MOLECULAR DETECTION OF METHICILLIN-RESISTANT
Staphylococcus aureus
ISOLATED FROM MILK AND CHEESE OF COW AND BUFFALOES
IN BASRAH CITY

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ABSTRACT

In the present study, 135 samples were collected from different animal's including: 75 samples were from milk and 60 samples were from cheese, 54 (40%) sample were found to be harbored with *Staphylococcus aureus*. The rate of *S. aureus* isolates was 50% in buffalo's cheese, 40.54% in buffalo's milk, 36.8% in cow's milk, and 33.33% in cow's cheese. 100% strains were Methicillin Resistance *Staphylococcus aureus*. The antibiotic sensitivity test was determined against 8 common antibiotics by the agar disc diffusion method on Muller-Hinton agar. These antibiotics were amoxicillin (25mcg), ampicillin (10mcg), Oxacillin (1mcg), chloramphenicol (30mcg), erythromycin (15mcg), gentamycin (10mcg), methicillin (5mcg), and tetracycline (30mcg). *S. aureus* strains were screened by PCR for 16S rRNA and *nuc* genes. 49 out of 54 *S. aureus* isolates were yielded products with molecular weight approximately (228 bp) corresponding to 16S rRNA gene, 42 out of 54 isolates were give products with molecular weight approximately (270bp) corresponding to *nuc* gene, 22 and 4 out of 30 *S. aureu*s isolates were give products with molecular weight approximately (310bp and 509bp) corresponding to *mecA* and *femA* genes, respectively.

INTRODUCTION

The genus *Staphylococcus* includes over 30 species and subspecies, the most important species for the human and veterinary medicine is *S. aureus* which is among the most frequent causal organisms in human and animal's bacterial infections. *S. aureus* is one of the leading causes

of foodborne disease outbreaks due to its ability to produce staphylococcal enterotoxins [1,2]. Antibiotics Methicillin resistant *Staphylococcus aureus* (MRSA) is important because, in addition to being methicillin resistant, most strains are also resistant to other β-lactam antibiotic, with the exception of glycopeptide [3].The genus *Staphylococcus* is Gram-positive bacteria, and particularly *S. aureus* is one of the most harmful species of staphylococci encountered. It is extremely a major important pathogens, and it's one of the most common causes of nosocomial infections, especially pneumonia, surgical site infections and blood stream infections, it's also the leading cause of bacteremia, myocarditis, acute endocarditis, pericarditis, osteomyelitis, encephalitis, meningitis, chorioamnionitis, mastitis in dairy animals, and scalded skin syndrome and continues to be a major cause of community-acquired infections [4].

More recently, Methicillin Resistance *Staphylococcus aureus* (MRSA) has been isolated from most animals and foods of animal origin. MRSA strains have been isolated from cows' or small ruminants' milk and various dairy products in many countries. The MRSA prevalence in milk and dairy products reported from different countries or even regions of the same country differs significantly [5,6,7,8,9].Depending on growth conditions, the colony pigmentation varies from grey, grey white with yellowish to orange shades and a typical β-haemolysis on the blood agar [10]. The methicillin resistance mechanism is well understood in MRSA strains [11]. It is caused by the production of a novel penicillin-binding protein, PBP-2a, with a decreased binding affinity for β-lactams [12]. This process is encoded by the chromosomal gene *mecA* that is found in the mec region. The sequence of *mecA* gene is conserved in all methicillin-resistant strains of *S. aureus* [13].The β-lactam antibiotics damage bacteria by inactivating penicillin-binding proteins that are essential in the assembly of the bacterial cell wall [14]. The aim of this study is to evaluation of the occurrence of S. aureus in milk, and cheese from cows and buffalos in Basrah city, antimicrobial-resistance pattern, and detection of the important genes 16S rRNA and *nuc* genes (*S. aureus* species specific determinants), and *mecA* and *femA* genes (methicillin resistance determinant).

