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MOLECULAR DETECTION OF STAPHYLOCOCCI ISOLATED FROM MASTITIS IN SHEEP AND COWS IN THI- QAR PROVINCE

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ABSTRACT

The present study aimed to investigate the prevalence of mastitis in sheep and cows in veterinary hospital during the period from August to December, 2014 in Thi-Qar province, Iraq. From a total of 700 milk samples collected from both animals (150 sheep and 100 cows), a positive number of clinical and subclinical mastitis cases in sheep recorded an infection rates of 46 (30.7%) and 60 (40.0%), respectively. On the other hand, in cows, clinical and subclinical mastitis recorded a percentages of (52.0%) and (18.0%), respectively ($p \leq 0.01$). According to the age distribution of the infected animals, the age groups of (2-4) and (4-6) years recorded the highest mastitis rates in sheep and cows with 75(70.8%) and 55(78.5%) infection, respectively ($p \leq 0.01$). The highest mastitis cases in sheep and cows was recorded in November with 39(88.6%) and 40 (86.9%), respectively. Rural areas showed the highest mastitis infections for both animals with 149 cases (59.6%) in comparison to Urban areas who recorded less mastitis cases with 101 cases (40.4%). Identification of common bacterial species isolated from all mastitis cases was done depending on morphological, cultural, microscopic characterization and biochemical tests, then confirmed by API system. The results of antibiotics susceptibility test for *Staphylococcus aureus* and Coagulase negative staphylococci isolates showed a high rate of resistance to Pencillin, Oxacillin, Ciprofloxacin, Amoxillin/ Clavulanic acid with a percentage of (100%), (82%), (75.5%), (74.5%). On the other hand, *Staph aurues* and CNS isolates showed high sensitivity to Vancomycin, Piperacillin, and Nitrofuration(80.1),(76.4%),(73.5%), respectively ($p \leq 0.01$). Polymerase chain reaction (PCR) was used, as a molecular technique, to detect the prevalence of *mec* A, *Plaz* and 16SrRNA genes in CNS (n=64) and *Staph. aureus* isolates (n=42). The results revealed that all *Staph. aureus* and CNS isolates were positive for the three genes, except *Staph. xylosis* which showed a percentage of (93.7%) for *mec* A gene.

KEYWORDS: Staphylococci, Bovine Mastitis, Polymerase Chain Reaction

INTRODUCTION

Mastitis is an inflammation of the mammary gland characterized by physical, chemical, bacteriological and cytological changes in milk. Pathological changes in glandular tissues of the udder and effects on the quality and quantity of milk have been observed [1]. This disease is mainly caused by microorganisms usually bacteria, including gram-negative and gram-positive bacteria, mycoplasmas, yeasts and algae [2]. Bovine mastitis (mast = breast; itis = inflammation), a disease affecting dairy cattle worldwide results from the inflammation of the mammary gland. the severity of the inflammation can be classified into clinical, sub-clinical and chronic forms, and its degree is dependent on the nature of the causative pathogen, age, breed immunological health and lactation state of the animal [3]. Clinical

mastitis (CM) inflammation that results in visible abnormalities of milk or the gland is defined as clinical mastitis. Most symptoms of clinical mastitis are quite mild and cannot be detected unless foremilk is observed, thus the perceived incidence of clinical mastitis on individual dairy farms is dependent on the intensity of detection [4]. on the other hand, subclinical mastitis (SCM), in absence of any clinical sign, usually remains unnoticed. As a result of subclinical mastitis affected animals produce reduced amounts of milk compared to their true production potential [5]. The increase of coagulase negative Staphylococci infections causes loss of capacity and damage of mammary gland [6]. *Staphylococcus aureus* have been considered the most important pathogen among staphylococci causing mastitis, whereas coagulase-negative staphylococci (CNS) were considered minor pathogens [7]. However, recently CNS have become a significantly predominant pathogens in bovine mastitis, and considered the most common cause of subclinical mastitis in many countries [8, 9, 10].

