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Identification of *Mycoplasma synoviae* by PCR and Culture Methods and Determination of the Prevalence of *P1*, *P30*, and *P116* Virulence Genes in the Isolates

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ABSTRACT

Aims: *Mycoplasma synoviae*, as one of the main pathogens of birds, causes a lot of economic losses to the poultry industry. This study aimed to identify *M. synoviae* strains in clinical samples by PCR and culture methods.

Materials & Methods: A total of 135 samples were randomly collected from the respiratory tracts of female broilers in industrial poultry farms in Kerman, Iran during the first six months of 2016. Samples were cultured on Frey and PPLO broth media. Then PCR method was performed to identify *Mycoplasma* genus and *synoviae* species. Finally, multiplex PCR was performed to determine the prevalence of *P1*, *P30*, and *P116* virulence genes.

Findings: In this study, 17 (32%) out of 53 poultry samples were positive for the presence of *Mycoplasma* genus by culture method, whereas according to the PCR results, 25 (47%) out of 53 samples were confirmed as *Mycoplasma* genus, among which 13 samples (25%) were identified as *M. synoviae* species. Among the strains confirmed as *M. synoviae*, the prevalence rate of *P1, P30*, and *P116* genes was 7 (53.8%), 6 46.1%), and 5 (38.46%), respectively.

Conclusion: According to the PCR and culture methods results, the prevalence of *M. synoviae* strains was high in industrial poultry farms, Kerman, Iran. The PCR results revealed a higher prevalence rate for this bacterium, suggesting that this method may be more reliable than culture method.

Keywords: *Mycoplasma synoviae*, Virulence genes, PCR.

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[1] Mycoplasma synoviae infection. Dis Poult... [2] Seropositivity, involvement in suspected cases of chronic respiratory diseases and comparative efficacy of various... [3] Avian encephalomyelitis. In: Swayne DE, glisson Jr, McDougald Lr, Nolan LK, Suarez DL, Nair V, editors. Diseases of poultry. 13th ed. Ames: Wiley-Blackwell; 2013. [4] The resistance and carrier status of meat-type hens exposed to *Mycoplasma synoviae*. Poult Sci J. 1976;55(1):268-73. [5] Aerosol-induced *Mycoplasma synoviae* arthritis: The synergistic effect of infectious bronchitis virus infection. Avian Pathol. 2004;33(6):591-8. [6] Egg transmission of infectious synovitis. J Comp ... [7] Test profiles of broiler breeder flocks housed in farms with endemic *Mycoplasma synoviae* infection. Braz J Poult Sci. 2003;5(1):37-43. [8] Safety and efficacy of the *Mycoplasma* ... [9] Development and evaluation of a diagnostic PCR for *Mycoplasma synoviae* using primers located in the intergenic spacer region and the 23S rRNA gene... [10] Laboratory manual for the isolation and identification of avian pathogens. American Association of Avian Pathologists...[11] Using PCR and culture methods for *Mycoplasma* testing in poliomyelitis vaccine... [12] Isolation and identification of *Mycoplasma agalactiae* by culture ... [13] Development and application of a polymerase chain reaction assay for *Mycoplasma synoviae*. Avian Dis. 1993;37(3):829-34. [14] Comparison of culture, PCR, and different serologic tests for detection of *Mycoplasma* ... [15] Isolation and detection of *Mycoplasma gallisepticum* by polymerase chain reaction and restriction fragment length polymorphism. Iran J Vet Res. 2005;6(2 (Ser. No. 12):35-96. [16] Application of culture and polymerase chain reaction (PCR) methods for isolation and identification of *Mycoplasma synoviae* on broiler chicken farms. Arch Razi Inst. 2011;66(2):87-94. [17] Detection of *Mycoplasma synoviae* infection in broiler breeder farms of... [18] Intraspecific variation in 16S rRNA gene of *Mycoplasma synoviae* determined by DNA... [19] Polymerase chain reaction optimization ... [20] On the evolutionary descent of organisms and organelles: A global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. Nucleic Acids Res. 1984;12(14):5837-52.

