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Prevalence of *Mycoplasma bovis* in Cattle in Nineveh Governorate, Iraq

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Abstract

THE CURRENT STUDY aimed to determine the prevalence of *Mycoplasma bovis* in cattle in Nineveh Governorate, Iraq, using the culture method, indirect enzyme-linked immunosorbent assay (i-ELISA)**,** and conventional polymerase chain reaction (c-PCR) technique, to evaluate the efficiency of the various laboratory methods used in this study. During the period from March 2022 to February 2023, a total of 352 nasal swabs were collected randomly from cattle and tested using the culture method and conventional polymerase chain reaction (c-PCR) technique (amplifying the *uvrC* gene) for isolation and molecular detection of *M. bovis* , respectively. Moreover, 352 blood samples were collected from the same animals for the detection of antibodies against *M. bovis* using i-ELISA. The results observed that the prevalence of *M. bovis* was 23.57% (83 out of 352) using the culture method, 30.68% (108 out of 352) using the c-PCR technique, and 47.15% (166 out of 352) using i-ELISA. According to the Kappa value (0.807), there was perfect agreement between the c-PCR technique and the culture method, with sensitivity, specificity, and accuracy were 98.79%, 90.33%, and 92.32% for c-PCR technique respectively. While there was moderate agreement between i-ELISA test and the culture method based on Kappa (0.444), with sensitivity, specificity and accuracy were 89.15%, 65.79%, and 71.30% for i-ELISA, respectively. This study was concluded that *M. bovis* is prevalent in Nineveh Governorate and the PCR technique is the more efficient tool than ELISA for detection of *M. bovis* in suspected samples.

Keywords: *Mycoplasma bovis*, Cattle, c-PCR Technique, i-ELISA, Kappa value, Nineveh-Iraq.

Introduction

There are more than 200 species in the *Mycoplasma* genus [1], It can cause numerous infectious diseases in ruminants, particularly cattle, and humans, that result in financial losses and significant higher mortality rates in cattle [2,3]. Among these marvels is the *Mycoplasma bovis* which was initially identified from an outbreak of epidemic mastitis in cows in California state in the USA at 1961, due to their great biochemical similarities to the *Mycoplasma agalactia* that cause epidemic mastitis in cows, they were originally known as *Mycoplasma bovimastitides* and were subsequently renamed *Mycoplasma agalactia sub. bovis* [4]. *Mycoplasma bovis* is classified in List (B), which infects cows and calves [5]. This bacteria belongs to the *Mycoplasma*taceae family and it is distinguished by its short genome, absence of a cell wall, and high nutritional needs for growing *in vitro* [6]. *Mycoplasma bovis* is disseminated

by direct or indirect animal-to-animal contact in herds. Direct transmission between cows can occur during milking or through nose-to-nose contact, but indirect transmission can also happen through sharing drinking and feeding troughs [7]. Timonen *et al*. [8] stated that certain strains of *M. bovis* were isolated from calves that had respiratory illnesses and cows with clinical mastitis, suggesting a potential pathogen transfer via tainted milk between dairy cows and calves. Furthermore, *Mycoplasma bovis* causes a number of infectious diseases in cattle, so-called bovine mycoplasmosis [8,9]. These diseases include chronic bronchopneumonia in calves and cows, polyarthritis in calves, infectious mastitis in cows [10], metritis and abortion in cows, vasculitis in bulls, vasculitis in cows and calves, and keratoconjunctivitis [11]. In addition, middle ear infection and pressure sore abscesses in calves [12]. Endocarditis was also recorded in calves [13].

Mycoplasma bovis is widely spread worldwide, especially in Europe, North America, Australia, and Asia [14]. In Iraq, *M. bovis* was detected in cows and calves infected with pneumonia, polyarthritis, and mastitis in Nineveh Governorate using an indirect enzyme linked immuno-sorbent assay (i-ELISA) [15]. Another study diagnosed *M. bovis* in calves infected with pneumonia using the polymerase chain reaction technique in Mosul city, Iraq [16].

Several laboratory techniques have been developed to detect *M. bovis* in infected cattle, such as the isolation of bacteria (culture method) and identification by electron microscope [17]. Serological tests are also used, such as indirect ELISA [18]. In addition, polymerase chain reaction (PCR) technique [19]. Immunohistochemistry (IHC) [20]. There is a limited information about the prevalence of *M. bovis* in Nineveh Governorate, Iraq using different diagnostic methods. Hence, this study targeted determining the prevalence of *M. bovis* in cattle using the culture method, c-PCR technique, and i-ELISA test and evaluating the efficiency of the laboratory methods used in this study.

