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Isolation, identification and sensitivity of *Mannheimia haemolytica* from Ewes udder with clinical mastitis

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ABSTRACT

The current study aims to isolate and identify *M. haemolytica* from ewes with clinical mastitis using conventional techniques with comparative study to their resistance to several types of antibiotics for the isolated bacteria from udder. The study was carried out in different regions of Nineveh governorate. Two Hundred sixty-six samples (133 milk samples and 133 udder skin swabs) were collected During December 2020, after the clinical examinations of the flocks. The samples were put in test tubes containing Brain Heart Infusion (BHI) Broth; then transferred into the Veterinary Public Health Laboratory, College of Veterinary Medicine, University of Mosul for different bacteriological, biochemical examination and sensitivity tests. The results showed that there is a matching between bacterial isolation ratios of milk and udder skin swabs of sheep affected by clinical mastitis at 51.12%. The results also showed a difference in the sensitivity test between *M. haemolytica* isolated from milk and skin swabs. It can be concluded that, the improper and indiscriminate use of veterinary medicines is one of the most important causes for the increase in bacterial resistance to antibiotics in treated cases without consulting a veterinarian.

Keywords: *M. haemolytica*, Nineveh governorate, sensitivity test, sheep.

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INTRODUCTION

Sheep are domestic animals reared for multiple economic purposes, ***i.e.,*** mutton, milk, wool, skin, and manure as a byproduct used for organic fertilizer. In Iraq, sheep are reared in wide regions highly concentrated in Nineveh governorate. According to the latest census (2020), the Animal Wealth directorate sheep population section is about 4825696 head. Most sheep are reared on fields, pastures and farms having the merit of low-cost raising. Hence, such a character supports the number and production of these animals. However, sheep may expose to different diseases such as mastitis which is regarded as an important and broadly distributed disease in several countries resulting in huge economic losses **(Amir, 2013).**

Mastitis is considered a crucial disease of sheep affecting meat and wool production and their adverse effect on sheep and human health welfare. Mastitis is one of the commonest contagious diseases causing high economic losses due to decreased milk production and quality and negative financial consequences **(Bourabah *et al.*, 2013; Kotb and Gafer 2020; Ahmed and Jwher 2019).** These undesirable effects may include remedies, therapeutic expenses and cost treatments, and their influence on the suckling lambs **(Zaninelli *et al.*, 2015; Bourabah *et al.*, 2013).** Mastitis is defined as the inflammation of the mammary gland or the udder, which sometimes may include the anatomical tissues and structures related to the mammary gland such as the teats, milk cisterns, teat canals leading to bacteriological, physical and chemical changes of both of the udder and the milk **(Contreras *et al.*, 2011).**

Additional losses caused by clinical mastitis are expenses of treatment, culling of diseased ewes due to the affected udder's entire damage, particularly in peracute and highly affected cases. However, gangrenous mastitis may lead to the death of the animal, resulting in sheep welfare and the country's economy **(Larsgard and Vaabenoe, 1993).** Also, mastitis may act as a reason for animal culling leading to increasing the average of ewe removal with frequent loss of high-quality sheep as well as the increased use

of antibiotic administration and other remedies applied as therapeutic or preventive, correlated with additional costs, labor and various drug withdrawal periods **(Portis, *et al.*, 2012; Contreras *et al*., 2011)** referred that there are 5% of annual new mastitis cases.

However, several studies recorded that such a percentage may reach up to 22% **(Bergonier and Berthelot, 2003; Onnasch *et al.*, 2002)**, especially when these cases are concurred with the poor environment or accompanied by other pathological infections **(Burriel, 1997).** Previous studies carried out by several workers stated that clinical mastitis is the commonest type of mastitis in dairy sheep systems caused by *Staphylococcus aureus* **(Bergenier *et al.*, 2003; Ebtsam *et al.*, 2020).**

On the other hand, *Mannheimia haemolytica* is classified as the most common bacteria causing ovine mastitis in mutton and wool sheep rearing systems. However, *M. haemolytica* has the same importance as Staph. aureus as a causative agent in clinical and subclinical mastitis of sheep **(Bergonier and Bertholet, 2003).** *M. haemolytica* is gram-negative rods, non-motile, non- sporulated bacteria, containing 39-40% of G + C DNA **(Bojesen, *et al.*, 2007).** *M. haemolytica* is one type of Pasteurella which is re-classified by the German bacteriologist Walter Mannhemia **(Zecchinon *et al.*, 2005).**

