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Isolation, identification and genetic analysis of *Mannheimia haemolytica* in ovine clinical mastitis in Mosul city

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Abstract

The aim of this study was to identify and diagnose of *M. haemolytica* strains as one of the most important causes of ovine clinical mastitis in Mosul city. One hundred and thirty-three milk samples were directly obtained from the udders of ewes infected by clinical mastitis from November 2020 to January 2021.Standard and conventional methods were followed for isolation and identification of *M. haemolytica*. Milk samples were cultured on blood agar 7% and MacConkey agar, then it was purified and was stained by Methylene blue. Later, different biochemical tests were conducted. Molecular identification of *M. haemolytica* depending on 16srRNA gene, followed by sequencing, similarity and phylogenetic tree was generated. The results showed that 62(46.61%) of samples were positive for bacterial isolation, biochemical tests and conventional PCR technique. Sequencing results showed that the positive samples were belonged to *M. haemolytica* strains. The similarity within strain Ib001 and within strain 39433 were 100%, and 99.47% respectively. Poor management was associated with the high level of mastitis caused by *M. haemolytica*, so the application of prophylactic programs should be followed to limit the spread of the disease.

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Introduction

Mastitis is known as inflammation of mammary glands including teats, teat canals and milk cistern of the udder. Its characterized by physical, chemical, and biological alteration of the udder and milk (1,2). Mastitis is regarded as a source of anxious for animal owners due to its adverse effect on animal hygiene and management as well as its influence on milk production, dairy manufacturing, wool and meat production. Mastitis caused by bacterial infection is the most common Infectious agents compared with other pathological agents (3,4). Several previous studies reported that subclinical and clinical mastitis caused mostly by *Staphylococcus aureus* (5,6). However, *Mannheimia haemolytica* was reported as etiological factor of subclinical and clinical mastitis in ewes (3,7). It was observed that *Mannheimia hemolytica* is the most important causes of mastitis of sheep (8). *Mannheimia haemolytica* is gram negative bacilli, non- motile, non-sporulated, the contents of G+C constitutes 39-40 % of the nuclear acid of DNA (9). *M. haemolytica* was considered as one of *Pasteuella* species after its re-classification by the German microbiologist Walter Mannheimia (10). It is naturally present in the upper part of the respiratory tract of ruminants. Most of common Mannheimia species are isolated from cattle, sheep and goats. Pathogenicity of *M. haemolytica* due to the ability of these bacteria for adhesion, presence of capsule and lipopolysaccharides, external protenous membranes iron-regulated proteins and leukotoxins. Shipping or transit fever is thought to be the main causative agent of mastitis in sheep and goats as well as the causation of pneumonia and enterotoxaemia syndromes and gangrenous mastitis in sheep and goats (11). According to the available information, there is a few studies on the role of *M. haemolytica* as a cause of

mastitis in sheep at Mosul city. The aim of current study was to identify, diagnose and determine *M. haemolytica* as one of the most important causes of ovine mastitis in Mosul city.

Materials and methods

Samples collection

Initially, 13 dairy ewe's farms, located in the Mosul city, Iraq, were selected for the milk sample collection. One hundred and thirty-three milk samples were directly obtained from the udders of sheep infected by clinical mastitis "warm, swollen, indurated, painful with abnormal milk secretion" from the period of Nov of 2020 to Jan, 2021. The milk samples were taken from the inflamed part in a sterile plastic container after the discarding the first drops of milk. Samples were put in cooled box and transported rapidly to the laboratory of veterinary public health department /college of veterinary medicine for bacteriological investigations and analysis (12).

Bacteriological investigation

Method described by Abdallah *et al.* (13) was followed, standard and conventional methods were followed for isolation and identification of *M. haemolytica*. Milk samples were cultured on blood agar 7% and MacConkey agar, then they were purified and stained by Methylene blue. Later, different biochemical tests (Indole-production, Methyl red, Voges-proskauer, Simmons Citrate, Kligler's Iron Agar, Urease, Motility, Maltose, Sucrose, Mannitol, Glucose, Cytochrome oxidase, Catalase) (11).