Material and Method

Ethical approval

The institutional animal care and use committee in the College of Veterinary Medicine, University of Mosul, was ethically permitted for this study (UM.VET. 2022.085) on February 15,

2022.

Animals and sampling size

This study was conducted on cattle of different sexes, ages, breeds, and origins, that were obtained from various regions in Nineveh Governorate, Iraq. Furthermore, for calculating the number of animals to be sampled was based on the previous study, the seroprevalence of *M. bovis* in Mosul was 76.09% [15]. Therefore, with the expected prevalence 70%, a confidence level of 95% and an absolute error of 5% [21], the following equation was used:

$$n = \frac{z^2 p(1-p)}{d^2}$$

where: n = number of sampled animals, Z = value of the normal distribution for a 5% confidence level, P = expected prevalence, and d = absolute error.

The minmum number of cattle required for this study was at least 322. However 352 samples were collected.

Samples collection that used for detection M. bovis

During the period from March 2022 to February 2023. A 352 nasal swabs were collected from cattle by rotating the swabs over any inflamed areas, visible exudate, or rhinorrhea from the anterior nares of the noses, the samples placed in phosphate buffered saline solution as transporting media, and kept in containers containing a sufficient amount of ice until delivered to a diagnostic laboratory. Then kept at -20°C until performed for the culture method and c-PCR technique [9,19]. In addition, 352 blood samples (5ml) were collected from the same animals via jugular vein puncture, then placed in a tube without anticoagulant for serum separation using a centrifuge at 2500 rpm for 15 min and stored at -20°C to be tested until use for i-ELISA test [22].

Culture method for M. bovis isolation

The nasal swabs (n=352) were placed in *Mycoplasma* broth medium, and transported immediately to a laboratory of the microbiology department, then incubated at 37°C with 5% Co2 for 7-14 days in a candle jar. When turbidity appeared, an inoculum of each broth was cultured on *Mycoplasma* agar medium: pleuropneumonia like organism (PPLO) medium (Hiehgmedialab.com, India) and incubated at 37°C with 5% Co2

for 7-14 days in a candle jar. The culture was checked daily for the first 7 days and weekly thereafter [22].

Conventional PCR technique for M. bovis detection

The DNA of *M. bovis* was extracted from nasal swabs (n=352), using the commercial HS Prime Taq Premix (2X) DNA extraction kit (GeNetBio, Korea). This was performed according to the manufacturer's instructions. Using the Nanodrop (BioDrop, Germany), the concentration of extracted DNA was estimated at wavelength 260nm, while the purity of extracted DNA was estimated by calculating the ratio of (A260 nm to A280 nm) as described by Morais *et al.* [23]. In addition, the c-PCR technique was used to amplify deoxyribodipyrimidine photolyase (*uvrC*) gene of *M. bovis*. Oligonucleotides specific primers provided by Macrogen Inc., South Korea. Further, the positive bands were at approximately 1626bp, that were used to amplify the *uvrC* gene; the primers *uvrC*-F(5'- TTACGCAAGAGAATGCTTCA-3'), and *uvrC*-R (5'-TAGGAAAGCACCCT ATTGAT -3') were designed by Perez-Casal and Prysliak, [24]. A clinically and laboratory-positive cow's DNA was used as a positive control. Additionally, all components of PCR reaction was added except the DNA, used as a negative control. A total volume of 20μl of the PCR reaction was used, comprising 10μl of the master mix (2X), 1μl (10 pmol) of each primer (*uvrC*-F and *uvrC*-R), 3μl of template DNA, and 5μl of nuclease-free water. Furthermore, the thermocycler was set with some modifications in steps according to Perez-Casal and Prysliak, [24]. Initial denaturation at 94°C for 2 min (1 cycle), followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min, then final extension at 72°C for 5 min (1 cycle). The Safe-Red™ dye-stained and 1.5% agarose gel were used to electrophorese the PCR products. Further, to visualize the resultant bands, UV transillumination (BIO-RAD/USA) was utilized.

Indirect ELISA test for M. bovis antibodies detection

The serum samples (n=352) were tested for detection the antibodies against *M. bovis* using ELISA kit BioX *Mycoplasma bovis* (BioX Diagnostics, Belgium). This test was done according to the instructions of the manufacturer.

