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Original Research Article

Detection of Drug Resistance *Staphylococcus Aureus* from Sheep Mastitis Milk

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Abstract: In Iraq, sheep play an important role in supplying the local market's requirements for milk and meat. The objective of this study, 117 milk samples collected from Awassi ewes put to sale in the animal sales yard in Al-Qasim District from Babylon Governorate during four weeks on December 2023. The udder and milk inspection by visual examination and indicator papers (BOVIVET) for detection of mastitis. Consequently, cultured mastitis milk sample on HiCrome™ Klebsiella Selective Agar Base for isolation and easy detection of *Klebsiella* spp and phenotypic detection of *Escherichia coli* (EC) on MacConkey agar. Consequently, *Staphylococcus aureus* is isolation by inoculating on Mannitol salt Agar, and confirmed by the Vitek2 system, then antibiotic resistance characteristics are studied by the Kirby-Bauer technique on Muller Hinton agar. The results showed phenotypic detection of 24(20.5%) mastitis milk sample out 117 milk samples and isolation of *Klebsiella* spp 5(20.8), *Escherichia coli* 7(29.1) and *Staphylococcus aureus* 12 (50%) then studying the characteristics of microbial resistance to *Staphylococcus aureus* isolates , it was shown that it possesses resistance to Ciprofloxacin 3(25%), Ampicillin 7(58.3%), Gentamycin 3(25%), Streptomycin 2 (16.6%), Amikacin 3(25%), Doxycycline 5(41.6%), Cefaxitin 2(16.6%), Ceftazidime 2(16.6%), Ceftazidime 2(16.6%) and Erthromycin 2(16.6%). In conclusion, data showed there are many causes of mastitis in sheep, and *Staphylococcus aureus* bacteria is still the most common cause among other causes, and the most important thing is that it acquires resistance due to mismanagement and indiscriminate use of veterinary medicines. Therefore, we recommend periodic follow-up of sheep, giving necessary vaccines, and educating farmers to limit the spread of diseases that effects animal and human health and cause significant economic losses.

Keywords: *Staphylococcus aureus*, sheep, mastitis.

INTRODUCTION

Sheep play an important role in supporting the requirements of the agricultural economy, and producing good quality milk is an important goal for raising ewes in Iraq (Al-Hubaety and Al-Radhwany, 2012).

Milk-producing animals, such as cows, buffalo, sheep, goats, and camels, are among the most important animals on which global production depends. The existence of any species depends on the nature of the region and country, availability of food, and appropriate climate, as well as the market demand for milk, and some social traditions (FAO, 2024).

Milk production and its components are affected by many factors, such as breed and environmental factors such as production year, including the impact of climate nutrition, pastures, health status, age of the ewes and type of birth, sex of newborns and organization of herd management (Al-Dabbagh, 2019).

Mastitis is one of the important diseases in sheep because the systemic signs are usually severe and painful, and the mammary glands are damaged and difficult to treat. There is usually a change in the components of the milk, in addition

to a lack of milk production in ewes that respond to treatment, which affects the weight and health of the newborns. Therefore, mastitis effects on economics and animal welfare (Ashley and Burim, 2022).

Subclinical mastitis occurs more frequently than clinical mastitis. Clinical mastitis leads to clear changes in the udder and an increase in somatic cells in the milk, while the changes are not noticeable in the subclinical type, both types lead to a change in the production and components of the milk of varying degrees, and this depends on the severity the disease (Bogni *et al.*, 2011).

More than thirty types of pathogens that cause mastitis in sheep have been isolated. Studies have indicated that *Staphylococcus aureus* is the most common type and has been discovered repeatedly (Christine, 2021).

