheese	40	2.0	3.69	2.93*	16 (40%)
Ice cream	40	2.3	4.08	3.40*	16 (40%)
yoghurt	40	2.0	4.65	3.23*	12 (30%)
All products	200	2.0	5.04	3.41*	100 (50%)

*No significant difference at $P \leq 0.05$.

† Maximum permissible limit is 2 log_{10} cfu/g or ml according to EEC (1992)

Table 3. Frequency of *S. aureus* count (log_{10} cfu/ml or g) in examined milk and dairy products

Intervals (log_{10} cfu/g)	Raw milk	Damietta cheese	Kareish cheese	Ice cream	Yoghurt
	No (%)	No (%)	No (%)	No (%)	No (%)
0 : < 2	10 (25)	14 (35)	24 (60)	24 (60)	28 (70)
2 : < 3	10 (25)	10 (25)	6 (15)	6 (15)	4 (10)
3 : < 4	10 (25)	4 (10)	10 (25)	4 (10)	8 (20)
4 : < 5	10 (25)	10 (25)	0 (0)	6 (15)	0 (0)
5 : < 6	0 (0)	2 (5)	0 (0)	0 (0)	0 (0)
Total	40 (100)	40 (100)	40 (100)	40 (100)	40 (100)

Acidification is a way to reduce growth of bacteria in food processing. *S. aureus* is reported to be quite acid tolerant. In general, bacteria posses a multitude of defense mechanisms to cope with a sudden drop in pH. *Chan et al. (29)* observed that *S. aureus* is killed at pH 2. Also, *Pazakova et al. (30)* proved that no *S. aureus* was detected in yoghurt produced from milk contaminated by 10^5 *S. aureus* cells after 48 h of cold storage. On the contrary, *Zúñiga Estrada et al. (31)* reported that enterotoxigenic strains of *S. aureus* were able to survive the fermentation of milk with a yoghurt starter culture and they were inhibited after several days during storage of fermented products.

Acidity in both Kareish cheese and yoghurt has played a role in low incidence of *S. aureus* in both types of dairy products. The high incidence of *S. aureus* species in Damietta cheese is indicative of poor hygienic measures during production, handling and distribution. On the other hand, the difference in the prevalence rates of *S. aureus* between the examined products may originate from the method of manufacture, storage and handling.

Molecular characterization of MRSA isolates

We have isolated 414 MRSA strains from the milk and dairy product samples. One hundred and thirty four of these isolates were derived from raw milk, 88 from Damietta cheese, 26 from Kareish cheese, 138 from ice cream and 28 from yoghurt. All of the 414 isolates were tested by PCR for the presence of 4 different genes including 3 marker genes, namely *nuc*, *coa*, and *mecA* in addition to one virulence genes (*hla*).

Detection of the marker genes; nuc, coa, and mecA in MRSA isolates

PCR analyses verified the existence of *nuc*, *coa*, and *mecA* genes at the expected molecular size of 660 bp (Fig. 2A), 1000 bp (Fig. 2B), and 1200 bp (Fig. 2C), respectively in all of the 414 isolates. Sequence analyses of the amplified genes were carried out, and the resultant sequences were subjected to the GenBank database for homology search using BLAST, Basic Local Alignment Search Tool.

Detection of the virulence gene; *hla* in MRSA isolates

The *hla* gene which encodes the alpha-hemolysin (Hla) toxin is one of the main virulence factors of *S. aureus* and is formed by the most of *S. aureus* strains. Interestingly, all of the 414 MRSA isolated were positive for *hla* gene, which was detected at the expected molecular size of 744 bp (**Fig. 3**).

