Journal homepage www.jzs.uos.edu.krd
Journal of Zankoi Sulaimani
Part-A- (Pure and Applied Sciences)

Isolation, Molecular Identification and in vitro Antibiotic Susceptibility Testing of Mycoplasma agalactiae From Goats in Two Provinces of Kurdistan Region-Iraq

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Article info

Original: 9 Feb. 2015
Revised: 27 Mar. 2015
Accepted:19 Apr. 2015
Published online:
20 Sep. 2015

Key Words:
Contagious agalactia; Mycoplasma agalactiae uvrC gene antibiotic susceptibility

Abstract

Mycoplasma agalactiae is one of the causal agents of classical contagious agalactia (CA). It occurs in many parts of the world and most notably in the Mediterranean Basin. The aims of this study were isolation and molecular identification of *Mycoplasma agalactiae* for the first time in Kurdistan region from goats showing contagious agalactia and *in vitro* evaluation of the activities of different antimicrobial against *Mycoplasma agalctiae*. During the period of January 2011 to November 2012 a total of 126 milk samples were collected (68 from Slemani governorate and 58 from Arbil governorate) from goats that had clinical signs of CA. *Mycoplasma agalactiae* was recovered from 102(81%) milk samples out of (126) divided as; 58 (85.3%) out of 68 milk samples in Slemani governorate were positive for *M. agalactiae* while 44(75.9%) out of 58 milk samples were positive in Arbil governorate. All isolates were confirmed by amplification of 1624 bp *uvrC* gene by PCR assay. GenBank accession number of the nucleotide and amino acid sequences of Slemani and Arbil isolates were reported in this study is (KC 594646), (KC 594647) respectively. The isolates in current work from both governorates were showed 99% homology to each other and the topology of the phylogenetic tree indicated that both field isolates were clustered together and they were belonging to sub linage that contain most of PG2 strains. Danofloxacine and Azithromycine showed a great effectiveness against all isolates and may be considered as standard treatment for CA in this region.

I. Introduction

Small ruminants are frequently infected with mycoplasmas but more commonly in goats [1]World Organisation for Animal Health (OIE) listed contagious agalactia (CA) syndrome as a serious disease of small ruminants and was listed as a list B . The most significant symptom in ruminants reared for dairy purposes is mammary gland infection, which may lead to complete loss of milk production as

well as loss of the mammary gland itself. Additional clinical findings in goat and sheep included arthritis and keratoconjunctivitis. CA occurs in Europe, Western Asia, USA and North Africa, and is mainly caused by *Mycoplasma agalactiae* [1, 2] and CA regarded as being endemic in most Mediterranean countries [3]. Other mycoplasmas that cause this disease: *Mycoplasma capricolum* subsp. *capricolum*, *Mycoplasma mycoides* subsp. *mycoides* large colony and *M. putrefaciens*. The disease that caused by *M. agalactiae* is clinically recognized by high temperature, diminution in appetite and changing in the consistency of the milk in lactating goats, with decline and subsequent failure of milk production, often within 2–3 days, as a result of interstitial mastitis[4-6]. The disease is present in Iraq especially in Kurdistan region and it has been established and confirmed for the first time in 1986 by conventional culturing methods in Rania district[7] after that in Duhok province by [6]. In Kurdistan region CA is endemic and is currently not controlled by vaccination except using antimicrobial therapy, however due to the unreliability of the current antimicrobial therapy ,this lead to emergence of antimicrobial resistant strains[8, 9]. The aims of this study were molecular isolation of *Mycoplasma agalactiae* for the first time from goats presenting contagious agalactia and *in vitro* evaluation activities of different antimicrobial against *Mycoplasma agalctiae*.

II. Materials and Methods

A. Animals and study area

A total of 2280 goats that represent 57 flocks distributed in both Slemani and Arbil Governorate were included in this study.