Staphylococcal infection occurs when it begins to multiply and secrete its toxins and enzymes after entering through the skin, damaged mucous membranes, and sores, causing severe local damage to tissues in the area of infection, leading to the formation of abscesses, consequently the bacteria spread into the tissues or haematogenous can spread to other areas of the host's body, but the problem of clinical and subclinical mastitis in dairy sheep remains the most important problem due to the lack of milk production, its poor quality, and its rejection due to the administration of antibiotics (Vasileiou *et al.*, 2019). Moreover, Small ruminants are infected with many environmental microorganisms, which are difficult to treat due to their resistance to antibiotics. *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Pseudomonas aeruginosa, and Proteus spp. Serratia spp., and Enterobacter spp.* are the most important of these environmental microorganisms (Manuele *et al.*, 2008).

The presence of microbial communities in the environment or udder such as *Escherichia coli*, transmitted through the milkers' hands, or from sheep to sheep, can cause clinical or subclinical mastitis, and even gangrenous mastitis (AHDB, 2018).

Recent studies have proven that mastitis infection due to *Klebsiella spp*. considered a new challenge and causes greater losses in the dairy industry than *Escherichia coli* due to the lack of sensitivity to treatment with antibiotics, which causes shock and death (Muhammad *et al.*, 2021).

Different types of antimicrobial agents are used periodically in animal breeding farms for treatment, to enhance production, and this leads to an increase in the emergence of resistant strains. According to the World Health Organization microbial, mutations have been highlighted as a global issue, not only do they affect animal health, but also they affect human health by neutralizing the action of medications and making them ineffective causing the formation of pathogens in the body and increasing risk of spreading to others (WHO, 2022). However, multiple theories explain the development of microbial resistance to antibiotics agents, including to the nature of the microbial origin, human nature, misuse of medications, and incorrect application in the agricultural and animal sectors (Michael *et al.*, 2014).

In any case, the incidence of mastitis due to *Staphylococcus aureus* remains in first place in this study due to the importance and danger of this bacteria to milk-producing animals, including sheep. Therefore, some points were focused on, such as the rate of isolation of this bacteria from an average group of sheep and the study of the resistance of this bacteria to some of the antimicrobials we have available.

MATERIAL AND METHOD

Experiment Animals:

Samples were collected from the lactating ewes present in the animal sales yard during four weeks in December 2023. The samples were divided into four groups based on the week in which they are were collected. The ages of the ewes more than four years old and have multiple births. The samples were taken after observing the basic rules for collecting samples, such as cleaning, washing with clean water, disinfection with 70% ethanol, excluding the first drops of milk and cooling, then transferred to the laboratory College of Veterinary Medicine/ Department of public health for complete other tests.

Laboratory Examination:

Sensitive Indicator Paper for Udder Control (BOVIVET):

A rapid examination was used to distinguish between mastitis and non-mastitis ewes by putting drops of the milk on the spots of the sensitive indicator paper, when the quarter is infected will change the color of the spot paper from yellow to green or bluish-green, but either not change the color or at the most to light green from the normal quarter.

Isolation of Bacteria

Only mastitis milk samples were inoculated as (10ml mastitis milk) to (90ml) nutrient broth (HIMEDIA) inoculated 24 hours at 37 °C, then (10 µL) spreading on HiCrome™ Klebsiella Selective Agar Base for isolation of

Klebsiella spp and (10 µL) spreading on MacConkey agar (HIMEDIA) for isolation of *Escherichia coli* and *Staphylococcus aureus* are isolation by (10 µL) spreading on Mannitol salt Agar (Oxoid, USA). And all cultures incubated overnight at 37°C hours. Consequently, isolated bacteria were confirmed according to colony morphology, Gram stain and VITEK 2 System (Ahmed and Yousif, 2021; BAM, 2017 and Megha *et al*., 2022).

Identification of Clinical Isolates:

Rapid identification of the purified clinical isolates obtained from MSA agar media plates were further examined by VITEK 2 System (Teresa *et al*., 2003 and Thomas *et al*., 2003).

