Iraqi Journal of Veterinary Sciences, Vol. 35, Supplement III, 2021 (121-126)
Proceedings of the 13th (2nd International) Scientific Conference, College of Veterinary Medicine, University of Baghdad

Iraqi Journal of Veterinary Sciences

www.vetmedmosul.com

Molecular and phylogenetic analysis of methicillin resistant *Staphylococcus aureus* isolated from subclinical mastitis in lactating ewes

Z.A. Ahmed and A.A. Yousif

Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

Article information

Article history:
Received October 11, 2021
Accepted November 24, 2021
Available online December 16, 2021

Keywords:
Subclinical Mastitis
Staphylococcus aureus
mec A
California mastitis test

Correspondence:
A.A. Yousif
afaf.a@covm.uobaghdad.edu.iq

Abstract

This study was conducted for estimation of subclinical mastitis prevalence caused by *Staphylococcus aureus (S. aureus)* in apparent normal lactating ewes in the areas around Baghdad (Al-Rasheed, Al-Yousifya, Al-Latifya). 400 milk samples from 200 ewes were collected and examined by California mastitis test (CMT), Bacteriological examination was done by culturing on Blood agar, Mannitol salt agar, Staph-110 agar and Chrome agar then bacteria examined by Gram stain and biochemical test. Antibiotic sensitivity test and molecular assay by PCR were done to detect methicillin- resistant strains with the phylogenetic study. The results showed that 49/400 samples gave positive to CMT in the field and positive to *S. aureus* isolation. The percentage of subclinical mastitis in ewes was 12.25% in this study. All isolated bacteria were resistant to methicillin 100%. The result of PCR revealed that 16.32 % (8/49) of the MRSA were possess *mecA* gene, while no isolate carried the *mecC* gene, PCR-product for 8 positive samples of *mecA* target gene were sequenced, analyzed, and reported in Genbank-NCBI and obtained accession number and became a reference to Iraq and the Middle East and the world. Seven samples that match the global results by 100%, and one sample contains a mutation at the site 1539, Nucleotide. Our conclusion revealed that detection of subclinical mastitis in sheep very important caused by *S. aureus* and all *S. aureus* was resistant to methicillin and some strains possess *mecA* gene. However, absence of *mec A* in other MRSA isolates requires detecting the alternative genetic possibilities related to the resistance profile and fragment of gene used.

DOI: 10.33899/ijvs.2021.131854.2013,

Introduction

Mastitis defined as a mammary gland infection characterized by physical, chemical, and usually bacteriological changes in milk and glandular tissue abnormalities (1). It is an important disease economically and its subclinical mastitis is difficult in the diagnosis, which makes it mitigation more challenging (2,3). *Staphylococcus aureus* is Gram-positive, non-spore-forming, facultative anaerobes that grow by aerobic or by fermentation, non-motile, arranged as irregular "grape-like clusters cocci, pairs or tetrads. It is characterized by production of golden pigment. Typical biochemical identification test includes positive catalase to differentiate from Streptococci and Enterococci. Coagulase test is a guide to differentiate between *Staphylococcus aureus* from coagulase-negative *Staphylococcus* spp and ferment Mannitol (4). There are numerous studies in Iraq on mastitis in sheep, a survey study by (5) revealed the prevalence of *Staphylococcus* subclinical ovine mastitis at a percentage 26.9%. Hassan and Yousif (6) found the 12.22% of subclinical mastitis in ewes in Al Anbar Province. Many articles showed that *S. aureus* is one of the most important bacteria causing mastitis in dairy animals including sheep (7,8). California mastitis test used as a quick field assay to determine subclinical mastitis of ewes and other animals, its

reagent lyses the membrane of somatic cells including WBCs in the milk and precipitate the protein and DNA as a gel, the amount of gel formation reflects the numbers of WBCs in the milk sample (9). Resistance of *S. aureus* to beta-lactam antibiotics was firstly seen in penicillin by the production of penicillinase which hydrolyzed the beta- lactam ring in penicillin. However, methicillin was introduced to substitute penicillin resistance, therefore, methicillin-resistant *S. aureus* (MRSA) strains developed and this has been mediated by the *mecA* gene (10).

