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Isolation, Antimicrobial Susceptibility and Molecular detection of *Staphylococcus aureus* from clinical mastitis in ewes

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ABSTRACT

The study aimed for isolation and molecular investigation of *Staphylococcus aureus* (*S. aureus)* isolated from clinical mastitis in ewes. 119 ewes located in Al-Rasheed, Al-Yousifya, and Al-Latifya in Baghdad city, were examined for systemic reaction and local examination of the udder. 238 ewes' milk samples were collected at aseptic conditions for bacterial isolation, antimicrobial susceptibility test and molecular assay by polymerase chain reaction (PCR) for *23srRNA* and *acc-aph* genes and phylogenetic analysis. Bacteriological isolation was done by culturing on Blood agar, Mannitol salt agar, Staph-110 agar, and selective Hi Chrome agar. The isolates were examined by Gram stain and different biochemical tests. From a total of 119 ewes and 238 teat halves examined, 10.08% and 7.31% were affected with clinical mastitis, respectively. Ten out of 238 (4.20%) samples were positive to *S. aureus.* The isolates were susceptible at 100% to Ciprofloxacin, Tetracycline and Neomycin, 80% to Amikacin, 50% Oxacillin, and highly resistance to Methicillin, Erythromycin and Penicillin at 100%, 80% and 70%, respectively. PCR assay revealed that all 10 *S. aureus* carried *23rRNA* and only two isolates (resist to amikacin) possess *acc-aph* gene. Sequencing of the 2 isolates positive to *acc-aph* contains a mutation and the evolutionary tree was drawn using MEGA and NCBI for analysis and compare of *aac-aph* gene, it appeared when compared with the world that two samples isolate from Iraqi have different from world as 99%.

1. INTRODUCTION

Mastitis is a mammary gland inflammatory infection described by physical changes, chemical changes, and bacteriological changes of milk andudder tissue abnormalities, it can be caused by several species of bacteria classified into 3 groups: environmental, contagious, and opportunistic pathogens [1]. Staphylococcus aureus is important contagiousmicro-organism causing mastitis due to combination of toxin-mediated virulence, invasiveness, and antibiotic resistance, It be a major causes of milk borne illnesses, raw milk is considered vehicles for Staphylococcus aureus which cause illness ranging in severity from a mild to severe dermal infections, such as septicemi, apneumonia to human and mastitis to animals

[2], [3]. Our previous studies in Iraq of clinical mastitis in ewes in Iraq revealed that the isolates rates of S. aureus were 57.60 %, 33.3% and 27.8% [4- 6]. [4] observed 3 types of clinical mastitis in ewes (acute, gangrenous and chronic) according to the causative pathogens. Antimicrobial resistance is a main problem obstructing treatment of an ever-rising range of infectious bacterial agent [7]. Numerous researchers reported resistance of Staphylococci for antimicrobials which used for control of mastitis in sheep and goats [7], [8]. The appearance of resistant bacteria in food manufacture may leading to the transmit resistance gene to indigenous microbiota to human gut [9]. A polymerase chain reaction (PCR) based method for identification of S. aureus was consisted of 4 parts: (a) DNA extraction (b) DNA amplification with primers against the gene 23S rRNA, and (c) evaluation of the specificity of the PCR by nested PCR and (d) Southern hybridization [10]. [11] isolate S. aureus by cultural methods and biochemical tests as well as PCR assay and sequencing of the 23S rRNA gene of Staph. aureus, they found that sequencing of 23S rRNA was a great appliance for the characterization S. aureus isolated from cows mastitis.

The aac (6) _ aph (2) gene, believed to be from fusion of aac and aph genes, and can be found on both plasmids and bacterial chromosome. The gene was initially found in S. epidermidis and Staph. aureus and then in clinical isolates of Enterococcus and Streptococcus, this gene encodes bi-functional enzyme aac – aph [12]. This study aimed for isolation, molecular detection with phylogentic analysis of S. aureus isolated from clinical mastitis in ewes.

