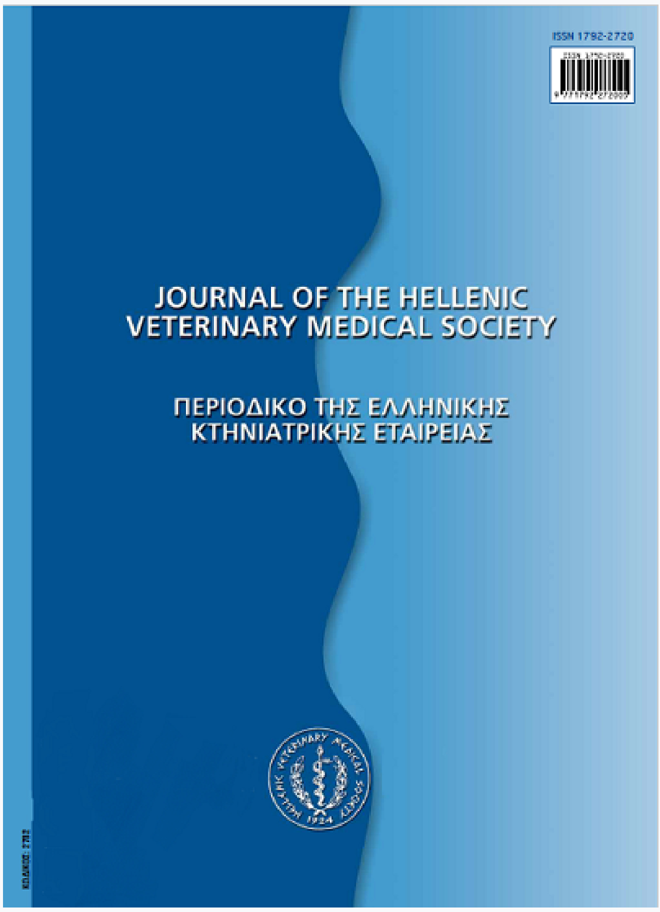
Journal of the Hellenic Veterinary Medical Society

Vol 68, No 4 (2017)



Department of Microbiology, Science and Research Branch, Islamic Azad University, Tehran, Iran

H MOOMIVAND, SA POURBAKHSH, M JAMSHIDIAN

doi: [10.12681/jhvms.16069](https://doi.org/10.12681/jhvms.16069)

To cite this article:

MOOMIVAND, H., POURBAKHSH, S., & JAMSHIDIAN, M. (2018). Department of Microbiology, Science and Research Branch, Islamic Azad University, Tehran, Iran. *Journal of the Hellenic Veterinary Medical Society*, *68*(4), 647–652. https://doi.org/10.12681/jhvms.16069

J HELLENIC VET MED SOC 2017, 68(4): 647-652
ΠΕΚΕ 2017, 68(4): 647-652

Research article
Ερευνητικό άρθρο

Isolation and identification of pathogenic mycoplasmas in ostrich farms using PCR and culture methods

Moomivand H.[1], Pourbakhsh S.A.[1], Jamshidian M.[1]

[1]Department of Microbiology, Science and Research Branch, Islamic Azad University, Tehran, Iran

ABSTRACT. In ostriches, mycoplasmas are generally associated with respiratory diseases and causes rhino-tracheitis, airsacculitis and inflammation of the upper respiratory tract. The aim of current study was the isolation and identification of pathogenic mycoplasmas in ostrich farms of Iran by the use of PCR and culture methods. In this study, mycoplasmas were isolated from ostrich slaughterhouse; 114 samples were collected from ostriches with respiratory signs and were cultured and PCR methods along with alignment were used to detect the mycoplasmas. For this purpose lung, trachea and air sacs were evaluated. The results indicated that 21.05% of samples were positive in PCR assayand from them 7.89% and 14% was *M. gallisepticum* and *M. synoviae*, respectively. The highest rate of *M. gallisepticum* and *M. synoviae* was detected in lung, airsacs and trachea. Alignment analysis demonstrated that the *M. gallisepticum* strains detected in our study have 97% homology to 06/14, 05/14 and 16S strains. In addition, *M. synoviae* strains have 99% and 98% homology to MSR-812, MSR-795 and MSR-1019 strains. One of the *M. synoviae* strains has 82% homology to ABSfsdMS2011 strain. The results of our study showed that ostriches in Iran were infected with chicken mycoplasmas but the pathogenesis of them in ostrich respiratory should be further evaluated.