B. Sampling

During the period of January 2011 to November 2012 a total of 126 milk samples were collected (68 from Slemani governorate and 58 from Arbil governorate) from goats that had clinical signs of CA. The samples were immediately placed in test tube containing transport Mycoplasma culture medium and were kept at 4°C until they have been transported to the laboratory within four hours as a maximum.

C. Culture of Milk Samples

According to [10]two types of media (broth and agar media with appropriate supplements) were used for the recovery of Mycoplasma from milk samples. The broth medium (Mycoplasma broth base M267) was prepared according to manufacturer's instruction (HiMedia, India). PPLO agar was made by adding 1.5% of noble agar to the PPLO broth medium. Mycoplasma enrichment supplements (FD075 HiMedia India) with 1% Potassium tellurite (FD052 HiMedia India) also were added. The cultured broths with milk samples were incubated for 3-5 days at 37°C in a humidified atmosphere with 5% CO_2 using CO_2 incubator, then Mycoplasma growth will appear between 3 and 5 days, and it was characterized by very fine turbid appearance then aliquots (100µl) of cultured broth were cultured on agar plates and incubated for 2-3 days under the same mention conditions for the cultured broths then examined using the light microscope under 40 X magnifications.

D. Identification and Sequencing

The colonies that resembled mycoplasma's colonies "fried egg" were all subjected to PCR assay for the identification of *Mycoplasma agalactiae* by amplification alignment of *uvrC* gene. According to[11] forward primer from set A and a reverse primer from set B were used to amplify 1624 *bp* of *uvrC* gene

as shown in table (1), in order to sequencing 1624 *bp* of *uvrC* gene two sets of designed primers for this study were used as indicated in table (1). Reactions were performed in an automated DNA thermal cycler. DNA has been extracted by commercial kit (Promega A1120). All extracted DNA were subjected to 35 cycles of amplification. An amplification cycle consisted of denaturation for 1 min at 94 °C, primer annealing to the template at 50 °C for 30 second and primer extension at 72 °C for 3 minutes. Finally 5 min incubation was performed at 72 °C. PCR products (10 µl) were examined by electrophoresis in 0.7% agar gels1 X TBE buffer (89mM Tris, 89 mM boric acid, pH 8.0), stained with ethidium bromide and examined by UV lamp in a gel documentation system.

Nucleotide sequencing: About 50µl of each 930 bp and 860 bp PCR products of amplified (*uvrC* gene 1624bp) from two isolates one from Slemani and other one from Arbil Governorates were purified using PCR purification kit (Qiagen, Germany), and sequenced with two forward and two reverse primers (table 1) in Innovations Biochemical Laboratory (IBL), Vienna-Austria.

GenBank accession numbers: GenBank accession number of the nucleotide and amino acid sequence of(uvrC) gene, partial CDs of MA01/Kurdistan/2012(Slemani isolate) reported in this study is (KC 594646) and (KC 594647) for MA02/Kurdistan/2012 (Arbil isolate).

Table -1: primer pairs used in amplification by polymerase chain reaction assays of *uvrC* gene specific for *Mycoplasma agalactiae* .

	Sequence	Position	bp
A	F 5-CTCAAAAATACACAACAAGC-3	263-283	930
	R 5-TTGTACCTATTTTTGGGCTA-3	1190- 1171	
B	F 5- CCATTAGGAATAAGTATTGA-3	1031- 1050	860
	R 5-CTTCAACTGATGCATCATAA-3	1889-1870	

E. Sequence comparison and phylogenetic analysis: The sequence identity of both MA02/Kurdistan/2012 PG2 and MA01/Kurdistan/2012 PG2 isolates were confirmed by blast method in National Center for Biotechnology Information (NCBI) Home page. The nucleotide sequences were subjected to multiple sequence alignment with Mycoplasma isolates in NCBI and the homology percentage were determined. Phylogenic tree was constructed among MA02/Kurdistan/2012PG2, MA01/Kurdistan/2012 PG2 isolates and all published Mycoplasma isolates that showed significant alignments in NCBI. The Phylogenic tree based on the neighbor-joining method using Kimura2-parameter model in Mega 6. The bootstrap values were determined from 1000 replicates of the original data[12].