2. Materials and methods

2.1 Animals and sample collection

This study were conducted on 119 lactating ewes located Al-Yousifya, Al-Rasheed, and Al-Latifya in Baghdad province (Iraq), during December (2019) to December (2020). Clinical systemic examination of ewes (temperature, pulse, respiratory rates and any other clinical signs), udders and supra mammary lymph nodes were inspected and palpated to detect any abnormality. Teats and udders washed by clean water, dried by clean towel and disinfected with alcohol 70%. Two hundred thirty eight ewes milk samples were collected from halves separately in sterile test tubes aseptically post discarding the fore milk and immediately transported in cool box to the laboratory of College of Veterinary medicine, Department of Internal and Preventive Veterinary Medicine [13].

2.2 Bacterial exanimation

The milk samples were streaked on blood agar enriched with 5-7% sheep blood, then incubated aerobically at 37°C for 24-48 hours, the suspected Staphylococcus colonies were subculture on selective media, Staph-110 agar, Mannitol salt agar and Chrome agar and incubated at 37 $\overset{o}{C}$ /24 hours. Suspected bacteria examined by gram stain, biochemical tests (Catalase test, Oxidase, Urease test, Gelatin, Oxidation / Fermentation glucose test and Coagulase test [13].

2.3 Antibiotic susceptibility test

Isolation of S. aureus was conducted to antibiotic susceptibility test for detection of sensitivity to different antibiotic by using disc diffusion test [13], the antibiotic inhibition zone estimated as mention by the Clinical and Laboratory Standards Institute [14].

2.4 PCR assay and sequencing analysis

The isolates were subjected to PCR assay in Internal and Preventive Veterinary Department Laboratory at College of Veterinary Medicine, University of Baghdad as following,

1. DNA was extracted from bacteria according to ABIO pure /protocol Extraction.

2. Quantification of DNA by using Quantus Fluorometer to detection the concentration of DNA.
3. Primers: Two primers were used 23sr RNA and aac-aph, supplied by Macrogen Company (Table 1).

Table (1): Primers applied to detect of S. aureus

Gene	Sequences (5`- 3`)	Product size	Reference
23srRNA	F: TCGGAATCTGGGAGGACCAT	350bp	Designed
	R: AATCGTAAGTCGGTTCGGTCC		
aac-aph	F: GAGCAATAAGGGCATACCAAAAATC	506bp	[15]
	R: CCGTGCATTTGTCTTAAAAAACTGG		

PCR program: thermo-cycle program for detection of the genes 23srRNA and acc-aph was performed: - 1 cycle /5 minutes at 95°C to denaturation of template. Then continues by 30 cycles, each cycle including the denaturation for 60 seconds at 95°C, 30 seconds for annealing: at 58°C, and extension for 60 seconds /at 72°C. Final extension was done 7 minutes at a degree 72°C, hold at 10 C for 10 minutes. After amplification, agarose gel electrophoresis was done to know existence of amplification. PCR was dependent on the DNA criteria.

PCR Product Analysis: this step used to complete of PCR assay, to analyses of the PCR product by the agarose gel electrophoresis, which stained with ethedium bromide, the bands were visualized by using the UV transilluminator and take a photographby digital camera.

2.5 Sequencing

PCR products of the isolates of S. aureus which resist Aminoglycosides antibiotic drug weresent for Sanger–sequencing by using ABI3730XL, the automated DNA sequences by the Corporation of Macrogen / Korea. The results were received by email then, analysis was done using the Geneious Software 9.

2.6 Ethical Approved

Information of animal owners about the aims o the study and consent was obtained from each animal owner before the examination of ewes for mastitis and collection of milk samples. The methodology for the study approved by ethics Veterinary Medicine College Committee / University of Baghdad.

2.7 Statistical analysis

Statistical analysis of the data was done by using the SAS [16]. Independent t test was used to assess the significant difference at $P < 0.05$.

3. Results

3.1 Clinical study of ewes

Clinical mastitis was detected in 12 out of 119 (10.08%) ewes, 2 with gangrenous mastitis and 10 ewes with acute mastitis. While bacterial examination of 119 lactating ewes (238 milk samples) showed the isolation percentage caused by S. aureus were 6.72% (8 out of 119) on ewes basis and 4.20% (10 out of 328 samples) on halves basis (Table 2)

Table (2): Isolation percentage of S. aureus mastitis

Total No. of examined ewes	Total No. of examined milk samples	No. of ewes with clinical mastitis	No. of examined milk samples from clinical mastitic halves	No. of infected ewes with *S. aureus*	No. of infected halves with *S. aureus*
119	328	12 (10.08%)	24 (7.31%)	8 (6.72%)	10 (4.20%)

Two types of clinical mastitis, the first was acute in 6 ewes which showed lameness because of painful udder swollen, redness, hotness and enlargement of supra mammary lymph node (Figure 1), only 8 halves from 6 ewes (2 with 2 halves and 4 with one halves) were infected with S. aureus. The second type was peracute gangrenous mastitis (Figure 2) in 2 ewes (1 half in each ewe), the signs were dullness, depression, and the udder was markedly swollen with red / black discoloration of skin (Table 3).