MATERIALS AND METHODS

Collection of Samples and Identification of *S. aureus:*

A total of 135 samples were collected; included 75 samples of raw milk: 38 cows' milk samples and 37 buffalo's milk samples, and 60 samples of cheese: 30 samples were from each of cows' cheese and buffalo's cheese). Samples were collected during the period from October 2017 to February 2018 from different area of Basrah City. All samples were

transported in an ice box to laboratory of microbiology. Milk samples were immediately cultured onto mannitol salt agar (MSA), and incubated at 37°C for 24-48 h. Mannitol fermenting colonies (i.e. those that were yellow or gold) were selected from the MSA and sub-cultured on Blood agar (BA. The colonies were subjected to Gram's staining, catalase test, coagulase test, oxidase test, and DNase test. [15]. Cheese samples were processed depending on [16]**.**

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility test for isolated *S. aureus* were determined by the agar disk diffusion method [17].in Mueller-Hinton agar (MHA) plate. The turbidity of the actively growing broth culture was adjusted to the 0.5 McFarland standard [18].

Antimicrobial susceptibility performed for 8 common antibiotics, amoxicillin (25mcg), ampicillin (10 mcg), Oxacillin (1mcg), chloramphenicol, (30mcg), erythromycin (15mcg), gentamicin (10mcg), methecillin (5mcg), and tetracycline (30mcg). The results were recorded after 24h incubation at 35°C according to the guidelines of the National Committee of Clinical Laboratory Standards guidelines [18].

DNA Extraction and PCR Assay

DNA template was extracted using the presto Mini g DNA Bacteria Kit (Geneaid/korea). A PCR reaction with specific primers was performed to identify (16S rRNA), *nuc*, *mecA*, and *femA* in each isolate. A total PCR reaction mixture was 25µl contained: 12.5µl Green Go *Taq* Master Mix pH 8 (Promega, USA) contained [(50unit/ml) of Go *Taq* DNA polymerase, (400Mm) of each dNTPs and (3mM) of MgCl$_2$], 1 µl for each primers, 5µl DNA template and the mixture was complete to 25µl by adding 5.5µl deionized distilled water. The oligonucleotide primers that used for generated bands for the genes detection are illustrated in the table (1) and reaction to each PCR reaction was listed in table 2.

Table 1: The oligonucleotide primer sets used for the genes.

Gene	Primer Sequences	Product size (bp)	Sources
16SrRNA	F;GTAGGTGGCAAGCGT TATCC R:CGCACATCAGCGTCAG	(228)	[20]
Nuc	F:GCGATTGATGGTGATACGGTT R:AGCCAAGCCTTGACGAACTAAAGC	(270)	[20]
mecA	F:GTAGAAATGACTGAACGTCCGATGA R:CCAATTCCACATTGTTTCGGTCTAA	(310)	[21]
femA	F:AGACAAATAGGAGTAATGAT R:AAATCTAACACTGAGTGATA	(509)	[19]

Table 2: Primer conditions sets used for the genes.

Primer	The condition
16SrRNA	Initial denaturation 94°C for 2min, 35 cycle (94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec) and final extension 72°C for 4 min.
Nuc	Initial denaturation 95°C for 2min, 35 cycle (94°C for1min, 50°C for 50sec, 72°C for 2min) and final extension 72°C for 5min.
mecA	Initial denaturation 95°C for 4min, 30 cycle (94°C for 30sec, 53°C for 45sec, 72°C for 4min) and final extension 72°C for 4 min.
femA	Initial denaturation 94°C for 5min, 30 cycle (94°C for 30sec, 45°C for 1 min, 72°C for 45sec) and final extension 72°C for 10 min.

RESULTS

Isolation and Identification

In the present study, 135 samples were tested (cows' milk, buffalo's milk, cows' cheese and buffalo's cheese). These samples Included 75 milk's samples and 60 cheese's samples. *S. aureus* isolated from 54 samples (40%). The highest rate of *S. aureus* isolation was observed in buffalo's cheese 15/30 (50%), followed by buffalo's milk 15/37 (40.54%), then cows' milk 14/38 (36.8%), and cows' cheese 10/30 (33.33%). Table (3).

