MULTIPLEX PCR DETECTION OF ERYTHROMYCIN RESISTANCE GENES IN COAGULASE NEGATIVE STAPHYLOCOCCI ISOLATED FROM COWS IN BASRAH, IRAQ

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ABSTRACT

This study was conducted to identify the erythromycin resistance genes in the coagulase-negative staphylococci (CoNS) and its molecular characterization after isolating the bacteria from the samples of domestic animals and their products during the period from September 2016 to March 2017 from different areas in Basra city. 200 samples were collected from animals including: 40 samples from meat, 50 samples from raw milk, 30 samples from treated milk, 40 samples from cow's nasal swabs and 40 samples from cow's teat swabs. Results showed that from 200 collected samples only 108 were CoNS distributed in 22(15.1%), 18(12.4%),10(6.8%), 26(17.9%),32(22%), from meat, raw milk, treated milk, nasal swabs and teat swabs respectively. Samples were planted on the selecting mannitol salt medium to isolate *Staphylococcus spp*. which had the ability to grow on the mentioned medium. When the coagulation test was performed, some isolates were not able to produce the coagulation enzyme, and the results showed that 108 isolates were coagulase negative (54%). Twenty-two isolates of minced meat 55%, 18 isolates of cow's milk (36%), 10 isolates of milk sold (33%), 26 isolates of the nose of the animal (65%) and 32 isolates of animal teat swabs (80%). Twenty-five isolates of these negative staphylococci were identified using VITEK 2 kit. The result showed that 10(40%) isolates identified as coagulase negative *Staphylococcus* and fall in

four species including 4 (40%) *Staphylococcus lentus*, 4 (40%) *Staphylococcus gallinarum*, 1 (10%) *Staphylococcus haemolyticus*, and 1 (10%) *Staphylococcus chromogen*. When the *ermA*, *ermB*, *ermC* and *msrA* genes were investigated by PCR the result showed that they contain the genes in a percentage 5%, 20%, 20% and 5% respectively. By using the multiplex PCR molecular weight technique (*ermA, ermC)* and (*msrA, ermC*), the ratio of both genes was 15% and 5% respectively.

INTRODUCTION

Staphylococci are ubiquitous bacteria that include different opportunistic pathogenic species, responsible for human and animal infections. These groups of microorganisms colonize skin, hair, nose and throat of people and animals and from these sources they can be transferred to food because both organisms are the main reservoirs (1). The most frequently isolated species of CoNS from bovine Intramammary inflammation are, *Staphylococcus haemolyticus,* and *Staphylococcus xylosus* (2,3), but a number of other species have also been reported. Some CoNS species have been associated with the bovine skin microflora or the close environment of the cow *S. haemolyticus* has been isolated from udder skin of cows (4,5). In this species two serovars have been defined, one from pigs and one from cows (6). *S. xylosus* and *S. sciuri* are often found to be a part of the skin flora of cattle as well as of other mammals and of birds (4,7,8,9,3). These species have,15 however, also been isolated from bedding material in cow stables (10). One of the important reasons for failure antibiotics is assumed to be disceamet antibiotic without testing in vitro sensitivity of causal organism, for suitable antibiotic therapy, bacterial isolation and antibiotic sensitivity studies are always essentials. Antimicrobial susceptibility tests help to guide the veterinarian in selecting the antimicrobial agent for treatment of Intra Mammary Infection (IMI) caused by *Staphylococcus* species (11). Most research concerning antibiotic resistance of Staphylococci isolated from food focuses on the *Staphylococcus aureus* species (12,13,14,15,16,17), whereas less attention is paid to the group of coagulase-negative staphylococci (CoNS) (18). Due to the fact that for many years CoNS were considered non-pathogenic, in routine laboratory tests, CoNS are very often identified only at the

genus level, while coagulase-positive strains are selected for further analyses. The spread of resistance to antimicrobial agents in Staphylococci is largely due to the acquisition of plasmids and/or transposons (19). In Staphylococci, the conjugative transfer of resistance determinants is usually mediated by conjugative plasmids which spread resistance determinants between species and genera (20). Besides transferring the resistance determinants, they can mobilize non conjugative plasmids, recombine with non conjugative plasmids to form new plasmids, or acquire and transfer resistance transposons (21). The present study aims to investigate the presence and frequency of Coagulase-Negative Staphylococci (CoNS) in domestic animal, some animal products and to determine phenotypic antimicrobial resistance profiles of CoNS isolates from these sources, with emphasis on antimicrobial of clinical relevance to assess associations between species and resistance profiles in addition to detection of resistance genes.