DNA extraction

The extraction of nucleic acid from isolates of bacteria was performed according manufacture instruction using special kits supplied by (Geneaid Corporation, Taiwan).

Conventional PCR amplification of DNA

Molecular identification of *M. haemolytica* depending on 16srRNA gene. The DNA of the isolates bacteria were amplification using conventional PCR technique. The specific oligonucleotide primers used to amplify the bacterial 16s rRNA gene for this study (14). A total volume of PCR reaction was 25 µL which consist of: (i) 1 µL forward primer, (ii) 1 µL of reverse primer, (iii) 12.5 µL of 2×Go Taq Green Mix Master containing (1-unit Gold Star DNA polymerase, 400 µM dNTPs, 3 µM $MgCl_2$, 20 µM (NH_4) $2SO_4$, 75 µM Tris HCl (pH 8.5), yellow and blue dyes which function as loading dye (Promiga USA), (iv) 8 µL of nuclease-free water (Promiga USA), and (v) 2.5 µL DNA template of *M. haemolytica*. The mixture was placed in PCR reaction tube (Promiga USA).

The DNA of *M. haemolytica* was amplified by using the thermocycler program which was set as the following: (i) 5 minutes at 95°C for the denaturation, (ii) 35 cycle. Where each cycle consists of denaturation (45 seconds at 94ºC); annealing (1 min. at 56ºC); and extension (1 min. ant 72ºC) and extension (1 min. at 72ºC) and (iii) 5 min.at 72ºC for the final extension (Table 1), finally, the PCR products were determined by gel electrophoresis together with DNA marker 100 bp ladder in 2% agarose gel (Bio Basic Inc., Canada).

Table 1: The utilizing PCR Primers for identification of *M. haemolytica* isolated from ovine mastitis.

Gene	Primer	Sequence (5`-3`)	Molecular weight	Reference
16srRNA	Forward Revers	GACCTCGGTTTAGTTCACAGA CACACGCTGACGCTGACCA	1350 bp	14

Sequencing and phylogenic analysis

From a total of 62 positive samples of *M. haemolytica* strains base on conventional PCR.10 PCR amplicons were submitted to (Immunogene Center, North Korea). Sequences of the DNA were analyzed by using blasted against Mannheamia GenBank sequences by using NCBI blast (BLASTn) from NCBI [available at]. In addition to similarity analysis of sequences within and between our obtained sequences were preform using online multiple sequences alignment-CLUSTALW (GenomeNet) software program [availabel at] then phylogenic tree was generated ClustalX (NCBI) software programs [available at].

Results

The results showed that out of 133 samples, 62of them were positive 46.61% by PCR technique that depended on bacterial culture of all samples obtained from ewes infected with clinical mastitis. The growth of microbial colonies of *M. haemolytica* on the blood agar, which were in the form of regular smooth gray colonies, with *β*-hemolysis surrounding and below the bacterial colonies. In McConkey agar can also be observed through its ability to ferment lactose and change the color of the medium from pink to yellow as a result of a change in the pH to acidic due to lactose fermentation. Microscopically, bacteria were stained with Methylene blue appear as a bipolar rods, and arranged either singly, in pairs, or as strings. Regarding the biochemical tests, the findings of all strains of *M. haemolytica* (Table 2). This study varied individual sequence analysis (BLASTn) of 10 sequences of 16SrRNA gene, 7 sequences for *M. haemolytica* strain Ib001 and 3 sequences for *M. haemolytica* strain 39433 out of 62 isolates of *M. haemolytica* samples. For the first time, the two strain were detected in Mosul city. The results also showed that the similarity within *M. haemolytica* strain Ib001 was 100%, and the similarity within *M. haemolytica* strains 39433 was 99.47%. while the similarity between two strains was from to 96.04% (Table 3).

Table 2: Biochemical tests for *M. haemolytica*

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

The current study showed that all 62 samples which were positive to standard microbiological techniques (Bacterial isolation and Biochemical tests), have been confirmed by the conventional PCR technique base on specific primers (Figure 1).