Table 3: prevalence of *Staphylococcus aureus* isolated from milk and cheese

Sample source of animal	No. of sample	No. of *S. aureus*	(%)
Cows' milk	38	14	(36.8)
Buffalo's milk	37	15	(40.54)
Cows' cheese	30	10	(33.33)
buffalo's cheese	30	15	(50)
Total	135	54	
p>0.05 no significant			

Screening for Methicillin Resistance *S. aureus* (MRSA)

By using agar disc diffusion method, 30 randomly selected *S. aureus* isolates were subjected for susceptibility toward methicillin. Total of the 30 (100%) *S. aureus* strains were MRSA which were isolated in this study.

Antimicrobial Susceptibility

Table (4) provides antimicrobial susceptibility results by agar disc diffusion test. Interestingly, 30 *S. aureus* isolates exhibited the Multi-Drug Resistance (MDR) trait against 8 commonly used antibiotics; the isolates were completely resistant (100%) for amoxicillin, ampicillin, Oxacillin , and methicillin, and (92%) of them were sensitive to chloramphenicol and gentamycin and (8%) resistant, while all the tested isolates were completely sensitive to tetracycline (100%), and (72. %) sensitive for erythromycin and 26% resistant (2% intermediate) p> 0.05, Figure (1).
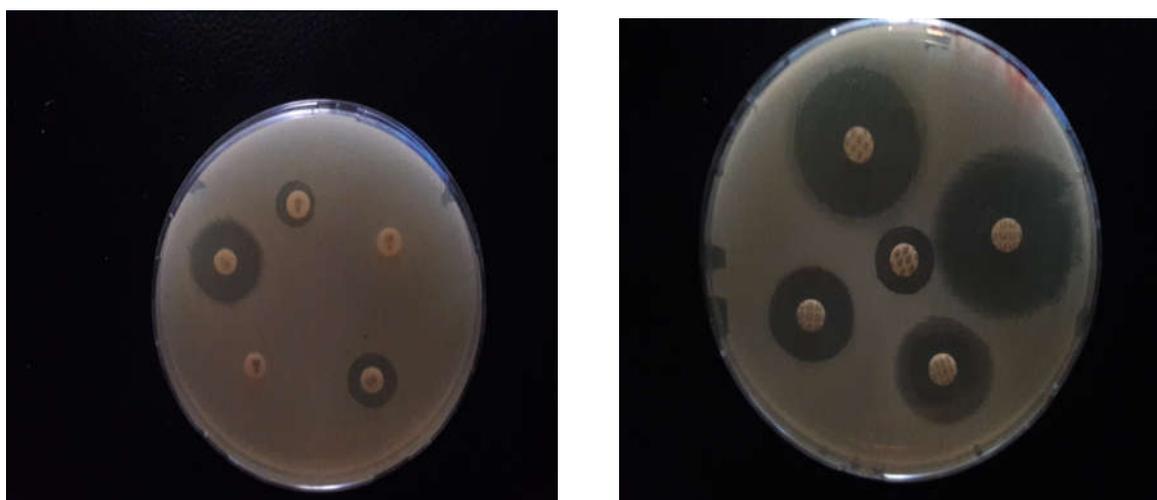
Figure 1: Disc diffusion method with inhibition zones for antibiotics against *S. aureus*.

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

Antibiotic	Concentration (mcg)	Resistance (%)	Intermediate (%)	Sensitive (%)
Amoxillin	(25)	100	-	-
Ampicillin	(10)	(100)	-	-
Oxacillin	(1)	(100)	-	-
Chloramphenicol	(30)	-	(8)	(92)
Erythromycin	(15)	(26)	(2)	(72)
Gentamycin	(10)	(8)	-	(92)
Methicillin	(5)	(100)	-	-
Tetracycline	(30)	-	-	(100)

Detection of the *16S rRNA, nuc, mecA* and *femA* Genes by PCR Assay

Results of PCR assay revealed that 49 (90.7%) isolates from total 54 isolated *S.aureus* were yielded products with molecular weight approximately (228 bp) corresponding to 16S rRNA gene, as showed in Figure (2), 42 isolates of them (54 isolates) were gave products with molecular weight approximately (270bp) corresponding to *nuc* gene, Figure (3).

