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Using Real-Time PCR to Investigate Some of Antibiotic Resistance Genes from *Streptococcus agalactiae* Isolates from ewe Mastitis cases in Nineveh province

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Abstract

In this study, from a total of 856 mastitis cases in lactating ewes, only 34 Streptococcus agalactiae isolates showed various types of resistance to three types of antibiotics (Penicillin, Erythromycin and Tetracycline). St. agalactiae isolates were identified according to the standard methods, including a new suggested technique called specific Chromogenic agar. It was found that antibiotic bacterial resistance was clearly identified by using MIC-microplate assay (dilution method). Also, by real-time PCR technique, it was determined that there were three antibiotics genes resistance (pbp2b, tetO and mefA). The high percentage of isolate carried of a single gene which was the Tetracycline (20.59%) followed by percentage Penicillin was (17.65%) and the lowest was in Erythromycin (11.77%). However, there were many isolates that carried two genes of antibiotics resistance represented by Penicillin and Erythromycin with collective present of 38.22%, and for the Penicillin and Tetracycline, the percentage was found to be 11.77%. In contrast, no common gene with two antibiotics (Erythromycin and Tetracycline) was detected. On the other hand, it was found that no bacterial sharing with three kinds of antibiotic resistance genes (pbp2b, tetO and mefA). This study has revealed that the St. agalactiae isolates did induce recurrent mastitis in lactating Iraqi's ewes.

Key words : Antibiotics resistance, Animal public health, Mastitis, Real time PCR , Streptococcus spp.

Introduction

Mastitis is one of the most crucial diseases in lactating farm animals causing a subsational lose to sheep owners around the world (1). *Streptococcus agalactiae i*s one of the significant bacteria that cause inflammation of the mammary glands in ewes resulting in decreasing milk yeild. It is a Gram positive bacteria that belong to the beta group and called a Group B Streptococcus or GBS (2, 3). Furthermore, Streptococci are the general group of bacteria causing mastitis in sheep after Staphylococci species (4, 5). *S. agalactiae*, have many virulence factors responsible for pathogenicity, including aggregation factor, glutamine synthetase and others, such as those resistant to antibiotic as (Penicillin, Erythromycin and Tetracycline). Antibiotic resistance is considered a critical issue in treating many cases of mastitis, and they possess genes resistance whcih are randomly accquaired. Usually, bacteria become resistant for certain antibiotics by horizontal procuration of resistance genes through or transposons plasmids. It can take place by integrating DNA-resistance gene into the chromosome, and also reassortment DNA-nucleotides in several chromosomal site, thus resulting in mutations (6). This is due to a misuse of antibiotics in

treating mastitits as well as the grwoth factor in sheep nutrition. The mechansim of antibiotic resistance is directly in charge of many cases of diseases treated with penicillin, tetracycline, erythromycin and others (5, 7). Usually, in Nineveh region the commmon traditional treatment of sheep and goat mastitis is performed using antbiotics without sensevity test. Evenually, the bacterial become more adapted and the relive, accordingly, is less secure and more virulance.

The purpose of this study is to detect the presence or abcence of three differnet antibiotic resistance genes (*pbp2b*, *tetO* and *mefA*), already decided for this inevstigation. *S. agalactiae* isolates were isolated from lactating ewes, suffering from recurrent mastitis. Moreover, the realization of a minimum inhibitory concentration (MIC) of antibiotic resistance is to be an existed gene that carrying whole information about the influence of antibiotics on mastitis.

Materials and methods

Collections Samples

About 856 samples of milk were obtained from ewes mastitis cases from vet medical clinics at Nineveh province. The milk samples were placed into sterile test tubes and conveyed directly to the lab under low temperture via the ice bucket. Then, each sample was inoculated on a standard microbiological media.

Isolation and Identification

Firstly, one drop of milk sample was streaked out on blood agar and incubated at 37 °C for 24 hours. Then, the culture was examined with regard to positive Gram's stain and morphology (hemolytic beta group). Moreover, all positive suspected colony for streptococcus species were recultured on Luria Bertani agar (LB) at 30°C for 24-48 hours with 5% CO2. Then, a single colony was streaked out on spesfic anaerobic chromogenic agar at 37 °C for 24 hours inorder to idetitifed *S. agalactiae* in accordance with chromogenic agar test kit (CHROMagarTM StrepB) (8). Furthermore, all isolates were asserted by Christie-Atkins-Munch-Petersen test (CAMP) (9). Finally, all isolates were added 20% DMSO and kept at -80°C ultra-freezer. The steps of the current experiment procedures are illustrated as in Fig. 1.

