IDENTIFICATION BY CULTURE, DGGE-PCR, OF *Mycoplasma* FROM RUMINANTS AND POULTRY

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ABSTRACT

A total of multiple random samples 88 milk sample from buffalo; 10 lung sample from cattle; 30 lung and trachea samples from sheep; 63 lung, nasal swabs, and milke samples from goats; 126 lung and trachea sample from layer and 177 lung and trachea samples from broiler, were immediately cultured for primary isolation. This report describes the development of a new diagnostic test based on PCR of the 16 Sr RNA gene with *Mycoplasma* specific primers and separation of PCR product according to primary sequence using denaturing gradient gel electrophoresis (DGGE) DNA product from ruminants and poultry were submitted to the Animal Health and Veterinary Laboratory Agency, UK, to confirm diagnosis by DGGE/ PCR.

INTRODUCTION

The term "*Mycoplasma"* (previously called pleuropneumonia like organisms or PPLO) is usually used as a synonym for the class of Mollicutes that represents a large group of highly specialized bacteria and are all characterized by their lack of a rigid cell-wall and instead they are bounded by a triple-layered "unit membrane, "*Mycoplasma* is the largest genus within this class, because of their small size (0.3-0.8um) and flexibility, these bacteria are able to pass through conventional microbiological filters 0.45nm pore size. *Mycoplasmas* can be seen as commensales, because of their reduced metabolic abilities which cause a relatively long generation time, and also they have a small genome size and simple structure that leds *Mycoplasma* species radically to economise genetic resources and biosynthetic capacities, and adapt to an obligate parasitic lifestyle (18).

The primary difference between *Mycoplasma* and other bacteria is that bacteria have a solid cell- wall structure and can grow in the simplest culture media, also bacteria are inhibited by penicillin (19) but Mycoplasma are completely resistant to penicillin but inhibited by tetracycline or erythromycin .The lack of cell- wall separate *Mycoplasma* in a class mollicutes (20) . Due to the lack of a rigid cell- wall they are osmotically fragile and pleomorphic, So they can contort a broad range of shapes, from round to oblong. They therefore cannot be classified as rods or cocci (25). Phylogenetically Mycoplasma related to low (G-C) Gram-positive bacteria (21).

This invesitgation is aimed to *Mycoplasma* are very small prokaryotes ,devoid of cell wall and bounded only by a plasma membrane. Diagnosis of *Mycoplasma* infection is normally based on culture and serological tests, which can be time–consuming and laborious. A number of specific PCRs have been developed but to date there has not been a single generic test capable of detecting and differentiating *Mycoplasmas* to a species level .

The objectives of this study was to identify the genus and species of *Mycoplasma* in the lung, trachea, nasal swabs, and milk samples of ruminants and poultry with pneumonia and mastitis using culture, and DGGE/PCR.

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The parasitic *Mycoplasma* appear to be strictly host-specific and potentially pathogenic only within a singl host species. *Mycoplasma* cause a wide range of diseases in both humans and animals and are commonly associated with pneumonia, arthritis, conjunctivitis , infertility and abortion (22) . *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. The classical methods for detecting and identifying *Mycoplasmas* are time consuming and complicated by serological crossreactions between the closely related organisms. Molecular diagnosis has improved their detection and identification, specifically the polymerase chain reaction (PCR) (23). PCR has been used to detect a number of *Mycoplasma* species over 102 species so, the recently introduced PCR and denaturing gradient gel electrophoresis (DGGE) method (24) has the ability to detect and differentiat 27 *Mycoplasma* of veterinary importance using universal primers for the V3 region of 16SrRNA (10). generic nature of the test lead to the detection of *Mycoplasma* infection in less than 24h compared with 1-2 weeks for traditional culture techniques. This method has ability to identify mixed infection which would have been difficult to detect by conventional methods (26).

MATERIALS AND METHOD

Sampling

The study material consisted of (396) samples obtained from poultry unit and clinical unit in central veterinary laboratory brought from different parts of Baghdad province for diagnosis. The samples were collected in the years 2015 - 2016, most of samples being callected by the end of summer or the end of winter.

Mycoplasma, Isolation and Enrichments

For the primary isolation, the specimens were diluted of up to 10^{-5} and filtered through 0.45µl syringe filter in fresh PPLO broth supplemented with 10% inactivated horse serum or swine serum, 10% yeast extract, 10% thallium acetate, 1% ampecillin. Inoculated broth incubated for 3 days under microaerophilic condition (5% CO2 at 37 °C).

The growth medium was checked daily for growth. A loopful of the broth culture showing *Mycoplasma* growth or color change were inoculated on *Mycoplasma* Agar Base media in a 95% N2 and 5% CO2 humidified atmosphere at 37° C, and the petri dishes xamined in stereomicroscopy at the end of incubation for appearance of typical *Mycoplasma* colonies (15).

DNA EXTRACTION

DNA was extracted from the enriched *Mycoplasma* broth culture using QIA amp DNA extraction kit (Qiagen), and performed according to the users instructions for the kit .