While 22 out of 30 *S. aureus* isolates were gave products with molecular weight approximately (310 bp) corresponding to *mecA* gene, Figure (4), and only 4 out of 30 *S. aureus* isolates were yielded products with molecular weight approximately (509 bp) corresponding to *femA* gene $p> 0.05$, Figure (5).
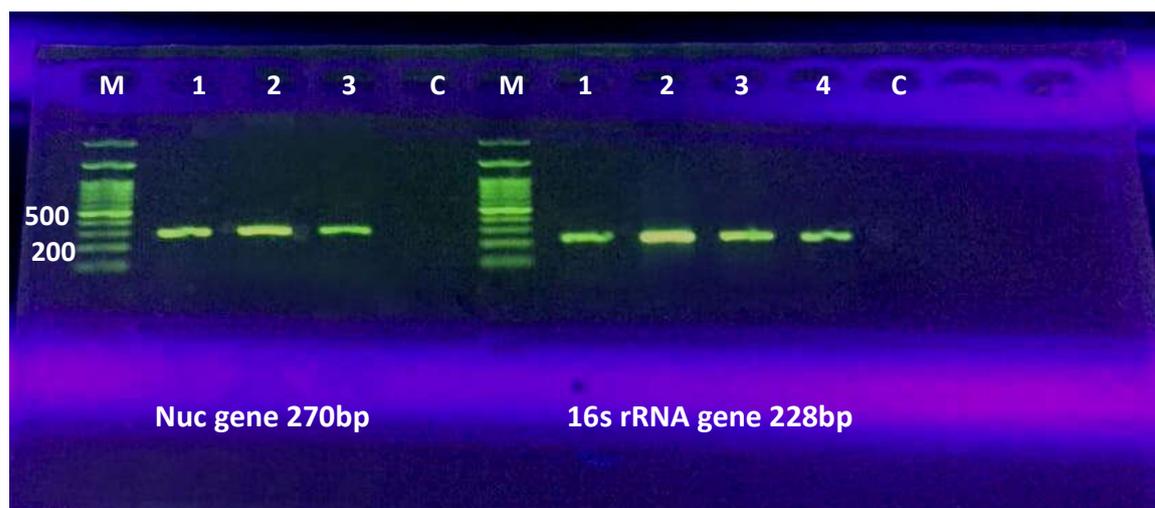

