# Molecular detection of *Mycoplasma agalactiae* and *Mycoplasma capricolum* in mastitic and non mastitic milk of goats by using Real Time Polymerase Chain Reaction

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## Summary

A total of 200 samples were collected from 100 goats from more than one regions within the three governorates (Sulaimani, Erbil and Duhok); all these samples were tested by California mastitis test and these showed the following results: 103 samples (51.50%) were negative in all governorates, 28(14.00%) were trace, 35(17.50%) were (+) mark and 34(17.00%) were (++) mark. Clinical mastitis was observed on 93 out of 200 half udder of goats, in the three governorates; 37(18.5%) were acute mastitis, 27(13.5%) were sub acute mastitis and 19(9.5%) were Chronic mastitis. Examination of extracted DNA by Real Time Polymerase Chain Reaction out of 200 DNA extracted from milk samples of goats only 10 (5.00%) were positive for *Mycoplasma agalactiae* in the three governorates, 8(80%) from mastatic milk samples and 2 (20%) from non mastatic milk samples in result of *M. agalactiae*. For *Mycoplasma capricolum* 6 (3.00%) were positive and all were from mastatic milk.

**Keywords: *Mycoplasma agalactiae*, *M. capricolum*, Goats, Mastitis, Real Time Polymerase Chain Reaction.**

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## Introduction

*Mycoplasma agalactiae* and *Mycoplasma capricolum* are the traditional etiological agents which particularly affect goats, sheep and several wild species (1). Clinically characterized by mastitis, arthritis, kerato-conjunctivitis and occasionally abortion (2 and 3), *M. agalactiae* is the main *mycoplasma* microorganism which impacts small ruminant; it's the main cause of 90%, 100% outbreaks of contagious agalactia syndrome in goats, and sheep respectively (4 and 5). In Iraq the disease that caused by *M. agalactiae* is present especially in Kurdistan region, and it was first diagnosed by (6) in fifteen herds of sheep and goats. All *Mycoplasma* isolates were identified as *M. agalactiae;* the diagnosis of this was established and confirmed for first time in 1986. It was isolated for the second time by (7) from Kurdistan region also. (8) isolated the *M. agalactiae* from milk of goats with clinical and sub clinical mastitis from Duhok governorate.

*Mycoplasma* diseases may not be diagnosed solely on the basis of clinical signs, pathological lesions or serological tests because of the close association among the *Mycoplasma* organisms. Isolation and identification are, therefore, required to confirm diagnosis, but this requires a specialist laboratory with experience of these very fastidious organisms (9). A real time PCR assays contain many benefits that can be utilized over conventional PCR, composed rise speed of the reaction, higher sensitivity and specificity, and the removal of post-PCR processing, thus decreasing the danger of impurity and false-positive outcomes (10). The aims of this study was to identify the percentages of clinical and subclinical mastitis of goats in Kurdistan governorate and the molecular detection of *M. agalactiae* and *M. capricolum* in mastitic and non mastitic milk of goats by using Real Time PCR.

## Materials and Methods

The studied animals were 100 goats from flocks of small ruminants distributed in the three governorates of Kurdistan region-Sulaimania (Dukan and Arbat), Erbil (Koya) and Duhok (Zaxo) during the period of beginning of January until end of February (2017) after the session of goats parturition in

Kurdistan provinces. A total of 200 samples of goat milk secretion were collected from Erbil, Sulaimania, and Duhok (80, 70, 50) samples respectively from normal and clinical mastitic females; clinical examination was made for those does by taking temperature, pulse, respiration, examination of udder and supra mammary lymph node. About 10 ml of milk was collected from each animal for DNA Extraction and identification by Real time PCR. Those samples were tested by CMT test immediately in field, and all samples refrigerated in (-20°C) and then transported with ice bag to the laboratory of ministry of agriculture in AL- Nahda, Baghdad.