PRIMERS

The primers used were *Mycoplasma* genus-specific primers GOP3(5'GGG AGC AAA CAC GAT AGA TAC CCT 3') MGSO(5' TGC ACC ATC TGT CAC TCT GTT AA CTC 3') derived from the 16SrRNA gene (2). PCR, Denaturing Gel Electrophoresis (DGGE) were performed at the Animal Health and Veterinary Laboratory Agency (AHVLA) (Weybridge) UK. Amplification of the V3 region of the 16S rRNA gene was performed according to the method of as described previously (M), using universal bacterial primers; GC-341F 50-CGC

CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG. and 534R 50 –ATT ACC GCG GCT GCT GG. DGGE was performed using the Ingen phorU 2x2 apparatus (GRI Molecular Biology). Samples (20 uL) were loaded onto 10%poly-acrylamide/bis(37.5:1) gels with denaturing gradient from 30-60 % {where 100% is 7 M urea and 40% (v/v) deionized formamide} in 1xTAE electrophoresis buffer (severn) .Electrophoresis performed at 100 V at a temperature of 60 °C for 18 h. Gels were then stained with SBYR Gold (Cambridge BioScience) in 1x TAE for 30 min at room temperature and visualized under UV illumination.

Inoculated broth and agar media were incubated under microaerophilic condition (5% CO2) at 37c with 28% humidity and checked for color change of broth and typical *Mycoplasma* colonies on agar. AS soon as the phenol red indicator changed to yellow, the subculture on to the fresh broth and agar were carried out (7) several passages until 21 to 28 days were subcultured. Identification of *Mycoplasma* samples was carried out by PCR after appearing of the specific colonies of *Mycoplasma*. DNA was extracted from broth culture with help of a DNA extraction kitQIAamp DNA Mini Kit (50), conventional PCR using genus-specific primers for *Mycoplasma* conventional PCR using genus-specific primers for*Mycoplasm*.

RESULTS AND DISCUSSION

In the current study, *Mycoplasma synoviae* and *Acholiplasma* laidlawii were isolated from 36 sample out of 303 poultry samples. The *Acholiplasma laidlawii* isolated from 24 out of 98 cattle and buffalo samples, and *Mycoplasma bovigentalium, Mycoplasma ovipneumoniae, Mycoplasma arginini and Acholiplasma laidlawii* were isolated from 24 sample out of 93 small ruminants samples using traditional culture techniques, the growth of mycoplasma in PPLO broth media was demonstrated by changes in color or turbidity due to biochemical activity and metabolism of the mycoplasma. The identified isolates were confirmed by molecular method DGGE (Table 1).

Table 1: Presence of *Mycoplasma* In the ruminant and poultry samples

Animal	Sample type	Sample NO .	positive	Results
Laying hens	Lung ,Trachea	126	22	*M. synoviae* *Acholiplasma laidlawii*
Broiler	Lung ,Trachea	177	14	*M. synoviae* *Acholiplasma laidlawii*
Buffalo	Milk	88	20	*Acholiplasma laidlawii*
Cattle	Lung , Milk	10	4	*Acholiplasma laidlawii*
Sheep	Lung ,Trachea	30	8	*M. bovigenitalium* *M . arginini* *Acholiplasma laidlawii*
Goat	Lung Nasal swab	29 34	4 12	*M. ovipneumoniae* *M. arginini*

These results strongly support the use of DGGE / PCR assay as an efficient alternative or supplement to culture and serological identification, which are labor-intensive extremely time-consuming and often provide confusing results. Overall, it is suggested that the DGGE/PCR could be an alternative method for accurate identification of *Mycoplasma* infection to cultures that can also make up

for negative aspects of bacteriology and serological tests, in diagnosis *Mycoplasma* infection and identifying this agent at the species level.

Efficient *Mycoplasma synoviae* microbiological and molecular methods are necessary for diagnosis, eradication and research goals of this important poultry pathogen, although respiratory infections with *Mycoplasma synoviae* are generally considered subclinical, but an increasing number of reports have documented economic losses related to respiratory infections and arthropathic strains (6,14). The eggshell pathology and the concomitant egg production losses that result from *M. synoviae* infection, further highlight the economic significance of these in commercial poultry (4).