Figure 2: Agarose gel electrophoresis of PCR-amplified for 16S rRNA gene and nuc gene of S. aureus isolates. LineM: DNA marker. Lines. 1-3: nuc gene 270bp and 1-4 16s rRNA gene 228bp and line C: Negative control.
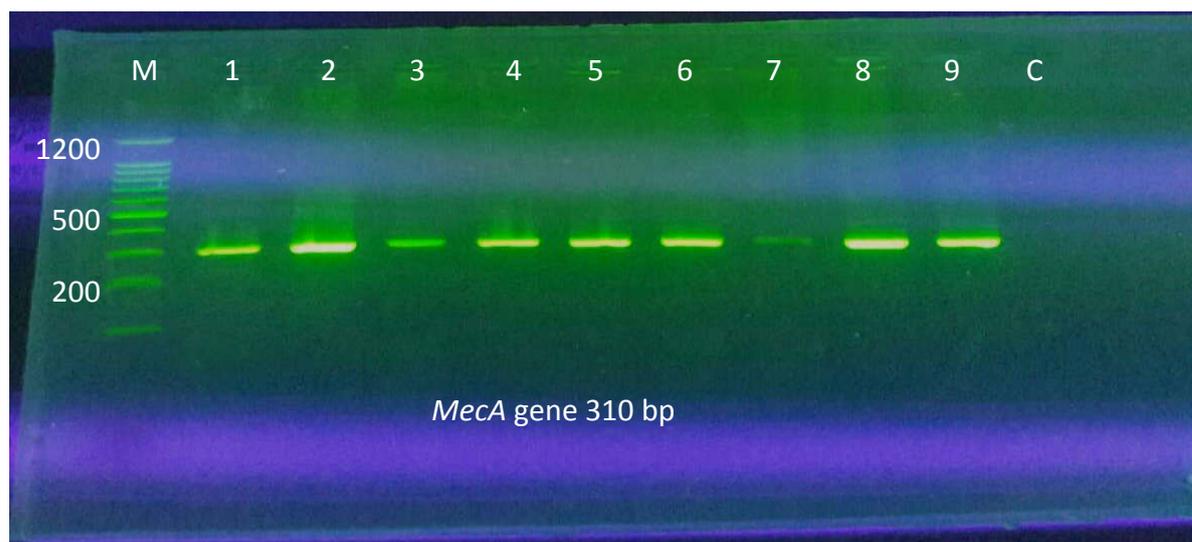
Figure 4: Agarose gel electrophoresis of PCR-amplified for *mecA* gene of *S. aureus* isolates. Line. M: DNA marker. Lines. 1-9: *mecA* gene 310 bp; Line. C: Negative control.
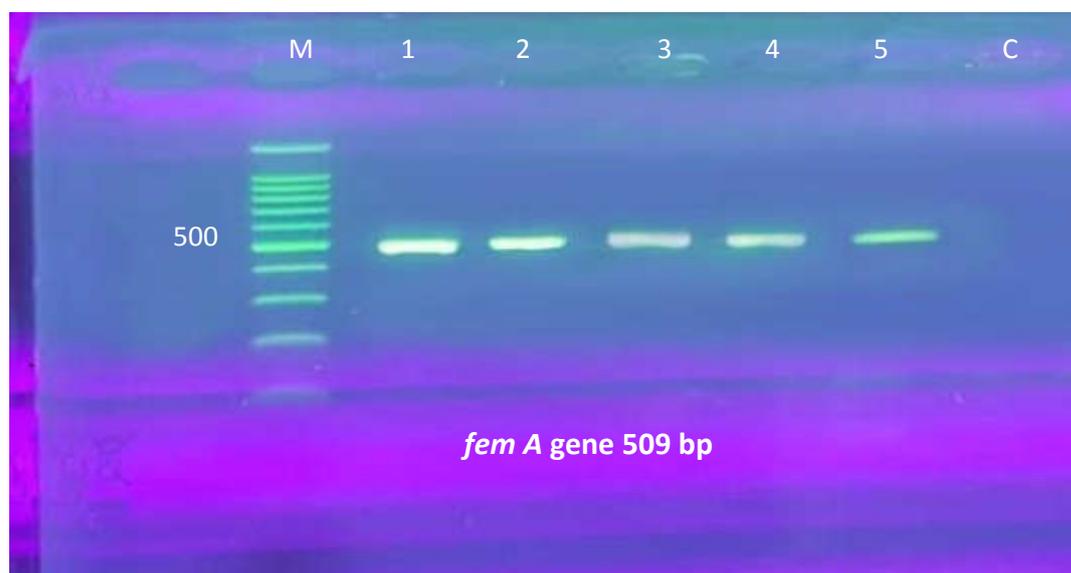

Figure 5: Agarose gel electrophoresis of PCR-amplified *femA* gene of *S. aureus* isolates. Line. M: DNA marker. Lines. 1-5: *femA* gene 509bp line C: Negative control.