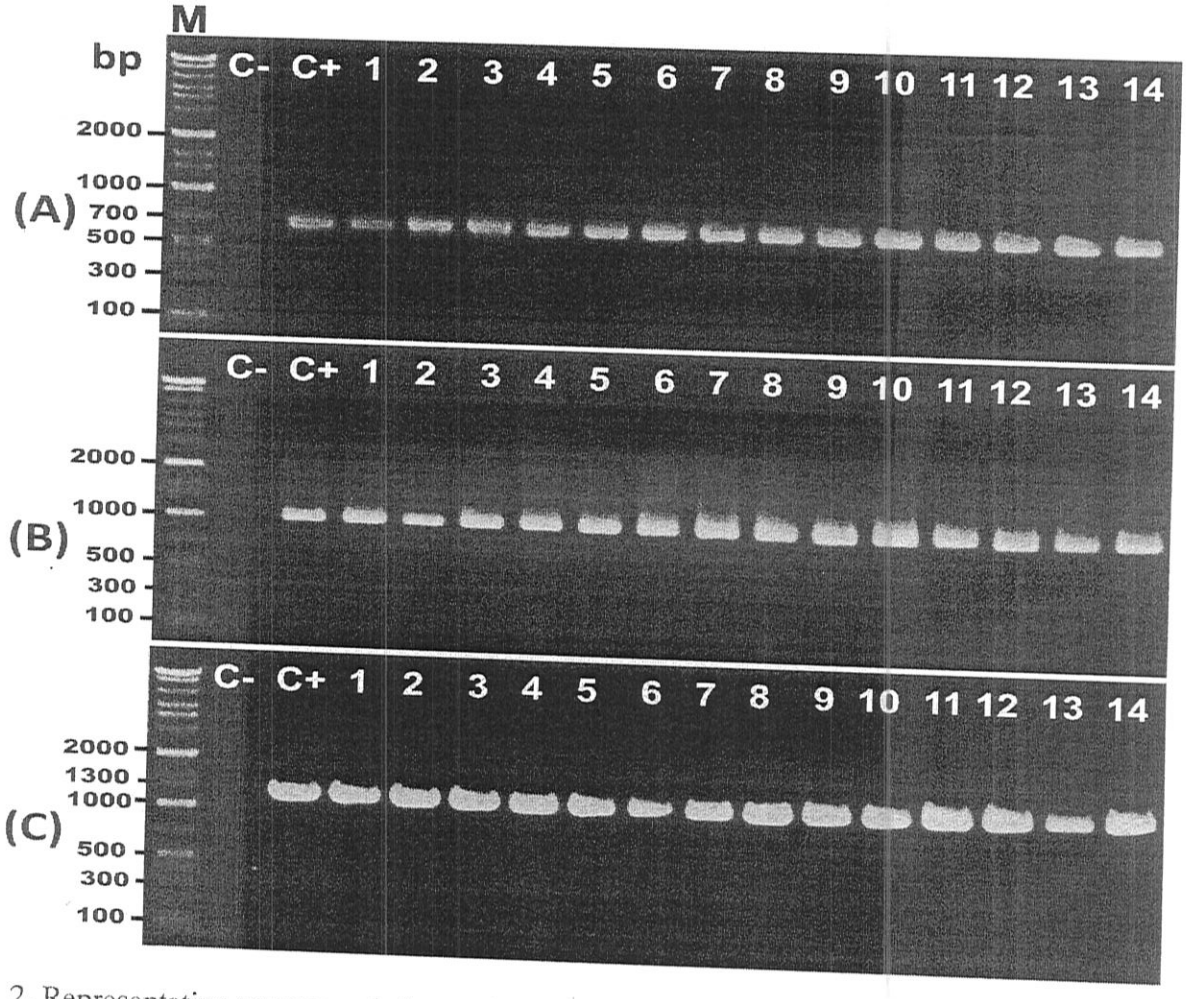

Fig. 2. Representative agarose gel electrophoresis for PCR products of the marker genes identified in the *S. aureus* strains (n= 414) isolated from milk and different dairy products. Three microliters from the PCR products were separated by electrophoresis on 1.2% agarose gel. Amplified DNA of the expected molecular size of 660 bp for *nuc* gene (A), 1000 bp for *coa* gene (B), and 1200 bp for *mecA* gene (C), were visualized under UV light. M: DNA marker (Gene Ladder Wide 1) used as a reference for fragment size; Lane C-: *E. coli* K12 DH5α as a negative control strain; Lanes with the key numbers from 1 to 14 are representative positive strains for the target genes.