M. haemolytica is naturally found in the upper part of the ruminants' respiratory tract, and most isolated bacteria are carried out from cattle, sheep, and goats. Virulence and pathogenicity factors of *M. haemolytica* are represented by the adherence, capsule, lipopolysaccharides, external proteinous membrane, iron-regulated proteins, and leukotoxin, the main causes of pasteurellosis or shipping fever in sheep and goats. Furthermore, *M. haemolytica* may cause pneumonia syndrome, hemorrhagic septicemia syndrome, and gangrenous mastitis in sheep and goats **(Quinn *et al.*, 2011).**

Certain meteorology and climates, various production regimes, and different farm management applications may interfere with epidemiology, bacteriology, and clinical manifestations of mastitis caused by multiple bacteriological etiologies, including *M. haemolytica*. Hence, the current study aimed to isolate and identify *M. haemolytica* from sheep infected by clinical mastitis, milk and the outer surface of the udder and teats, as well as its confirmation using conventional techniques with comparative study to their resistance to several types of antibiotics.

MATERIALS AND METHODS

The study was carried out on eight different Nineveh governorate regions, which represent sheep rearing centers. Two hundred sixty-six samples were collected During December 2020, after the clinical examinations of the flocks. The obtained samples included 133 milk samples taken from sheep who suffered from clinical mastitis. The samples were placed in sterile containers at the time of collection; 133 skin swab samples were taken from the udder and teats of the same ewes. The samples were poured in test tubes containing Brain Heart Infusion (BHI) Broth. They were transferred into Veterinary Public Health Laboratory, College of Veterinary Medicine, University of Mosul for different bacteriological examinations.

Bacterial isolation:

The bacteria were isolated according to the method described by (**Macfaddin, 2000 and Borkar, 2017).** Test tubes containing the media were inoculated after the gentle shaking of the milk container sample. One loopful was taken using a sterile bacteriological loop from the intended milk spread on 7% sheep blood agar and incubated at 37c° for 24-48 hours. The bacterial colonies were further purified. Skin swabs of the udder and teat skin were cultured in heart-brain infusion broth, which was incubated for 2-3 hours and was treated in the same manner as previously mentioned. The results were verified according to gross and microscopical findings of the microorganisms such as shape, texture, contour, size, and color of the colony smears. These colonies were stained by methylene blue and those bacteria were stained were preserved while the others were excluded.

Biochemical Test:

Different biochemical tests include Indole-production, Glucose, Methyl red, Voges-Proskauer, Simmons Citrate, Mannitol, Kligler's Iron Agar, Sucrose, Urease, Cytochrome oxidase, Catalase, Maltose, and Motility were carried out according **(Quinn, 2011).**

Sensitivity test:

Bacterial sensitivity to antibiotics was carried out for all bacterial isolates applying the modified Kirby-Bauer method, the standard method recommended by the World Health Organization **(Bauer *et al.*, 1996)** to detect the sensitivity or resistance of the bacterial isolates for antibiotics. *M. haemolytica* were cultured in heart-brain broth for 4 hours; then the suspension was spread on Muller-Hinton agar using sterile swabs. The cultured Petri dishes were left for desiccation for 10-15 minutes; later, the antibiotic discs were put using sterile forceps the plates were incubated for 24 hours at 37c°. The results obtained depending on the inhibition zone caused the growth of the bacteria on the media and were accompanied by the standard diameter, which is 8mm. The results classified into:

1- Sensitive bacteria: i.e., the antibiotic has the ability to inhibit the growth of the isolated bacteria.

2- Resistant bacteria: i.e., the antibiotic is not able to inhibit the growth of the isolated bacteria.