Statistical analysis

Descriptive statistics on the Excel program 2010 was used to calculate the prevalence, and IBM SPSS Version 22 (Inc., Chicago, USA) was used to determine the agreement between the different diagnostic methods used in this study based on the Kappa value [25], and calculating the sensitivity, specificity, and accuracy of diagnostic tests was used to evaluate the efficacy of the applied laboratory methods [26].

Results

Results of this study observed that the prevalence of *Mycoplasma bovis* in Nineveh Governorate was 23.57% (83 out of 352), 30.68% (108 out of 352), and 47.15% (166 out of 352) using the culture method, c-PCR technique, and i-ELISA, respectively (Table 1). According to the Kappa value (0.807), there was a perfect agreement between the c-PCR technique and the culture method, with a sensitivity 98.79%, specificity 90.33%, and an accuracy 92.32% for the c-PCR technique. While there was a moderate agreement (Kappa=0.444) between the i-ELISA test and the culture method, with a sensitivity 89.15%, specificity 65.79%, and accuracy 71.30% for i-ELISA test (Tables 2, 3).

Based on the culture method of *Mycoplasma* colonies on pleuropneumonia like organism (PPLO) medium, the growing organism colonies appeared as small, pearlescent, with a matte center and a white to colorless aura halo, with an egg-like (fried egg) appearance (Fig. 1). Using the Nanodrop, the concentration of DNA extracted from the nasal swabs ranged between 70.6 - 277.2 ng/μl, and the purity was ranged between 1.7 - 1.9. Based on the c-PCR technique for amplifying the *uvrC* gene of *M. bovis* using specific primers, the positive bands were at approximately 1626bp. (Fig. 2).

Discussion

In this study, the prevalence of *M. bovis* infection in cattle in Nineveh Governorate was 23.57%, 30.68% and 47. 15% using culture method, c-PCR technique and i-ELISA test respectively. These results are lower than other studies addressing the prevalence of *M. bovis* in Iraq, Mhmood and Rhaymah, [14] and Hamad *et al.* [15] who were mentioned that the popularity of *M. bovis* in calves in Mosul city were 76.09% and 86.5% using i-ELISA test and PCR techniques, respectively. There are several studies that have detected the prevalence of *M. bovis* in cattle in various countries, including Turkey, was 23.3% using direct fluorescent antibody testing (DFAT) [27], In Iran, was 88% using the nested PCR

(n-PCR) technique [28], in Jordan, was 27.3% using conventional PCR (c-PCR) technique [29], in Saudi Arabia, was 24% using c-PCR technique [30], in China, was 48.7% using i-ELSA [31], in Egypt, was 67.5% and 8.3%, using culture method and c-PCR technique respectively [32,33], in Sudan, was 7.2% using i-ELISA test [34], in Algeria, was 69.0% and 58.0% using i-ELISA test and Real time PCR technique respectively [35], in United States of America, was 100% and 87.5% using LAMP and real-time PCR (RT-PCR) technique respectively [36], in Brazil, was 91.4%, 1.1% and 62.3% using (IHC), RT-PCR technique, and i-ELISA test respectively [1,37,38], and in Australia, was 42.5% using i-ELISA test [39]. The differs in the prevalence of *M. bovis* among counties may be due to different management approaches, environmental conditions, efficient diagnostic techniques, types of samples that were tested, and the presence or absence of additional factors, such as the host's age, physical characteristics, and immunological status. The variations in *M. bovis* prevalence across different countries was recorded [35,40-45]. Based on the culture method of *M. bovis* on PPLO medium, it appeared as small, pearlescent, with a matte center and a white to colorless aura halo, with an egg-like (fried egg) appearance. This result was similar to those of Niu *et al*. [31]; Ismael *et al*. [46]; Quinn *et al*. [47]; and Gioia *et al*. [48]. Despite some significant drawbacks with the culture method, such as its ability to identify *Mycoplasma* organisms to the genus level only, the process is laborious and time-consuming, *M. bovis* cannot be detected at low concentrations of less than 100 cfu/mL (less than one colony in 10ml of milk), and it cannot distinguish between closely related species [6,46,49,50]. The culture method is considered a gold standard method for detection *M. bovis* in this study, which is used for evolution of new techniques, and it is simple and inexpensive method as well [6,9].

In this study, the *uvr*C gene was selected for the PCR technique to detect *M. bovis* in cattle because it is one of the most commonly targeted in epidemiology, sequencing, and phylogenic analyses studies, and it is available in various molecular databases [1,24,51,52].