Antibiotic Susceptibility Tests:

Antibiotic analysis was performed on all *Staphylococcus aureus* isolates to determine their antibiotic resistance. Available antibiotic discs were used on the disc diffusion method. Testing was performed by pure colonies prepared from freshly inoculated overnight cultures, transferred from the top of these colonies to 4-5 ml nutrient broth and incubated for 2 hours at 37°C. Then a sterile cotton swabs were immersed into the suspension and spread on the surfaces of Muller-Hinton agars(HIMEDIA). 9 antibiotic discs were gently placed on the surface of the inoculated agar to ensure good adhesion to the surface, then the plates were incubated at 37 °C for 18 hours - 24 hours and the plates reading and interpretation the results by diameters zone of inhibition (Bauer *et al*., 1966 and CLSI, 2021).

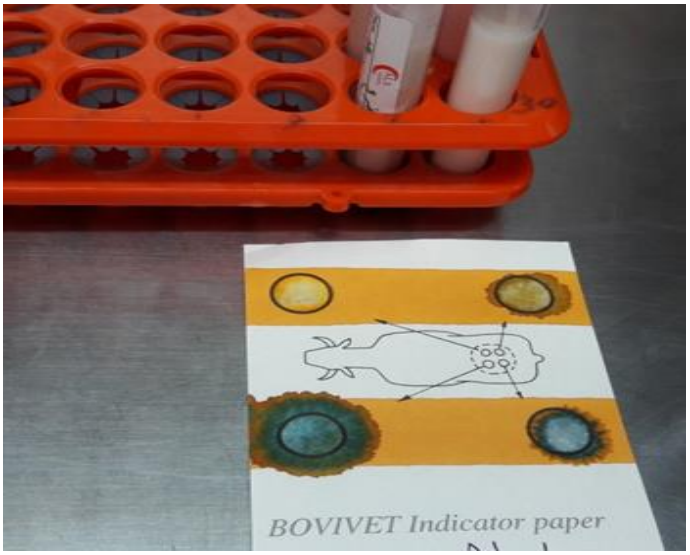
RESULT AND DISCUSSION

The results of this study showed that milk samples collected from ewes offered for sale in the animal sales yard within four weeks, out of 117 milk samples detection of (24 (20.5%) had positive test results for BOVIVET indicator paper and most of these ewes were suffered from systemic signs present in the mammary glands, unilateral or bilateral. Bacteriological examinations of inflamed milk samples showed isolation of *Staphylococcus aureus* 12 (50%) positive, *E.coli* 7 (29.1), and *Klebsiella pneumoniae* 5(20.8) in Table 1.

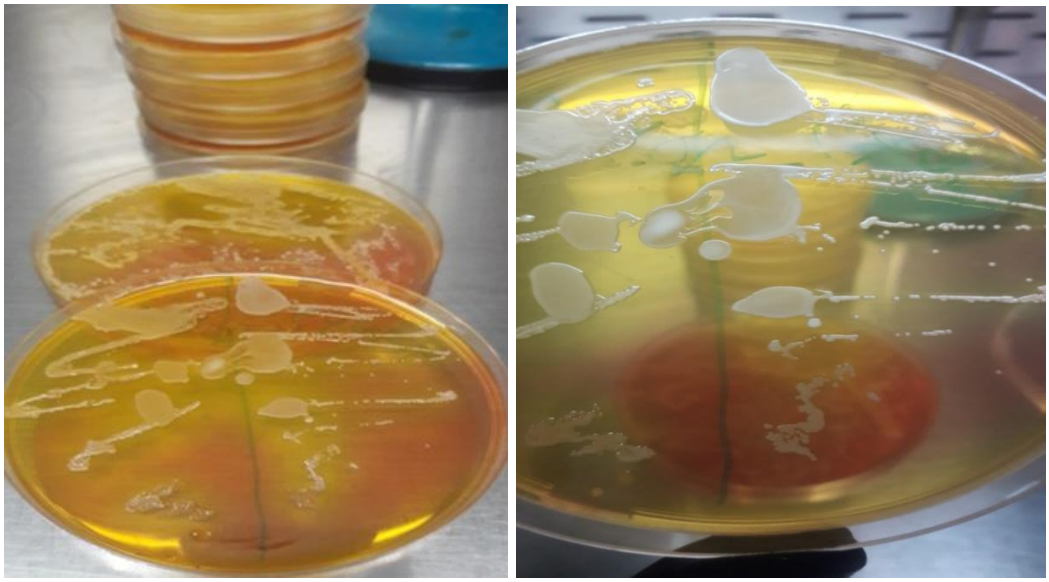
Table 1: Prevalence of *Staphylococcus aureus, E.coli,* and *Klebsiella pneumoniae* in ewes mastitis milk in Al-Qasim District

