Molecular identification of *Mycoplasma agalactiae* from mastitic milk of lactating ewes in Al-Muthana province\Iraq

Lugien Abed Al-Khudher Qassim Haleem Kshash
Coll. of Vet. Med. / Univ. of Al-Qadisiyah
email: qassim.kshash@qu.edu.iq
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Abstract

Mastitis is the main signs of contagious agalactia syndrome in sheep and goats, which is caused by *Mycoplasma agalactiae* and induces heavy economic losses. The aim of this study was to isolate and identify *M. agalactia* from mastitic lactating ewes at Al-Muthana province. Clinical mastitic milk collected from infected animals. The collected samples were transferred in a transport medium which contained PPLO broth, horse serum and yeast extract at cool conditions to the lab. All the samples were carried out by PCR and cultured on PPLO agar too. The results showed that, from the total of 150 mastitic milk samples, 40% was positive by culture for growth of mycoplasma. Out of 60 positive culture there was 35(58.3%) positive for *Mycoplasma spp.* PCR, and 4\35 (11.4%) were identified as *Mycoplasma agalactiae* by PCR. The lactating ewes with mycoplasmas mastitis exhibited non-significant variation in temperature, respiration and pulsation rates when compared with healthy ewes group.
Key words: *Mycoplasma agalactiae*, ewes, mastitis, PCR, milk.

التشخيص الجزيئي للمايكوبلازما اكالاكشيا من حليب النعاج المصابة بالتهاب الضرع في محافظة المثنى- العراق

لجين عبد الخضر قاسم حليم كشاش
جامعة القادسية \ كلية الطب البيطري

الخلاصة

التهاب الضرع هو أبرز علامات متلازمة انقطاع اللبن المعدية في الأغنام والماعز، حيث تعد المايكوبلازما اكالاكشيا المسبب الرئيسي له والذي يؤدي إلى خسائر اقتصادية فادحة. ان الهدف من الدراسة هو عزل وتحديد المايكوبلازما اكالاكشيا من النعاج المصابة بالتهاب الضرع في محافظة المثنى. تم جمع عينات الحليب من الحيوانات المصابة و تم نقل العينات التي تم جمعها في وسط PPLO والذي يحوي مصل الحصان وخلاصة الخميرة في ظروف باردة إلى المختبر. زرعت جميع العينات على وسط PPLO الصلب وفحصت بواسطة اختبار متعدد السلسلة المتبلمرة PCR. وأظهرت النتائج أن من مجموع 150 عينة حليب مصابة بالتهاب الضرع انه 40٪ موجبة للزرع وظهر من بين الستين (60) عينة الموجبة للزرع كانت هناك 35 (58.3٪) شخصت موجبة لجنس المايكوبلازما بواسطة تقنية PCR ، و4 \ 35 (11.4٪) تم تشخيص المايكوبلازما اكالاكشيا على إنها المسبب لالتهاب الضرع. النعاج المصابة بالتهاب الضرع لم تظهر أي تباين مميز إحصائيا في درجات الحرارة ومعدلات التنفس والنبض بالمقارنة مع مجموعة من النعاج السليمة.
الكلمات المفتاحية: المايكوبلازما اكالاكشيا ، التهاب الضرع ، اختبار متعدد السلسلة المتبلمرة ، النعاج ، الحليب.

Introduction

Mastitis associated with *Mycoplasma* has been reported worldwide (1). Mastitis caused by mycoplasma is the main important signs of contagious agalactia syndrome affected small ruminants which included kerato-conjunctivitis, arthritis as well as signs of pneumonia and abortion (2). Moreover, contagious agalactia is important infectious disease of small ruminants, it has been established for about two centuries (3) and was distinguished in sheep and goat firstly (4). So, the contagious agalactia is a noti-fiable worldwide diseases in sheep, goat and bovine and it reported from Mediterranean