Keywords: Mycoplasma, Ostriches, isolation, PCR, sequencing

Corresponding Author:
Pourbakhsh S.A.
Science and Research Branch of Islamic Azad University,
E-mail: pourbakhs@gmail.com

Date of initial submission: 4-12-2016
Date of revised submission: 12-1-2017
Date of acceptance: 11-3-2017

INTRODUCTION

Mycoplasmosis is an infectious disease and causes economic losses due to respiratory signs, leg disorders, growth retardation, losses of egg production and reduction of hatch rate (Bradbury and Morrow, 2008; Calnek and Barnes, 1997). Mycoplasmas are widespread in nature and differ from other bacteria, because of their small size and the lack of cell wall (Bradbury and Morrow, 2008). Mycoplasmas are host-specific and completely resistant to antibiotics that affect cell wall synthesis (Al-Ankari and Bradbury, 1996). Pathogenic avian mycoplasmas include the species *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in chickens and turkeys as well as *Mycoplasma meleagridis* (MM) and *Mycoplasma iowae* (MI) in turkeys (Panangala *et al.*, 1992).

In ostriches, mycoplasmas are generally associated with respiratory diseases and causes rhino-tracheitis, airsacculitis and inflammation of the upper respiratory tract (Huchzermeyer, 1994; Verwoerd, 2000). Rhino-tracheitis, usually appeared in cold, windy weather during winter or in summer following heat stress (Botes *et al.*, 2005; Verwoerd, 2000). MS have been isolated from respiratory infected ostriches, with respiratory symptoms (Huchzermeyer, 1994). Although serological response to poultry mycoplasmas (MG, MS and MM) were reported from northern Italy, infection of ostriches with poultry mycoplasmas was not determined (Peccati *et al.*, 1996). It was reported that the concurrent infections in ostriches cause production loss, carcasses downgrading and thus considerable economic losses in ostrich industry (Botes *et al.*, 2005).

When ostriches were kept near poultry in northern Italy, they had positive immunologic response to poultry mycoplasmas (including MG, MS, and MM), although infection with that mycoplasmas was not recognized (Peccati *et al.*, 1996). In Zimbabwean ostriches slaughterhouses antibodies against MG and MS were reported (Cadman *et al.*, 1994).

Concurrent infections of mycoplasmas and other pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Pasteurella* species and occasionally *Avibacterium paragallinarum* were reported from clinical syndromes of infected ostriches (Blackall *et al.*, 2005; Verwoerd, 2000).

Experimental infection of M.G with clinical signs in ostriches was previously reported (Cline *et al.*, 1997). Researchers indicated that the mycoplasmas occured in ostriches, were not typical mycoplasmas of poultry which infected respiratory system in poultry (Shivaprasad, 1993).Other investigations suggested that unique ostrich-specific mycoplasmas, causes infections in ostriches (Shane, 1998; Smith, 1993). Previous studies reported isolation of M. synoviae from ostriches in Iran (Tebyanian *et al.*, 2014). Generally, Mycoplasma transmitted within species or between closely related species, with rare exceptions (Kleven, 2008; Nascimento *et al.*, 2005).

The aim of current study was the isolation and identification of pathogenic mycoplasmas in ostrich farms of Iran using molecular and culture methods.

MATERIALS AND METHODS

Samples: For this study, 114 samples were collected from ostrich slaughterhouse and investigate the presence of mycoplasma by culture and PCR. From all ostriches with respiratory signs serum samples were collected for antibody evaluation against MS and MG. The samples were collected from lung, trachea and air sacs of ostriches with respiratory signs transported to the laboratory.