Table (3): Percentage of Staphylococcus aureus mastitis according to type of mastitis

Types		Total No. of examined ewes	No of infected ewes with *S. aureus*	Total No. of examined milk samples	No of infected halves with *S. aureus*
Clinical mastitis	Gangrenous	2	2	4	2
	Acute	10	6	20	8
Total of clinical		12	8 (66.66%)	24	10 (41.66%)

Figure (1): Acute mastitis

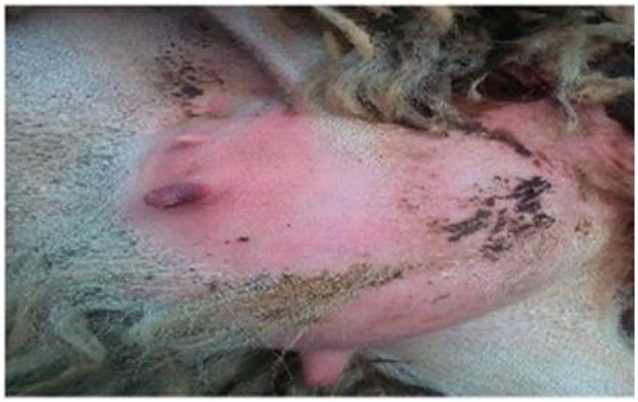

Figure (2): Gangrenous mastitis

Ewes with clinical mastitis (acute and gangrenous) showed systemic reactions such as increase in temperature (41.60±0.05), respiratory (92.83±0.36) and pulse rates (33.58±0.45) (Table 4).

Table (4): Systemic reactions in clinical mastitis and normal ewes

Reactions	Animals	Mean	P-value*
Temperature ºC	Normal apparent ewes	38.83±0.18	<0.0001
	Clinical mastitic ewes	41.60±0.05	
Pulse / minutes	Normal apparent ewes	26.00±1.21	<0.0001
	Clinical mastitic ewes	33.58±0.45	
Respiration	Normal apparent ewes	76.41±1.23	<0.0001
	Clinical mastitic ewes	92.83±0.36	

*Independent t test

3.2 Antibiotic susceptibility test

IsolatedStaph. aureusfrom ewes with clinical mastitis were sensitive 100% to Ciprofloxacin, Neomycin and Tetracyclin, 80% to Amikacin and Cefotaxime, 50% to Oxacillin, and 70% to Ampicillin. S. aureus were highly resistance to Methicillin and Penicillin at 100% and 70%, respectively. All isolates were susceptible to Tetracycline 100% (Table 5)

Table (5): Isolated S. aureus against antimicrobial disc

Antimicrobial group		Antimicrobial disc	Resistant (%)	Intermediate (%)	Susceptible (%)
Aminoglycosides		Neomycin (NE)	-	-	10 (100%)
B-Lactam	Cephalosporin	Amikacin (AK)	2(20%)	-	8 (80%)
	Synthetic Penicillin	Cefotaxime (CFM)	2(20%)	-	8 (80%)
		Oxacillin (OX)	5(50%)	-	5 (50%)
		Ampicillin (AM)	3(30%)	-	7 (70%)
		Methicillin (ME)	10(100%)	-	-
	Penicillin	Penicillin (P)	7(70%)	-	3 (30%)
Tetracycline		Tetracycline	0	-	10 (100%)
Macrolides		Erythromycin (E)	8(80%)	-	2 (20%)
Flourquinlone		Ciprofloxacin (CIP)	-	-	10 (100%)

3.3 Molecular assay

Confirmatory detection of Staph aureus by 23srRNA gene

All DNA extracted from isolates of S. aureus (10) were carried 23srRNA gene revealed that the amplified DNA was 350bp fragments (Figure 3).