MATERIALS AND METHODS

Samples Collection: In order to obtain *Staphylococcus* spp. different samples were collected from several regions in Basrah province. 200 samples were collected between 20 September 2016 to March 2017. 50 milk samples were collected directly from the cows other 30 milk samples were collected from markets in addition to 40 from cow's meat samples, 40 cow's teat swabs and 40 cow's nasal swabs. were collected. Milk samples collected directly from cows after cleaning the udder by water and drying by a piece of towel then using cotton moistened by alcohol 70% and removing the first flowage of milk and collecting 5 ml in sterile tube, transported with ice box according to (22). Samples treated aseptically, the swab samples and the milk samples were pre-enriched in appropriate amount of Peptone Buffered Water (PBW) in 1:9 ratio and incubated at 37°C for 24 hrs. according to the standard methods of ISO 6579(1993). Swabs of teat and nose samples were inoculated into 10 ml. of PBW then incubated for 24 hrs. at 37°C (23). Hands of milker samples were done by moistening the sterilize cotton swab by BPW then rolled over the palm of hands, area between fingers tips and

nails then incubated for 24 hrs. at 37°C according to the standard methods of ISO 6579 (1993).

Laboratory Diagnosis: The specimens were transported to the laboratory directly. The diagnosis was performed by directly inoculated on to plated of Mannitol Salt Agar (MSA) and blood agar then incubated at 37 ºC for 24 hrs. All colonies from primary cultures were purified by subculture onto MSA medium and incubated at 37 ºC for 24- 48 hr (24). Gram stain and other biochemical test such as, catalase test, oxidase test, coagulase test, clumping factor test and heamolysin production; were done according to (25,26). The VITEK 2 is an automated microbiology system utilizing growth-based technology. The system accommodates the same colorimetric reagent cards that are incubated and interpreted automatically. It is focused on the clinical microbiology laboratory and provide increased levels of automation and capacity for higher volume laboratories. It also provides an option of automatic pipetting and dilution for antimicrobial susceptibility testing.

Antibiotics susceptibility testing: The antibiotics susceptibility testing done according to method of Kirby and Bauer (27) using disc diffusion method.

Table (1): Types of antibiotics and their concentrations

No	Antibiotic	Code	Concentration	Origin
1	Ampicillin	AM	25 mcg	Bioanalys, Turkey
2	Amoxicillin	AX	25 mcg	Bioanalys, Turkey
3	Erythromycin	ER	15 mcg	Bioanalys, Turkey
4	Ceftriaxone	CRO	10 mcg	Bioanalys, Turkey
5	Clindamycin	CD	10 mcg	Bioanalys, Turkey
6	Ciprofloxacin	CIP	5 mcg	Bioanalys, Turkey
7	Tetracyclin	TE	30 mcg	Bioanalys, Turkey
8	Vancomycin	VA	10 mcg	Bioanalys, Turkey
9	Rifampin	RA	5 mcg	Bioanalys, Turkey
10	Trimthropin Sulphamethoxide	SXT	25 mcg	Bioanalys, Turkey

Molecular Study using PCR technique: Bacterial DNA extracted according to manufacture of bacterial extraction kit (Geneiad). All *Staphylococcus* isolates had been grown in 5ml. of LB broth overnight at 37°C for DNA extraction. Genomic DNA was amplified by using the primers given in table 2. These primers were used to amplifying the *ermA, ermB, ermC and msrA* genes. From each sample extracted bacterial DNA 5µl. were amplified by PCR technique with specific primers and cycling conditions as described previously (28). Detection of the PCR amplified product, was done by electrophoresis on the agarose gel at 1%. 4 µl of PCR product inoculated in each well of agarose gel. The molecular weight of PCR amplified product, was determined according to 100bp ladder after 60 min at 70 volts. The PCR amplificated product examined under UV light (29). Sequences of Primers for genes were used in this study in table(2), according to (28).