DISCUSSION

The results of bacterial isolation and identification from milk and cheese of cows and buffaloes revealed that 54 out of 135 (40%) samples were implicated with *S. aureus*. Among 135 samples, 75 milk's samples showed (40.54%) buffalo milk and (36.8%) cow milk, and 60 cheese samples showed (50%) buffalo cheese and (33.33%) cow cheese. The percentage of *S. aureus* infection in the present study agreed with the study conducted in Iraq [5]. The present study results are in line with study of [22].who reported that the percentage of *S. aureus* was (48.57%) from bovine's milk. But it's not in agreement with the results that recorded by [6, 23].which were (10.23%) and (21.25%), respectively. Several studies have demonstrated that pathogenic bacteria like *S. aureus* may responsible for vomiting, abdominal cramps and diarrhea like diseases in humans [24,25,26,27,28]

Thirty *S. aureus* isolates were subjected towards different antibiotics. The percentage of susceptibility toward methicillin and oxacillin was (100%). The present study is along with the study of Nusrat [23].who found that coagulase positive *S. aureus* was found to be highly resistant against oxacillin (100%). In addition to that occurrence of methicillin resistant *S. aureus* in food samples has been a major concern worldwide [29].In developing countries more than 70% of infecting bacteria have been accounted as multi drug resistant strain (MDR) [30,31].noticed the least effective drugs were tetracycline, and amoxicillin with bacterial resistance percentages of 65.2%, and 55.6%, respectively.

S. aureus isolates were analyzed by PCR assay. DNA from *S. aureus* isolates of milk and cheese were extracted by purification kit to detect the presence of *16S rRNA, nuc, mecA, and femA* genes. In the present study, 90.7 % *S. aureus* isolates that identified were confirmed by amplification of the 16S rRNA gene [32] confirmed that 100% of S. aureus isolates from cow and buffaloes were produced 16S rRNA gene. The present study agreed with the study of Al-Ashmawy [31] found that all recovered *S. aureus* isolates were genetically verified as MRSA strains by molecular detection of the *mecA* gene.

CONCLUSION

The high percentage of methicillin resistant *S. aureus* isolates which also resistant to the most of the antibiotics used, which may be milk and milk products the main source of transmission to humans and these are a high risk to human health.

الكشف الجزيئي للمكورات العنقوديه المقاومه للمثيسيلين المعزوله من الحليب والاجبان للابقار والجاموس في مدينة البصره

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الخـــلاصـــة

في هذه الدراسة، تم جمع ١٣٥ عينة من الحيوانات المختلفة بما في ذلك: ٧٥ عينة من الحليب و٦٠ عينة من الجبن، ووجد أن ٥٤ عينة (٤٠ ٪) تحتوي على المكورات العنقودية الذهبية. *S.aureus* حيث كان معدل عزلات المكورات العنقودية 50٪ في جبن الجاموس ، و ٤٠.٥٤ ٪ في حليب الجاموس ، و ٣٦.٨ ٪ في حليب البقر ، و ٣٣.٣٣ ٪ في جبن البقر. اظهرت جميع عزلات المكورات العنقودية الذهبية ١٠٠ ٪ مقاومة للمضاد الحيوي الميثيسيلين. تم تحديد اختبار الحساسية للمضادات الحيوية تجاه ثمانية انواع من المضادات الحيوية الشائعة من خلال طريقة agar disc diffusion method على وسط مولر-هينتون اكار. هذه المضادات الحيوية هي أموكسيسيلين (25mcg)، أمبيسيلين (10mcg)، أمبيسيلين / كلوكساسيللين (30mcg)، كلورامفينيكول (30mcg)، إريثرومايسين (15mcg)، جنتاميسين (10mcg)، ميثيسيلين (5mcg)، وتتراسيكلين (30mcg). وبعدها تم فحص سلالات المكورات العنقودية الذهبية بواسطة تقنية التفاعل التسلسلي للبلمرة الـ PCR لجينات nuc و 16S rRNA. تم الحصول على ٤٩ عزلة من أصل ٥٤ عزلة من المكورات العنقودية الذهبية كانت ذات وزن جزيئي ٢٢٨ قاعدة نايتوجينية تقريبًا ،تعبر عن جين الحامض النووي الريبي النووي 16 ،بينما أعطت ٤٢ من اصل ٥٤ عزلة حزم ذات وزن جزيئي ٢٧٠ قاعدة نايتوجينية تعبر عن جين nuc ، في حين اعطت ٢٢ و ٤ من ٣٠ عزلة من المكورات العنقودية الذهبية حزم ذات اوزان جزيئية ٣١٠ اي تحمل mecA gene و٥٠٩ قاعدة نايتوجينية تقريبًا اي تحمل femAgene.