There are several techniques were developed for the detection of MRSA including phenotypic (disk diffusion test) (11) and genotypic assay, such as *mecA* and *mecC* genes detection by Polymerase chain reaction (PCR), PCR is a reliable, accurate and confirmatory technique for the identification of pathogens especially *S. aureus* recovered from mastitic milk samples from animals and the genes *mecA* and *mecC* was the code for methicillin-resistant *Staphylococcus aureus* (12,13). Phylogenetic analysis was used to determine the evolutionary history of the species, the genes or proteins. Understanding the phylogenetic relationships between the organisms is a prerequisite of almost any evolutionary studies, as contemporary spp. all share a history through their ancestry. Moreover, Phylogenetic analysis is important because of its wide range of applications that includes understanding genomic organization, epidemiological studies, protein functions, and deciding genes to be analyzed in comparative studies (14, 15).

This study aimed to isolate MRSA from Subclinical mastitis in lactating ewes and molecular study of these isolates with recorded phylogenetic properties of *mec A* gene of the isolates to compare it with strains in the world.

Materials and methods

Animals and samples collection

This study was conducted on 200 lactating ewes located around Baghdad (Al-Rasheed, Al-Yousifya, Al-Latifya) during a period between December, 2019 to December, 2020.

The Ewes were examined clinically, the udders were washed with clean water then dried by clean tool, later the teat disinfectant with 70% ethanol, the foremilk was discarded and then the milk samples (400) were collected from each half separately in Labeled sterile test tubes under aseptic condition. Then California mastitis test was done on all collected samples at the field (4).

The samples were transported immediately to the laboratory of the College of Veterinary Medicine/ Department of Internal and Preventive Medicine in Baghdad by ice box. Milk samples were examined physically to record the changes in color, odor and consistency (4).

Bacteriological examination

All bacterial examination was done according to (4). 400 milk samples were cultured on blood agar and Mannitol salt agar, then incubated at 37ºC / 24 hours. Identification of *S. aureus was* done according to colony morphology and Gram stain finally suspected colony sub-cultured on different selective media, Staph-110 agar and Chrome agar (Biomedia company). Then Biochemical tests were done **(**Catalase test, Oxidase test, Urease test, Coagulase test, Gelatin medium and O/ F glucose test) were done to confirm the diagnosis of *S. aureus.*

Antibiotic sensitivity test

Isolated *S. aureus* isolates were examined for detection of sensitivity to methicillin by using disc diffusion test (4), the antibiotic inhibition zone estimated as mention by the Clinical and Laboratory Standards Institute (11).

DNA Extraction

The isolation of DNA genomic from bacterial growth done according to ABIO pure protocol extraction. The purity and concentration of the final template DNA after extraction were measured by Quantus Fluoro meter.

Primers

Two primers were used *mecA* and *mecC* were used in this study (Table 1). These primers were supplied by (Macrogen Company) in a lyophilized form. Dissolved in a nuclease-free water, to give a final concentration of (100 pmol/µl) as a stock. The working solution of primers was prepared by adding 10µl of primer stock solution which (stored at freezer -20◦ C) to 90µl of nuclease- free water to get a working primer solution of 10pmol/µl.

Table 1: Primers used in this study

Primers	Sequences	Size
mecA	F AAAAGATAAATCTTGGGGTG R CCTTGTTTCATYTTGAGTTC	525
mecC	F CAGCCAGATTCATTTGTACC R AACATCGTACGATGGGGTAC	486

PCR Program

Steps of PCR cycle/s were recorded in tables 2 for different primers (*mec A, mecC*). Only one difference in the annealing time at 55ºC for primer *mecC*. After PCR amplification -Agarose gel electrophoresis was adopted to know the presence of amplification, PCR was dependent on the extracted DNA criteria completely.

Standard Sequencing

PCR products were sent for (Sanger sequencing) using ABI3730XL, the automated DNA sequences, by (Macrogen Corporation)/ Korea. The results were received by email then, analyzed using geneious software 9.