Table (2) Sequences of Primers for genes used in this study:

Primer	**Primer sequences (5'-3')**	**Product size (lengh)**
ermA	F: AAGCGGTAAACCCCTCTGA R: TTCGCCATTTGGGGAGACT	190
ermB	F: CTATCTGATTGTTGAAGAAGGATT R: GATAGACTAACAACTTCTTCCTAA	142
ermC	F: AATCGTCAATTCCTGCATGT R: TTAGCAGTTAAGGACGTACA	299
msrA	F: TCCAATCATTGCACAAAATC R: AGGTTAGTAACGTGTTTTAG	163

Laboratory Protocol :

The reagents which were added for each PCR tube of master mix displayed in table (3).

Table (3): PCR reaction (25µl).

Mixture content	Volume
Blue master mix	5 µl
Primer forward	1µl
Primer reverse	1µl
DNA template	5µl
Nuclease free water	13µl
Total	25 µl

To avoid contamination; all reagents should be taken with separate tip. The contents were mixed thoroughly by shaking and spin, a lighted vortex was used. PCR tubes were transferred to preheated thermo cycler under reported condition in (Table 4).

Table (4): PCR amplification conditions for genes.

No	Step	Temperature	Time	Cycle
1.	Denaturation	95 ºC	3 Minutes	1cycle
2.	Denaturation Annealing Extension	95 ºC 54 ºC 72 ºC	30 Second 30 Second 30 Second	35cycle
3.	Extension	72 ºC	4 Minutes	1cycle

RESULTS

Isolation of CoNS strains: The results of this study obtained by cultural characteristics and bacterial identification, that's based on culture, the suspected colonies of CoNS were smooth, round, raised, glistening, gray to deep golden yellow and white in color plate, While the colonies on blood agar plates appeared large, round creamy/buff colored colonies which appear β or Alpha-haemolysis, after that by Gram stain the smear of colonies showed clusters or different irregular shape Gram positive cocci. All isolates were positive to catalase test and negative for oxidase and coagulase tests (table 5).

Table (5): The biochemical tests and their results for CoNS

Test	Results of 145 Isolates
Gram stain	100% positive
Catalase	100% Positive
Oxidase	100% Negative
Coagulase	88% Nagative
DNase	100% Positive
Hemolysis behavior	100% Positive

Out of 200 different samples 108 isolates were coagulase negative (54%). Twenty-two isolates of meat 55%, 18 isolates of cow's milk (36%), 10 isolates of treated milk (33%), 26 isolates of the nose of the animal (65%) and 32 isolates of animal teat swabs (80%), table (6).

Table (6): Number and percentage of staphylococcal positive according to type of samples

Samples type	No. of samples	Posative results	Posative results %	Percentage%
Meat	40	22	55	15.1
Raw milk	50	18	36	12.4
Treated milk	30	10	33.3	6.3
Nasal swap	40	26	65	17.9
Teat swap	40	32	80	22.0
	Chi-square (χ^2	---		

** (P<0.05).

Twenty five coagulase-negative staphylococci, according to biochemical tests, were subjected to Vitek 2 kit. There were 4 species of CoNS according to this test (table 7).

Table (7): Number and percentage of CoNS isolates which identified by Vitek 2 system

Species	Number of isolates	Percentage
S. lentus	4	40%
S. gallinarum	4	40%
S. chromogen	1	10%
S. haemolyticus	1	10%
Total	10	40%

Antibiotics susceptibility test: Twenty fife isolates of CoNS from different sources in the current study were examined for their susceptibility to 10 different antibiotics. Resistance were recorded toward different antibiotics like cip, te, and ra (table 8).