***Mycoplasma ovipneumoniae* is known as the cause of atypical or ovine nonprpgressive pneumonia, which is well recognized in different parts of the world. This research reports that finding of *M.ovipneumonia* in the respiratory tract of goats, although *M.ovipneumoniae* considered to be one of the most important *Mycoplasma* involved in the respiratory diseases of sheep, and the primary infection with it may predispose sheep to invation of lower respiratory tract by other organisms such as *Parainfluenza-3 virus* and *Mannheimia heamolytica* (11, 12). There are few reports incriminating *M. ovipneumoniae* as a cause of sever respiratory disease in goats. *Mycoplasma ovine/caprine serogroup 11* affect goat and sheep induces vulvovaginitis, cervicitis, endometritis, epididymitis and oophoritis (16), is pathogenic to the udder of lactating sheep and produces marked biochemical alterations in the milk (8). *Mycoplasma bovigentalium* is commonly isolated from the reproductive tracts of cattle and buffaloes and some strains are implicated in mastitis, arthritis, and genital discards (1) .*M. bovigentalium* first classified by (5), is biochemically very similar to *M. serogroup 11* as neither ferments glucose, hydrolyses arginine possesses phosphatase activity; however, both digest inspissated serum, reduce tetrazolium and produce film and spots. these similarities leading the suggestion that these might in fact be strains of the same species (13), also there is similarity In 16S and 23S rRNA gene sequence and DNA –DNA hybridization, and it is impossible to distinguish between *M. bovigentalium* and *M. serogroup 11*. isolation of *M. bovigentalium* from the respiratory tract of sheep needs furthery study to research the potential pathogenicity for the sheep respiratory tract.**

***Mycoplasma arginini* is an important pathogen by it self in sheep and goats, coinfection by other agents intensifies the pathologic injury of pneumonia (9) *Acholiplasma laidlawii* considered anon-pathogenic microorganism, it causes clear-water disease in the gill of Asian Mud carb *Scylla serata* (3, 16). Almost any animal, vertebrate or invertebrate is the potential host (3). *A. Laidlawii* creates survival bodies called ultramicroforms that enhance pathogenic factors in the organism due to stressors, such as starvation or other infections.**

CONCLUSION:

DGGE enable the rapid detection and differentiation of *Mycoplasma* species and can be used to diagnosis infections either directly from tissues or from culture isolates. It is capable of detecting mixed cultures or even new *Mollicutes* species with help of using *Mycoplasma*-specific primers, and suitable for routine use in the diagnostic laboratory with its high speed and specificity.

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تشخيص المايكوبلازما بطريقة الزرع البكتيري وتقنية البلمرة من المجترات والدواجن

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الملخص

ان الهدف من هذه الدراسة استخدام تقانات حديثة ودقيقة وخاصة في تشخيص المايكوبلازما جنساً ونوعاً وذلك لصعوبة تشخيصها بالطرق الكيموحيوية **Biochemical tests** لوجود تشابه بين بعض الأنواع بالتالي لمعرفة أمراضيتها حسب نوع المضيف ولإتخاذ الإجراءات اللازمة في علاج الأمراض التي تسببها والسيطرة عليها.

المايكوبلازما كائنات بدائية النواة صغيرة الحجم ليس لها جدار الخلية ولكنها محاطة بغشاء الخلية. يعتمد تشخيص الاصابة بالمايكوبلازما عادة على الزرع البكتيري وعلى الفحوص السيرولوجية التي تستغرق وقتاً طويلاً وجهداً كثيراً، ونتيجة للتطورات العلمية والبحثية فقد تم استخدام تقنية البلمرة **Specific- PCR** لتشخيص المايكوبلازما، الا ان هذه التقنية لم تكن بالتقنية النوعية الوحيدة لتشخيص انواع المايكوبلازما ***Mycoplasma species*** ولكن اعتمد تشخيص جنس ونوع المايكوبلازما في هذه الدراسة على تقنية أكثر تطوراً شملت بلمرة الجين **PCR(of the 16SrRNA gene)** مع بوادئ خاصة بالمايكوبلازما وفصل مستخلص الدنا **DNA** بالإعتماد على التسلسل الجيني الأولي وتسمى هذه الطريقة **Denaturing gradient gel electrophoresis DGGE**، إذ تم تشخيص جراثيم المايكوبلازما المرضية وغير المرضية بطريقة الزرع البكتيري وتقنية البلمرة **PCR** من المجترات الكبيرة والصغيرة ومن الدواجن باستخدام مسحات انفية وعينات رئة وقصبة هوائية وحليب من الأبقار، الجاموس، الأغنام، الماعز التي ظهرت عليها اعراض تنفسية والتهاب ضرع، وكذلك من مسحات انفية وعينات رئة وقصبة هوائية وقناة بيض وسائل زلالي اخذت من دجاج اللحم والبياض ،والتي ظهرت عليها ايضا اعراض تنفسية.

أجري الزرع البكتيري على العينات المذكورة آنفاً مباشرة بعد جمعها باستخدام وسط **PPLO** السائل لغرض عزل جراثيم المايكوبلازما، ثم تم استخلاص **DNA** بعد التأكد من وجود مستعمرات المايكوبلازما الأنموذجية على وسط الاكار باستخدام عدة تشخيصية خاصة لاستخلاص الدنا **DNA**، وبتقنية **Conventional PCR** وتم تشخيص جنس المايكوبلازما باستخدام بوادئ خاصة بجنس المايكوبلازما، في قسم المختبرات والبحوث/دائرة البيطرة.

ارسل مستخلص **DNA** الى مختبرات مرجعية **Animal Health and Veterinary Laboratory Agency (AHVLA)** في ويبرج/المملكة المتحدة لتأكيد التشخيص.

دائرة البيطرة، وزارة الزراعة، بغداد، العراق.