Staphylococcus aureus colonize the nipple skin, advance through the mammary gland canal into the gland. The Intramammary infection (IMI) with *S. aureus* predominantly cause subclinical mastitis resulting in a chronic infection lingering lifelong [11, 12].

Staphylococcus aureus is one of the main pathogens isolated from contagious bovine mastitis cases in many countries. Although *S. aureus* is isolated less frequently from heifers than coagulase-negative staphylococci [13].The former continues to be an important pathogen due to its difficult control and high prevalence of antimicrobial resistance [14]. antimicrobials therapy has been an effective strategy for controlling CNS IMI, it is important to monitor antimicrobial susceptibility of CNS causing mastitis. β-lactams are important antimicrobial agents used for the prevention and treatment of mastitis in dairy cows [15], and penicillin is recommended as the first choice for bacteria that inherently sensitive to it [16]. However, efficacy of this treatment could be compromised by staphylococci through the production of *blaZ*-encoded β-lactamases and through the production of *mecA*-encoded alternative penicillin binding protein 2A (PBP2A), which shows a reduced binding to all β-lactam antibiotics [17].

MATERIALS AND METHODS

Sample Collection

A total of 700 milk samples were collected from 250 animals (150 Sheep, 100 Cows) during the period from August to December 2014 in Thi-Qar Province after a quarter had been cleaned up by removing any possible dirt and washed with tap water, the teat end was dried and swabbed with cotton soaked in ethyl alcohol. Approximately 100 ml of milk was collected aseptically into sterile bottles after discarding the first 3 milking streams. Milk sample from each quarter were transported to the animal production research center laboratory in ice cooled box at 4 ºC and analyzed immediately (max.4h after collection) either for identification of the clinical mastitis pathogen or subclinical mastitis [18].

Electronic Detector: Electronic detector (Drasminski, Mastitis detector) was used according to the manufactures' instructions. The Drasminski mastitis detector is electronic device used for testing the milk from animals. [19].

Ph Detector: As described by the manufactures' pH reading was recorded by pH meter for checking the alkalinity milk samples. The pH level more than 6.8 is an indicator for sub-clinical mastitis [19].

Identification of *Staphylococcus* Spp. *Staphylococcus* spp. was identified depending on the morphological features, culture media and biochemical test [20,21].

A. Microscopic Examination: The isolates were stained by Gram stain to detect their response to stain, shapes and their arrangement [22].

B. Growth on Mannitol Salt Agar: The plates were streaked from a pure colony of tested bacteria and then incubated at 37°C for 24 hr. This medium is used for selective isolation of *staph. aureus* [23].

C- Biochemical Tests

- **Catalase Test**: A drop of catalase reagent (3% H2O2) was placed on a slide. A colony of tested bacteria was mixed with the reagent on the slide, and positive results were indicated by air bubbles formation [24].
- **Coagulase Test:** Citrated rabbit plasma diluted 1:5 was mixed with an equal volume of BHI broth culture then incubated at 37°C. A tube of plasma mixed with sterile a broth is included as a control. For mating clots in 1-4 hr. indicates that the test is positive test. The negative result is re-examined for for 24 hr. [25].
- **Api Staph System:** The Api Staph was used as identification system for *Staphylococcus* and *Micrococcus*. This test is applied according to the company instructions (Company, organ).