Table (8): Antibiotic susceptibility patterns of CoNS isolates

Susceptibility	SXT	RA	VA	TE	CIP	CD	CRO	ER	AX	AM	total
Resistance no.	5	6	4	6	13	3	1	5	1	1	45
Moderate no.	4	17	13	7	5	12	14	16	6	10	104
Sensitive no.	16	2	8	12	7	10	10	4	18	14	99

Resistance of CoNS strains toward erythromycin were investigated also. The results showed 20% of strain were resistant to this antibiotic (table 9).

Table (9): Results of CoNS susceptibility to erythromycin

Type	No.	Percentage (%)
Resistance-R	5	20%
Moderate-M	16	64%
Sensitive –S	4	16%
Total	25	100%
Chi-square ((χ^2	---	

** (P<0.05).

PCR results: The DNA was extracted from (25) CoNS isolates using the conventional methods, The DNA extraction results were accepted, concentration and purity were determined using nano drop 1000 spectrophotometer at 260/280nm. When the *ermA*, *ermB*, *ermC* and *msrA* gene were investigated by PCR the result showed that they contain the genes in a percentage 5%, 20%, 20% and 5% respectively. By using the multiplex PCR molecular weight technique (*ermA, ermC)* and (*msrA, ermC*), the ratio of both genes was 15% and 5% respectively (fig. A & B).

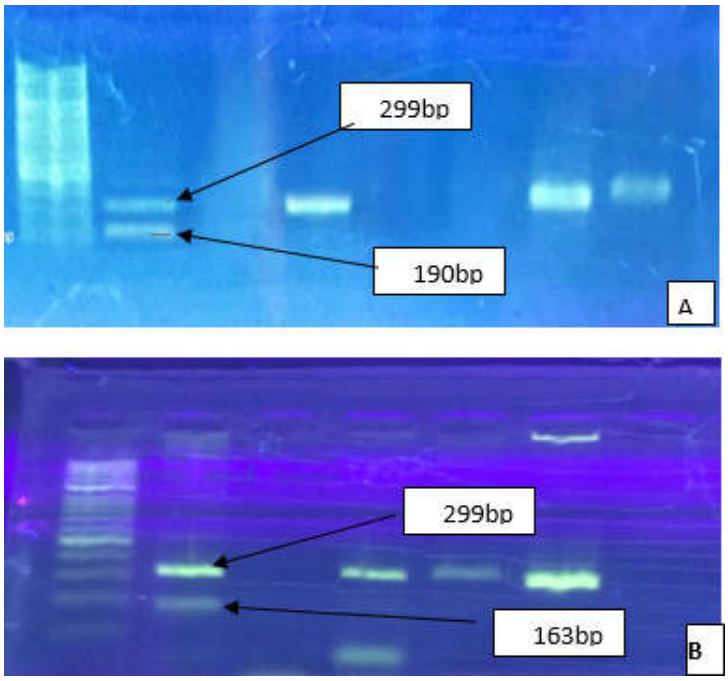

Figure. Agarose gel electrophoresis of PCR amplification products of CoNS (1%). A. ermA 190 bp, *ermC* 299 bp. B. *msrA* 163bp , *ermC* 299 bp with 100bp DNA ladder.

DISCUSSION

Several biochemical tests were carried to identify the CoNS the results showed that all the isolates of CoNS 100% positive for Catalase. In order to support the previous biochemical test, DNase tests and tube coagulase tests were carried out. DNase production was detected by culturing the isolates on DNase agar, DNase is an extracellular enzyme that cleaved DNA into subunits composed of nucleotides (Oligonucleotides). The appearance of clear zone around bacterial growth was considered as the positive activity that indicated the presence of Deoxyribonuclease enzyme hydrolyses DNA (30). Most of isolates of *Staphylococcus* produced DNase enzyme. Generally DNase degrades the host DNA and that increases the invasiveness and pathogenicity of staphylococci that possess it (31). Identified species level by the VITEK 2 system allows the identification of medically important CoNS organism in 15 hours due

to a sensitive fluorescence-based technology and allows a result to be generated without the need for a morphological assessment (32).