Table 2: Steps of PCR cycles for the two primers

Steps	ºC/ Time	Cycles
Initial Denaturation	95 / 5 minutes	1 cycle
Denaturation Annealing Extension	95 / 30 second 55/50 second 72 / 30 second	30 cycles
Final extension Hold	72 / 7 minutes 10 minutes	1 cycle

DNA loading

PCR products were loaded. 10µl was loaded in well. Ethidium bromide has stained the bands in gel were visualized using camera.

Results

Bacteriological examination

The cultural results showed different morphological shapes and color of *s. spp*. on different media, on blood agar the colonies appeared round and opaque colonies with β-hemolysis. On Staph 110, the colonies were convex, round, mucoid and white in color, while on mannitol salt agar appeared mucoid, round, convex and change the color of media to yellow. On the selective Chrome agar *S. aureus* produce green colonies. On microscopic examination the bacteria appeared as Gram-positive cocci, irregular clusters either pairs or tetra, these bacteria were non spore-forming and non-motile.

Percentage of subclinical mastitis

A total of 400 milk samples were examined for detection of a percentage of subclinical mastitis by CMT and bacteriology, the results showed that 49 samples were positive for both *S. aureus* isolation and CMT. The distribution of subclinical mastitis on ewes and halves basis were 14.5% and 12.25% respectively (Table 3). In addition, all isolates of *S. aureus* (49 samples) were resistant to Methicillin antibiotic.

DNA extraction

The 49 isolates of *S. aureus* were successfully extracted to obtain genomic DNA and this DNA which loaded on the agarose 1.2%, produced sharp, clear and pure bands (Figure 1).

Confirmatory detection of MRSA by PCR

Results of amplification on the extracted DNA by PCR by using two types of primers *mecA, and mecC* revealed that the 8 out of 49 MRSA gave positive results to *mecA* (16.32%) (Figure 2) while *mecC* gene not detected in any isolates of *S. aureus* (Figure 3).

Table 3: Percentage and number of subclinical mastitis on ewes and halves basis

Mastitis	No. ewes	No. halve	No. (%) subclinical mastitis	
			ewes basis	Halves basis
Subclinical	200	400	29/200 (14.5%)	49/400 (12.25%)

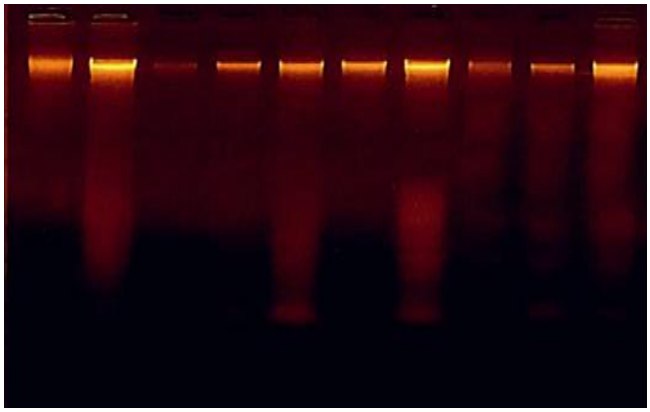

Figure 1: DNA expulsion and quantitation.