Figure (3): PCR product analysis of 23srRNA showing amplification of 350bp fragments in S. aureus isolates on agarose gel electrophoresis.

Where Lane (M): Marker 2000-100bp, Lane (1-10): Positive isolates for 23srRNA gene

Detection of aac-aph gene

The results showed 2 isolates of S. aureus possess the gene aac-aph at amplification 506 bp fragments (Figure 4).

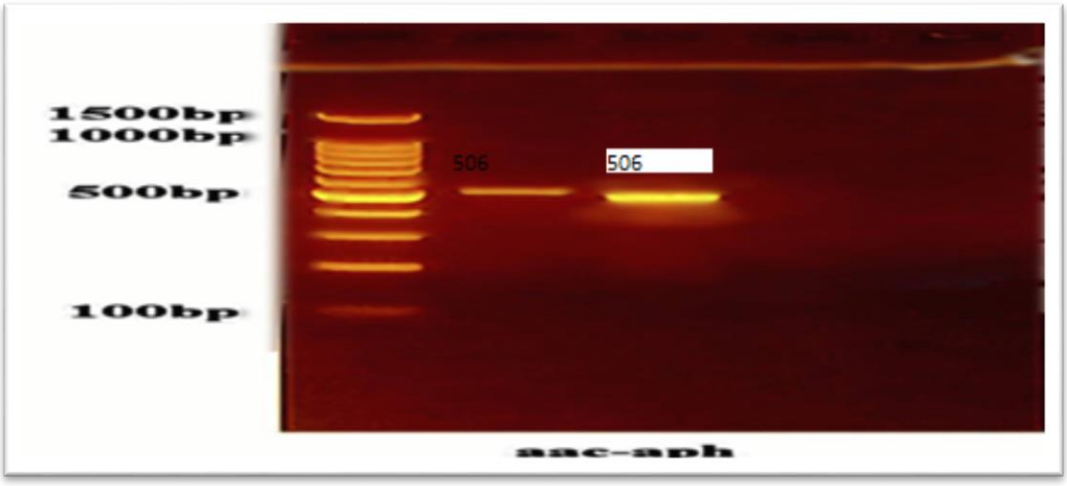

Figure (4): Agarose gel electrophoresis showing amplification of 506 bop fragments of aac-aph gene give two positive result to amplificated S. aureus

Sequencing

Two samples were taken for aac-aph gene analysis, and the result was both of them contains a mutation and have identities as 99% (Table 4).

Table (4): Represent type of polymorphism of S. aureus aminoglycoside acetyltransferase aac-aph gene

Source: *S. aureus*									
No. of sample	**Type of substitution**	**Location**	**Nucleotide**	**Nucleotide change**	**Amino acid change**	**Predicted effect**	**Sequence ID with compare**	**Gene**	**Identities**
1	---------	-------	--------	-------	-------	-------	**ID: LC377539.1**	**AAC**	**99%**
2	---------	-------	--------	-------	-------	-------	**ID: LC377539.1**	**AAC**	**99%**

The evolutionary tree was drawn using mega and ncbi program for analysis and compare of aac-aph gene, it appeared when compared with the world that Tow samples isolate from Iraqi have different from world as 99% (Figures 5 and 6).