Most the isolates were sensitive and some its moderate to most of the antibiotics used in this study. The resistance may be due to the fact that some antibiotics cannot penetrate the outer membrane which might decrease the permeability of the drug (33) stated in his report that the resistance rate of CoNS from to ampicillin, were 91.4 , 74.4 , 60.0 and 2.8 % respectively. However, all isolates of CoNS were resistant to ampicillin corroborating the findings of (34) who documented that CoNS from often produce β-lactamases and are resistant to ampicillin and that multiply resistant strains may limit antibiotic choice. The resistance of CoNS from to amoxicilline may be due to the common use of this antibiotic in treatment to most clinical infections. This result is in line with that of (35) who stated that 50% of CoNS was sensitive to Amoxicillin while most of the isolates in our study were sensitive and moderate. The study by ceftriaxone agreed with (36) who reported multi-drug resistant bovine CoNS has a chromosomal class C β-lactamase that makes treatment outcomes with most cephalosporins unpredictable , and that conifer ampicillin resistance (37).

In molecular study PCR negative control was carried out in each experiment to determine any contamination and due to careful employment of laboratory techniques all negative controls appeared as empty gel lanes through all experiments of optimization. Each reaction was repeated twice and only reproducible bands were considered for analysis (38,39,40). *ermB* gene investigated in the present work exhibited typical gene attribute could be used for the genotypic characterization of isolates of this species and resistance. The protein *ermB* gene segments encoding the region are known to consist of a variable number of small repeats (41). It is thought that extend the N-terminal *ermB* binding portion of the protein through the cell wall. It was interesting to note that isolates from the same farm exhibited polymorphism and resistance in among the CoNS. The ability of CoNS to adhere to extracellular matrix proteins is thought to be essential for the colonization and the establishment of infections (42). In the present study, CoNS isolates from Cow a were found to and human differ in their gene patterns. Phenotypic and genotypic characterization might provide a better understanding of the distribution of the prevalent CoNS clones among isolates. The results of multiplex pcr were agree with

more recently study by (43) that MRSA gene was detected in 39/39 (100%) of CoNS isolates and *ermB, ermC, ermA* was detected in 35/39 (89.7%) and all strains were positive for *msrA* gene 35/35 (100%) expressed this gene during infection. (45) found that the presence of the Erm A, gene was detected in 53.9% of all strains, while the *msrA* gene was present in 5.3% which are much lower than present findings.

كشف مضاعف للجينات المقاومة للاريثرومايسين في المكورات العنقودية السالبة التجلط والمعزولة من الابقار في البصرة، العراق

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الخلاصة

أجريت هذه الدراسة للتعرف على جينات مقاومة الاريثرومايسين في المكورات العنقودية السالبة التجلط (CoNS) وتوصيفها الجزيئي بعد عزل البكتريا من عينات الحيوانات الأليفة المتمثلة بالابقار ومنتجاتها خلال الفترة الممتدة من سبتمبر ٢٠١٦ إلى مارس ٢٠١٧ من مناطق مختلفة في مدينة البصرة. تم جمع ٢٠٠ عينة من الحيوانات منها: ٤٠ عينة من اللحوم و ٨٠ عينة من الحليب (٥٠عينة من الحيوان مباشرة و ٣٠ عينة من الحليب المباع في الاسواق) و ٤٠ عينة من مسحات انف الحيوان نفسه و ٤٠ عينة من مسحات حلمة ثدي الحيوان.