Culture: Samples were cultured on pleuropneumonia-like organisms (PPLO) broth and agar media without crystal violet (21 g/l), 20% heat-treated horse serum, 10% fresh yeast extract, 0.2% glucose, 0.4% sodium pyruvate, 0.04% ampicillin and 1% agar noble. For the evaluation of MS by culturing samples 1%, nicotinamide adenine dinucleotide was supplemented to above medium. Firstly, the samples were enriched in PPLO broth at 37°C for 24 hours and then, for PCR investigation, they transported to PPLO agar (Swayne, 1998), in 5% CO2 for 7 to 10 days. The plates were checked daily for the appearance of the characteristic colonies. The mycoplasma specific stain was used for the evaluation of cultured bacteria. A single colony from each isolate and the samples that was collected from various organs was used for DNA extraction and mycoplasma identification.

DNA extraction: For DNA extraction the Genomic DNA extraction Kit (Gene Transfer Pioneers, GTP) was used. Following the instructions to manual 1-4 ml of bacterial culture centrifuged in 8000g for 1 min and the supernatant was discarded, 250 µl RNase A was added and vortexing for 15 seconds (s). Then 400 µl wash buffer and 400 µl chloroform were added, vortexing for 30s and centrifuged at 12000g for 5 min.400 µl of supernatant was transferred in new microtube and 400 µl guanidine hydrochloride was added and mixed gently. The solution was transferred to DNA spin column assembled in a clean collection tube and centrifuged at 10000g for 1 min. 500µl wash buffer were added to DNA spin column and centrifuged until remove residual ethanol. Then prewarmed elution buffer added onto column membrane and stand for 3 min. The extracted DNA was electrophoresed on 1% agarose gel and visualized by staining with ethidium bromide (1.5 µg ml−1).

Primers for the amplification of 16S rRNA: To confirm the presence of mycoplasma in samples, general and specific primers of 16S rRNA were used: Primers GPO3F and MGSO to detect the genus (Van Kuppeveld *et al.*, 1992), primers M.syn and M.Syn-R the detect MG (Bagheri et al., 2011) and primers MS-1 and MS02 to detect MS (Lauermanet al., 1993). Primers are listed in Table 1.

PCR amplification of 16S rRNA gene: PCR amplification carried out in 0.5ml tubes in a final reaction volume of 20 µl. The PCR mixture consisted of 5 ng of template DNA, 1 mM MgCl2, 0.8 µl of each primers, 2 µl of 10X PCR buffer, 100 mM dNTPs, and 1 U Taq DNA polymerase, was amplified according to Botes et al., (2005): 95°C (5min) of initial denaturation and then 35 cycles of denaturation at 95°C (40s), annealing at 52°C (60s) and elongation at 72 °C (60s) and final extension in 72°C (5min). Amplified DNA was electrophoresed on 1% agarose gel and visualized by staining with ethidium bromide (1.5 µg ml−1).

Table 1: Primers used for amplification and detection of mycoplasmas from ostrich samples

Primer	Sequence	References
	General mycoplasma primers	
GPO3F (F)	5′-TGGGGAGCAAACAGGATTAGATACC-3′	(Van Kuppeveld *et al.*, 1992)
MGSO (R)	5′-TGCACCATCTGTCACTCTGTTAACCTC-3′	
	16S rRNA primers developed for detection MG	
M.Syn (F)	5′- GCGATGACGTGTAGTTATGCTG -3′	(Bagheri *et al.*, 2011)
M.Syn-R (R)	5′- CCAATGCATACAATCGTTAAGC -3′	
	16S rRNA primers developed for detection MS	
MS-1	5′- GAAGCAAAATAGTGATATCA -3′	(Lauerman *et al.*, 1993)1993
MS-2	5′- GTCGTCTCCGAAGTTAACAA -3′	

SEQUENCING OF THE MYCOPLASMA 16S rRNA GENE

The PCR products of GPO3 and MGSO primer pairs, purified as templates for sequencing reactions. PCR products were electrophoresed on a 0.5% agarose gel. DNA containing bands were excised under a UV light and purified, then samples were concentrated and subsequently analyzed by electrophoresis, as described previously by researchers (Botes *et al.*, 2005). For sequencing, the samples were sent to Macrogen Korea co, (Republic of Korea). Data from sequencing were aligned to and compared on the NCBI nucleotide sequence database.