The current study indicated that perfect agreement was showed between the c-PCR technique and culture method, based on the Kappa value, with a sensitivity 98.79%, specificity 90.33%, and accuracy 92.32% for the c-PCR technique. According to the type of PCR technique, the agreement, sensitivity, and specificity differed for detection of *M. bovis* when compared with the culture method, such as the perfect agreement between the LAMP PCR technique and the culture method (Kappa value = 0.8231), with 97.2% sensitivity and 90.9% specificity, and the substantial agreement between the c-PCR technique and the culture method (Kappa value = 0.7767), with 86.1% sensitivity and 92.9% specificity [53]. Moreover, Parker *et al*. [6] and Scott *et al*. [54] noted that PCR techniques have greater efficiency, specificity, and sensitivity in laboratory detection of *M. bovis* when compared with traditional culture methods. The results also showed moderate agreement between the i-ELISA test and the culture method based on Kappa (0.444), with sensitivity 89.15%, specificity 65.79%, and accuracy 71.30% for the i-ELISA. This finding may be due to the different targets for the two methods (in i-ELISA, the target is the antibodies, while in the culture method, the target is the antigen), cross-reactivity of *M. bovis* with other pathogens, seroconversion, which may take 2-3 weeks before antibodies can be detected, the only detected *M. bovis* in serum, plasma, or milk, and suggestions of poor sensitivity of i-ELISA test [6,36,55]. Furthermore, Szacawa *et al*. [56] stated that i-ELISA test had the lowest level of compatibility with antigen ELISA, PCR techniques, and culture method for diagnosing *M. bovis*, but a high degree of correlation between these methods.

Conclusions

This study was stated that *M. bovis* is circulated among cattle, with a higher prevalence in Nineveh Governorate, Iraq. There were perfect agreements between the c-PCR technique and culture method, while there were moderate agreements between the i-ELISA test and culture methods. This indicates that the c-PCR technique is more efficient than ELISA for diagnosing *M. bovis*. Cattle management practices through responsible cattle ownership and applying a carefully planned program for *M. bovis* control measures were advised.

Conflict of Interest
The authors claim no conflicts of interest.

TABLE 1. Prevalence of *Mycoplasma bovis* in cattle using the culture method, conventional-PCR technique, and indirect-ELISA

Type of test	No. of examined samples	No. of Positive samples (%)
Culture method		83(23.57)
Indirect-ELISA	352	166 (47.15)
Conventional-PCR		108 (30.68)

TABLE 2. The agreement between culture and c-PCR technique based on Kappa value, with the calculating the ratio of the c-PCR technique sensitivity, specificity, and accuracy for *Mycoplasma bovis* diagnosis

		Culture method		
		Infected	Uninfected	Total No.
c-PCR technique	Infected	82[a]	26[b]	108
	Uninfected	1[c]	243[d]	244
Total		**83**	**269**	**352**

(a) True positive samples, (b) False positive samples, (c) False negative samples, (d) True negative samples. Kappa value was (0.807). Sensitivity = a/(a+c) x 100= 98.79%. Specificity = d/(b+d) x 100 = 90.33%. Accuracy= (a+d)/ (a+c+ b+d) x 100 = 92.32%.

TABLE 3. The agreement between culture and indirect ELISA based on Kappa value, with the calculation of the ratio of the indirect ELISA sensitivity, specificity, and accuracy for *Mycoplasma bovis* diagnosis

		Culture method		
		Infected	Uninfected	Total No.
i-ELISA	Infected	74 [a]	92 [b]	166
	Uninfected	9 [c]	177 [d]	186
Total		83	269	352