DNA extraction: First steps of DNA extraction were according to (11). Other steps were according to gsync[tm] DNA extraction kit.

The Statistical Analysis System- SAS (2012) program was used to detect the effect of different factors in the study parameters. Chi-square test was used to detect the significance in the percentages of this study. Real-time PCR was used in this study for detection of both *Mycoplasma agalactiae* and *Mycoplasma capricolum* and used for this detection Real time PCR kits specific for each species of them. Real-time PCR was performed in 96-well plate use applied 2010 Genekam Biotechnology AG Real-time PCR system using *Mycoplasma agalactiae* and *Mycoplasma capricolum* applied Genekam Biotechnology AG (GERMANY) (Tagman), Real-time PCR is based on fluorogenic dyes, in these kits there were 2 dyes used for detecting the target; they are FAM (reporter) 6-carboxy tetramethyl rhodamine and TAM (quencher) carboxy-fluorescein (Table, 1).

**Table, 1: Real time PCR program.**

| Step | Enzyme activation | PCR | |
|---|---|---|---|
| | Initial denaturetion | CYCLE (45 ) | |
| | | Denature | Anneal/ extend |
| Temp. | 95 °C | 95 °C | 60 °C |
| Time | 2 minutes | 15 seconds | 60 seconds |

## Results and Discussion

Clinical examination for the goats included temperature, pulse and respiration and the statistical analysis of these data as in (Table, 2). Out of 200 samples, 103 (51.50%) showed negative result for CMT test in all governorates. The trace mark of CMT was recorded in 28(14.00%) out of 200 sample in all animals of the three governorate. The one (+) mark were recorded in 35(17.50%) out of 200 sample in the three governorates. The two (++) mark were recorded in 34(17.00%) samples in all governorates. There are no three (+++) mark recorded in the three governorate samples (Table, 3). According to the clinical examination of the goats, clinical mastitis was observed on 93 out of 200 half udder of goats, in the three governorates: 37(18.5%) were acute mastitis, 27(13.5%) were sub acute mastitis and 19(9.5%) were chronic mastitis (Table, 4).

The DNA test was done on all (200) milk samples and amplified them by Real time PCR assay for the detection of *M. agalactiae* and *M. capricolum*. Ten (5.00%) out of 200 DNA extracted from milk samples of goat were positive for *M. agalactiae* in the three governorates; the higher rate was in Duhok (8.00%) then in Erbil (6.25%) the lower rate was in Sulaimania (1.42)%. The positive result in (Table, 5 and Fig. 1-A, B). Six (3.00%) out of 200 DNA extracted from milk samples of goat were positive for *M. capricolum* in the three governorates, the higher rate was in Duhok (8.00%) and then in Erbil (2.50%), and it was negative in Sulaimania. The positive result are shown in (Table, 6 and Fig. 2-A, B).

According to clinical signs and CMT test and Real time PCR test of all 200 milk samples there were 8(80%) positive sample from mastatic milk samples and 2 (20%) positive sample from non mastatic milk samples for *M. agalactiae* (Table 6). In case of *M. capricolum* according to clinical signs and CMT test and Real time PCR test of all 200 milk samples there were 6 (100%) positive sample from mastatic milk samples and non from non mastatic milk samples for *M. capricolum* (Table, 7).

**Table, 2: Shows the Statistical Analysis of clinical examination goats in the three Governorates.**

| Governorate | Mean ± SE | | |
|---|---|---|---|
| | Respiratory | Pulse | Temperature |
| Erbil | 31.88±0.49 a | 91.24±0.25 b | 40.03±0.09 |
| Sulimania | 32.40±0.44 a | 91.45±0.31ab | 40.13±0.09 |
| Duhok | 32.47±0.55 a | 92.33±0.44 a | 40.00±0.15 |
| LSD value | 3.941 NS | 1.084 * | 1.539 NS |
| * (P<0.05), NS: Non-Significant. | | | |