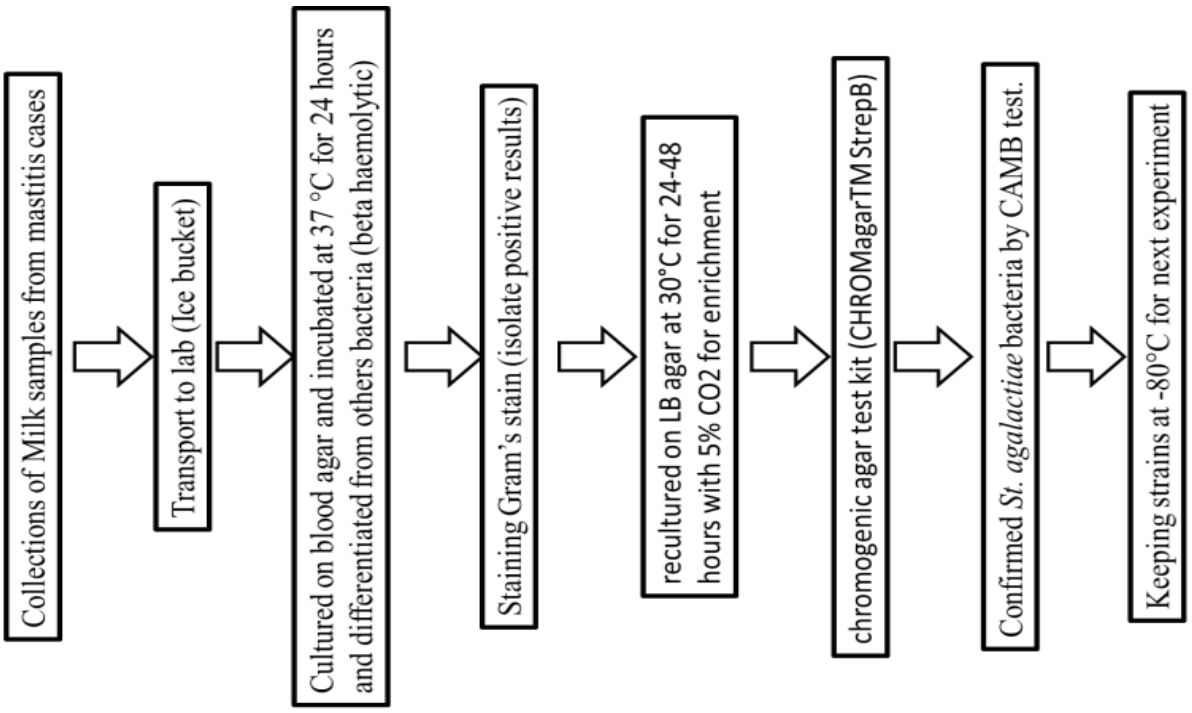

Figure 1: Step by step of the collection sample, bacteriological standards identified and preservation of the isolation of *S. agalactiae.*

Bacterial Activation

The entire 34 strains samples were directly taken from frozen -80°C, isolated from sheep suffered from mastitis cases across Nineveh province. All strains were reactiviated in 5mL LB broth and incubated at 30°C for 24-48 hours with 5% CO2 environment and shaked at 150 rpm.

Microplate MIC Assay

Microplate minimum inhibitory concentration assay for detecting isolates poossed antibiotic resisitance ablility by using the microdilution method according to Clinical & Laboratory Standards Institute protocol (CLSI) (10, 11). Cultures were prepeared to start growth at an OD600 = 0.01 in 3 ml of LB broth (5%CO2) and allowed to grow until reaching OD600= 0.15. Each microplate well was prepared with 10 µl of each of penicillin, tetracycline and erythromycin, starting dilution from (0.125,0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 µg/mL), then, they were compared to the control (without adding antibiotic). Each well was added to 190µl culture and the final volume was 200µl per well. The whole microplate was sealed with a gas-permeable membrane and incubated 30°C for 24 hours. The plate reader (Bio-Rad) was adjusted to measure the optical density. all results were analyzed with Graphpad Prism 6 program.

Real-time PCR technique

Genome extraction and purification

All strains were cultured on LB plate at 30°C (5% CO2) for 24 hours and then inoculate single colony into the 3 mL LB broth with specific antibiotic and grown up until reached OD600 =0.5 (12). Genomic DNA was extracted and purified using Promega genomic DNA and Purification kit in accordance with the manufacturer's protocol. The DNA concentration was valued by using Nanodrop NA1000.