(a) True positive samples, (b) False positive samples, (c) False negative samples, (d) True negative samples. Kappa value was (0.444). Sensitivity = a/(a+c) x 100= 89.15%. Specificity = d/(b+d) x 100 = 65.79%. Accuracy= (a+d)/ (a+c+ b+d) x 100 = 71.30%.
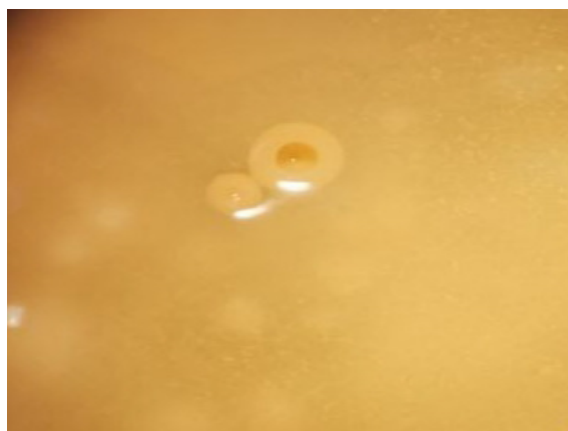
Fig. 1. Fried egg appearance of *Mycoplasma* colonies growing on medium (PPLO Medium) using a light microscope.
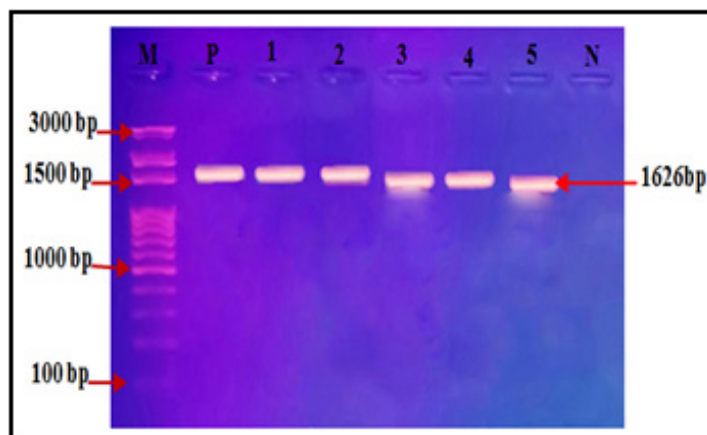
Fig. 2. The conventional PCR image: Lane M) DNA ladder; Lane P) DNA extracted from infected cattle used as positive control for *Mycoplasma bovis*; Lanes (1-5) positive samples of *M. bovis* using specific primers for *uvrC* gene in approximately band size 1626bp.; Lane N) add all of PCR component except DNA used as negative control.

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انتشار المفطورات البقرية في الابقار في محافظة نينوى، العراق

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المستخلص:

هدفت الدراسة الحالية إلى تحديد مدى انتشار المفطورات البقرية في الأبقار في محافظة نينوى، العراق، باستخدام طريقة الزرع الجرثومي واختبار الممتز المناعي غير المباشر وتقنية تفاعل البلمرة المتسلسل التقليدي ، مع تقييم مدى كفاءة الطرق المخبرية المختلفة المستخدمة في هذه الدراسة. من خلال الفترة الممتدة من أذار ٢٠٢٢ إلى شباط ٢٠٢٣، تم جمع ٣٥٢ مسحة أنفية بشكل عشوائي من الابقار وفحصها باستخدام طريقة الزرع الجرثومي وتقنية تفاعل البلمرة المتسلسل التقليدية (تضخيم الجين uvrC)، لغرض العزل والكشف الجزيئي لتلك الجراثيم على التوالي. فضلا عن، جمع ٣٥٢ عينة دم من نفس الابقار للكشف عن الأجسام المضادة لجراثيم المفطورات البقرية باستخدام اختبار الممتز المناعي غير المباشر. أظهرت النتائج أن معدل انتشار المفطورات البقرية كانت ٢٣،٥٧٪ (٨٣ من ٣٥٢) باستخدام طريقة الزرع الجرثومي، و٣٠،٦٨٪ (١٠٨ من ٣٥٢) باستخدام تقنية تفاعل البلمرة المتسلسل التقليدي، و٤٧،١٥٪ (١٦٦ من ٣٥٢) باستخدام اختبار الممتز المناعي غير المباشر. ووفقا لقيمة كابا (٠،٨٠٧)، لوحظ ان هناك توافق تام بين تقنية تفاعل البلمرة المتسلسل التقليدي وطريقة الزرع الجرثومي، وكانت الحساسية والنوعية والدقة ٩٨،٧٩٪، ٩٠،٣٣٪، و٩٢،٣٢٪ لتقنية تفاعل البلمرة المتسلسل التقليدي على التوالي. في حين كان هناك توافق معتدل بين اختبار الممتز المناعي غير المباشر وطريقة الزرع الجرثومي اعتمادا على قيمة كابا ((٠،٤٤٤)، وكانت الحساسية والنوعية والدقة ٨٩،١٥٪، ٦٥،٧٩٪، و٧١،٣٠٪ لاختبار الممتز المناعي غير المباشر على التوالي. استنتج من هذه الدراسة إلى أن المفطورات البقرية منتشرة في الابقار في محافظة نينوى ،العراق، وأن تقنية تفاعل البلمرة المتسلسل التقليدي أكثر كفاءة من اختبار الممتز المناعي غير المباشر في الكشف عن المفطورات البقرية.

الكلمات المفتاحية: الميكوبلازما البقرية، الأبقار، تقنية c-PCR ،i-ELISA، قيمة كابا، نينوى-العراق.