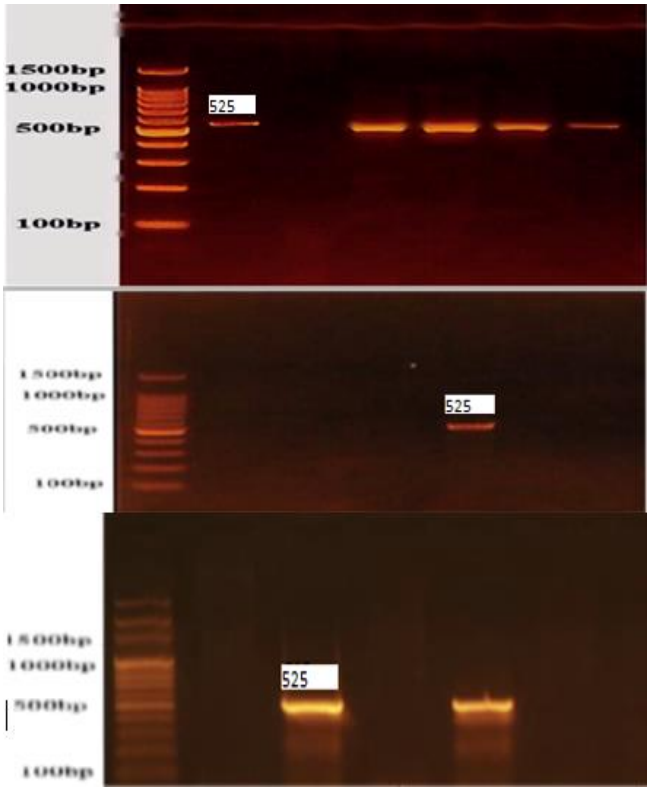

Figure 2: Agarose gel electrophoresis/ showing amplification of 525bp fragments of mecA gene gave eight positive result to amplificated *S. aureus.*

Figure 3: Agarose gel electrophoresis/ showing the amplification of 586bp fragments of gene *mecC* which give negative result to amplificated *Staph aureus.*

Sequencing

Eight samples positive to *mecA* were taken for gene analysis, and the result was seven samples that match the global results by 100%, and one sample contains a mutation at the site 1539, Nucleotide A/G, Nucleotide change ATA/GTA, Amino acid change Isoleucine/ Valine and Predicted Effect Missense; the second at the site 1626, nucleotide A/G, nucleotide change AAA/AAG, Amino acid change Lysine/ Lysine and Predicted Effect Silent. The evolutionary tree was drawn using mega and NCBI program, and it appeared when compared with the world that there were seven samples that matched the results of the mentioned countries by 100%, while the similarity of one Iraqi isolate with Russia- Kazan was 99 % (Figure 4).

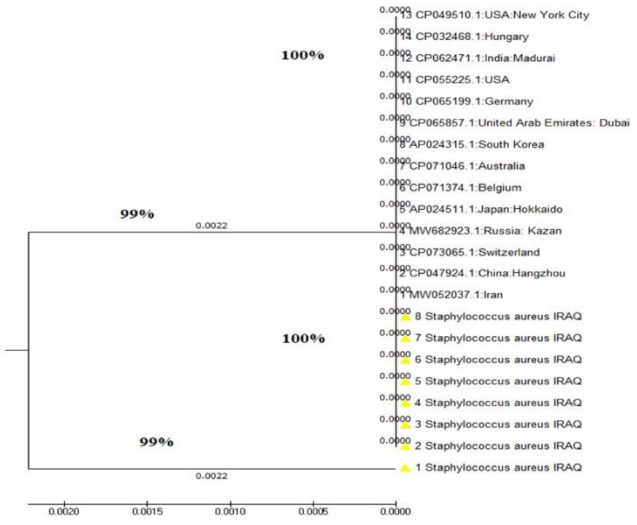

Figure 4: Neighbor-joining tree *Staphylococcus aureus* aminoglycoside acetyltrans ferase (mecA) gene.

Discussion

Mastitis is one of the important diseases of sheep which reduces the productivity of dairy animals. The dominant form of mastitis was Subclinical in sheep, which resulted in decreased milk yield, which lead to growth retardation and a higher mortality rate among suckling kid and lambs (2,16). In this study, the prevalence of Subclinical mastitis was 12.25%. according to CMT and bacterial isolation. Similar results were recorded by (6) who found a percentage 12% of SCM in ewes. In contrast, our finding was lower than those reported by (17) who found the prevalence of subclinical mastitis in ewes 51.1%. Gebrewahid and colleagues (17) reported a high prevalence of SCM reached 28.14% in sheep by using CMT positive milk samples and bacterial isolation. *Staphylococcus aureus* isolates were found in 49 out of 400 (12.25%). A Similar isolation rate was also recorded 12.22% previously (6). Cortimiglia and coworkers (18) found the most prevalent species was *Staphylococcus aureus* 20.14% in sheep milk and isolated *S. aureus* prevalence 43% in bulk tank milk in sheep, while (5) found that the isolated *Staph. aureus* bacteria a higher percentage 26% of SCM in sheep.