Primers Design

Real-time PCR primers were designed By the National Center for Biotechnology Information website (NCBI). Each of the Penicillin *(pbp2b*), Erythromycin *(mefA*) and Tetracycline *(tetO*) genes was identified in the bacterial genome (Table 1). All primers were synthesized by Integrated DNA Technologies Company (IDT).

Table 1: Real time PCR primers for antibiotic resistance genes.

Gene	Primer Forward Sequence	Primer Reverse Sequence	References	Gene length(bp)
Erythromycin *(mefA)*	ATCACTAGTGC	ACTAAAAGTGG	In this study	1,218 pb
Tetracycline *(tetO)*	ATTCTGGCTCAC	ATATCGTCA	In this study	1,920 pb
Penicillin (*pbp2b)*	ATTCTCAGGTGG	ATAGGTGTTGG	In this study	2,058 pb

Diagnosis of antibiotic resistance genes (Real time PCR)

An experiment was carried out, using a Bio-Rad real time PCR machine to determine antibiotic gene resistance. Each well volume was 20 µl (1 µl forward and reverse primers 20 pmol / µl, 9 µl SYBR Green Promega Master Mix and 10 µl DNA).

Then, a standard curve was determined by using different serial dilutions of genomic (0.00032, 0.0016, 0.08, 0.04, 0.02, 0.1), in order to set a template DNA concentration inside the linear zone for unknown samples. Analysis of samples was used by ΔCT Real time PCR technique depending on the initiation frequency of template of DNA detection (13). All experiments were performed in biological triplicate bases. (statistical analysis)

Results

Isolation and Identification

Out of 856 diagnosed mastitis, it was observed that 112(13%) cases were identified as *Streptococcus agalactiae*, with 78(9%) sensitive for antibiotics. While, 34(4%) were resistant for the three different included 655(76%) Staphylococcus genus, whereas, 89(11%) identified as another sorts of bacteria. antibiotics (Penicillin, Tetracycline and Erythromycin). However, there were 744(77%) isolates(Table 2).

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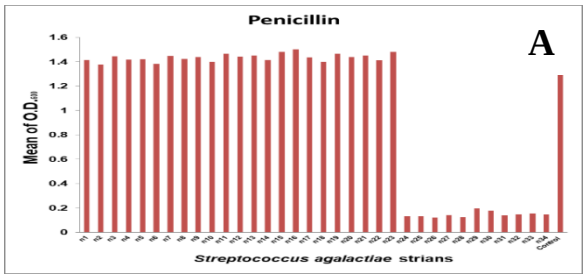
Table 2. Classification and the percentage of diagnosis

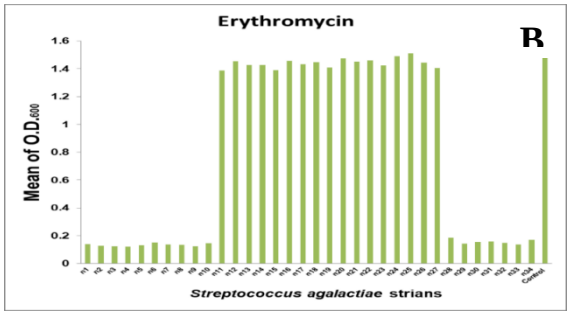
Diagnosed mastitis = **856 cases**	***Streptococcus agalactiae* =112 (13%)**		**Others causative agents =744(87%)**	
	Sensitive for Antibiotics	Resistance for Antibiotics	*Staphylococcus* genus	Other sorts of bacteria
	78(9%)	34 (4%)	655 (76%)	89(11%)

Microplate MIC Assay

Thirty four strains S*t. agalactiae* were tested for their ability to resistance and sensitivity, by using three different antibiotics (Penicillin, Erythromycin, Tetracycline). Microplate assay (MIC) has shown how some of strains have susceptible and resistance trends against one or two of the experimental antibiotics, simultaneously. The results have revealed 6(17.65%) of samples were Penicillin resistance, while, 4(11.77%) were resistance Erythromycin. Also, 7(20.59%) of samples were
resistance Tetracycline (Fig.2).

Furthermore, there were samples characterized by having resistant for more than one antibiotic. In terms of resistance Penicillin and Erythromycin, there were 13(38.22%) samples, whereas resistance Penicillin and Tetracycline were just 4(11.77%) from samples. In contrast, no resistance was found in terms of sharing Erythromycin and Tetracycline together. Also, as for the resistance the three types of antibiotics (Penicillin, Erythromycin, Tetracycline), no such case in the field of this study has been found (Fig.3).