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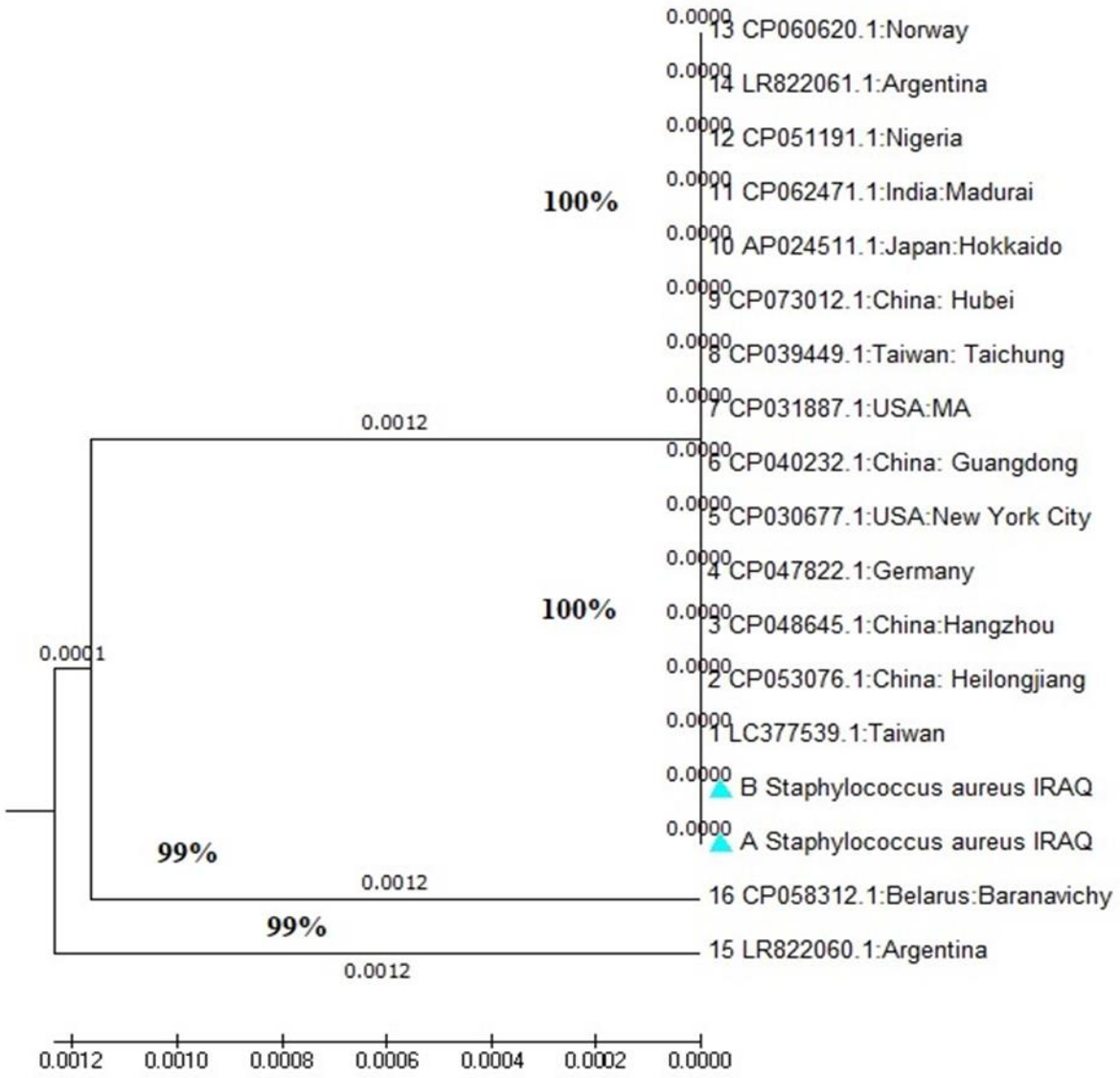

Figure (5): Neighbor-joining tree S. aureus aminoglycoside acetyltransferase aac-aph gene

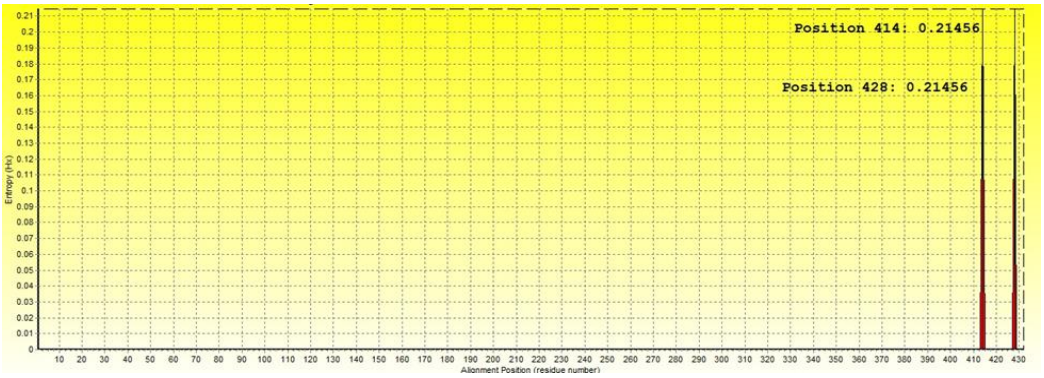

Figure (6): Position of mutation

4. Discussion

Mastitis is an economic disease in ewes and S. aureus is the most important causative agent of the clinical mastitis in lactating ewes [17]. The present results showed that clinical mastitis was detected in 12 out of