Our conclusion this difference in the prevalence of SCM in ewes may be due to many factors such as management especially in Iraq they used an open system of living for sheep, also environmental condition which was hot for long time of year and ewes were not highly producing milk. In this study, all isolated *S. aureus* strains were conducted to methicillin antibiotic sensitivity test, and for detection of *mecA and mecC* genes using PCR assay which are considered a genetic marker used for rapid and direct confirmation of MRSA. The genotypic analysis of 49 S. *aureus* isolated from ewes milk, showed that 8 (16.32%) had *mecA* genes. These results in agreement with (19) who found that not all *Staph. auerus* were carry *mecA.* Also this finding is in compatible with data recorded by (20) in Turkey who isolate 93 *Staph. aureus*, 16 isolate (17.2%) were MRSA. All 49 isolates of MRSA showed negative results to *mecC*. These results different from the study by (21) they represents the detection of *mecC*-positive in MRSA isolation and would confirm that sheep might was a *mecC* MRSA reservoir, they thought *mecC*-positive MRSA is difficult to confirm by routine diagnostic methods which employed for mecA-positive MRSA, so diagnosis laboratories should be aware of searching for the *mecC* gene. Worthwhile the *mecA* and *mecC* negative MRSA in the current study may contribute to the presence of other ß lactam resistances such as found of blaZ genem slao may depend on the fragment of sequencing. Our suggestion for control of SCM due to MRSA, must be screened lactating ewes by chemical test for detection of SCM and must treat these animals on the basis of antibiotic sensitivity tests to reduce resistance to the antibiotics. Furthermore, other studies on the *Staphylococcus spp*. explore resistant genes to antibiotics in addition to the *mecA* gene due to its importance in public health.

Our results of the phylogenetic analysis showed that all *Staph aureus* were compatible 100% with other rescuers, strains in japan (22) with ID: P024511.1 found methicillin resistant *S. aureus* isolates were isolated from dogs, environment, dog patients and humans. In Hungary, MRSA

with ID: CP032468.1 revealed that Hungarian *Staph. aureus* isolates 60% were recovered previously from wounds, blood, tracheas, ears, lungs, nostrils, skins and throats while in German *S. aureus* isolates 40% were recovered from body sites (23). Compatibility showed 99% with strains of *S. aureus* with ID: MW682923.1 isolated in Russia: Kazan by (24) they isolated *Staphylococcus aureus* from the patients with chronic infections of the upper respiratory tract and the middle ear.

Conclusion

The finding of these results demonstrate the importance of *S. aureus* in the causes of sheep subclinical mastitis. All isolated *Staph. aureus* was resistant to methicillin antibiotic but only 8 strains possess *mecA* gene. However, the detecting of *mec A* in some isolates and the absence in other MRSA isolates requires detecting the alternative genetic possibilities related to the resistance profile and fragment of gene used.

Acknowledgments

This work was supported by Dept. of Internal and Preventive Vet. Medicine, College of Vet. Medicine, University of Baghdad, Iraq.