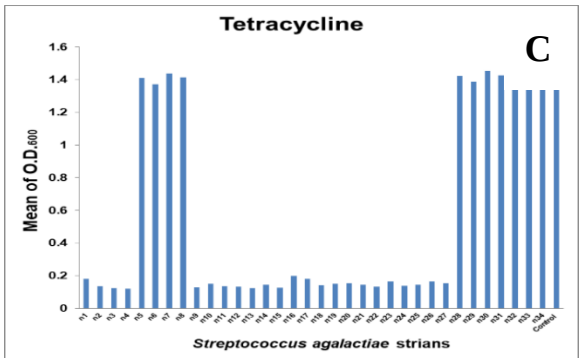


Figure :2 Micro-plate assay for testing 34 isolates of *S.agalactiae* about their ability to resistance or sensitivity against different concentrations of antibiotics. (A). 23 strains have resistance against Penicillin (B). 17 strains have resistance against Erythromycin (C). 11 strains have resistance against Tetracycline

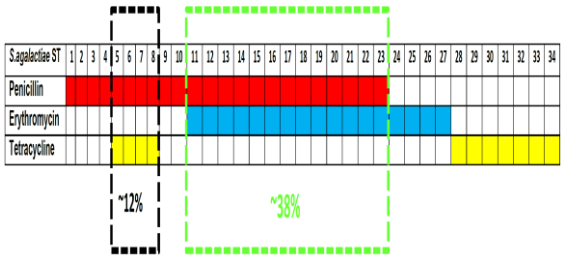


Figure : 3 The colour-codes illustrate some of S.agalactiae isolates have shared with two antibiotics resistance in particular Penicillin with Erythromycin (~38%) and penicillin with Tetracycline (~12%). However, there are no isolates have resistance Erythromycin and Tetracycline. While, no presence was observed any of isolations having resistance for three antibiotics together.

detection, through determining the antibiotic gene resistance (*pbp2b*, *tetO* and mefA) for 34 S.agalactiae isolates. DNA genome dilution series were examined as standard curves to identify the best of an average phase dilution (0.04), which gave the

optimal expression for this experiment. The gene *pbp2b* existed in 23 strains, while *tetO* gene was found in 11 strains. In terms of *mefA* the gene was present in 17 strains (Fig.4). Astonishingly, the results of Real time PCR for three genes were 100% compatible with Microplate MIC assay results for detection Penicillin, Tetracycline and Erythromycin resistance genes versus the minimal inhibitory concentration of antibiotics, respectively.

Moreover, the expression of stability for each antibiotic gene differs from others genes when compared to each other.. In terms of Penicillin, the time gene expression comes up after 4 minutes, while, the magnitude was 120 rate fluorescent units (RFU). In comparison the Pencillin, the period of Tetracycline gene expression was at 12 minutes with magnitude 60 RFU. On the other hand, in Erythromycin, the time of gene expression at 8 minutes with amplification size was 24 RFU (Fig.4)

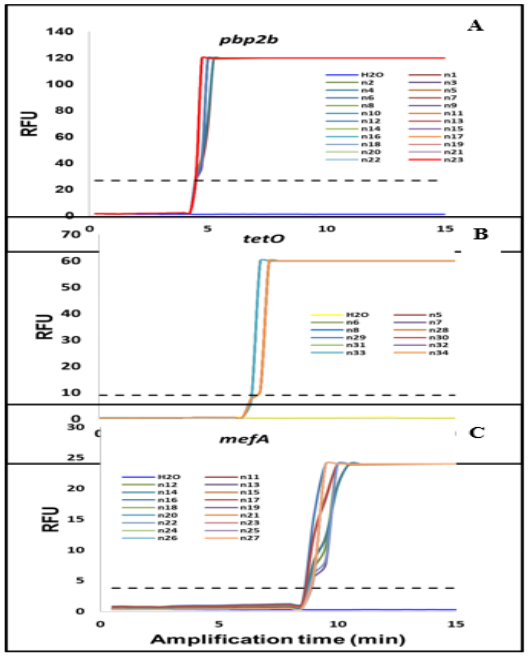


Figure :4 Real time PCR assay of three antibiotics resistance genes (*pbp2b, tetO and mefA*). A). 23 strains were found *pbp2b* gene. B) 11 strains were detected *tetO* gene. C) 17 isolates were detected *mefA* gene. Overall, there are different of log-phase for each gene that give us indication for detection specific antibiotic gene based on which concentration of genome used, and the amplification time.