**Table, 3: The CMT test to all goats' milk samples in all governorate.**

| CMT mark | Erbil | Duhok | Sulaimania | Total | % |
|---|---|---|---|---|---|
| Negative | 42 (52.50%) | 23 (46.00%) | 38 (54.28%) | 103 | 51.50 |
| Trace | 11 (13.75%) | 8 (16.00%) | 9 (12.85%) | 28 | 14.00 |
| + | 15 (18.75%) | 10 (20.00%) | 10 (14.28%) | 35 | 17.50 |
| ++ | 12 (15.00%) | 9 (18.00%) | 13 (18.57%) | 34 | 17.00 |
| +++ | _ | _ | _ | _ | _ |
| Chi-square | 10.483** | 9.516** | 10.852** | --- | 10.625** |

** (P<0.01).

**Table, 4: Shows the clinical forms of mastitis in goats in the three governorates.**

| Clinical form | Acute mastitis | Sub acute mastitis | Chronic mastitis |
|---|---|---|---|
| Erbil (80) | 15(18.75%) | 12(15%) | 10(12.5%) |
| Sulimania (70) | 13(18.57%) | 8(11.42%) | 4(5.71%) |
| Dohuk (50) | 9(18.0%) | 7(14.0%) | 5(10.0%) |
| Total (200) | 37(18.5%) | 27(13.5%) | 19(9.5%) |
| Chi-Square | 0.339 NS | 1.529 NS | 4.09 * |

* (P<0.05), NS: Non-Significant.

**Table, 5: Shows the number and percentage of *M. agalactiae* on Real time PCR in milk samples.**

| Governorate | Milk samples | Positive | Percentage |
|---|---|---|---|
| Erbil | 80 | 5 | 6.25% |
| Duhok | 50 | 4 | 8.00% |
| Sulaimania | 70 | 1 | 1.42% |
| Total | 200 | 10 | 5.00 % |
| Chi-square | --- | --- | 4.295 * |

** (P<0.01).

**Table, 6: Shows the number and percentage of *M. capricolum* on Real time PCR in milk samples.**

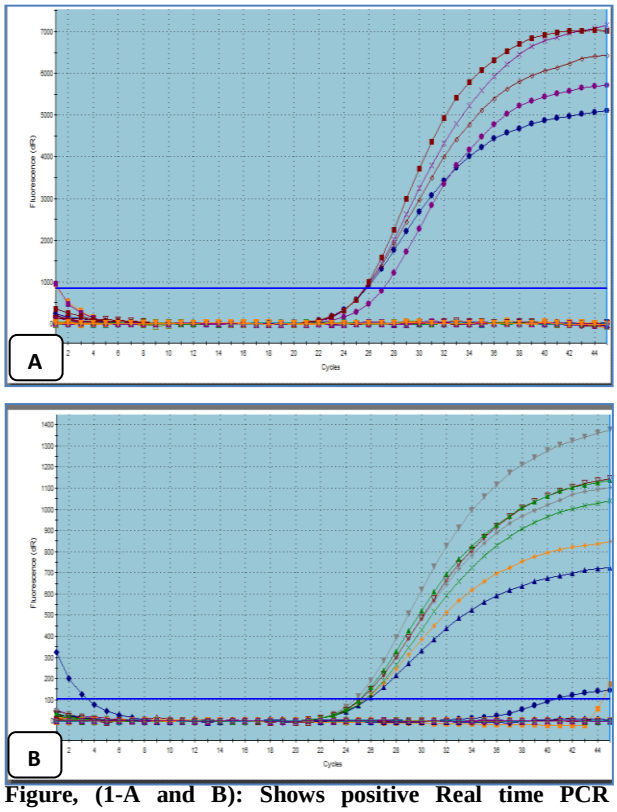
| Governorate | Milk samples | Positive | Percentage |
|---|---|---|---|
| Erbil | 80 | 2 | 2.50 % |
| Duhok | 50 | 4 | 8.00 % |
| Sulaimania | 70 | _ | 0.00 % |
| Total | 200 | 6 | 3.00 % |
| Chi-square | --- | --- | 4.471 * |

* (P<0.05).