F. Antimicrobials:

All the antimicrobial agents were obtained from Sigma as a powder; the antimicrobials were quinolones (Danofloxacin, Norfloxacin, Ciprofloxacin and Enrofloxacin), aminoglycosides (Streptomycin, Spectinomycin, Amikacin, Kanamycin and Tobramycin), Doxycycline, Oxytetracycline, macrolides (Erythromycin, Tylosin, Spiramycn and Azithromycin). The concentration ranges of the antimicrobials to be tested were determined by taking into account the active ingredient of each as shown in table (2). The MIC for each field isolate, reference strain and antimicrobial was determined according to the

recommendations of Hannan[13]. Cultures were grown in PPLO broth with necessary supplements for the growth of mycoplasma also supplemented with 1% phenol red in 96-well round-bottom microwell plates. The antimicrobials were added to achieve each of the pre-established final concentrations and a final concentration of the mycoplasma cultures of 105 to103 colour-changing units/ml. As well as positive controls lacking antibiotics and negative controls lacking mycoplasma isolates were prepared. The plates were then sealed and aerobically incubated at 37 ° C until the positive control (antibiotic-free) changed colour from red to yellow and the negative control (mycoplasma-free) remained red. The MIC was defined as the lowest concentration at which no bacterial growth (no colour change) was observed. Depending on the isolate, the colour change occurred after 24 to 72 h of incubation.

III. Results

Mycoplasma agalactiae was recovered from 102(81%) milk samples out of (126) divided as; 58 (85.3%) out of 68 milk samples in Slemani governorate were positive for *M. agalactiae* while 44(75.9%) out of 58 milk samples were positive in Arbil governorate. All isolates were confirmed by partial amplification of 1624 bp *uvrC* gene using PCR assay as shown in figure (1), later in order to sequence the 1624 bp of uvrC gene, the PCR products of isolates were purified and amplified using two sets of designed primers for this study, first set were amplified 930bp and second set were amplified 860bp as indicated in figure (2). Blast result of MA02/Kurdistan/2012 PG2 and MA01/Kurdistan/2012 in NCBI genebank showed alignment of 15 isolates of *Mycoplasma agalactiae*, 4 isolates of *Mycoplasma bovis* and 3 isolates of *Mycoplasma fermentus*. . Both Kurdistan isolates showed (99%) homology with each other and they showed (99%) homology with most *Mycoplasma agalactiae*. However they have (82%) and (67%) identities with *Mycolpalsma bovis* and *Mycolpalsma fermentus* respectively. According to phylogenetic tree construction based on the partial uvrC nucleotide sequence alignment of the 25 genomes (Fig 3), the isolates were distinctly divided into three groups, namely *Mycolpalsma agalactiae*, *Mycolpalsma bovis* and *Mycolpalsma fermentus*. The topology of the phylogenetic tree indicated that the field isolates MA02/Kurdistan/2012 and MA01/Kurdistan/2012 was clustered together and they were belonging to sub linage that contains most of PG2 strains of *Mycolpalsma agalactiae.*