The study revealed that the homology between isolates of both *M. haemolytica* strain sequences obtained from acute mastitis in ewes and GenBank database demonstrated that *M. haemolytica* strains Ib001 (n=7) and *M. haemolytica* strains 39433 (n=3) were highly related 100% and 98% identity respectively with comprising to those sequences previously published of *M. haemolytica* strain USMARC_2286 (NC_021883.1) in USA (Figure 2 and Table 4).

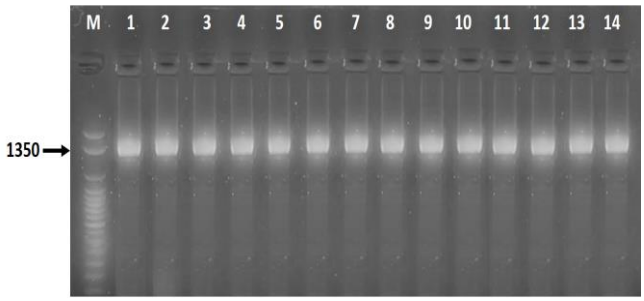

Figure 1: Amplification products for 16srRNA gene (1350 bp) of *M. haemolytica*. isolated from ovine mastitic milk.

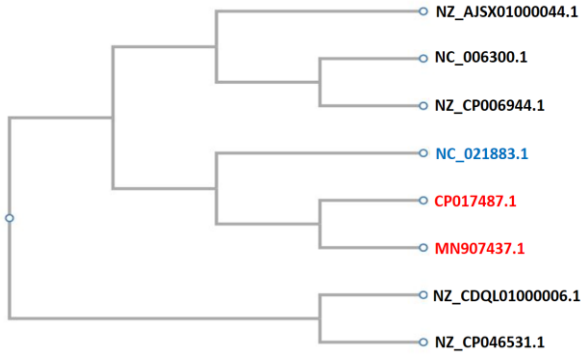

Figure 2: Simplified phylogenetic tree of *M. haemolytica* obtained with partial sequences of 16S rRNA gene (our strains, USA strain).

Table 3: Similarity within and between isolated *M. haemolytica* strains using multiple sequence alignment-CLUSTALW

Category	Strain	No. of sample	Similarity (%)
Within strains	Ib001	7	100%
	39433	3	99.47%
Between two strains	Ib001: 39433	7:3	96.04%

Table 4: Homology between obtained sequences of *M. haemolytica* strains and GeneBank data

Sequence accession number	GeneBank-NCBI number	Query cover	Identity	Gap
Ib001	NC_021883.1	99%	556/558(99.64%)	0/558(0%)
39433	NC_021883.1	97%	537/558(96.04%)	0/555(0%)

Discussion

Mastitis is regarded as an important productive disease contracting ranged-reared sheep with particular concern of those dairy and nursing ewes. Several studies have approved and confirmed the effective role of *M. haemolytica* as a primary and crucial etiology of mastitis in sheep which similar to those caused by *Staphylococcus aureus*, however, the infection percentages of the former microorganism are relatively higher (2,4,15). Consequently, the current work supports such type of mastitis in sheep of Mosul city. Although the occurrence of such inflammation had significantly decreased in countries which had elaborate and developed agricultural and dairy herd manufacturing processes particularly in the last decades, it may act as challenge and obstacle in countries who's their types of animal raising systems are classical, primitive and traditional. However, these environments and ecology hinder the development and prevention which ultimately participate in prevailing ovine mastitis (16). Apart of being an infectious disease, mastitis is regarded as an economic problem causes greater losses with unprofitable consequences in the most