Discussion

All positive isolates *S. agalactiae* showed resistance to different antibiotics phenotype (Penicillin, Erythromycin and Tetracycline). Also, the tests were confirmed by the Real time PCR technique based on Yuexia's method (5). Only three different genes (*pbp2b, tetO and mefA*) of antibiotics resistance were identified in 34 isolates distributed for high percentage starting Penicillin then Erythromycin and Tetracycline. Regarding Penicillin, 23 samples were first identified by using Microplate MIC assay that showed positive results. The 23 samples, after that, they were confirmed by Real time PCR technique where *pbp2b* gene was recognized, which is (*pbp2b* gene) certainly responsible for Penicillin resistance phenomena. It was found that 6 samples (17.65%) which possessed only one gene were resistant to the Penicillin antibiotic. This might be due to the fact that the sheep owners are over using Penicillin antibiotic to treat their sheep even for trivial reasons or for enhancing growth rate and milk production in such cases (14, 15). It appears that the genetic entity of the farm sheep under this study has gained some resistance to the Penicillin antibiotic (16-18). The 13 samples (38.22%) had positive resistance results to another antibiotic, the Penicillin shared resistance alone with Erythromycin. In turn, only 4 samples (11.7%) showed resistance to Tetracycline in conjunction with Penicillin, too. These results confirmed the fact that Penicillin antibiotic was over used by the owners which created bacterial resistance to this antibiotic (Penicillin). In this regards and from personal communication, according to (19, 20) it was observed that most mastitis cured cases by penicillin were ineffective.

Regarding the Tetracycline antibiotic, results of this study indicated that 20.59% of cases were positive which contained *tetO* gene only. It suggested that the dissimilarity in Tetracycline resistance genes might be due to possible variation in versions of Tetracycline available in the market. With respect to Erythromycin antibiotics, it is common that there are two erythromycin resistance genes *erm* and *mefA* (5). However, it was found in this study that only *mefA* gene was detected. The results of this study coincide with other findings (21).

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.

Ethical Clearance: The project was approved by the local ethical committee in Mosul University.

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استخدام تقنية RT-PCR للتحري عن الجينات المقاومة لبعض المضادات الحيوية من بكتريا *Streptoccous agalactiae* المعزولة من حالات التهاب الضرع في الاغنام في محافظة نينوى

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نبذة مختصرة
هذه الدراسة ، من بين مجموعه 856 حالة من حالات التهاب الضرع في النعاج المرضعة ، أظهرت 34 عزلة فقط من المكورات العقدية اكالكشيا أنواعًا مختلفة من المقاومة لثلاثة أنواع من المضادات الحيوية (البنسلين والإيثروميسين والتتراسيكلين). تم التعرف على عزلات المكورات العقدية اكالكشيا وفقًا للطرق القياسية, بما في ذلك تقنية جديدة مقترحة باستخدام الاكار الكروموجيني المتخصص. حيث حددت المقاومة البكتيرية للمضادات الحيوية باستخدام مقايسة الصفيحة الميكروية (بطريقة التخفيف). أيضا، تم استخدام تقنية تقنية الوقت الحقيقي لتفاعل سلسلة البوليمر, حيث تم تحديد أن هناك ثلاثة مقاومة لمورثات الجينات (*pbp2b* و*tetO* و *mefA*). اظهرت النتائج نسبة التتراسيكلين (20.59%) مرتفعة من بين العزلات التي تحمل جين واحد، يليها نسبة البنسلين (17.65%), بينما أدنى نسبة كانت في الإريثروميسين (11.77%). مع ذلك, كان هناك العديد من العزلات التي تحمل جينين مقاومة للمضادات الحيوية يتمثل بـ البنسلين والاريثروميسين حيث تشكل (38.22%), في حين كانت النسبة المئوية للبنسلين والتتراسيكلين(11.77%). في المقابل ، لم يتم الكشف عن أي جين شائع مع اثنين من المضادات الحيوية (الاريثروميسين والتتراسيكلين). من ناحية أخرى ، لم يتم العثور على اي نتائج بالنسبة لمشاركة مع ثلاثة أنواع من الجينات المقاومة للمضادات الحيوية معا *(pbp2b و tetO و mefA)*. كشفت هذه الدراسة أن عزلات المكورات العقدية اكالكشيا هي السبب الحقيقي وراء التهاب الضرع المتكرّر في النعاج العراقية المرضعة وخصوصاً في هذه الدراسة للبكتيريا التي تحمل جينات مقاومة للمضادات الحيوية المختلفة.

الكلمات المفتاحية : (مقاومة المضادات الحيوية ، الصحة العامة للحيوان ، التهاب الضرع ، تقنية الوقت الحقيقي لتفاعل سلسلة البوليمر, انواع المكورات العقدية.