RESULTS

Out of 133 milk samples, 68 samples were positive for *M. haemolytica* according to the results of bacterial culture, blue methylene stain and biochemical tests. Also, the results obtained from skin swab samples obtained from sheep's udder suffered from clinical mastitis from different regions of Nineveh governorate similarity, identity and homogeneity with a percentage of isolation from sheep's milk and sheep affected by clinical mastitis in a percentage of 51.12% as shown in Fig. (1a) showed the growth of bacterial colonies of *M. haemolytica* on blood agar. The colonies appeared with 2-3mm diameter, having pearly-grayish colour, medium-size, of mucous texture. The bacteria caused hemolysis type, *i.e.*, the appearance of transparent zones around the colonies. Fig. (1b) showed a microscopic manifestation of *M. haemolytica* characterized by bipolarity in the bacilli arranged either alone or in groups.

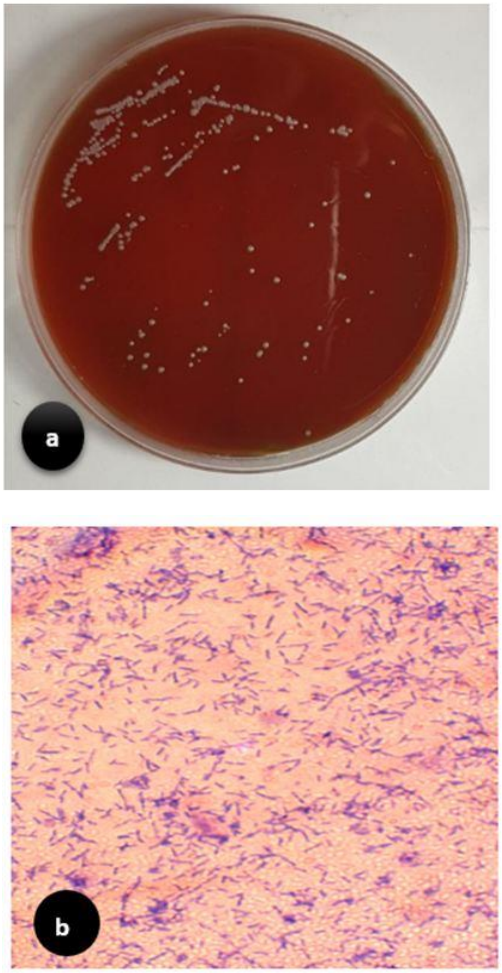

Fig. 1: (a) Growth of *M. haemolytica* colonies on blood agar (b) blue methylene staining smear (1000X).

Also, the findings obtained from the current study showed that all the isolates of *M. haemolytica* bacteria were non-indol production positive for methylene blue test, negative for Fox-Proscar, Simmons citrate and urease production, non-motile, positive for red methyl, iron test, tri-saccharides, oxidase test and catalase test. Also positive for mannitol fermentation, glucose, maltose and sugar without gas formation as shown in Table. 1.

Table 1: Biochemical tests for *M. haemolytica* isolates.

Reaction	Indole-production	Methyl red	Voges-proskauer	Simmons Citrate	Kligler's Iron Agar	Urease	Motility	Maltose	Sucrose	Mannitol	Glucose	Cytochrome Oxidase	Catalase
Result	-	+	-	-	+	-	-	+	+	+	+	+	+

Regarding the sensitivity test, it was found that different isolates of *M. haemolytica*_from the milk samples were **sensitive** to ampicillin and gentamicin. In contrast, *M. haemolytica* isolates from swabs (skin and teats of the udder) were sensitive for neomycin, norfloxacin, ampicillin, gentamicin, ciprofloxacin and levofloxacin, as shown in table (2) and Fig. (2).

Table 2: Results of the antibiotic sensitivity tests for *M. haemolytica* isolated from different samples

No.	Type of antibiotic	Conc. Mg/disk	Milk	skin
1	Doxycycline	30	R	R
2	Carbenicillin	25	R	R
3	Oxytetracycline	30	R	R
4	Neomycin	10	R	S
5	Fosfomycin	50	R	R
6	Norfloxacin	10	R	S
7	Ampicillin	25	S	S
8	Tiamulin	30	R	R
9	Trimethoprim	5	R	R
10	Tilmicosin	15	R	R
11	Lincomycin	2	R	R
12	Chloramphenicol	10	R	R
13	Gentamicin	10	S	S
14	Spiramycin	30	R	R
15	Erythromycin	10	R	R
16	Ciprofloxacin	10	R	S
17	Enrofloxacin	5	R	R
18	Florfenicol	30	R	R
19	Thiamphenicol	30	R	R
20	Levofloxacin	5	R	S
21	Nitrofurantoin	100	R	R
22	Tylosin	15	R	R
23	Trimethoprim-sulfamethoxazole	25	R	R