Time of sample	No of samples	No. and % of mastitis samples	No. and % of *S.aureus* positive(24)	No. and % of *E.coli* positive(24)	No. and % of *Klebsiella spp* positive(24)
First week	25	5 (20%)	2 (8.3%)	2 (8.3%)	1 (4.1)
Second week	27	5 (18.5%)	3 (12.5%)	1 (4.1)	1 (4.1)
Third week	30	6 (20%)	3 (12.5%)	2 (8.3%)	1 (4.1)
Fourth week	35	8 (22.8%)	4 (16.6%)	2 (8.3%)	2 (8.3%)
Total	**117**	**24 (20.5%)**	**12 (50%)**	**7 (29.1)**	**5 (20.8)**

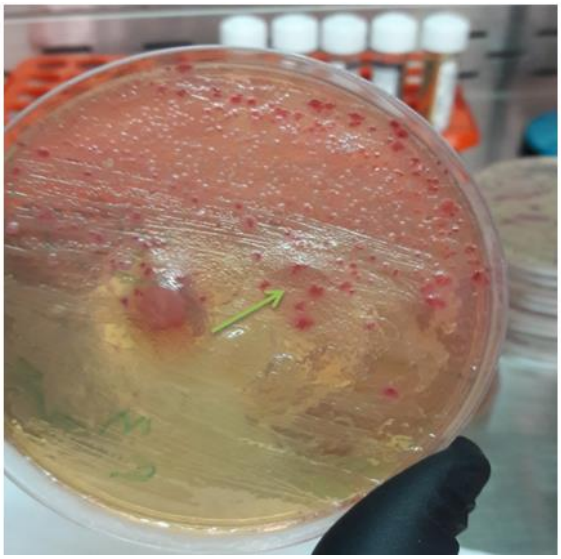
Case of ewe mastitis



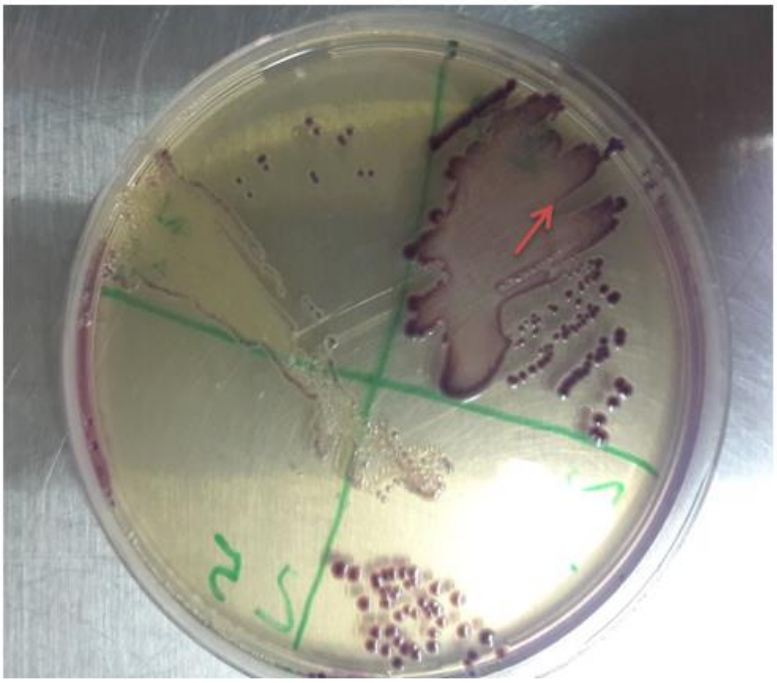
Milk samples change the color of the spot paper from yellow to green