countries of the globe. Such harm and disadvantages are not limited to developing countries. Several hundred tons of various antibiotic remedies are used as cure, therapy and prevention of mastitis as well as production of un healthy and un wholesome milk which is harmful and unfit for human consumption (17). Mastitis is frequently related with poor hygienic practices and animal management which lead to bruising, trauma and contusion in the udder parenchyma or the teats. Damage of the udder tissue can be due to shocks, violent abnormal suckling or lactation or fly's stings or other kinds of superficial wounds that affect skin providing a suitable environment or media for possible infection (9,17). Our findings were in very close to the isolation ratios recorded by others Omaleki *et al.* (18) which reached 40.3% and 44.5% respectively. In a study carried out on approximately three thousand ewes including seventy dairy herds of England and Wales, *M. haemolytica* was observed as an etiology forming 40.3% (17) which was somewhat less than those recorded in the present study. In Australia, another study confirmed these findings of ovine mastitis occurrence which was 64% (15), whereas, in Netherlands, a rate of 86% was recorded in a study conducted by Radostits *et al.* (17) on lactating ewes in the first six weeks of lactation, which is rather higher than the percentage obtained in our study.

The aforementioned workers attributed their causes to the suckling lambs that play an important role in transmitting infection because they carry the pathogen in their nasopharynx (2,15). However, the outcome of the present work is in accordance with those authors Omaleki *et al.* (15) and Van den *et al.* (19), who isolated and identified other species of Mannheamia (*M. haemolytica*, *M. ruminalis* and *M. glucosida*) from multiple cases of acute mastitis. Burriel (6) mentioned that fields, paddocks and ranges could possibly be contaminated by *M. haemolytica* due to previous grazing of such places by lambs infected with pneumonia caused by these bacteria with the higher probability of *M. haemolytica* isolation from grass, drinking water and straw bedding. The role of *M. haemolytica* was also referred to by Al Salihi (20). Differencing the geographical nature and the method of breeding system that depends on open grazing, unlike the animal rearing in Mosul city, which relies on semi-open grazing and sheltering, as well as the difference in temperature with subsequent impact on the incidence of *M. haemolytica* infection.

Nonetheless, ambient climatic conditions *i.e.* lowered atmospheric temperature, high relative humidity prevail in places surrounding the animals play a serious impact in maintaining, sustaining, accelerating, perpetuation or even the survival of the causative microorganisms. It was observed that such circumstances may be an additional agent for preserving the etiology of the disease with cumulative effect in places highly grazed by intensive flocks of sheep. However, these situations predispose sheep for possible mastitis (6,21). It is possible that these bacteria may establish and colonize ewe's teats with consequent entering or later introducing of microorganisms inside the teat during suckling, nursing or lactation. The most acceptable interpretation of mastitis occurrence cases is the typical requirements of climate *i.e.* cool and humid weather and during grazing which is actually noticed in our study Giadinis *et al.* (22). According Giadinis *et al.* (22) and Bergonier *et al.* (23), poor or unbalanced feeding may contribute in the development of mastitis particularly in ewes fed on deficient vit A and selenium rations causing lowered immunity of localized epithelial cells of the udder. These observations were confirmed by AL-bayati *et al.* (24) who found an isolation rate of 12.8% in ovine mastitis.

Most animal suffer from these conditions especially during drought and desiccation periods reflected by malnutrition. Notably, the current investigation was carried out on the same prerequisites in which feed sources (*i.e.* quantity and quality) is quite rare and scarce which was apparently seen in the manifestations of the examined sheep, suffering from malnutrition. Other plausible causes of mastitis concerned with milking practice followed including rough and violent approach and handing of animal during milking such as excessive lactation, dirty and filthy udder and teats which may increase or exaggerate the condition make the udder more prone to the risks, of mammary gland infection (6,24). In the same context, exposure of the sheep to the cold weather may cause abnormal pathological lesions in the teat with subsequent increase in the bacterial counts on the teat's skin or orifice leading to increase number of mastitis cases (24). Furthermore, the common practice of washing and rinsing of the udder with sufficient clean water is absent due to shortage of water itself or due to application or use of dirty and impure water which may produce the worse state (25). Strategic applications of udder with antibiotics should be followed in lactating female's prior of being dry (26). Interestingly, such scientific tradition is not practiced in local farms which is regarded as an essential element of successful udder hygiene (27). This practice aims to treat inflammation occurring preceding to lactation period as well as avoidance and prevention of new udder infection in the next dry periods as recommended by other Fthenakis *et al.* (28).