area, Balkan, south west Asia countries like Turkey, Iran, Iraq, center east of Africa, and United State of America in endemic form (5). Mycoplasmas are micro-organisms that are widely distributed in nature and they are able to colonize animals, humans, plants and soil. *Mycoplasma spp.* are grouped under the class Mollicutes (soft skin), they do not have the a cell wall (6).There are four types of mycoplasma that causes disease in sheep and goat, such as *M. agalactia, M. capricolum spp., capricolum (M. cc), Mycoplasma mycoides spp. mycoides LC (LC = large colony), and Mycoplasma putrefaction* that produce a clinically similar disease (7) and all these types have been reported by Europe, western Asia, the United States of America (USA), and north of Africa (8). In most cases, infected hosts spontaneously recover from acute clinical signs within a few weeks, but develop a chronic infection accompanied by shedding of *Mycoplasma agalactiae* in milk or other body secretions for years without presenting any symptoms (9). The major causal agent of the disease in both sheep and goats are *Mycoplasma agalactia*, in lactating female, it is usually manifested by mastitis, which clinically characterized by, in appetence, alternation in consistency of the milk in lactating ewes with decline and subsequent failure of milk production (9).

Materials and methods

One hundred and fifty (150) milk samples were collected from mastitic lactating ewes which had signs of clinical mastitis (abnormalities in udder and milk features). Before sampling and according to (10) all the teat was disinfected by 70% of alcohol, and after withdrawing 2-3 drops of milk, some drops were collected in a screwed tube that contain transport medium which included (PPLO broth+ 20% horse serum and yeast extract) were transferred to laboratory in ice box for culture on PPLO agar after filtering and incubated at 37 º C with humid and 10% CO_2 environments for 10 days, and checking the growth for fried egg shape colony each alternate day (11), then the positive mycoplasma culture were submitted for PCR technique. The positive culturing sample was used for DNA extraction by DNTP kit (Cinagen Company) and multiplex PCR assay, after preparation of master mixture by using primers MGSo and Gpo3 for detection of *Mycoplasma genus* according to (5), and specific primers for *Mycoplasma genus* with163 bp and for *M. agalactia* strain with 375 bp, the sequences of primers are as follow. FS1(5- AAAGGTGCTTGAGAAATGGC- 3) FS2 (5- GTTGCAGAAGAAAGTCCAATCA- 3). The PCR procedure was processed according to (12) by used 1ml of incubated cultured mycoplasmal cells in a Eppendorf DNA thermo cycler under an optimized program which is consisted of denaturation at 95 °C for 5 minutes followed by 35cycles of 94 °C for 30 seconds, 60 °C for 40 seconds and 72°Cfor 40 seconds for denaturing, annealing and extension phases respectively. The process was followed by an additional period of 10 minutes for final extension at 72°C. PCR products were run on 1% agarose gel and subjected to electrophoresis for about 1 hr. at 95 volts. After staining the gel with 0.5µg/ml of ethidium bromide, bonds of amplified fragments were visualized and photographed. Clinical examination of infected ewes including temperature, respiratory and pulse rates were measured.

Statistical analysis: The obtained data was statically analyzed for means and significances between groups using ANOVA according to computerized SPSS program (version 7).

Results

Out of 150 mastitic milk 60 (40%) gave positive in mycoplasma culture (table 1) by appeared characteristic fried egg colony on PPLO agar (fig. 1). So, 35/60 (58.3%) were identified as *Mycoplasma spp.* by PCR (fig. 2) and 4/35 (11.4%) as *Mycoplasma agalactiae* (fig. 3). Moreover, the clinical examination of ewes that exhibited positivity to mycoplasma spp. by PCR, there was no significant differences in temperature, respiration and pulsation rate when compare with twenty (20) healthy lactating ewes (table 2).