119 (10.08 %) ewes. These results are considered higher to other researchers who found that incidence rate is less than 7% in the lactation in ewes [18], [19]. Also, it lower than the results of [20] who reported the incidence of clinical cases of mastitis in lactation ewes is less than 5%. Our results showed that the percentage of S. aureus was (41.20%). These results were similar to study of [19] who reported that Staphylococci were responsible for 40% of cases of mastitis especially in meat production sheep flocks. [21] reported a percentage 22% of the clinical mastitis which affected by S. aureus. [22] in Norway mentioned that S. aureus was more prevalent as a cause of clinical ovine mastitis revealed a higher percentage 65.3% from clinically affected mammary glands. [23] reported a lower percentage of S. aureus isolation 5.3% of bacterial positive samples from ewes with mastitis. The current study revealed two form of clinical mastitis in ewes, acute and gangrenous mastitis, ewes with acute form showed signs of lameness, swollen painful udder, redness, hotness and enlargement of supra mammary lymph node. Similar findings recorded unilateral clinical mastitis systemic signs in ewes with moderate or severe systemic signs and in other ewes with a temperature above 40.0°C [24]. Gangrenous mastitis occurred in two ewes from 12 ewes affected with clinical mastitis, which showed markedly swollen udder with red/ black discoloration of skin. This compatible with other findings, [25] reported that gangrenous mastitis was one of the important form affecting ewes, it occurs sporadically especially in the first stage of lactation and affecting one or both halves of the mammary glands and characterized by anorexia and fever.

All staphylococcal isolates were susceptible 100% to Ciprofloxacin, neomycin and tetracycline 80% to Amikacin. In this study, the results showed multiple resistances to four antimicrobials, Oxacillin, Methicillin, Penicillin, Erythromycin. This is compatible with other study record that Staphylococcus isolates from milk samples of ewes with mastitis were characterized by multi-drug resistance [26]. In the current study, PCR assay was applied for characterization of S. aureus based on the amplification of 23S rRNA and acc–aph genes. No previous study reported the use of 23S rRNA and acc–aph genes in ewes affected with clinical mastitis, except studies in bovine by [27] depended on the amplification 16S-23S rRNA / intergenic-spacer by (RS-PCR) which was used for genotyping of S. aureus isolated from bovine in Switzerland.

In China, [28] observed aac-aphD genes in 23% of S. aureus isolates from clinical mastitis in cows between years 2014 and 2017. In addition, [29] detect the gene aph (3′)-IIIa in 3 isolates of S. aureus and the aac (6′) / aph (2″) combined with aph (3′)-IIIa gene in 1 isolate of S. aureus from cows mastitis in Turkey.

The results of phylogenetic analysis of the 2 S. aureus / isolates (MZ359749.1 and MZ359748.1) which resist Aminoglycosides (Amikacin) showed that these were compatible 100% with other rescuers: Taiwan, China: Heilongjiang, China: Hangzhou, Germany, USA: New York City, China: Guangdong, USA: MA, Taiwan: Taichung, China: Hubei, Japan: Hokkaidom India: Madurai, Nigeria, Norway, Argentina and 99% with Argentina, Belarus: Baranavichy [30]. Concluding that characterization of S. aureus genotypes in lactating ewes might assessed in planning strategies for decreasing of infection spread and for the treatments.

5. Conclusion

The results of the current study showed that characterization of S. aureus by cultural and biochemical tests in addition to amplification of the 23S rRNA specific to Staph. aureuswas a good tool for diagnosis for all 10 isolates from ewes clinical mastitis, sequencing of aac-aph gene showed identity 99% with the world. Regarding antimicrobial resistance genes, isolates resist Amikacin exhibited the aac-aph gene. The widespread of the antibiotics uses resulted in emergence of the multi-resistance bacteria.

6. Authors' contributions

Zainab Alaa Ahmed was responsible for clinical examination, collection of milk samples and bacterial examination, and Afaf Abdulrahman Yousif was responsible for diagnosis and statistical interpretation of obtained results. Both authors contributed equally in writing this paper.

7. Acknowledgment

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8. Conflict of interest

Reports of the author revealed no conflicts of interest in this paper.

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