***Staphylococcus aureus* colonies changes the color of the Mannitol salt Agar to yellow and seen as round and convex**



***Escherichia coli* on MacConkey agar**



HiCrome™ Klebsiella Selective Agar Base for isolation of *Klebsiella* spp

The results of this research were generally consistent with a several of researchers in Iraq and other countries, and if there was some difference in proportions, this was due to some factors affecting the animal, such as the method of rearing, surrounding environment, and annual season.

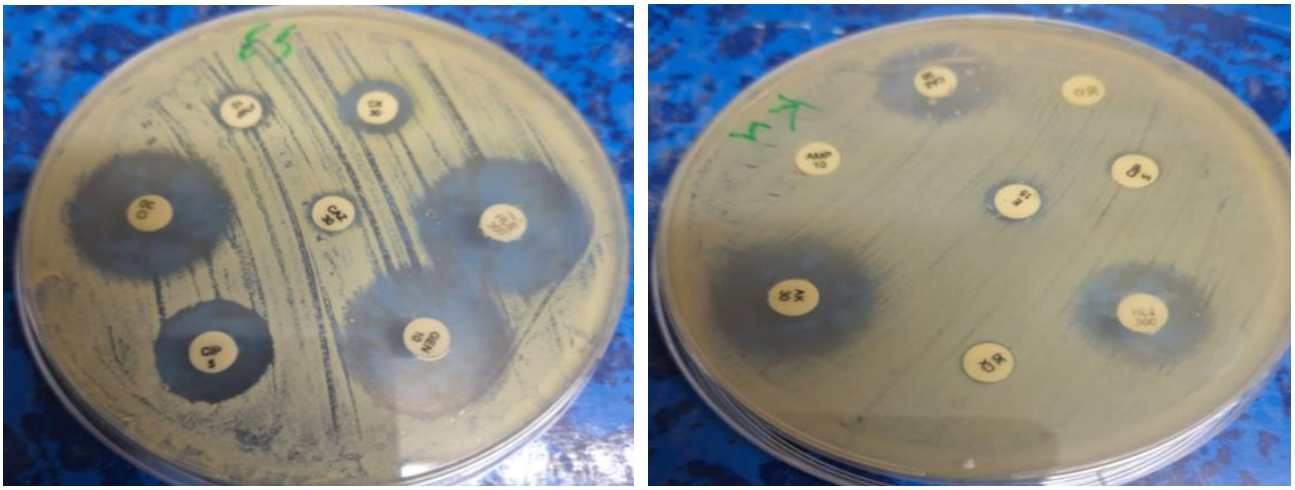
The rate of mastitis cases in the third and fourth weeks was higher than the first and second weeks, and rate of bacterial isolation was also higher these may be due to the increase in new births as well as an increase on rain in these days of the year. These are consistent with what some researchers have interpreted, including for several reasons births, frequent sucklings by the offspring, physical stress, and physiological changes in the mammary gland, all of which helps the entry of bacterial pathogens into the udder and increase the infection rate (Luiz *et al*., 2018) isolation rate of *Staphylococcus aureus* was higher than *E.coli* , and *klebsiella pneumoniae* 12(50%), 7(29.1), and 5(20.8), respectively.

This study was in agreement with a several of researchers and showed that *Staphylococcus aureus* is the main causative agent and most common cause of mastitis in small ruminants. *Staphylococcus aureus* was responsible for approximately 40% of mastitis in ewes suckling lambs, and responsible for approximately 80% of mastitis in milking ewes. In addition, studies have indicated that subclinical cases are difficult to treat and cure, and therefore for the safety of the herd, animals infected with *Staphylococcus aureus* mastitis must be slaughtered or the milk of infected ewes must be disposed of and carried out of necessary treatment (Christine, 2021).