24	Spiramycin	100	R	R

* R= Resistant, S= Sensitive

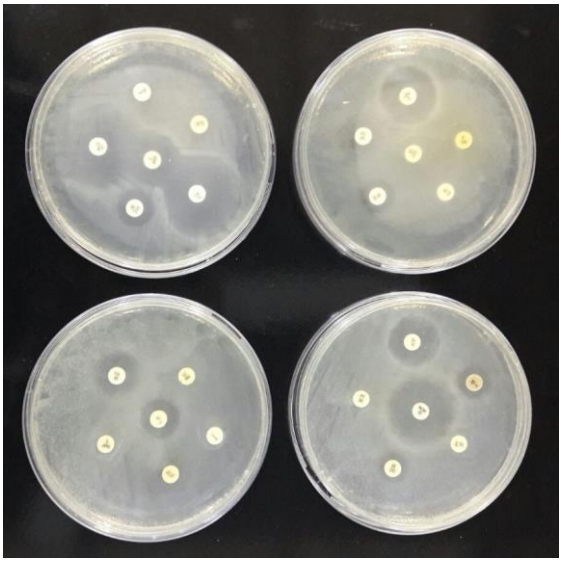

Fig. 2: Results of sensitivity test for *M. haemolytica* isolates.

DISCUSSION

Mastitis is an important productive and reproductive disease infecting sheep, especially the ewes reared for milk and nursing ewes. Several works approved and confirmed the effective role of *M. haemolytica* as the primary elementary and determinative etiology of Ovine mastitis, closely resembling *Staphylococcus aureus*. However, mastitis caused by the latter agent is less than those caused by the former **(Arsenault *et al.*, 2008, Omaleki *et al.*, 2010).** Consequently, the current study supports such hypothesis that mastitis generally prevails in Iraq, particularly in the Nineveh government. However, the percentage of mastitis decreased in the previous decades in several countries that applied developed strategies in flock management as well as dairy production. However, such systems represent challenges and obstacles in developing countries where animal rearing systems and management are traditional and primitive. In those countries, mastitis may be exaggerated by poor hygiene, ineffective prophylactic measures and severe environment initiating and creating a suitable atmosphere for increasing udder inflammation **(Peeler *et al.*, 2002).**

Regardless mastitis is an infectious disease, a disease that may constitute an economic problem. Leading to huge losses with unprofitable sequels. However, such disadvantages and defects do not confine to developing countries that use hundreds of different remedies as antibiotics either for protection or treatment of mastitis. Furthermore, in the esthetic and hygienic points of view, milk secreted from inflamed udders is unfit for human consumption because it is unwholesome with rejection and condemnation of milk **(Radostits *et al.*, 2000).** Mastitis is mostly correlated with administrative and hygienic malmanagement with poor manipulation and handling of the animals such as overcrowding and trauma and injuries caused by physical damage and affection of the teats or udder, or due to destruction or harm in the udder tissue resulting from inappropriate milking or violent suckling or stings of different flying or crawling arthropods and insect or due to superficial wounds of the skin leading to initiate favorable circumstances for the occurrence of infection **(Bergonier *et al.*, 2003; Radostits, *et al.*, 2000).**

A similar observation was explained by **Hein, (2000).** It is noteworthy to refer that abstaining from the hygienic measures and precautions related to the soundness of the udder, *i.e.*, intramammary injections of antibiotics during the beginning of the dry period, has an adverse and harmful effect. Unfortunately, the previous conventional practice is scientific, which has approved an essential factor for healthy and intact udder is not followed in local sheep farms. The fore-mentioned practice is precautionary aims to treat the inflammation before milking, nursing and lactating, and an avenue to avoid and protect the udder from the possible recurrence of mastitis, especially in the dry period, as stated by **Fthenakis *et al.*, (2012).** There is little information and data about the treatment of mastitis caused by *M. haemolytica*.