D-Molecular Detection

- **DNA Extraction and Purification**: The DNA was extracted and purified according to the instructions of the manufacturing company (Geneaid/ Korea).
- **Nano Drop:** The nano drop system was used in the present study for DNA has been supplied from (Bioneer, Korea)
- **16S RRNA, PLAZ And MECA Genes Amplification:** A single amplification of 16SrRNA gene was done according to [26] Table 1. As previous, the final volume of reaction tubes is 20µl, containing Green Master Mix. tube contents, 1µl of both F. and R. of the primer specific for the 16S rRNA gene, 5µl of DNA template and complete the volume by adding free water to 20µl. The thermo cycling conditions were set at initial denaturation 94°c for 5min followed by 10 cycles of denaturation 94°C for 45sec., annealing 55°C for 45sec., extension 72°C for 75sec, denaturation 94°C for 45°c., annealing50°c for 45sec., extension 72°c for 45sec followed by 25cycle and final extension 72°c for 5min. The expected PCR amp icons were 756 bp for the 16SrRNA gene. A single amplification of *mec*A gene was done using primer described by [26] Table 1. The final volume of reaction tubes is 20µl, containing Green Master Mix. tube contents, 1.25µl of both F. and R. of the primers specific for the *mecA* gene, 5µl of DNA template and complete the volume by adding free water to 20µl. The thermo cycling conditions were sited at initial denaturation 94°c for 5min followed by 30cycles of denaturation 94°c for 45sec., annealing 50°c for 45sec., and extension 72°c for 1min. and final extension 72°c for 2min. The expected PCR amplicons was 310bp for the *mecA* gene. A single amplification of *PLAZ* gene was done using primer described by[27] Table 1. The final volume of reaction tubes is 20µl, containing Green Master Mix. tube contents, 1µl of both F. and R. of the primers specific for the *PLAZ* gene, 5µl of DNA template and complete the volume by adding free water to 20µl.The thermo cycling conditions were sited at initial denaturation 94°c for 5min followed by 35cycles of denaturation 94°c for 30sec., annealing 55°c for 30sec., and extension 72°c for 30sec. and final extension 72°c for 10min. The expected PCR amplicons was 518bp for the *PLAZ* gene.

- **Agarose Gel Electrophoresis:** The agarose gel was prepared according to the method of [28].Two concentrations of agarose gel were prepared (1 % and 1.5%). The concentration of 1% agarose was used in the electrophoresis after DNA extraction process, while 1.5 % agarose was used after *mecA* and 16SrRNA gene by PCR detection. A 25ml of 1X TBE buffer and 0.5 µl ethidium bromide were added into a beaker, 0.25 g agarose was added to the buffer The mixture was heated for boiling by hot plate until all gel particles were dissolved and allowed to cool down to 50-60ºC.

RESULTS AND DISCUSSIONS

Study Samples: As shown in table 2, a positive number of clinical and subclinical mastitis cases in sheep recorded an infection rates of 46 (30.7%) and 60 (40.0%), respectively. On the other hand, in cows, clinical and subclinical mastitis recorded a percentages of (52.0%) and (18.0%), respectively, (P≤ 0.01). Inside Iraq, this results disagreement with [29,30] who recorded CM (13.15%),(26%), respectively. outside Iraq, the results was disagreement Jordan and Egypt, who recorded (15.7%),(12.8%), respectively[31]. On the other hand, the result of SCM disagreement with [32,33]. Who found the periodic prevalence rate of SCM (9.23%) and (38.89%), outside Iraq, with similar results performed who noticed the prevalence of Subclinical mastitis 5-30% in goats.[34].

Age Distribution in Sheep and Cows

As shown in table 3, 4, The age groups of (2-4) and (4-6) years recorded the highest mastitis rates in sheep and cows with 75(70.8%) and 55(78.5%) infection, respectively, (P≤ 0.01). This result was in agreement with the study of [33] who recorded (38.89%) from the total of (72) cattle and [35] in Egypt, who recorded (15.43%) from (54) infected cows. That due to prevalence of (IMI) increased with age in agreement with other studies[36] It may be due to increased length of exposure to pathogens in older animals compared to younger animals. Additionally, where the duration of infection is long and the spontaneous cure rate low, prevalence will increase [37].