Conflict of interest

Authors declares, no conflict of interest

References

1. Constable PD, Hinchcliff KW, Done SH, Grünberg WA. Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs, and goats. 11th ed. St. Louis: Elsevier; 2017. 1904-2001 p.
2. Knuth RM, Stewart WC, Taylor JB, Bisha B, Yeoman CJ, Van Emon ML, Murphy TW. Relationships among intramammary health, udder and teat characteristics, and productivity of extensively managed ewes. J Anim Sci. 2021;99(4):59. DOI: 10.1093/jas/skab059
3. Mohammed ZA. Association between clinical and subclinical mastitis and reproductive performance of cows at Nottingham dairy center. Iraqi J Vet Sci. 2021;35(2):343-50. DOI: 10.33899/ijvs.2020.126843.1398
4. Markey B, Leonard F, Archambault M, Cullinane A, Maguire D. Clinical veterinary microbiology. NY: Elsevier Health Sciences; 2013. 105-119 p.
5. Hammadi KM, Yousif AA. Prevalence of clinical and subclinical ovine mastitis caused by *Staphylococcus aureus*. Al-Anbar J Vet Sci. 2013;6 (1):57-64. [available at]
6. Hassan MS, Yousif AA. Alteration of some enzymatic activities in whey of ewe's milk Suffered from Staphylococcal mastitis. MRSVA. 2013;2(2):8-15. [available at]
7. Al-Manning, Vasileiou N, Crilly J. Control of mastitis in dairy sheep and goats. Livestock. 2021;26 (3):161-8. DOI: 10.12968/live.2021.26.3.161
8. Al-Graibawi M, Hassan I, Yousif A. Intramammary and systemic antibiotic therapy of bacterial clinical mastitis in cows. Iraqi J Vet Med. 2002;26(2):153-60. [available at]
9. Pradiee J, da Rosa Moraes C, Goncalves M, Vilanova MS, Correa GF, Lauz OG, Osorio MTM, Schmidt V. somatic cell count and california mastitis test as a diagnostic tool for subclinical mastitis in ewes. Acta Sci Vet. 2012;40(2):1-7. [available at]
10. Guo Y, Song G, Sun M, Wang J, Wang Y. Prevalence and therapies of antibiotic-resistance in *Staphylococcus aureus*. Frontiers in cellular and infection microbiology. 2020;10:107. DOI: 10.3389/fcimb.2020.00107
11. CLSI (Clinical laboratory Standard Institute), Performance Standards for Antimicrobial Susceptibility Testing", Twenty seven edition; CLSI Supplement M100.Waye. PA; clinical laboratory standard institute, 2017.
12. Sheet OH, Hussein SA, Al-Chalaby AY. Detection of methicillin-resistant *Staphylococcus aureus* from broiler carcasses in Mosul city. Iraqi J Vet Sci. 2021;35(3):489-493. DOI: 10.33899/ijvs.2020.127052.1451
13. Saeed ZK, Abbas BA, Othman RM. Molecular identification and phylogenetic analysis of lactic acid bacteria isolated from goat raw milk. Iraqi J Vet Sci. 2020;34(2):259-63. DOI: 10.33899/ijvs.2019.125896.1176
14. Som A. Causes, consequences and solutions of phylogenetic incongruence. Brief Bioinformat. 2014;16(3):536-548. DOI: 10.1093/bib/bbu015
15. Soulieman N, Al-Mariri A, Al-Atrash F. Detection of Shigella in raw bovine milk by polymerase chain reaction. Iraqi J Vet Sci. 2020;34(1):9-16. DOI: 10.33899/ijvs.2019.125758.1146
16. Moroni P, Pisoni G, Varisco G, Boettcher P. Effect of intramammary infection in bergamasca meat sheep on milk parameters and lamb growth. J Dairy Res. 2007;774(3):340-4. Doi: 10.1017/S0022029907002506.
17. Gebrewahid TT, Abera B, Menghistu H. Prevalence and etiology of subclinical mastitis in small ruminants of Tigray regional State, north Ethiopia. Vet World. 2012;5(2):103-9. DOI: 10.5455/vetworld.2012.103-109
18. Cortimiglia C, Bianchini V, Franco A, Caprioli A, Battisti A. Prevalence of *Staphylococcus aureus* and methicillin-resistant S. aureus in bulk tank milk from dairy goat farms in Northern Italy. J Dairy Sci. 2015;98(4):2307-11. DOI: 10.3168/jds.2014-8923
19. Shrestha A, Bhattarai RK, Luitel H, Karki S, Basnet HB. Prevalence of methicillin-resistant *Staphylococcus aureus* and pattern of antimicrobial resistance in mastitis milk of cattle in Chitwan, Nepal. BMC Vet Res. 2021;17(239):1-7. DOI: 10.1186/s12917-021-02942-6
20. Turkyilmaz S, Tekbiyik S, Oryasin E, Bozdogan B. Molecular epidemiology and antimicrobial resistance mechanisms of methicillin-resistant *Staphylococcus aureus* isolated from bovine milk. Zoonosis Public Hth. 2008;57:197-203. DOI: 10.1111/j.1863-2378.2009.01257.x
21. Giacinti G, Carfora V, Caprioli A, Sagrafoli D, Marri A, Giangolini A, Amoruso R, Iures cia M, Stravino F, Dottarelli S, Feltrin F, Franco A, Amatiste S, Battisti A. Prevalence and characterization of methicillin-resistant *Staphylococcus aureus* carrying mecA or mecC and methicillin-susceptible *Staphylococcus aureus* in dairy sheep farms in central Italy. J Dairy Sci. 2017;100(10):7857-7863. DOI: 10.3168/jds.2017-12940
22. Ishihara K, Shimokubo N, Sakagami A, Ueno H, Muramatsu Y, Kadosawa T. Occurrence and molecular characteristics of methicillin-resistant *Staphylococcus aureus* and methicillin-resistant *Staphylococcus pseudintermedius* in an academic veterinary hospital. Applied Envron Microbiol. 2010;76(15):5165-74. DOI: 10.1128/AEM.02780-09
23. Naorem RS, Urban P, Goswami G, Fekete C. Characterization of methicillin-resistant *Staphylococcus aureus* through genomics approach. Biotech. 2020;10(9):1-19. DOI: 10.1007/s13205-020-02387-y
24. Tyurin YA, Lysovskaya SA, Kulikov SN, Reshetnikova ID, Isaeva GS, Morzunov SP. Nucleotide polymorphism in the iron utilization system gene isdB NEAT domain affects heme-binding ability of IsdB protein in various human strains of *Staphylococcus aureus*. Bio Nano Sci. 2018;8:390-393. DOI: 10.1007/s12668-018-0501-z