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Introduction

Mycoplasma synoviae is one of the most important pathogens in poultry and turkey, which annually causes a lot of economic losses to the poultry industry. This bacterium has only one serotype; however, there are heterogeneous strains with different tissue tropisms and pathogenicity. Although *M. synoviae* was first introduced by Olsten as a cause of infectious sinusitis, today, in addition to infectious sinusitis, it is known to be the causative agent of respiratory and airway infections [1]. Despite recent advances in the control and eradication methods, *M. synoviae* continues to be of great importance in poultry industry, especially in broilers with vertical and horizontal transmission [2]. Broilers and turkeys are considered as the natural hosts of this bacterium. Natural infection occurs in poultry at an early age (even at one week of age), but acute infection usually occurs at the age of 4 to 7 weeks. In poultry flocks, the prevalence rate of sinusitis caused by *M. synoviae* varies between 2 and 75% and typically ranges from 5 to 15%. Respiratory tract infection is usually asymptomatic; however, 90 to 100% of poultry may be infected by *M. synoviae* with a mortality rate of 1-10% [3-4]. The combination of this infection with Newcastle disease, infectious bronchitis, or both, may lead to airway lesions. In other cases, *M. synoviae* may cause systemic infections in poultry, leading to infectious sinusitis and acute-to-chronic infectious diseases, in which the joint synovial membrane and the tendon plates become involved and ultimately lead to exudative sinusitis, tenovaginitis, or burcitis [5]. Laminitis and respiratory diseases following this infection could lead to reduced growth, reduced egg lying, and economic losses in industrial poultry. About 6 to 21 days after inoculation, *M. synoviae* could lead to tracheal infections, infertility, and fetal death [3]. Apparently, following the female broilers infection during egg laying, the highest vertical transmission occurs 4-6 weeks post-infection [6]. The timely diagnosis of *Mycoplasma* infection is important for female broilers, breeders, and veterinary organizations.

Objectives: The purpose of this study was to identify *M. synoviae* by PCR and culture methods and to determine the prevalence rate of *P1*, *P30*, and *P116* virulence genes in the isolates.

Materials and methods

Study population and sampling methods: Samples were randomly collected from the respiratory tracts of female broilers in industrial poultry farms in Kerman, Iran during the first six months of 2016. Samples were then sent to Pasargad Science and Research complex, Tehran, Iran to confirm the bacterial strains and genera.

Culture and isolation: Specific media including Frey and PPLO broth were prepared for *M. synoviae* culturing [10]. Specimens sent to the laboratory were first turned into three layers, and 5 mL of the resulting fluid was transferred to the PPLO broth medium and incubated at 37 °C for 24 hrs. All the sample-carrying PPLO broth was filtrated. Using sterile syringes, 2 mL of the solution was introduced into PPLO broth culture medium (pH=7.6-8) [11]. The medium was then placed in a CO_2 incubator and monitored for 3-5 days. After the required time, any color change or turbidity was recorded, and in any case, the media were passaged to the PPLO broth. After 24 to 48 hrs, 0.2 mL of each broth media was cultured on solid PPLO culture media and transferred to a CO_2 incubator at 37 °C for 21 days. The

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solid PPLO culture media were examined each day with light microscopy with x40 magnification in terms of growth and formation of special colonies [11].

Primer design, DNA extraction, and PCR: Genomic DNA extraction was performed using the phenol chloroform method. In this study, a fragment of *Mycoplasma 16S rRNA* gene was used to trace *M. synoviae* strains. Then the presence of *P1, P30*, and *P116* genes was investigated using specific primers (Table 1). PCR reaction was performed in a final volume of 20 µL (Table 2) in 35 cycles including: initial denaturation at 94 °C for 1 min, annealing (according to Table 1), extension at 70 °C for 1 min, and final extension at 72 °C for 7 min. PCR products were then loaded on 1% agarose gel and evaluated by electrophoresis. Finally, DNA was purified by purification kit, and final purified products were sent for sequencing. Sequencing results were then aligned, and phylogenetic analysis was performed.