The Study Period

The highest mastitis cases in sheep and cows was recorded in November with 39(88.6%) and 40 (86.9%), respectively, Fig (1). The season variation is an important factor that directly affects the occurrence of mastitis [38]. In USA study, the result showed an increase in the somatic cell count during the cold seasons [39].

Distributed of Animals at Residential Area

sheep and cows were recorded highest infections in rural with a percentage of 64 (77.1%) and 49 (72.2%), respectively. On the another hand, urbans recorded a percentage of 42(62.6%) and 21(61.7%), respectively,(P≥0.05). Fig (2).

Antibiotic Susceptibility of *Staphylococcus* Isolates

The results of antibiotics susceptibility test for *Staph. aureus* and CNS isolates showed that there were a high rate of resistance to Pencillin, Oxacillin, Ciprofloxacin, Amoxillin/ Clavulanic acid, with a percentage of (100%), (82%), (75.5%), (74.5%), respectively.

Table 1: Oligonucleotide Primer Sequences for PCR Amplified of *Meca*, 16srrna and *PLAZ* Genes

Genes	Orientation	Primers {Oligonucleotide Sequence (5'→3')}	Size (Bp)	Referance
***Staph* 756**	Forward	AACTCTGTTATTAGGGAAGAACA	756	McClure., 2006
	Reverse	CCACCTTCCTCCGGTTTGTCACC		
***Mec*A**	Forward	GTAGAAATGACTGAACGTCCGATAA	310	McClure., 2006
	Reverse	CCAATTCCACATTGTTTCGGTCTAA		
PLAZ	Forward	AAG AGA TTT GCC TAT GCT TC	518	Haveri *et al*., 2005
	Reverse	GCT TGA CCA CTT TTA TCA GC		

Table 2: The Percentages of Clinical and Subclinical Mastitis in Sheep and Cows at Quarter Level

Age Group	Number	Positive	Percentage (%)
2-4	86	75	70.8
5-7	64	31	29.2
Total	**150**	**106**	**100**

Table 3: Distribution Rates of Mastitis in Sheep at Relation to Age

Age Group	Number	Positive	Percentage (%)
2-4	86	75	70.8
5-7	64	31	29.2
Total	**150**	**106**	**100**

Table 4: Distribution Rates of Mastitis in Cows at Relation to Age

Age Group	Number	Positive	Percentage (%)
1-3	9	3	4.2
4-6	58	55	78.6
7-9	15	8	11.4
10-12	18	4	5.8
Total	**100**	**70**	**100**

On the other hand, *Staph aurues* and CNS isolates showed high sensitivity to Vancomycin, Piperacillin, and Nitrofuration (80.1), (76.4%), (73.5%), respectively. ($p \leq 0.01$)Fig (3). These results was in agreement with the study of [40],who recorded that isolates showed highest resistance to methicillin (100%), amoxicillin (91.7%), followed by penicillin (83.3%), the resistance of *Staph.* spp. isolates to gentamycin (CN) was (63.2%). This results was disagreement the highest sensitivity of *Staph.* spp. to gentamycin (100%) was obtained by [41]. *Staph.* spp. Isolates resistant to Ciprofloxacin (75.5%), This result was disagreement with the result of [42]. Who mentioned that the sensitivity to Ciprofloxacin was (93.04%). The present study showed that the resistance of *Staph.* spp. isolates to erythromycin was (63.2%), this result is in agreement with[43].Which revealed that erythromycin resistance for *Staph.* spp. isolated from human and animals were (75% and 62.5%), respectively, this result was disagreement with the result of the study of [41]. That erythromycin resistance for *Staph. aureus* isolated were (89% and 90%), respectively. Antimicrobial resistance represents a serious problem in the treatment of infectious diseases including mastitis. In recent times, an increasing antimicrobial resistance rate has been recognized in *S. aureus* from bovine mastitis [44,45]. This resistance may be due to

the structural modification of enzymatic action (β-lactame action) or the prevention of access to target by altering the outer membrane permeability and may be due to the alternation of the antibiotic target site and sometimes the resistance is due to efflux pump which pumps out the antibiotic [46].