The results showed that the *Staphylococcus aureus* isolated from mastitis milk are resistant to 9 antibiotics, as shown in Table 2.

Table 2: Antibiotic resistance pattern, and sensitive of *Staphylococcus aureus* isolation from sheep mastitis milk

Antibiotics	Resistance	Intermediate	Sensitive
Ciprofloxacin (CIP) 5 mcg	3 (25%)	2 (16.6%)	7 (58.3%)
Ampicillin (AMP) 10 mcg	7 (58.3%)	1 (8.3%)	4 (33.3%)
Gentamycin (GN) 10 mcg	3 (25%)	1 (8.3%)	8 (66.6%)
Streptomycin HLS 300 mcg	2 (16.6%)	2 (16.6%)	8 (66.6%)
Amikacin (AK) 30 mcg	3 (25%)	2 (16.6%)	7 (58.3%)
Doxycycline (D) 30 mcg	5 (41.6%)	1 (8.3%)	6 (50%)
Cefaxitin (CX)30 mcg	2 (16.6%)	2 (16.6%)	8 (66.6%)
Ceftazidime (CAZ) 30 mcg	2 (16.6%)	2 (16.6%)	8 (66.6%)
Erthromycin (E)15	2 (16.6%)	2 (16.6%)	8(66.6%)



Show Zone of inhibition on Muller-Hinton agars by drug resistance *Staphylococcus aureus*

After diagnosing the percentage of ewes infected with mastitis and isolating some of pathogens, another focus of this study was to know the characteristics of microbial resistance *Staphylococcus aureus* isolated from the milk of ewes suffering from mastitis were identified using 9 antimicrobial disc. It was observed in this study that there was a relative increase in resistance to Ampicillin 8 (66.6%), which belongs to the penicillin family, and Doxycycline 5 (41.6%) which belongs to the tetracycline family, this low frequency of resistance to Ampicillin and Doxycycline are strong evidence of the repeated and random use of these treatments in small ruminants, and these are consistent with other studies conducted in previous years (Charalampos *et al.*, 2021).

Staphylococcus aureus had relatively moderate resistance to the Aminoglycosides group; Amikacin 3 (25%),, Gentamycin 3 (25%), and Streptomycin 2 (16.6%), this group is widely used in veterinary medicine. However, Studies have shown the limited use in case of mastitis by infusing into the udder. Therefore, it is used once a day at a lower concentration and more frequent intervals against *Staphylococcus aureus* to reduce the risk of nephrotoxicity (Melissa *et al*., 2022).

Resistance to Ciprofloxacin 3(25%) Erythromycin 2(16.6%), Ceftazidime 2(16.6%) and Cefaxitin 2(16.6%) was relatively weak, and these may be due to the limited use of drugs in treating sheep.

The results of this research paper are compatible within the general pattern of many previous studies in a some of countries, and if there is a difference in some results, this is due to the nature of the policy followed in the use of antibiotics in treating milk-producing animals from one country to another. In any case, the strict policy to limit the indiscriminate use of antibiotics reduces the emergence of resistant strains and thus reduces the chance of these pathogens being transmitted to humans (Michael *et al*., 2023; Andrade *et al*., 2021; Azzi *et al*., 2020 and Abdalhamed *et al*., 2018).

CONCLUSIONS

This study indicated that approximately (20.5%) of the ewes offered for sale are infected with mastitis, and this indicates poor management of milk-producing herds and the failure to use modern methods in breeding. The study also showed that many ewes infected with mastitis due to Staphylococcus aureus bacteria are resistant to more than one type of antibiotic. This is a dangerous indicator that warns of an increase in antibiotic-resistant strains and thus affects the agricultural sector and human health. We recommend the use of modern breeding methods and restriction of the use of veterinary drugs by regulatory authorities.

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