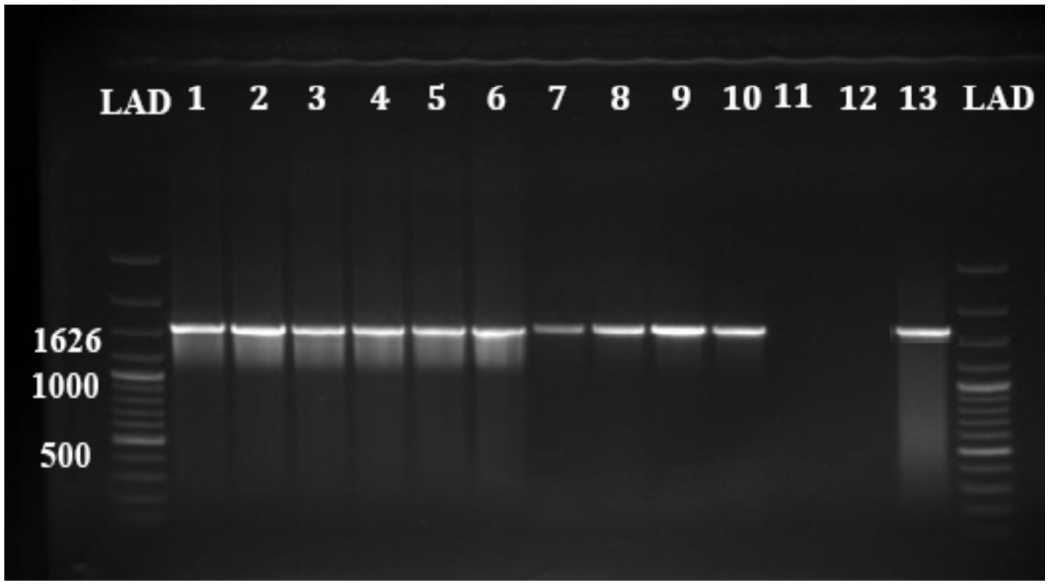

Fig (1) PCR amplification of colonies from milk samples obtained from goats with CA. The products were loaded on 1.2 % agarose gel containing ethidium bromide. Lanes 1-10 were 1624 bp *Mycoplasma agalactiae* positive samples, whereas Lanes 11 and 12 were negative, Lane 13 is positive control.

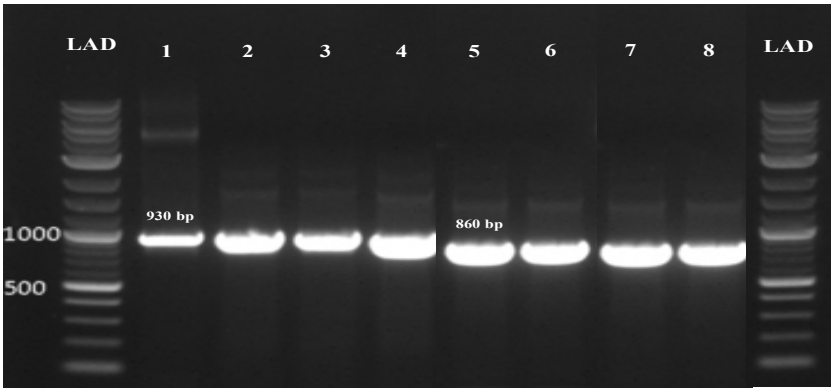

Fig (2) PCR amplification from PCR product of *uvrC* gene. The products were loaded on 1.2 % agarose gel containing ethidium bromide. Lanes 1-4 amplified with primers set (A) and lanes 5-8 amplified with primers set (B) were produce 930 bp and 860 bp respectively.