وأظهرت النتائج أنه من بين ٢٠٠ عينة تم جمعها كانت ١٠٨ عينه هي مكورات عنقودية سالبة لانزيم التجلط موزعة على ٢٢ (١٥.١٪) و ١٨ (١٢.٤٪) و ١٠ (٦.٨٪) و ٢٦ (١٧.٩٪) و ٣٢ (٢٢٪) لكل من اللحوم والحليب المباع وحليب الحيوان نفسه، مسحات الأنف ومسحات الحلمة على التوالي. زرعت العينات على وسط المانيتول الملحي لعزل المكورات العنقودية *Staphylococcus* والتي كانت قادرة على النمو على الوسط المذكور.عندما تم إجراء اختبار التجلط، كانت بعض العزلات غير قادرة على إنتاج انزيم التجلط، وأظهرت النتائج أن ١٠٨ عزلة كانت سالبة (CoNS)(٥٤٪). وعشرين عزلة من اللحم المفروم ٥٥٪ و ١٨ عزلة من حليب البقر (٣٦٪) وعشر عزلات من الحليب المباع (٣٣٪) و ٢٦ عزلة من أنف الحيوان (٦٥٪) و ٣٢ عزلة من مسحات حلمة الحيوانات ٨٠٪). تم تحديد ٢٥ عزلة من هذه المكورات العنقودية السالبة باستخدام جهاز VITEK 2 kit وقد اظهرت النتائج ان ١٠ عزلات (٤٠٪) تم تحديدها على أنها مكورات عنقودية سالبة التجلط وتمثلت في أربعة أنواع منها ٤ (٤٠٪) *S. lentus* و ٤٪ ٤٠ *S. gallinarum* و ١٠٪١ *S. hemolyticus* و ١٪ ١٠ *S. chromogen*). وعند اجراء فحص تفاعل سلسلة البوليمريز(PCR)على الجينات *ErmA, ErmC, ErmB* , و *MsrA* المحمولة على البلازميد والمسؤولة عن مقاومة الايرثرومايسين أظهرت النتائج أنها تحتوي على الجينات في نسبة ٥٪، ٢٠٪، ٢٠٪ و ٥٪

على التوالي،وباستخدام تقنية وزن الجزيئات المتعددة (*ErmA* ، *ErmC*) و (*MsrA*، *ErmC*)، كانت النسبة لكلا الجينين ١٥٪ و ٥٪ على التوالي.

REFERENCES

1. **Anacarso, I; Condò, C; Sabia, C; Messi, P; de Niederhausern, S ; Bondi, m and Iseppi R**(2013). Antimicrobial Resistance and Other Related Virulence Factors in *Staphylococcus* spp isolated from Food, Environmental and Humans in Italy. Universal Journal of Microbiology Research 1(1): 1-9 Pp.
2. **Matthews, K. R.;Harmon, R. J. and Langlois, B. E.** (1992). Prevalence of *Staphylococcus* species during the periparturient period in primiparous and multiparous cows. J. Dairy .Sci. 75:1835-1839.
3. **Taponen, S.;Simojoki, H.; Haveri, M. ; Larsen, H. D. and Pyörälä, S.**(2006). Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. Vet. Microbiol. 115, 199-207.
4. **Devriese, L. A. and H. Dekeyser.** (1980). Prevalence of different species of coagulase-negative staphylococci on teats and in milk samples from dairy cows. J. Dairy. Res. 47:155-158.
5. **Baba, E.; Fukata, T. and Matsumoto, H.** (1980). Ecological studies on coagulase-negative staphylococci in and around the bovine udder. Bull. Univ. Osaka Pref. B 32:69-75.
6. **Chesneau, O.; Morvan, A.; Aubert, S.; and El Solh, N**. (2000**)**. The value of rRNA gene restriction site polymorphism analysis for delineating taxa in the genus *Staphylococcus*. Internal. J. Systematics and Evoltionary Microbiol., 50: 689-697.
7. **Kloos, W.E.** (1980) Natural populations of the genus Staphylococcus Annual Review of Microbiol, 34:559–592.

8. White, D.G.; Harmon, R.J.; Matos, J.E.S. and Langlois, B.E. (1989). Isolation and identification of coagulase negative *Staphylococcus* species from bovine body sites and streak canals of nulliparous heifers. J. Dairy Sci., 72:1886-1892,

9.Nagase, N.; Sasaki, A.; Yamashita, K.; Shimizu, A.; Wakita, Y.; Kitai, S. and Kawano,J.(2002). Isolation and species distribution of staphylococci from animal and human skin. J .Vet. Med Series A 64, 245-250**.**

10. Matos, J.S.; White, D.G.; Harmon, R.J. and Langlois , B.E. (1991). Isolation of *Staphylococcus aureus* from sites other than the lactating mammary gland. J. Dairy Sci. 74:1544-1549.