Interestingly in our results demonstrated that *M. haemolytica* strains Ib001 and *M. haemolytica* strains 39433 were highly related with *M. haemolytica* strain USMARC_2286 (NC_021883.1) USA at identity rat of 99.64% and 96.04% respectively that are proven by the results of homology and phylogenetic analysis (29). The results of this study showed that *M. haemolytica* strains Ib001was significantly more detected than *M. haemolytica* Strains 39433 in Mosul city. This result is inconsistent with previous studies in other countries Tewodros and Annania (30); Tabatabaei and Abdollahi (31), where the geographical location plays an important role in the relationship between living organisms, as the genetic types that are from similar or neighboring areas are closely related (32). Results like this could be used in designing primers for the diagnosis of *M. haemolytica* and vaccine production.

The obtained findings promoted a reasonable discussion to develop acceptable principles and bases to perform farther observation for recently detected isolates. Also, such study could provide valuable information about the diversity of several strains infecting sheep dairy herd in Mosul city. Consequently, these findings may contribute to facilitate and developed efficient and practical programs for prophylaxis of ovine mastitis caused by *M. haemolytica*.

Conclusion

Agents predispose mastitis occurrence with *M. haemolytica* of sheep is probably great, particularly in nursing ewes. It is possible that these microorganisms may be transferred from the mouth cavity of the lamb to the mammary gland of ewe through nursing. There is an evidence that *M. haemolytica* of the udder may spread horizontally. Farm equipment and facilitates should be designed to lower the spread of the diseases *i.e.* altering animal surroundings and ambient circumstances. Lambs should be weaned early for possible reduction of the infections. Measures followed to reduce *M. haemolytica* of the respiratory tract may have preventative influence. It should be noted that there is an identity between *M. haemolytica* strains in the studied areas, this helps to control mastitis by vaccines manufacturing. So, precautionary steps, hygiene practice and better attention must be taken for sheep as well as the application of defensive programs to limit the spread of the disease.

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Conflict of interest

The authors reported no potential conflict of interest.

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عزل وتشخيص وتحليل وراثي للمانيميا الحالة للدم المسببة التهاب الضرع السريري عند الأغنام في مدينة الموصل

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الخلاصة

هدفت الدراسة الحالية للتعرف على سلالة المانيميا الحالة للدم المعزولة من حالات التهاب الضرع عند الأغنام. لهذا الغرض، جمعت ١٣٣ عينة من حليب الأغنام من النعاج التي تعاني من درجات مختلفة من التهاب الضرع السريري من تشرين الثاني ٢٠٢٠ إلى كانون الأول ٢٠٢١. اتبعت الطرق القياسية والتقليدية لتحديد وعزل المانيميا الحالة للدم، حيث زرعت عينات الحليب على أكار الدم بنسبة ٧٪ وأكار الماكونكي، ثم صبغت بصبغة المثلين الأزرق. وأجريت عليها الاختبارات الكيموحيوية المختلفة. شخصت المانيميا الحالة للدم جزيئيا بالاعتماد على جين 16Sr RNA وأجريت عليها عملية تطابق وتشابه للتسلسلات الجينية ورسم شجرة النشوء والتطور. أظهرت النتائج أن ٦٢ عينة أي بنسبة ٤٦,٦١٪ كانت موجبة للعزل الجرثومي والاختبارات الكيموحيوية وتقنية تفاعل البلمرة المتسلسل التقليدية. وبينت نتائج التسلسل أن العينات الإيجابية التي تنتمي إلى سلالات المانيميا الحالة للدم للتطابق الجيني ان هناك تشابها ضمن عزلات السلالة Ib001 بنسبة ١٠٠٪ وعزلات السلالة ٣٩٤٣٣ بنسبة ٩٩,٤٧٪. ارتبط سوء الوضع الصحي بالمستوى المرتفع من حالات التهاب الضرع الذي تسببه المانيميا الحالة للدم، لذلك يجب اتخاذ التدابير الوقائية والاهتمام بالممارسات الصحية للأغنام وتطبيق البرامج الوقائية للحد من انتشار المرض.