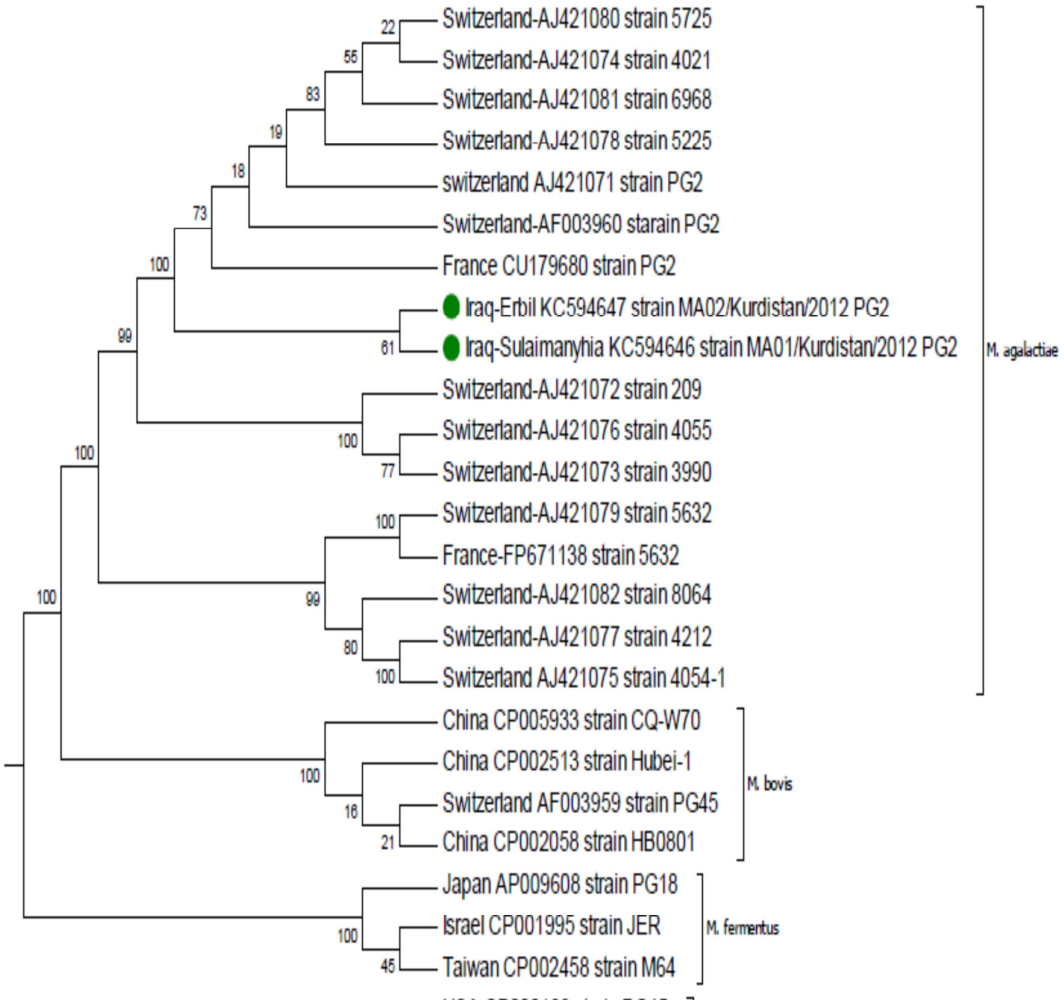

Fig. 3: Phylogenetic tree of MA02/Kurdistan/2012 PG2 and MA01/Kurdistan/2012 isolates. Analyses of phylogenetic tree indicate 3 clusters; *Mycolpalsma agalactiae*, *Mycolpalsma bovis* and *Mycolpalsma fermentus*. Kurdistan isolates were clustered together and they were belonging to sub linage that contains most of PG2 strains of *Mycoplasma agalactiae*.

The MIC_{50}, a MIC90 and MIC range obtained for the antimicrobials in the field strains and the reference strain is provided in table (2). Danofloxacin and Azithromycin were shown to be the most effective agents the isolates from both governorates, with a MIC_{50} of 0.5 µg/ml and MIC_{50} of 10 µg/ml MIC values ranging from 0.3 to 1 and 2.2 to 15 µg/ml, respectively, followed by Norfloxacin with a MIC_{50} of 3.7 µg/ml and MIC values ranging from 0.3 to 13.1 µg/ml while other remaining antibiotics beneath MIC_{50} were failed to inhibit the growth of microorganism. According to the MIC_{90} values Danofloxacin remaining the most effective (MIC_{90} 1.0 µg/ml) followed by Azithromycin (MIC_{90} 15 µg/ml), Norfloxacin (MIC_{90} 13.1 µg/ml), Ciprofloxacin (MIC_{90} 0.7 µg/ml), Spectinomycin (MIC_{90} 12.5 µg/ml) and Amikacin (MIC_{90} 13.9 µg/ml). All isolates were resistant to Enrofloxacin, Streptomycin, Doxycycline, Oxytetracycline, Erythromycin, Tylosin and Spiramycin with MIC_{50} and MIC_{90} values ranging from (0.09 to 82 µg/ml). No obvious differences have been recorded between isolates from Slemani and Arbil governorates in their susceptibility or resistance for testing antibiotics.