11. Owens, W. E.; Ray, C. H.; Watts, J. L. and Yancey, R. J.(1997). Comparison of success of antibiotic therapy during lactation and results of antimicrobial susceptibility test for bovine mastitis. J. Dairy Sci. 80: 313–317.

12-Khudor, M.H. ; Abbas, B.A. and Idbeis, H.I. (2012). Detection Of Enterotoxin Genes Of *Staphylococcus aureus* Isolates From Raw Milk . *Bas. J. Vet. Res.,* 11(1):254.

1**3-Abbas B. A. ;Khudor, M.H. and Idbeis, H.I.** (2013). Iinvestigation of the activity and pathogenecity of *Staphylococcus aureus* enterotoxin c by ligated ileal loop assay in rabbits. Bas.j.vet.Res. 12,.2,(2):104.

14-**Khudaier ,B.Y.; Abbas, B.A. and Khudaier,A.M.(**2013). Detection of Methicillin Resistant *Staphylococcus aureus*Isolated from Human and Animals in Basrah Province / Iraq.MRVSA , 2(3):12-21.

15-Abbas, B.A.;Khudor, M.H. and Hanoon, B. M.(2014). Isolation And Identification Of *Staphylococcus Aureus* From Bovine And The Detection Of Its Coagulase Gene (*Coa*) Using Polymerase Chain Reaction (PCR).*Sc. Res. Assays.*, 9(20):864-868.

16-Abbas, B.A.;Khudor, M.H. andHanoon, B. (2016).The Relationship Between Biotype,Serotype, Antibiotic Susceptibility And Coa Gene Polymorphism In *Staphylococcus aureus* Isolated From Bovine.*Vet. Med., Assiut Univ., Egypt*,17:33.

17. Abbas, B. A. B. Y. Khudaier, A. M. Khudair.(2017). Studies on *mecA* gene in methicillin resistant Staphylococcus aureus isolates. Jokull Journal, 58-65.

18. Gao, J; Ferreri, M; Yu, F; Liu, X; Chen, L; Su, J and Han, B.(2012). Molecular types and antibiotic resistance of *Staphylococcus aureus* isolates from bovine mastitis in a single herd in China. Vet. J. 192, 550-552.

19. Ortegad, C; Revilloc, M.J; Zarazaga, M and Torresa, C.(2012). Identification of novel vga(A)-carrying plasmids and a Tn5406-like transposon in meticillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* of human and animal origin. Int. J. Antimicrob. Ag. 40, 306-312.

20. Malachowa, N and DeLeo, F.R.(2010). Mobile genetic elements of *Staphylococcus aureus*. Cell. Mol. Life Sci. 67, 3057-3071.

21. Khan, S.A; Nawaz, M.S; Khan, A.A and Cerniglia, C.E.(2000). Transfer of erythromycin resistance from poultry to human clinical strains of *phylococcus aureu*s. J. Clin. Microbiol. 38, 1832-1838. of Sta

22. Honkanen – Buzalski, T.; Myllys,v; and Pyrolla .S .(1994).Bovine clinical mastitis due to Coagulase- Negative Staphylococci and their susceptibility to antimicrobials .J .Vet .Med., 41:344-350.

23. Quinn, P.J. ; Marky, B.K. ; Carter, M.E. ; Donnelly, W.J. and Leonard, F. C. (2004).Veterinary microbiology and microbial disease. Printed and bound in Great Britain by international Ltd. padstow-Cornwall.

24. Talan, D. A.; Staatz, D. ; Staatz, A. ; Goldstein, E. J. C. ; Singer, K. and Ocrturf, G. D. (1989**).** *Staphylococcus intermidius* in canine gingival and canine-infected human wound infections: Laboratory characterization of newly recognized zoonotic pathogen. J. Clin. Microbiol. 27:78-81.

25. Macfaddin, J. F. (2000). Biochemical tests for identification of medical bacteria. 3rd Ed. Lippincott Williams and Wilkins USA.