Molecular Detection of *Staphylococcus aureus* and CNS in Bovine Mastitis

Polymerase chain reaction (PCR) was used, to detect the prevalence of *mec* A, *Plaz* and 16SrRNA genes in CNS (n=64) and *Staph. aureus* isolates (n=42). The results revealed that all *Staph. aureus* and CNS isolates were positive for the three genes, except *Staph. xylosis* which showed a percentage of (93.7%) for *mec* A gene. Staphylococci develop resistance to ß-lactam antibiotics with two mechanisms: the production of ß-lactamases encoded by the *bla*Z gene and by the production of altered form of penicillin binding protein, PBP2a, encoded by the *mec*A gene . All penicillin and ampicillin resistant CNS strains were also ß-lactamase producers and carried *blaZ* gene in this study. The presence of *mec*A mediated methicillin resistance has been reported in different species of staphylococci by[47].

Relationship between Penicillin Resistance and the Presence of the *Blaz* Gene

The detection of the *blaZ* (code for β-lactamase) and *mecA* (code for alternative penicillin-binding proteins) genes is considered the gold standard for the determination of penicillin and finding is consistent with those of [48,49]. Who reported high resistance rate to penicillin in staphylococci from clinical and subclinical bovine mastitis in other different of Iran, a possible explanation for this might be the frequent use of β-lactams in intra-mammary infusions for mastitis treatment in Iranian dairy farms. However, the reported percentage of *blaZ* carrying for CNS isolated from bovine mastitis that is disagreement with other similar study such as in Switzerland [50] and in Egypt [51]. Who recorded (23.8%, 28.6%), respectively. On the other hand an agreement with other studies [52], Netherlands ,who recorded (65.7%, 80.6%), respectively. Presence of resistance genes may not be always indicative of resistance phenotypes, and vice versa. Also, the relationship between presence of resistance genes and clinical response to treatment is largely unexplored terrain [53].

Relationship between Methicillin Resistance and the Presence of the *Meca* Gene

Treatment of staphylococcal infections with antibiotics is becoming increasingly difficult in view of the widespread presence of *Staph.* spp. strains resistant to multiple antibiotics, the conventional control/therapy measures for MRSA using antibiotics were shown to be part of the problem. The emergence of infectious diseases caused by drug resistant bacteria requires alternatives to conventional antibiotics and phages are one potential solution that will help to replace, curb, or promote judicious use of antibiotics in farm animals [54]. Several Iraqi studies have shown an increase in MRSA prevalence in milk and cheeses and MRSA is considered endemic in most areas of Iraq [55,56].

CONCLUSIONS

The present study results revealed a high prevalence of subclinical and clinical mastitis in sheep and cows, respectively. *Staph. aureus* and CNS can be considered as one of the most frequent bacteria in mastitis in Sheep and Cows.

Figure 1: Percentages of Mastitis in Sheep and Cows According to the Study Period

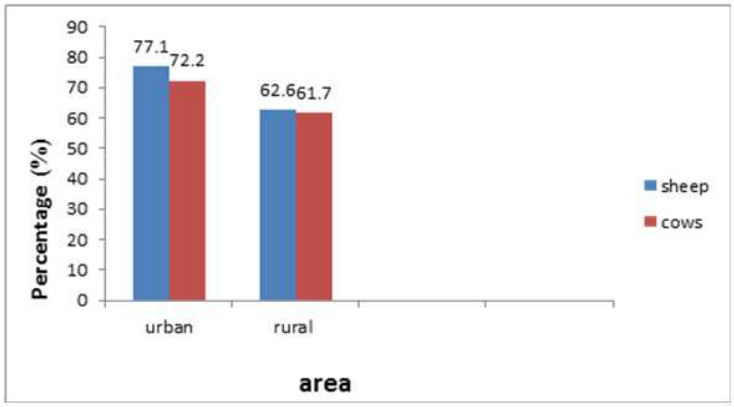

Figure 2: Distribution of Mastitis in Sheep and Cows According to Residential Areas