Findings

Culture method: Fried egg-shaped colonies of *Mycoplasma* were observed on PPLO agar under the light microscope (Figure 1). Also, 17 out of 53 studied

Table 1) Primers used in this study to determine the presence of the studied genes

Primer	Target Gene	Sequence	Length (bp)	Annealing Temperature	Reference
MS-1 MS-2	*16S rRNA*	F: GCTGCGGTGAATACGTTCT R: TCCCCACGTTCTCGTAGGG	163	56	(12)
	16S rRNA	F: GAAGCAAAATAGTGATATCA R: GTCGTCTCCGAAGTTAACAA	207	56	(13)
Virulence genes	*P1*	F:AGCTTGACAGGTATACAACTGG R:AGAAGTGGGATCAGTTTGTTCC	245	62	This study
	P30	F: CTACAGTTGCTGGTTGCTTTGG R: TTGGGCAGAAATGCGTT GTAGG	151bp	62	This study
	P116	F: TGTATCGGTTGTAGTTGACTCCCG R: CT GA GA GACT ACCAGA TACT	675bp	64	This study

Table 2) PCR mixture components and their volumes used in this study

PCR Mixture Components	Volume
PCR Buffer (10X)	2 µL
$MgCl_2$ (25 mM)	1.5 µL
dNTPs (10 mM)	1.5 µL
Primer Forward (10 pmol/µL)	1 µL
Primer Reverse (10 pmol/µL)	1 µL
Taq DNA Polymerase (5U/µL)	0.3 µL
Template DNA	2
ddH_2O	10.7
Total Volume	20 µL

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industrial broiler samples were confirmed as *Mycoplasma* genus.

Results of PCR for *16S rRNA* gene: According to the PCR method results, 25 (47%) out of 53 poultry samples were positive for *Mycoplasma* genus, among which 13 samples (25%) were considered as *M. synoviae* species (Table 3).

Comparing the results of PCR and culture methods: Identification of samples based on the PCR and culture methods are illustrated in Table 4.

Results of PCR for virulence genes: The prevalence rate of *P1*, *P30*, and *P116* virulence genes in 13 *M. synoviae* isolates confirmed by

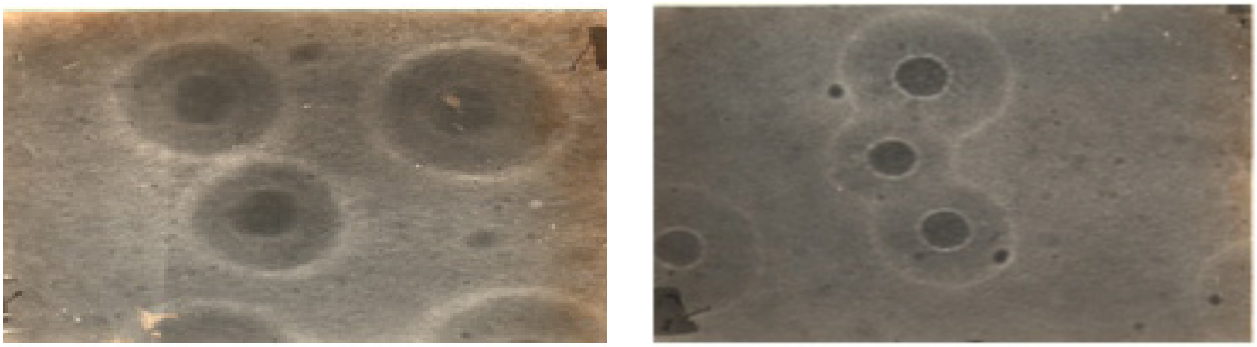

Figure 1) Fired egg-shaped colonies of *Mycoplasma* under the light microscope (40×).