26. Collins; C.H.(2004). A text book of microbiological methods 8th edition .Arnold London UK.

27. Bauer AW, Kirby WM, Sherris JC, Turck M. (1966). Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 45:493-496.

28. Duran, N., Ozer, B. Duran, G.G., Onlen, Y. and Demir C. (2012). Antibiotic resistance genes & susceptibility patterns in staphylococci. Indian J Med Res 135:389-396 (2012).

29. Sambrook, J; Fritsch, E. F.; and Maniatis, S. (1989). Molecular cloning 2nded Cold spring Harbor Laboratory press, N. Y.

30. Gaasbeek, E.J.; Wagenaar, J.A.; Guilhabert, M.R.; Wo¨sten, M.M.S.; van Putten, M.J.P. ; van der Graaf-van Bloois, M.L. ; Parker, C.T. and van der Wal , F.J.(2009). A DNase encoded by integrated element CJIE1 inhibits natural transformation of Campylobacter jejuni. J. Bacteriol. (191) pp: 2296–2306.

31. Brooks, G.F.; Carroll, K.C.; Butel, J.S. and Morse,S.A.(2007). (Jawetz) Melnick and Adelbergs Medical Microbiology, 24 th .ed. The McGraw-Hill Companies, Inc., New York, 224-232.

32. Graf B., Adam T., Zill E., Göbel U.B.(2000). Evaluation of the VITEK 2 system for rapid identification of yeasts and yeast-like organisms. J Clin. Microbiol. 38(5):1782-1785.

33. Ghostaslo R, Ghorashi Z, Nahaei MR.(2007). Klebsiella pneumonia in neonatal sepsis :A 3 –year study in the pediatric hospital of Tabriz, Iran. *Jpn. J. Infect.* Dis.; 60 :126-128.

34. Elliot T, Hastings M, and Desselberger U.(1997) Lecture Notes on Medical Microbiology. 3rd ed. Blackwell Science.p.53.

35. Al-Bayaa YJ. (2005) Bacteriological Septicemia in Newborn infants. University of Baghdad/College of medicine, M .Sc. Thesis .Baghdad.

36. Saraswathi K, De A,(1995) Gogate A, and Fernandes AR. Citrobacter Sepsis in Infants. *Ind Pediatr*. 1995; 32 : 359-362.

37.Norskov-Lauristen N, Sandvang D, Hedegaard J, Fussing V, Mortensen KK, Sperleng-Petersen HU and Schonheyder HC.(2001). Clonal Origin of

Aminoglycosides –resistance of *Citrobacer freundii* Isolates in a Danish Country. *J. Med. Microbiol*.; 50 : 636-641.

38. McPherson, M.J. and MØller, S.G. (2001). PCR The basic background to bench. Cornwell Press, Trowbridge, UK.

39. Sambrook, J. and Russell, D.W. (2001). Molecular Cloning. In: "A Laboratory Manual". Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

40. Roux, K.H. (2009). Optimization and Troubleshooting in PCR. *Spring Harb. Protoc.* doi:10.1101/pdb.ip66.

41. El-Sayed A, Alber J, Lmmler C, Bonner B, Huhn A, Kaleta EF, Zschۏck M. PCR-(2005) based detection of genes encoding virulence determinants in Staphylococcus aureus from birds. J Vet Med B Infect Dis Vet Public Health, 52, 38-44.

42. Fitzgerald JR, Hartigan PJ, Meaney WJ, Smyth CJ.(2000). Molecular population and virulence factor analysis of Staphylococcus aureus from bovine intramammary infection. J Appl Microbiol, 88, 1028-1037.

44. Contreras, A., D. Sierra, A. Sanchez, J. C. Corrales, J. C. Marco, M. J. Paape, and C. Gonzalo. (2007). Mastitis in small ruminants. Small Ruminant Res. 68:145-153.

45. Taponen, S., J. Björkroth, and S. Pyörälä. (2008). Coagulase-negative staphylococci isolated from bovine extramammary sites and intramammary infections in a single dairy herd. J. Dairy Res. 75:422-429.