Table -2 MIC50, MIC90 and MIC[1] ranges of the antimicrobial agents.

Antimicrobial	Field isolates (n=102)			Reference strain
	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC range (µg/ml)	[2]PG2 MIC (µg/ml)
Quinolones				
Danofloxaacin (DAN)	0.5	1.0	0.3-1	0.8
Norfloxacin (NOR)	3.7	13.1	0.3-13.1	1.2
Ciprofloxacin (NOR)	0.4	0.7	0.015-0.7	0.09
Enrofloxacin (ENR)	0.5	1.2	0.075-1.2	0.075
Aminoglycoside				
Streptomycin (STP)	4.0	82	4-82	4.0
Spectinomycin (SET)	1.0	12.5	0.7-12	0.7
Amikacin (AMK)	3.0	13.9	1-13.9	1.0
Tetracycline				
Doxycycline (DOX)	0.09	1.0	0.045-1	0.045
Oxytetracycline(OXT)	0.35	0.5	0.25-0.5	0.25
Macrolides				
Erythromycin	80	140	20-140	20
Tylosin	0.16	1.0	0.08-1	0.08
Spiramycin	0.8	14.6	0.4-14.6	0.4
Azithromycin	10	15	10-15	12

[1]MIC50 and MIC90 = lowest concentration at which growth of 50% and 90% of the isolates, respectively, is inhibited;
[2]PG2 = reference strain of *Mycoplasma agalactiae* (NCTC 10123) from UK.

IV. Discussion:

Mycoplasma agalactiae is the main causal agent of contagious agalactia syndrome in goats, it occurs in the Mediterranean region and the Balkan Peninsula in Europe, in western Asia and in northern, central and eastern Africa [14-17]. The organism for the first time was established and confirmed in1986 by conventional culturing methods at Rania district in (Slemani governorate) as a cause of CA by Al-Aubaidi and his colleagues [6]. All countries surrounding the Iraqi Kurdistan region have Contagious agalactia caused by *Mycoplasma agalactiae* . Kheirabadi, and Ebrahimi, in Iran, investigated the prevalence of *Mycoplasma agalctiae* in milk and conjunctival swab samples of sheep flocks in shahrekord and lordegan districts of west central Iran by using PCR method. They detected *Mycoplasma agalactiae* in 20(19.8%) out of 101 animals[18]. In Turkey *Mycoplasma agalactiae* had been detected in goats by culturing on PPLO