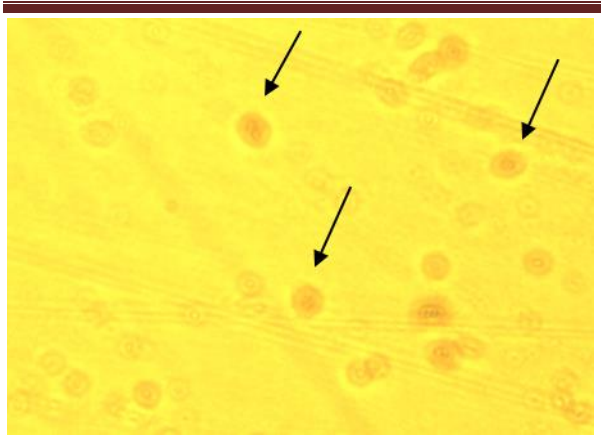

Fig. (1): *Mycoplasma agalactiae* with fried egg colonies on PPLO agar (black arrows) X4

Table (1): Percentage of positive results of milk samples for culture, *Mycoplasma spp.* PCR and *M agalactiae* PCR.

Samples no.	+ve for culture	%	+ve for *Mycoplasma spp.*	%	+ve for *M. agalactiae*	%
150	60	40	35	58.3	4	11.4

Table (2): Physical examinations (temperature, respiratory and heart rates) in examined infected and healthy ewes groups

Animal group	Temperature M±SE	Respiration M±SE	Pulse M±SE
Infected	39.5±0.81 a	29.9±1.44 a	75.8±0.54 a
Control	39.2±0.30 a	29.6±0.19 a	74.8±2.54 a

Different small letters refers to significant variations at ($p \leq 0.05$)

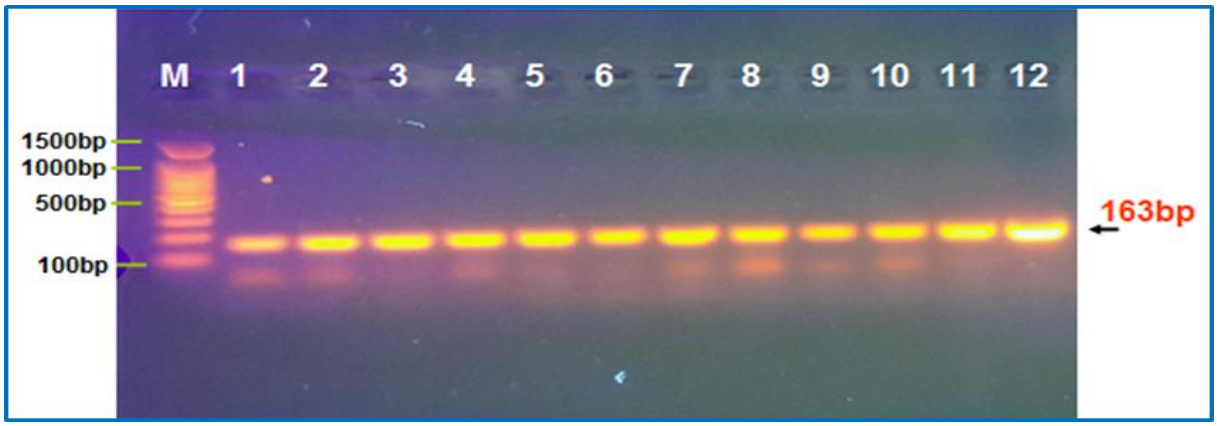

Fig. (2): Agarose gel electrophoresis image that show the PCR product analysis of 16S rRNA gene in Mycoplasma genus positive isolates. Where M: marker (1500-100bp), lane (1-12) positive *Mycoplasma genus* at (163bp) PCR product.