Table 3) The prevalence of *Mycoplasma* genus and *M. synoviae* species based on the PCR and culture methods results

	***Mycoplasma* Genus (PCR)**	***synoviae* Species(PCR)**	***Mycoplasma* (Culture)**
	Prevalence No. (%)	**Prevalence No. (%)**	**Prevalence No. (%)**
Positive	13 (25)	25 (47)	17 (32)
Negative	12 (22)	28 (53)	36 (68)
Total	25 (47)	53 (100)	53 (100)

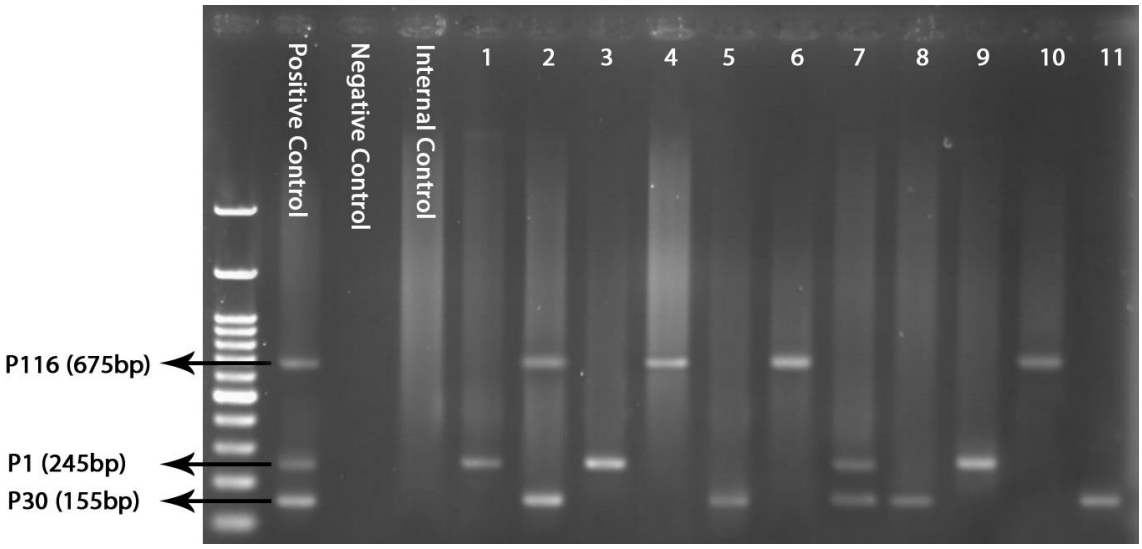

Figure 2) Agarose gel electrophoresis showing band patterns of *P1*, *P30*, and *P116* genes

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Table 4) Prevalence of *Mycoplasma* genus based on the PCR and culture methods

No. of Samples	PCR	Culture
14	+	+
25	-	-
3	-	+
11	+	-
53		Total

PCR method was 7 (53.8%), 6 (46.1%), and 5 (38.46%), respectively (Fig. 2).

Discussion

Considering the importance of infections caused by *M. synoviae* strains, including respiratory and articular diseases, as well as the imposition of treatment costs on community and the lack of proper response to treatment and loss of optimal performance of infected poultry, direct DNA-based identification of *M. synoviae* strains in tissues or among the laboratory isolates seems to be necessary [14]. Although a probe could be used to detect *M. synoviae* strains, the most common method is to replicate the specific region of bacterial genome [14]. *Mycoplasma* has been identified in Iran for several years by serological and culture methods. Molecular analysis of *Mycoplasma* has also been carried out by Iranian researchers with different methods, including *16S rRNA* gene detection to identify *M. synoviae* [15-17]. In these studies, PCR has been introduced as an effective and time-saving method which could be considered as a proper alternative to culture method with more sensitivity, speed, and reliability.