media after that identification of the organism by PCR method[19]. In the current study the identification of *Mycoplasma agalactiae* had been done by partial amplification of 1624 bp of *uvrC* gene. The constitutive genes are suitable candidates for genetic differentiation of species that, which are expressed in all living organisms because they provide basic functions such as replication, transcription and translation [20]. One of these essential genes, *uvrC*, encodes deoxy-ribodipyrimidine photolyase (EC 4.1.99.3). Deoxy-ribodipyrimidine photolyase is an enzyme of the excision DNA-repair system, Uvr ABC, which removes damaged DNA segments via concerted dual incisions by an ATP-dependent enzyme system with an essentially infinitive substrate range[21]. This system has been found in all free-living species tested, from the smallest known self-replicating organism *M. genitalium* to humans [22]. According to a study has been done by Subramaniam and his co-workers , they were confirmed that *uvrC* gene can be used as species specific diagnostic tool due to the lack of amplification in heterologous reactions among *Mycoplasma agalactiae* and *Mycoplasma bovis*. Also, no amplification of *uvrC* gene was observed in other related Mycoplasma species such as *Mycoplasma bovigenitalium, M. alcalescens, M. arginini, M. dispar, M. californicum, M. bovoculi, M. bovirhinis, My ovipneumoniae, M. verecundum and M. conjunctivae*[11]. Both isolates MA02/Kurdistan/2012 PG2 and MA01/Kurdistan/2012 were isolated from the two neighbor Arbil and Slemani provinces, had (99%) identity and by phylogenetic tree analysis they clustered together, it mean that they had the same genomic characteristic and it suggested circulation of the same *M. agalactiae* strain in Kurdistan region. On other hand, genomics analysis in this study supported by many researchers[23, 24] who reported the correlation between phylogenetic and geographical distribution of the isolates as well as these results are in agreement with the data published in a global VNTR and MLST typing study[20]. *M. agalactiae* was considered to be the classical etiological agent of contagious agalactia[25], in current study a high percentage 102(81%) of *M. agalactiae* was verified from 126 milk samples, this result is very close with the results recorded in areas endemic with CA[2, 3] in Spain, as well as greatly similar with the situation in Europe where the majority of isolates from cases of CA in sheep and goats were *M. agalactiae*[9], while its lower than recorded in northern Jordan in mixed flocks of sheep and goats[26], in Turkey 17 isolates of *M. agalactiae* was obtained from 47 milk samples collected from the sick goats[19]. In a survey in Brazil 11 milk samples of caprine have been cultured and reported that 4 of milk samples have *M. agalactiae*[27] and in a study have been done in Kurdistan province of Iran they were showed that 5(25%) out of 20 isolated mycoplasma from 367 milk samples were positive with *M. agalactiae* primers[28]. Recently *M. capricolum* subsp. capricolum and *M. mycoides* subsp. Capri has also been isolated in many countries from sheep and goats with mastitis and arthritis [14]that explain why not all samples were gave positive results for primers of *M agalactia* even there were clinical findings of CA. The control of CA is complex and mainly based on antimicrobial treatment and preventive handling procedures[29]. Because Mycoplasmas lack a cell wall, they are not susceptible to the β-lactam class of antimicrobials. In addition, the chronic nature of CA infection in endemic areas has given rise to uncontrolled antimicrobial treatment, which could lead to the appearance of resistant strains[9, 30]. The antimicrobial drug families known to be effective against Mycoplasmas are macrolides, fluoroquinolones, tetracyclines, and aminoglycosides[31], macrolides and quinolones being widely used in endemic areas. However, some resistance to macrolides and fluoroquinolones has been observed and attributed to several gene mutations[32, 33]. The diversity of genetic populations of M. agalactiae isolates from goats detected in endemic areas and the wide use of antimicrobials could lead to inefficient control therapy. It is well known that in vitro sensitivity of antimicrobials does not always correspond to the effectiveness of treatment in the field, but the MIC values in all its variations are of great help, so an ineffective drug in vitro is not very useful on the affected animal[23]. Regarding the antimicrobial activity, these results demonstrate the effectiveness of quinolones in vitro against *Mycoplasma agalactiae*, which are included as standard treatments against CA, and show agreement with many workers [9, 13, and 34] on the susceptibility of field isolates of *Mycoplasma agalactiae*. One possible explanation for these resistance to other antibiotics is that a cocktail of antimicrobials may have been used ineffectively at the farm before these *M. agalactiae* were isolated.

V. Conclusion:

The isolates of *Mycoplasma agalactiae* in current work from both governorates were showed 99% homology with each other and the topology of the phylogenetic tree indicated that the field isolates MA02/Kurdistan/2012 PG2 and MA01/Kurdistan/2012 is clustered together and they were belonging to sub linage that contain most of PG2 strains of *Mycolpalsma agalactiae*. Danofloxacine and Azithromycine showed great effectiveness against all isolates and may be considered for standard treatment for CA.

Competing interests: I'm declaring that I had no competing interests.

Acknowledgements: I would like to thanks Zana H. Mahmood for technical assistance and Peshnyar M. A. Rashid for constructing a phylogenetic tree.

VI. References:

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