RESULTS:

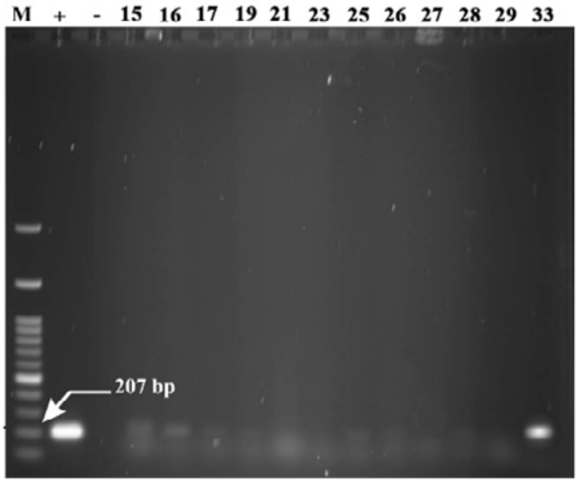
Mycoplasmas culture: After 48-96 hours incubation, mycoplasma colonies were visible. Typical colonies of mycoplasmas, were identified detecting the fried egg morphology in well-developed colonies. From the 114 ostrich samples, 24 were positive for mycoplasmas. Results are listed in Table 2.

Table 2:The Clinical origin of mycoplasma positive samples

Tissue	Mycoplasma	MG	MS
Lung	14	6	8
Trachea	4	1	2
Air sacs	6	2	4
Total	24	9	14

Polymerase Chain Reaction: The growth of *Mycoplasmas* in PPLO broth media causes changes in color due to the biochemical activity of the bacteria. One hundred and fourteen (114) suspected samples were analyzed by PCR test. In 24 samples *Mycoplasma* isolates were detected (Figures 103). All positive samples were evaluated by PCR test for the detection of MG and MS. From the 24 positive *Mycoplasma* samples, 14 (58.33%) were identified as M.S and 9 (37.5%) as MG. Microbiological methods indicated 7 MG and 8 MS positive samples. Results are listed in Table 3.

Fig 2: PCR results using Specific MS Primers

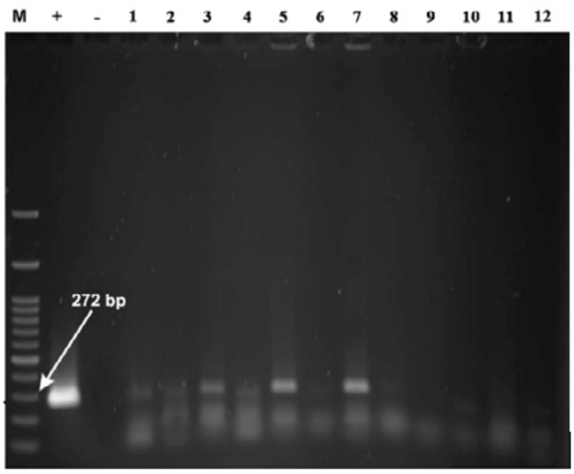


M, marker 100 bp; positive control (+); negative control (-); 15 to 33 samples; a 207bp band characteristic for MS.