However, based on the some studies results, genotypic differences resulting from the removal, addition, or substitution of nucleotides in *16S rRNA* gene have been observed in different strains of *M. synoviae* [18]. The PCR results are determined within one or two days, while it takes one to three weeks to identify an organism through culturing [17]. PCR could provide accurate results in the presence of mixed infections with several *Mycoplasma* strains; secondary bacterial infections; and *Mycoplasma* growth inhibitors such as antibodies, antibiotics, or other host factors [8]. Another advantage of PCR method is the ease of sampling. While specimens are necessary for culturing and isolation, in PCR test, even a single swab could be used for DNA extraction [12]. Several studies have been conducted to identify *M. synoviae* strains, and several methods have been proposed. Ricardo et al. (1996) examined various methods, such as SPA, HI, ELISA, and PCR, and introduced PCR as the most efficient method for the identification of this bacterium [19]. In this study, PCR and culture methods were performed simultaneously for the identification of *M. synoviae* strains. The results showed poultry infection caused by *M. Synoviae* strains. In this study, 47% of the samples were positive for *Mycoplasma* genus by PCR, and 25% of the specimens were positive for *M. synoviae* species, indicating a high prevalence of *M. synoviae* contamination and a serious warning for rapid and direct identification of this microorganism in industrial poultry farms. This study provided a very fast diagnostic method for *M. synoviae*, compared to culture method. In this study, three samples were reported as negative by PCR method but positive by culture method. Factors such as inhibitors of proper functioning of Taq

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DNA polymerase enzyme, all materials used in DNA extraction using phenol-chloroform method, user's error, non-compliance with the conditions of molecular tests, or changes in the gene sequences could lead to the false negative results. Another reason for PCR testing error in identifying *M. synoviae* strains could be the occurrence of genetic mutations in the target region of the primer, induced by frequent passaging of the isolates. In addition, 11 samples were reported as positive by PCR method but negative by culture method. This could be attributed to several reasons, including errors and inaccuracy in culturing; inadequate growth requirements in the complex PPLO broth/ agar culture media, such as insufficient CO2, serum, NAD, and other materials; as well as non-compliance with transfer conditions that sometimes could result in the death of *M. synoviae* isolates at room temperature. According to the culture and PCR methods results in this study, the prevalence rate of *M. synoviae* strains was significantly high in poultry farms in Kerman, Iran. Comparing these two techniques, the prevalence of *M. synoviae* strains was reported as very high by PCR test. This study indicated that industrial poultry farms actual contamination rate reported by PCR test could be more reliable than that reported by culture method. Other studies also confirmed the results of this study. Gray et al. (1984) concluded that the ribosomal rRNA molecule could be selected as the main target of PCR. Since rRNA naturally has many copies, it is therefore a suitable target for highly sensitive PCR test [20]. Lauerman et al. (1993) designed specific primers based on *16S rRNA* gene sequence for *Mycoplasma* species and called them MS-1 and MS-2 primers [13]. Their results showed that the sensitivity of PCR test using these two primers was 82%, and its specificity was 100% compared to other tests including culture, SPA, HI, and ELISA. In this study, among the *Mycoplasma* positive samples, 15.5, 13.3, and 11.11% harbored *P1, P30*, and *P116* genes, respectively, among which one sample carried *P1* and *P30* genes, and another sample carried *P30* and *P116* genes, simultaneously.

The presence of these genes in *M. synoviae* strains suggests the pathogenicity of these microorganisms, induced by their ability to attach respiratory tract epithelium. *P1* gene was the most prevalent and major gene contributing to attachment and pathogenicity. Among the *Mycoplasma* isolates, 4.44% carried two of the studied virulence genes, suggesting their greater ability in attachment and pathogenicity.

Conclusion

Despite attempts for infection treatment and vaccine usage in flocks, little success has been obtained in eradicating and eliminating *Mycoplasma* infections. Hence, timely and accurate identification of these isolates is of great importance. Although serum diagnosis with conventional methods (HIRSA, ELISA) has been very useful and promising, there is a possibility of obtaining false positive results. Therefore, the necessity of using more accurate methods has been proposed in recent years. As the results of this study and other studies show, PCR method could be used as an effective method to identify *Mycoplasma* strains.

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korani N. (First author), Original researcher, Methodologist / Discussion author (50%); Kheirkhah B. (Second author), Original researcher, Methodologist / Discussion author (50%).

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