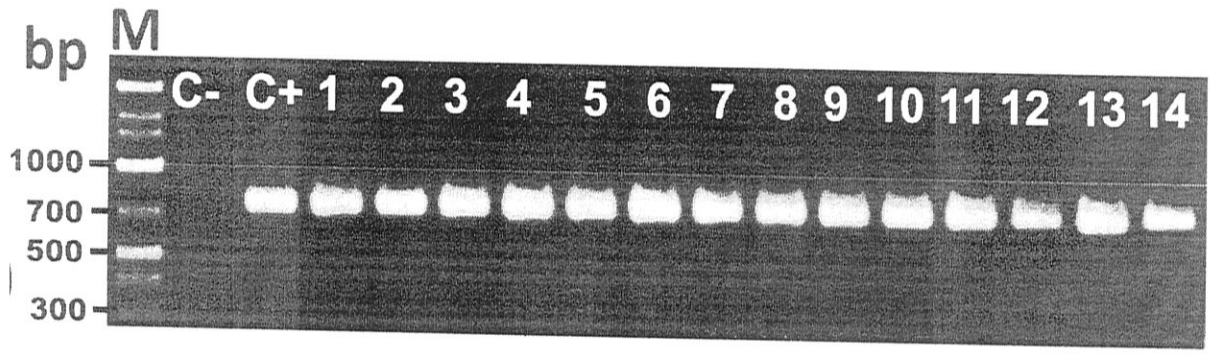

Fig.3. Representative agarose gel electrophoresis for PCR products of the virulence gene *hla* identified in the *S. aureus* strains (n= 414) isolated from milk and different dairy products. Three microliters from the PCR products were separated by electrophoresis on 1.2% agarose gel. Amplified DNA of the expected molecular size of 744 bp for *hla* gene was visualized under UV light. M: DNA marker (Gene Ladder Wide 1) used as a reference for fragment size; Lane C-: *E. coli* K12 DH5α as a negative control strain; Lanes from 1 to 14 are representative positive strains for the target genes.

Antimicrobial susceptibility of isolated MRSA strains

The antimicrobial drug susceptibility pattern for the 414 isolated MRSA strains is shown in table (4). Of the 13 tested antimicrobials, the least effective drugs were penicillin G, cloxacillin, tetracycline, and amoxicillin with bacterial resistance percentages of 87.9%, 75.9%, 65.2% and 55.6% respectively. Less effective drugs also included streptomycin, rifampicin, amikacin, chloramphenicol, which revealed bacterial resistance percentages of 44.9%, 36.7%, 35.3%, and 30.9%, respectively). The most effective antimicrobials against MRSA isolates were vancomycin, sulphmethazole/trimethoprim, ciprofloxacin, netilmicin, and Gentamicin, which exhibited bacterial sensitivity percentages of 76.3%, 75.45 70.1%, 69.1%, and 63.3%, respectively.

Table 4. Antimicrobial sensitivity pattern of MRSA isolated from milk and dairy products

Type of Antimicrobials	No. of MRSA isolates (n = 414)		
	S (%)	I (%)	R (%)
Tetracycline (30 µg)	80 (19.3)	64 (15.5)	270 (65.2)
Netilmicin (30 µg)	286 (69.1)	50 (12.1)	78 (18.8)
Amoxycillin (10 µg)	114 (27.5)	70 (16.9)	230 (55.6)
Cloxacillin (5 µg)	4 (0.97)	96 (23.2)	314 (75.9)
Streptomycin (10 µg)	134 (32.4)	94 (22.7)	186 (44.9)
Sulphamethazole/trimethoprim (25 µg)	312 (75.4)	44 (10.6)	58 (14.0)
Gentamicin (10 µg)	262 (63.3)	38(9.2)	114 (27.5)
Penicillin G (10 IU)	26 (6.3)	24 (5.8)	364 (87.9)
Rifampicin (5 µg)	222 (53.6)	40 (9.7)	152 (36.7)
Chloramphinicol (30 µg)	206 (49.8)	80 (38.3)	128 (30.9)
Ciprofloxacin (5 µg)	290 (70.1)	60 (14.5)	64 (15.5)
Amikacin (30 µg)	238 (57.5)	30 (7.3)	146 (35.3)
Vancomycin (30 µg)	316 (76.3)	62 (15.0)	36 (8.7)

Of the 414 MRSA strains tested, 348 (84.1%) were multidrug resistance (resistant to 3 or more antimicrobials). The high frequency of multi-drug resistant MRSA isolated from milk and dairy products in this study spotted light and suggests needs to change sanitary regulation with regards to milk and most of dairy products consumed in Egypt. One idea is that multi-drug resistant bacteria, particularly Staphylococci associated with milk samples and its related products might be related to human contamination rather than contamination with animal origins *(32)*. The antimicrobial susceptibility patterns observed for MRSA isolates may reflect the microbial adaptive response to the use and overuse of antimicrobials.