Table 3: Results of microbiological and PCR methods in detection of Mycoplasma from ostriches samples

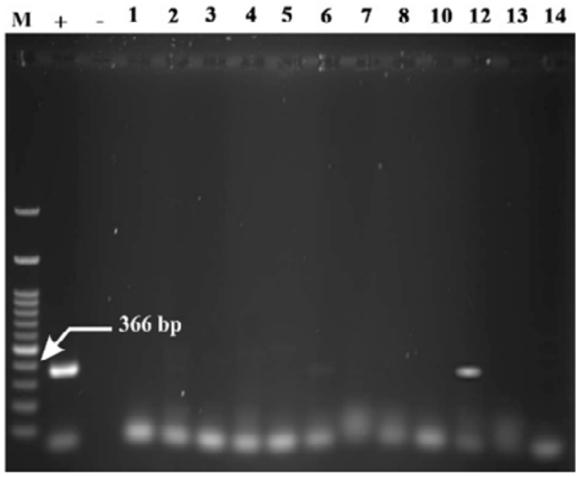
	Mycoplasma, PCR	MG PCR, No (%)	MG culture method, No (%)	MS PCR, No (%)	MS culture method, No (%)
Positive	24 (21.05)	9 (7.89)	7 (6.14)	14 (12.28)	8 (7.01)
Negative	90 (78.94)	105 (92.10)	107 (93.85)	100 (87.71)	106 (92.98)

Fig 1: PCR results using Genus Mycoplasma Primers



M, marker 100 bp; positive control (+); negative control (-); 1 to 12 samples; a 272bp band characteristic for Mycoplasma genus.

Fig 3: PCR results using Specific MG Primers



M, marker 100 bp; positive control (+); negative control (-); 1 to 14 samples; a 366bp band characteristic for MG.

Sequencing: Alignment data showed that the MG strains detected in our study had 97% homology to 06/14, 05/14 and 16S strains. MS strains had 98 -99% homology to MSR-812, MSR-795 and MSR-1019 strains. One of the MS strains had 82% homology to ABSfsdMS2011 strain.

Serological results: Serologic survey with rapid serum agglutination test demonstrated 22.80% and 30.70% of serum samples have antibodies against *M. gallisepticum* and *M. synoviae*, respectively.

DISCUSSION

In ostriches, mycoplasmas cause respiratory diseases, rhino-tracheitis, airsacculitis and upper respiratory tract inflammation (Huchzermeyer, 1994; Verwoerd, 2000). In current study pathogenic mycoplasmas from lung, trachea and air sacs of ostrich farms in Iran were isolated and identified using PCR and culture methods. The results of our study indicated that the *M. gallisepticum* and *M. synoviae* were isolated and identified in ostrich from Iran.

M. gallisepticum and *M. synoviae* isolated from South Africa with respiratory symptoms, regularly in winter from ostriches (Verwoerd, 2000). In northern Italy a serological survey, using the rapid plate test with specific antigen for M. *gallisepticum,* M. *synoviae* and M. *meleagridis*, demonstrated positive sera to all above mycoplasmas (Peccati *et al.*, 1996). Also, it was reported that the experimental infection of young ostriches with *M. gallisepticum* causes colonization in trachea.(Cline *et al.*, 1997).

It was indicated in ostrich farms of South Africa the new mycoplasma named as *Mycoplasma struthiolus* had infected ostriches and further studies on them showed three various *M. struthiolus* with 88.4, 88.7 and 93.1% sequence homology (Botes *et al.*, 2005).

Shivaprasad (1993) reported that there were not clinical signs for the majority of mycoplasmas isolated from lung and trachea and that none of the 32 reported isolates were *M. gallisepticum* or *M. synoviae*). *M. synoviae* was isolated from respiratory tract infection of ostriches (Verwoerd, 2000), and immune response to *M. gallisepticum* and *M. synoviae* was documented previously (Cadman *et al.*, 1994; Peccati *et al.*, 1996). Experimental infection with *M. gallisepticum* with clinical sign also was reported (Cline *et al.*, 1997).

It was indicated that the identification of mycoplasma species in ostriches and the evaluation of their infectivity and pathogenicity is important (Peccati *et al.*, 1996). Due to the susceptibility of ostriches to poultry mycoplasma, it was suggested that ostrich farms should be free of poultry species (Verwoerd, 2000).