دراسة جزيئية وتحليل التطورات الجينية للمكورات العنقودية الذهبية المقاومة للميثاسلين المعزولة من التهاب الضرع تحت السريري في النعاج الحلوب

زينب علاء احمد و عفاف عبدالرحمن يوسف

فرع الطب الباطني والوقائي البيطري، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة

أجريت هذه الدراسة للكشف عن انتشار التهاب الضرع تحت الإكلينيكي الذي تسببه المكورات العنقودية الذهبية في نعاج طبيعية ظاهريا في المنطقة المحيطة ببغداد (الرشيد واليوسفية واللطيفية). تم جمع ٤٠٠ عينة من الحليب من ٢٠٠ نعجة وفحصها بواسطة اختبار كاليفورنيا لالتهاب الضرع والفحوصات البكتريولوجية (باستخدام وسط الدم ووسط المانتول الملحي ووسط المكورات١١٠ ووسط الكروم، وبعدها فحصت البكتريا بصبغة جرام والاختبارات البايوكيميائية)، اختبار الحساسية للمضادات الحيوية والمقايسة الجزيئية باستخدام تفاعل البلمرة المتسلسل مع دراسة لنشوء والتطور. أظهرت النتائج ٤٠٠/٤٩ عينة أعطت نتائج إيجابية لاختبار كاليفورنيا في الحقل وموجبة لعزل المكورات العنقودية الذهبية باستخدام الاختبارات البكتيرية. وكانت النسبة المئوية لالتهاب الضرع تحت السريري ١٢,٢٥%، وكانت جميع العزلات مقاومه للمضاد الميثاسلين. أظهرت نتائج فحص البلمرة التسلسل بان ٤٩/٨ (١٦,٣٢%) من العزلات تمتلك مك أي جين بينما لم يتم الكشف عن جين مك سي في أي من العزلات. تم أرسال المنتج في تفاعل البوليمرات المتسلسل لثماني عينات إيجابية من الجين المستهدف مك أي لتحليلها والإبلاغ عنها فيبنك الجينات والحصول على رقم الانضمام وأصبح مرجعًا للعراق والشرق الأوسط والعالم. سبعة عينات تطابق النتائج العالمية بنسبة ١٠٠٪، وعينة واحدة تحتوي على طفرة في الموقع ١٥٣٩. أظهرت الاستنتاجات بأن الكشف عن التهاب الضرع تحت الإكلينيكي في الأغنام مهم جدا الذي تسببه المكورات العنقودية الذهبية وان جميع المكورات العنقودية الذهبية المقاومة للميثيسيلين وبعض السلالات تمتلك الجين مك أي. ومع ذلك، فإن غياب مك أي في العزلات الأخرى للمكورات الذهبية المقاومة للميثاسلين يتطلب الكشف عن الاحتمالات الجينية البديلة المتعلقة بملف المقاومة وجزء الجين المستخدم.