The present study concluded that milk and dairy products marketed in Mansoura, Egypt were highly contaminated with multidrug resistant MRSA, and such microbial contaminants harbor virulence gene, which have potential to cause severe infection in humans. This study presents the first genetic characterization of MRSA isolated from milk and dairy products in Egypt.

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الملخص العربي

تواجد ميكروبات المكور العنقودي الذهبي المقاوم للميثيسيلين (MRSA) في اللبن و بعض منتجاته و الكشف عن الجينات المميزة لها و جينات الضراوة و حساسيتها للمضادات الميكروبية

مها عبده محمد العشماوى[1] – خالد ابراهيم سلام[1]

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هذا العمل تناول دراسة مدى تواجد ميكروبات المكور العنقودي الذهبي المقاوم للميثيسيلين (MRSA) و الملف الجيني له و مدى حساسيته للمضادات الميكروبية في اللبن و بعض منتجاته بمدينة المنصورة – مصر.

تم الكشف عن وجود ميكروبات المكور العنقودي الذهبي المقاومة للميثيسيلين (MRSA) بنسبة ٥٣% من اجمالي عينات اللبن و منتجاته محل الدراسة حيث تواجد بنسب ٧٥%، ٦٥%، ٤٠%، ٥٠% و ٣٥% في كل من اللبن الخام ، الجبن الدمياطي ، الجبن القريش، الأيس كريم و اليوغورت على التوالي. و قد كان متوسط العدد البكتيري لميكروب المكور العنقودي الذهبي ٣,٤٩ ، ٣,٧١، ٢,٩٣، ٣,٤٠ و ٣,٢٣ لوغاريتم ١٠ مستعمره بكتيرية /جم في كل من عينات اللبن الخام، الجبن الدمياطي ، الجبن القريش، الأيس كريم و اليوغورت على التوالي. وكان متوسط العدد الإجمالي ٣,٤١ لوغاريتم ١٠ مستعمره بكتيرية /جم لكل العينات. و على غير المتوقع فقد كانت كل العترات المعزولة لميكروب المكور العنقودي الذهبي و عددها ٤١٤ عتره مقاومة للمثيسيلين. أكد اختبار البلمره المتسلسل وجود الجينات nuc ، coa ، mecA و hla في كل العترات المعزولة.

كما تم اختبار حساسية هذه العترات لعدد ١٣ من المضادات الميكروبية و كان أقلهم تأثيرا هم بنسيلين ج، كلواكسيسيلين، تيتراسيكلين، و أموكسيسيلين بنسب مقاومة ٨٧,٩% ، ٧٥,٩%، ٦٥,٢% و ٥٥,٦% على التوالى. بينما كان أشدهم تأثيرا على العترات المعزوله لميكروب المكور العنقودى الذهبى المقاوم للميثيسيلين (MRSA) هم الفانكومبسين، السلفاميثازول/الترايميثوبريم، سيبروفلوكساسين، نيتلميسين، و الجينتاميسين بنسب حساسية ٧٦,٣% ، ٧٥,٤٥%، ٧٠,١%، ٦٩,١%، و ٦٣,٣% على التوالى. و كان عدد ٣٤٨ عتره من إجمالي ٤١٤ عتره بنسبة ٨٤,١% من ميكروبات المكور العنقودي الذهبي المقاوم للميثيسيلين (MRSA) مقاومة للعديد من المضادات الميكروبية,

تعتبر هذه الدراسة الأولى التي تدرس الصفات الجينية لميكروب المكور العنقودي الذهبي المقاوم للميثيسيلين (MRSA) و المعزول من اللبن و منتجاته بمدينة المنصورة – مصر. وقد تم مناقشة الأهمية الصحية و الاقتصادية لتواجد تلك الميكروبات في اللبن و منتجاته و الإجراءات الوقائية لحماية المنتج من التلوث بها.