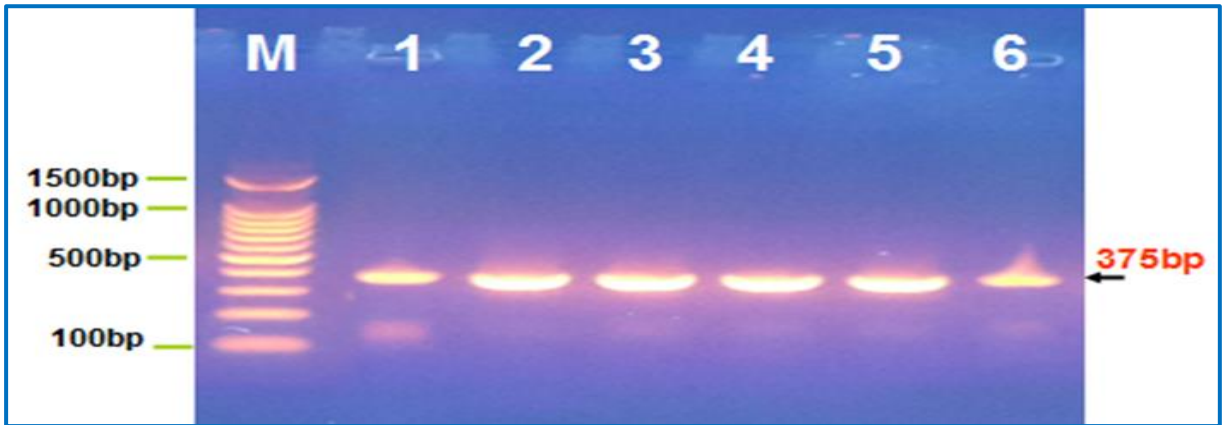

Fig (3): Agarose gel electrophoresis image that show the PCR product analysis of P80 lipoprotein gene in *Mycoplasma agalactiae* positive isolates. Where M: marker (100-

1500bp), lane (1 and 6) standard Ma strain (2, 3, 4, 5) positive *Mycoplasma agalactiae* at (375bp) PCR product.

Discussion

According to classical microbiologic diagnosis 40% of the causes of mastitic milk were regarded as *Mycoplasma spp*. and about 60% were another causes (bacteria, fungus, viruses) that in agreement with (2) and (13) about the recordation numerous causative agents of mastitis in sheep in the world and mycoplasmas with high percentage in recent years due to numerous and sometimes randomly using of antibiotic in therapy of animal diseases also the mixing type of pasturing and rearing system (sheep, goat, cows) increase incidence and distributions of mycoplasmas diseases. The characteristic colony shape of mycoplasma on sold agar of PPLO media is supported by (14) whom reported this characteristic colonies appeared after 5-7 days on PPLO agar with specific micro-environments. Microbiological culture from a milk sample is still the standard for the diagnosis of mycoplasma mastitis (15). *Mycoplasma agalactiae* (*Ma*) was identified by using 16S rRNA gene with 375 bp by conventional PCR as (11.4 %) which that is higher than findings by (16) as (9.6%) and lower than by (17) whom found *Ma* in milk (14.6%) as well as that by (18) in their study in Kerman\Iran whom showed *Ma* (29%) from milk samples, so as report by (12) with higher identification from milk (28%). *Mycoplasma spp.* was identified as 58.3% in milk samples, while (*Ma*) as 11.4% by conventional PCR that confirm that contagious agalactia syndrome which characterized by mastitis, keratoconjunctivitis, arthritis and sometimes abortion, it is possible caused by any of the other *Mycoplasma* species as well as (*Ma*) associated with this disease, as *Mycoplasma mycoides sub-spp. mycoides LC* (*large colony), Mycoplasma capricolum sub-spp. capricolum*, *Mycoplasma mycoides sub-spp. Capri* and *Mycoplasma putrefacients* (19, 20). There were no significant differences in temperature, respiration and pulsation rates which in similar to resultant by (21) that fever is common in acute cases and may be accompanied by alteration of pulsation and respiration rates, but theses general physical signs are with normal levels in the more frequently observed sub-acute and chronic infections, and this finding refer to most of infected ewes in chronic form and that supports by the udder of mastitic ewes could be flaccid, filled with connective tissue and atrophy develop and milk turn to yellowish or bluish in color with characteristic changes in their consistency.

In conclusion: *Mycoplasma agalactiae* was detected for the first time from mastitic ewes in Al-Muthana province-Iraq. So, *M. agalactiae*, isolation and identification are notoriously difficult and time consuming in classical microbio-logist methods. Thus use of DNA-extraction method to identify with specific gene in molecular assay reduces the assay time to hours as opposed to days or weeks, with high specificity.

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