However, according to the current study's findings, the sensitivity test of different antibiotics showed some resistance to certain antibiotics. The Table manifests that *M. haemolytica* bacteria have resistance to several antibiotics except for neomycin, norfloxacin, ampicillin, gentamicin, ciprofloxacin and levofloxacin from bacteria isolated from the external swabs. The bacteria are resistant to ampicillin, gentamicin, which was isolated from milk, which was inconsistent with those of **Omaleki *et al.*, (2010), w**ho carried out a sensitivity test on the isolates resistant to erythromycin while all the isolates were sensitive to trimethoprim, tetracyclin and Ciphtufor. However, this disagreement was interpreted by **(Burrows, 1980)** who ascertained that the sensitivity of bacteria isolated from outer swabs to antibiotics is not necessarily the same as those isolated from milk.

The study results are somewhat in harmony with a study carried out by **Sanchis and Abadie, (1992)** to test the extent of sensitivity of *Pasteurella haemolytica* isolated from ovine and caprine pneumonia. They found that 50% of these bacteria were resistant to penicillin, streptomycin, chloramphenicol and sulphonamides, while all the isolated bacteria were sensitive to gentamicin. In their interpretations, these authors attributed that the bacteria have acquired resistance for isolates and concluded that the resistance could be due to random, haphazard and unplanned administration of different medicines for

mastitis curing as well as other probable causes. The main mechanisms of bacterial resistance largely depend on the limitation of drug absorption, blocking and hindering the drug's action, stoppage of the active flow of the drug.

However, these means and systems may either actually exist in these microorganisms or are acquired from other adjacent microorganisms. Some workers postulated that the resistance might act as a bacterial accommodation or adaptation alongside the ambient environment based on a proposal that the continuous existence of antibodies may maintain and preserve such resistance. At the emergence of a resistant generation of certain bacteria, the "nascent" criterion is considered "adaptive technology" with adverse ambient conditions. Other possibilities that the resistance may submit to the selective mutation occurrence depending on the pressure subjected to bacteria will determine the manner followed to express such resistance.

Other thoughts include that beneficial bacteria "flora" having resistant genes may crossly transmit these genes or mutations to other bacteria which will influence the osmolality of the cellular membrane, which is known as the "expeller" mechanism **(Sahay, *et al.*, 2020; Yousif and Jwher 2021).** The frequent application of antibiotics such as penicillin, tetracycline, chloramphenicol, and macrolides for respiratory systems affected by *M. haemolytica* results in increased bacterial resistance among several isolates of the organism. These observations were confirmed by many researchers **(Michael *et al.*, 2012; Portis *et al.*, 2012).** However, (**Garrod *et al.*, 1981)** mentioned that the bacteria might display antibiotic resistance by multiple mechanisms, *i.e.*, selection, adaptation and mutation. These postulations promote and support the belief that these bacteria have other resistant mechanisms, otherwise *ß*-lactamase. This hypothesis promotes the belief that these bacteria have other resistant mechanisms rather than ß lactamase. These genes are mostly present in the Pasteurella class with movable genetic elements, including plasmids, transposons, and combined-integrated elements. It is possible that a fraction of chromosomes of DNA may be exposed to the "transplantation process".

A transplantation process can occur between whole DNA or between nuclear acid chromos and phagic chromosome and plasmid DNA in case of the absence of integrated series of the nucleic acid of the host **(Betermier *et al.*, 1993; Surrett *et al.*, 1987).** Micro-plasmids are regarded as the commonest in species of Pasteurellaceae. The availability of more than one plasmid is considered an efficient agent for the acquirement of multiple resistance. The resistance was described as transposons and integrative and conjugative (ICEs) elements in species of *M. haemolytica* and *P. multocida* isolated from respiratory tract infections of cattle **(Michael *et al.*, 2012).**

CONCLUSION

The work proves the important role of *M. haemolytica* in the occurrence of mastitis in lactating ewes, with 51.12% of the infected cases. Poor management, wrong and indiscriminate use of veterinary medicines is one of the most important causes for the increase in bacterial resistance to antibiotics in treating disease cases without consulting a veterinarian, was associated with the high level of mastitis caused by *M. haemolytica*. Therefore, it is necessary to conduct sensitivity tests before starting treatment for mastitis and other cases.

Declaration of competing interest

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

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