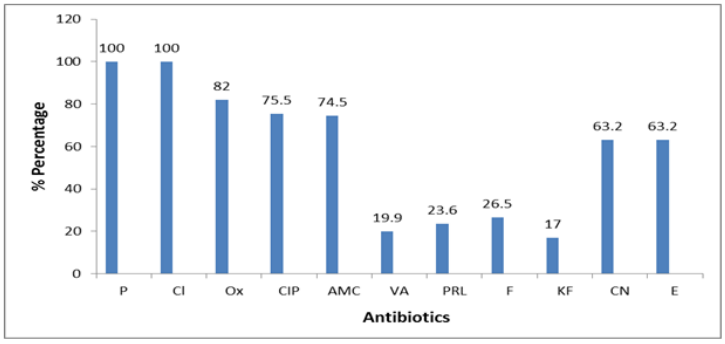

Figure 3: Antibiotic Resistant Patterns of *Staph*. Sp

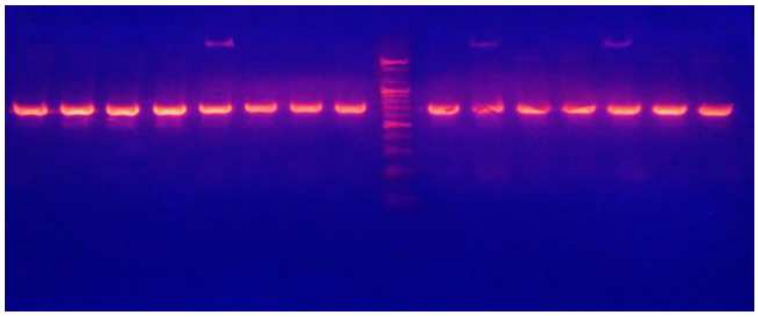

Figure 4: Agarose Gel Electrophoresis Showing Representative PCR Products after16srrna Genes Amplification

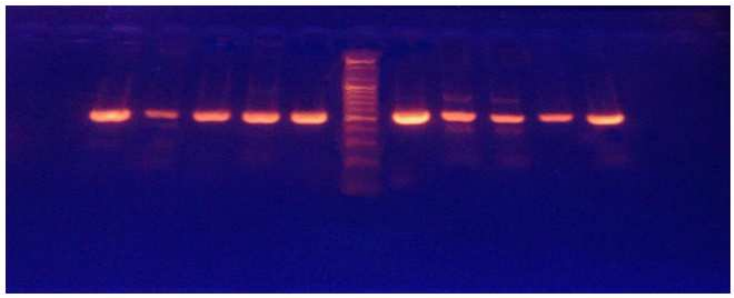

Figure 5: Agarose Gel Electrophoresis Showing Representative PCR Products after *Plaz* Gene Amplification

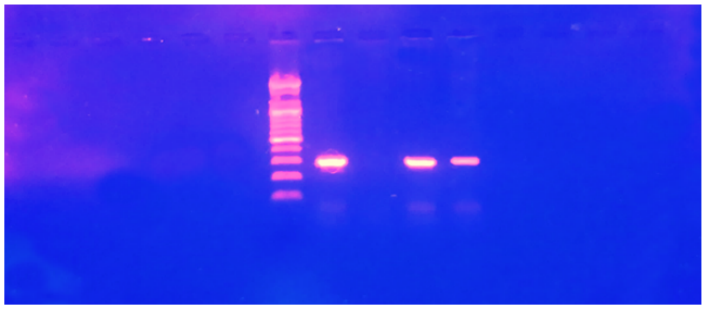

Figure 6: Agarose Gel Electrophoresis Showing Representative PCR Products after *Meca* Gene Amplification

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