The results of our study have been demonstrated that the ostrich farms from Iran were infected with *M. gallisepticum* and *M. synoviae*. According to our results, 58.33% and 37.5% of the positive samples in PCR were identified as *M. synoviae* and *M. gallisepticum*, respectively. Studies on ostrich farms in Kerman province of Iran indicate that 52 % of mycoplasma positive samples was *M. synoviae* and 48% of other mycoplasmas that was unidentified (Tebyanian *et al.*, 2014). Generally, *M. synoviae* is considered as subclinical respiratory infection in poultry, but economic losses were reported (Elhamnia *et al.*, 2016). The results of alignment analysis indicated that the majority of the MS strains, which was isolated from Iranian ostriches, had 81% and 97-99% homology to poultry origin strains of MS.

M. gallisepticum was isolated and detected from chicken farms by PCR and RFLP in the Fars province of Iran, while *M. synoviae* was not detected from samples (Ghaleh Golab Behbahan *et al.*, 2005). In addition, *M. gallisepticum* was identified by RAPD test from different geographical areas of Iran, while *M. synoviae* was not identified (Peighambari *et al.*, 2006).

The results of our study indicate that only *M. gallisepticum*, and *M. synoviae* were isolated, the frequency of *M. synoviae* being higher than this of *M. gallisepticum*. In addition, our results demonstrated that the mycoplasmas isolated from ostriches have a great homology to poultry origin mycoplasmas, thus keeping poultry away from ostrich farms is necessary. Although studies in South Africa indicated that ostriches kept close to poultry were not infected with poultry mycoplasmas and they were only infected with *M. struthiolus* some researchers documented infections with poultry mycoplasmas in ostriches. please add the references (Botes *et al.*, 2005).

From the samples collected from ostriches in slaughterhouse was found high rate of infections with *M. gallisepticum* and *M. synoviae* in respiratory system of ostriches. Lung was highly infected with *M. gallisepticum* and *M. synoviae*. It was indicated that mycoplasmas have infected upper respiratory tract of ostriches (Botes *et al.*, 2005) but infections with poultry mycoplasmas and their clinical signs in ostriches was not documented clearly. It should be investigated if poultry origin mycoplasmas cause clinical signs in poultry as the samples analyzed in current study could not be demonstrate the clinical signs of the disease, clearly. In addition, other organs should be evaluated to demonstrate the pathogenesis of mycoplasma in ostriches.

PCR is a precise and ideal method for the diagnosis of mycoplasmas (Kiss et al., 1997), and the detection of infectious agent before onset of signs (Moalic et al., 1997). PCR assays could be used to early detection of infections and evaluation of ostriche-farms. To determine the prevalence of the ostriches mycoplasma infection in Iran, more study is necessary.

REFERENCES

Al-Ankari, A R S, Bradbury, J M (1996) Mycoplasma Iowae: A Review. Avian Pathol 2: 205-229.

Bagheri, H, Doosti, A, Arshi, A (2011) Detection of Mycoplasma Gallisepticum in Chaharmahal Va Bakhtiari Province Poultry Using Pcr. Global Vet 1: 54-59.

Blackall, P J, Christensen, H, Beckenham, T, Blackall, L L, Bisgaard, M (2005) Reclassification of Pasteurella Gallinarum,[Haemophilus] Paragallinarum, Pasteurella Avium and Pasteurella Volantium as Avibacterium Gallinarum Gen. Nov., Comb. Nov., Avibacterium Paragallinarum Comb. Nov., Avibacterium Avium Comb. Nov. And Avibacterium Volantium Comb. Nov. Int J Syst Evol Microbiol 1: 353-362.

Botes, A, Peyrot, B, Olivier, A, Burger, W, Bellstedt, D (2005) Identification of Three Novel Mycoplasma Species from Ostriches in South Africa. Vet Microbiol 3: 159-169.

Bradbury, J M, Morrow, C J (2008) Avian Mycoplasmas. In: Poultry Disease. ed. Saunders Elsevier Philadelphia, USA: pp **220-234.**

Cadman, H F, Kelly, P J, Zhou, R, Davelaar, F, Mason, P R (1994) A Serosurvey Using Enzyme-Linked Immunosorbent Assay for Antibodies against Poultry Pathogens in Ostriches (Struthio Camelus) from Zimbabwe. Avian Dis 3: 621-625.