815

REFERENCES

1-Hennekinne, JA.De Buyser, ML. and Dragacci, S. (2012). *Staphylococcus aureus* and Its Food Poisoning Toxins: Characterization and Outbreak Investigation. FEMS Microbiol. Rev. 36(4): 815–836.

2- Tenhagen, BA. Vossenkuhl, B. Käsbohrer, A. Alt, K. Kraushaar, B. Guerra, B. Schroeter, and Fetsch, A.(2014). Methicillin-Resistant *Staphylococcus aureus* in cattle food chains - prevalence, diversity, and antimicrobial resistance in germany. J. Anim. Sci. 92: 2741-51.

3-Chambers, HF. (1997). Methicillin resistance in Staphylococci: molecular and biochemical basis and clinical implications.Clin. Microbiol. Rev. 10: 781-791

4- Kumar, R. Yadav, BR. and Singh, RS. (2010). Genetic determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitis crossbred cattle. Curr. Microbiol. 60(5): 379-386.

5- Khudaier, BY. Abbas, BA. And Khudaier, AM. (**2013**). Detection of methicillin resistant *Staphylococcus aureus* isolated from human and animals in Basrah Province / Iraq. MRVSA 2(3): 12-21.

6-Khudaier, BY. Anad, IT. and Abbas, BA. (**2014**). Isolation of   *Staphylococcus aureus* from Buffalo milk in Basra Governorate and   Detection of their Antibiotic Susceptibility. **Bas. J. Vet. Res**. 13(1): 1-12.

7-Pexara, A., N. Solomakos, and A. Govaris. (2013). Prevalence of Methicillin Resistant *Staphylococcus aureus* in Milk and Dairy Products. J. Hellenic Vet. Med. Soc. 64 (1): 17-34.

8- Khaleel, DA. Uthman, RM. and Khudaier, BY. (2016). Isolation and Identification of *Staphylococcus aureus* from Buffaloes Milk Infected with Subclinical Mastitis and Milk Workers. Bas. J. Vet. Res. 16(2): 304-312.

9- **Abbas, BA. Khudaier, BY.and Khudaier, AM. (2017**). Studies on mecA gene in methicillin resistant *Staphylococcus aureus* isolates. Jokull. 67(6): 58-65.

10-**Medveďová, Alžbeta, and Ľubomír Valík. (2012).** *Staphylococcus   aureus*: Characterisation and Quantitative Growth Description in Milk and Artisanal Raw Milk Cheese Production. In Tech. 32: 71-102

11-**Lowy, FD. (2003).** Antimicrobial Resistance: The Example of *Staphylococcus aureus*. J. Clin. Invest. 111(9): 1265-1273.

12-**Hartman, BJ. and Tomasz, A. (1984).** Low-Affinity Penicillin- Binding Protein Associated with R-Lactam Resistance in *Staphylococcus aureus*. J. Bacteriol. 158 (2): 513-516.

13- **Weller, Timothy MA. (1999).** The Distribution of mecA, mecR1 and mecI and Sequence Analysis of mecI and the Mec Promoter Region in Staphylococci Expressing Resistance to Methicillin. J. of Antimicrobl. Chemother. 43: 15-22.

14- **Pinho, Mariana G., Hermínia de Lencastre, and Alexander Tomasz(2001).** Acquired and a Native Penicillin-Binding Protein Cooperate in Building the Cell Wall of Drug-Resistant Staphylococci. PNAS. 98 (19): 10886-10891

15-**Macfaddin, JF. (2000).** Biochemical tests for identification of medical bacteria. 3rd Ed. Lippincott Williams and Wilkins USA.