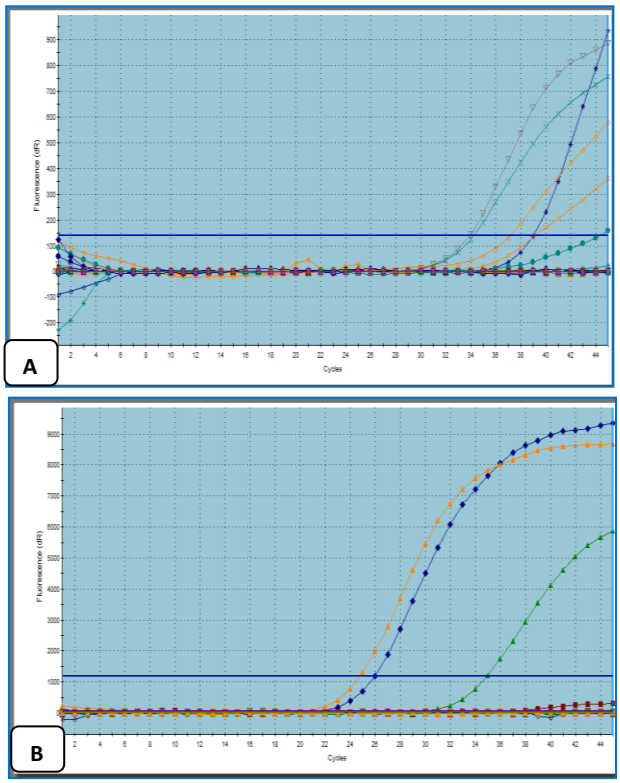
**Table, 7: Shows the relationship between mastatic and non mastatic milk samples and positive *M. agalactiae* and *M. capricolum* .**

| | No. | Mastatic | Non mastatic milk |
|---|---|---|---|
| **Positive *M. agalactiae*** | 10 | 8 samples (80%) | 2 samples (20%) |
| **Positive *M. capricolum*** | 6 | 6 samples (100%) | 0 samples (0.00%) |
| **Chi-square** | --- | 7.250 ** | 7.250 ** |

**** (P<0.01).**




**Figure, (1-A and B): Shows positive Real time PCR amplification for *M. agalactiae* for 200 DNA extract from 200 milk samples.**




**Figure, (2-A and B): shows positive Real time PCR amplification for *M. capricolum* for 200 DNA extract from 200 milk samples.**

In this study the identification of small ruminant *M. agalactiae* and *M. capricolum* was achieved from clinically affected goats by mastitis and asymptomatic

goats for the first time by real-time PCR assay .This identification is significant because *M. agalactiae* and *M. capricolum* are the two species of the major causes of contagious agalctia (CA) (12) and have gone unnoticed or attributed to other causes due to the difficulties in their diagnosis in addition the threat of CA by *M. agalactiae* and *M. capricolum* that should be kept in mind in a region which is open to animal import and serves as a transit area. According to (13), clinical mastitis due to mycoplasmas is highly prevalent in some areas and cause financial losses. The main clinical signs observed in this study were mastitis, change in milk color and consistency with agalactia. In most cases, infected hosts spontaneously recover from acute clinical signs within a few weeks but develop a chronic infection accompanied by shedding of *M. agalactiae* in milk and/or other body secretions for years without presenting any clinical signs (14). These (asymptomatic) carriers can transmit the bacteria to other susceptible animals and cause acute disease (15). There are reports of excretion of organisms in milk even after 8 years of infection with mild and with or without clinical signs (16 and 17) and this is in agreement with the result of identification if *M. agalactiae* in normal milk in this study. In this study the examination of milk by CMT test for detection of sub clinical mastitis for herds with no available individual SCC data, the CMT can help select the best samples for processing. The result was, negative (51.50 %), trace (14.00 %), + (17.50 %), ++ (17.00 %) in the three governorates.