Calnek, B W, Barnes, H J (1997). Diseases of Poultry. 10th Ed., Iowa State University Press, Ames, pp.

Cline, J. L.,, Turner, K S, O'connor, R J, Gomez, J, L. B.,, Kleven, S H (1997) Determination of Colonisation of Mycoplasma Gallisepticum and Mycoplasma Synoviae in Ostriches. Am. Ostrich, Special Annu. Res.: 12-13.

Elhamnia, F, Banani, M, Shokri, G, Pourbakhsh, S, Ashtari, A (2016) Detection of Mycoplasma Synoviae Infection in Broiler Breeder Farms of Tehran Province Using Pcr and Culture Methods. Archives of Razi Institute 2: 75-81.

Ghaleh Golab Behbahan, N, Asasi, A, Afsharifar, A, Pourbakhsh, S (2005) Isolation and Detection of Mycoplasma Gallisepticum by Polymerase Chain Reaction and Restriction Fragment Length Polymorphism. Iranian Journal of Veterinary Research of Univiversity Shiraz 1: 35-41.

Huchzermeyer, F W (1994). Ostrich Diseases. Onderstepoort Veterinary Institute, pp: 23.

Kleven, S H (2008) Control of Avian Mycoplasma Infections in Commercial Poultry. Avian Dis 3: 367-374.

Lauerman, L H, Hoerr, F J, Sharpton, A R, Shah, S M, Van Santen, V L (1993) Development and Application of a Polymerase Chain Reaction Assay for Mycoplasma Synoviae. Avian Dis 3: 829-834.

Nascimento, E R, Pereira, V, Nascimento, M, Barreto, M (2005) Avian Mycoplasmosis Update. Revista Brasileira de Ciência Avícola 1: 1-9.

Panangala, V S, Gresham, M M, Morsy, M A (1992) Antigenic Heterogeneity in Mycoplasma Iowae Demonstrated with Monoclonal Antibodies. Avian Dis 1: 108-113.

Peccati, C, Grilli, G, Gallazzi, D, Rampin, T (1996). ***Serological Survey in Ostriches in Northern Italy. Improving our Understanding of Ratites in a Farming Environment, Dalton-Ellis Hall, University of Manchester, England.***

17 - Peighambari, S, BozorgmehrifardÙ, M, Hosseini, H, Pourbakhsh, S, Razzazian, M (2006) Random Amplified Polymorphic DNA (Rapd) Fingerprinting of Mycoplasma Gallisepticum Isolates from Chickens. Archives of Razi Institute 2: 67.

Shane, S M (1998) Infectious Diseases and Parasites of Ratites. Veterinary Clinics of North America: Food Animal Practice 3: 455-483.

Shivaprasad, H (1993) Neonatal Mortality in Ostriches: An Overview of Possible Causes. Annual Conference of the Association of Avian Veterinarians, Proceedings: 282-293.

Smith, C (1993) Ostrich Chick Survival Presents Challenge. Journal of the American Veterinary Medical Association (USA).

Swayne, D E (1998). Laboratory Manual for the Isolation and Identification of Avian Pathogens. American Association of Avian Pathologists, University of Pennsylvania, pp.

Tebyanian, H, Mirhosseiny, S H, Kheirkhah, B, Hassanshahian, M (2014) Isolation and Identification of Mycoplasma Synoviae from Suspected Ostriches by Polymerase Chain Reaction, in Kerman Province, Iran. Jundishapur journal of microbiology 9.

Van Kuppeveld, F, Van Der Logt, J, Angulo, A, Van Zoest, M, Quint, W, Niesters, H, et al. (1992) Genus-and Species-Specific Identification of Mycoplasmas by 16s Rrna Amplification. Appl Environ Microbiol 8: 2606-2615.

Verwoerd, D (2000) Ostrich Diseases. Revue scientifique et technique (International Office of Epizootics) 2: 638-661.