16- -**Jaber N.N.(2011)**.Isolation and boityping of staphylococcus aureus from white cheese in Basrah local markets. Bas.J.Vet.Res.Vol.10, No.2

17- **Kirby, WM. and Bauer, AW. (1966).** Antibiotic susceptibility testing by a standardized single disc method. Amer. J. Clin. Path. 45: 493-496.

18-**National Committee on Clinical Laboratory Standards (NCCLS), (2003)**. Performance standards for antimicrobial susceptibility tests. Approved standard. NCCLS document M2-A8. National committee on clinical laboratory Standards, Wayne, P.A.

19- **Chikkala, R., George, N. O., Ratnakar, K. S., Iyer, R. N., & Sritharan, V. (2012).** Heterogeneity in femA in the Indian isolates of Staphylococcus aureus limits its usefulness as a species specific marker. *Advances in Infectious Diseases*, *2*(03), 82.

20- KarmakarA, DuaP, and Ghosh C, 2016. Biochemical and Molecular Analysis of Staphylococcus aureus Clinical Isolates from Hospitalized Patients, Hindawi Publishing Corporation Canadian Journal of Infectious Diseases and Medical Microbiology Article ID 9041636, 7 pages.

21- Othman H.E, Merza,N.S, and Salih Jubrae J.M .(2014). Nucleotide sequence analysis of methecilin resistant staphylococcus aureus in kurdistan region –Iraq, Journal of University of Zakho, Vol. 2(A), No.1, Pp 65-73,

22- Hanon, BM. (2009). Comparative study to the *Staphylococcus aureus*, isolated from the bovine and human with detection of virulence (coa) gene in these isolates by polymerase chain reaction. M.Sc. Thesis, College of Veterinary Medicine, University of Basrah.

23-Nusrat J., Ifra, TN. and Mrityunjoy, A. (2015). Detection of methicillin-resistant Staphylococcus aureus within raw milk and cheese samples. Inter. Food Res. J. 22(6): 2629-2633.

24- Aly, SA.and Galal, EA. (2002). Effect of milk pretreatment on the keeping quality of Domiati cheese. Pakistan J. Nutr. 1: 132-136.

25- Robinson, RK. (2002). The microbiology of milk and milk products. In Dairy Microbiology Hand Book, p. 765. New York: John Wiley and Sons.

26- Soomro, AH. Arain, MA. Khaskheli, M. and Bhutto, B. (2003). Isolation of *Staphylococcus aureus* from milk products sold at sweet meat shops of Hyderabad. Online J. Biological Sci. 3(1): 91-94.

27- Chye, FY. Abdullah, A. and Ayob, MK. (2004). Bacteriological quality and safety of raw milk in Malaysia. Food Microbiol. 21: 535-541.

28-Marjan, S. Das, KK. Munshi, SK. and Noor, R. (2014). Drug-resistant bacterial pathogens in milk and some milk products. Nutr. Food Sci. 44(3): 241-248.

29- Shanehbandi, D., Baradaran, B., Sadigh-Eteghad, S. and Zarredar, H. (2014). Occurrence of methicillin resistant and enterotoxigenic *Staphylococcus aureus* in traditional cheeses in the north west of Iran. ISRN Microbiology. http://dx.doi.org/10.1155/2014/129580

30- Prescott, JF. (2000). Antimicrobial drug resistance and its epidemiology. In Prescott, JF. Baggot, JD. and Walker, RD. (Eds.). Antimicrobial Therapy in Veterinary Medicine, P. 27-49. Ames: Lowa State University Press, Ames.

31- Al-Shmawy, M. Sallam, KI. And Elhadidy. (2016). Prevalence, Molecular Characterization, and Antimicrobial Susceptibility of Methicillin-Resistant Staphylococcus aureus (MRSA) Isolated from Milk and Dairy Products. Foodborne Pathogens Dis. 13(3). 1-7.

32- Elsayed, MS. El-Bagoury, AM.and Dawoud, MA. (2015). Phenotypic and genotypic detection of virulence factors of Staphylococcus aureus isolated from clinical and subclinical mastitis in cattle and water buffaloes from different farms of Sadat City in Egypt. Vet. World. 8: 1051-1