The researcher (18) showed that the results out of 98 milk samples 74.48% were positive for *mycoplasma* genus by PCR and in total only 28 samples (14.65%) were shown positive for *M. agalactia* in milk samples are 18.36%. Also (19) showed that 158(69.6%) out of 227 samples were positive for the presence of *Mycoplasma spp.* by PCR. As well the presence 51(32.27%) and 4(2.53%) positive samples of *M. Mycoides* cluster and *M. agalactiae* were demonstrated respectively.

In Kurdistan province (20) out of 300 goats milk samples, 82samples were positive, the highest rate was in Duhok (zaxo) 21(35.00%), in Erbil (koya) was 33(27.50%) and in Sulaimania was 28(23.33%). The results of the above research are not in agreement with the result of this study for following reason: In the above study the samples were collected from mastitis milk (clinical and subclinical), while in this study normal (non mastatic) and abnormal milk (mastatic) were both collected and some normal samples revealed *M. agalactiae* in them. This is the first study on the level of Iraq for detection of *M. agalactiae* and *M. capricolum* by Real-time PCR, and for the first time identification of *M. capricolum* in Iraq. In this study the result of real-time PCR for detection of *M. agalactiae* 8(4%) out of 200 samples were positive in the three governorates; the positive result appeared on (25Ct, 27Ct, 34Ct, 36Ct and 39Ct). This finding was in agreement with that of (21) who reported on the design and assessment of a real-time PCR assay for the quantitative detection of *M. agalactiae* and its evaluation and in-house validation in the analysis of milk samples. It can be concluded from this research that the *Mycoplasma agalactiae* is endemic in all Kurdistan region, while *Mycoplasma capricolum* was sporadic in existence in Kurdistan region. Identification of *M. agalactiae* and *M. capricolum* by Real time PCR assay was easy and had a high sensitivity and specificity.

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# التحري الجزيئي عن *Mycoplasma agalactiae* و *Mycoplasma capricolum* في الحليب المصاب بالتهاب الضرع وغير المصاب في الماعز بواسطة تقنية تفاعل البلمرة التسلسلي الآني

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**الخلاصة**

تضمنت هذه الدراسة جمع 200 عينة حليب من 100 انثى ماعز في أكثر من منطقة ضمن المحافظات الثلاث (أربيل والسليمانية ودهوك). جميع هذه العينات أجري عليها فحص التهاب الضرع (اختبار كاليفورنيا) وكانت النتائج كالآتي: 103 (51.50%)عينة كانت سالبة (-)، 28(14.00%) اثري، 35(17.50%) + , 34(17.00%) ++ في جميع المحافظات. شوهد التهاب الضرع السريري في 93 من مجموع 200 نصف ضرع في المحافظات الثلاث وكانت النتائج كالآتي: 37(18.5%) كان التهاب ضرع حاد، 27(13.5%) كان التهاب ضرع تحت الحاد و19(9.5%) التهاب ضرع مزمن. بفحص الحمض النووي الرايبوزي منقوص الاوكسجين المستخلص من عينات الحليب كانت النتائج كالآتي: 10(5.00%) عينة موجبة لـ *Mycoplasma agalactiae* و8(80%) كانت من عينات مصابة بالتهاب الضرع السريري و 2(20%) كانت من عينات حليب غير مصابة بالتهاب الضرع للمحافظات الثلاث، و6(3.00%) كانت موجبة لـ *Mycoplasma capricolum* جميعها من عينات حليب مصابة بالتهاب الضرع السريري من المحافظات الثلاث .

**الكلمات المفتاحية: *Mycoplasma capricolum*، *Mycoplasma agalactia* ، ماعز، التهاب الضرع، تقنية تفاعل البلمرة التسلسلي الآني.**