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RESEARCH ARTICLE

Diagnosis of mycoplasmosis in chicks by pathological and Real Time-PCR methods

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Özet

Tuzcu M, Özmen M, Karakoç SR, Tuzcu N, Yoldaş A. Tavuklarda mikoplazmozisin patolojik ve Real Time-PCR metotları ile teşhisi. **Eurasian J Vet Sci, 2012, 28, 2, 82-86**

Amaç: Bu çalışmanın amacı tavuklarda mikoplazmozisin teşhisinde Real Time-PCR'ın kullanılabilirliğini araştırmak ve mikoplazma tespit edilen tavuklardan alınan akciğer, hava keseleri, trake, kalp, karaciğer ve böbrek dokularındaki patolojik bulguları belirlemektir.

Gereç ve Yöntem: Solunum problemi şikayeti olan 3 ayrı işletmeye ait 3 kümesten, hastalık bulgusu gösteren 10'ar adet broyler piliçten toplanan konjuktival ve trakel svap örnekleri ile aynı piliçlerden nekropsiyi takiben alınan trake, hava kesesi, akciğer, karaciğer, böbrek ve kalp dokuları patolojik, Real Time-PCR (RT-PCR) ve mikrobiyolojik metotlarla incelenmiştir.

Bulgular: Çalışmada, klinik olarak burun ve gözyaşı akıntısı ile hırıltılı solunum, makroskobik olarak trake ve bronşlarda kataral eksudat görüldü. Mikroskobik olarak trake ve bronş epitellerinde ve goblet hücrelerinde hiperplazi ile lamina propriada mononükleer hücre infiltrasyonlarının şekillendiği belirlendi. Sekiz broyler pilicin trake dokusundan *Mycoplasma* ssp. izolasyonu yapıldı. RT-PCR ile 22 broylere ait doku ve svap örneklerinde *Mycoplasma gallisepticum* nükleik asitleri amplifiye edildi.

Öneri: Tavuklarda mikoplazmozisin teşhisinde, etken izolasyonunun geciktiği ya da yapılamadığı durumlarda RT-PCR'nin önemli bir alternatif olabileceği kanısına varılmıştır.

Abstract

Tuzcu M, Ozmen M, Karakoc SR, Tuzcu N, Yoldas A. Diagnosis of mycoplasmosis in chicks by pathological and Real Time-PCR methods. **Eurasian J Vet Sci, 2012, 28, 2, 82-86**

Aim: The purpose of this study was to investigate the suitability of the Real Time-PCR in the diagnosis of mycoplasmosis and to determine the pathologic findings in lungs, air sacs, trachea, hearth, liver and kidney tissues.

Materials and Methods: Conjunctiva and tracheal swab samples were taken from broiler chicks with respiratory disease complaints from 3 different breeders were used. Ten chicks from three separate flock in each breeders were collected. Trachea, air sac, lung, liver, kidney, and heart samples were also collected from the same chicks after necropsy in order to perform pathological, microbiological and Real Time-PCR analyses.

Results: Clinically, nasal and conjunctival discharge and wheezing were observed. Macroscopic examination illustrated gross catarrhal exudation in trachea and bronchus. In microscopically, hyperplasia in trachea and bronchus epithelia, mucus producing cells and mononuclear cellular infiltration in lamina propria were observed. *Mycoplasma* spp. were successfully isolated in the tracheal tissue of 8 broiler chicks. *M. gallisepticum* specific nucleic acid was amplified from tissue and swab samples of 22 broiler chicks by RT-PCR.

Conclusion: RT-PCR seems to be an alternative method when microbiological analyses are laborious or fails in diagnosis of mycoplasmosis.

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▸ Introduction

M. gallisepticum infection causes serious problems all over the world (Zanella 2007). The symptoms of mycoplasmosis are nasal and conjunctival discharge, wheezing and coughing. Clinical findings in broilers are more severe than that of layer chicken. If environmental conditions and coop hygiene are bad and secondary infections are also noted, mortality rate in diseased flocks get really higher (Ley 2003, Zanella 2007).

The catarrhal exudates in trachea and sinus bronchus are known as the defining macroscopic finding during the necropsy of *M. gallisepticum* infection in chicks. Besides, caseous substance in air sacs and fibrin bulks in liver and hearth are stated in the mycoplasmosis. Epithelial hyperplasia, increase in number of the cells that secrete mucus and macrophage in lamina propria and also lymphocyte and mononuclear cell infiltrations are detected. In lamina propria and submucosa, lymphoid follicle formations are observed microscopically and fibrin exudation in air sacs and heterophile, lymphocyte and macrophage cell infiltrations and degeneration and hyperplasia in epithelial cells are observed (Rodriguez and Kleven 1980, Levisohn et al 1986, Nunoya et al 1987, Gaunson et al 2000).

Bacteriological isolation methods and serologic tests (RSA, HI and ELISA) are mostly used in the diagnosis of *M. gallisepticum* infection (Levisohn and Kleven 2000). With the advent in molecular technology, different Polymerase Chain Reaction (PCR) techniques can also be used in diagnosis of mycoplasmosis (Nescimento et al 1991, Garcia et al 2005, Callison et al 2006, Grodio et al 2008). Previous research reported that primer pairs derived from genes encoding 16S rRNA and bacterial surface proteins can also be used in the molecular diagnosis of *M. gallisepticum* infection (Papazisi et al 2002, Garcia et al 2005).

Garcia et al (2005) used 4 different primer pairs and showed that mgc 2, LP and gapA primers amplified only *M. gallisepticum* DNA. However, 16S rRNA primers amplified both *M. gallisepticum* and *Mycoplasma imitans* in trachea and bronchus. Mekkes and Feberwee (2005) used 16S rRNA primers in RT-PCR analysis in trachea swabs and determined the *M. gallisepticum* quantitatively as low as 10 CFU/ml. Garcia et al (2005) also reported that bacteria identification limit in RT-PCR is lower than classical PCR and culture methods. Çarlı and İyigör (2003) were able to diagnose the *M. gallisepticum* infection in chick's tracheal swabs by using mga-0319 lipoprotein primers in RT-PCR analysis. In this study (Çarlı and İyigör 2003), the identification limits of the agent was as low as 3 CFU/ml for pure *M. gallisepticum* culture and 3000 CFU/mL for contaminated samples. Grodio et al (2008) quantitatively detected *M. gallisepticum* using mgc 2 primers and Taqman probe in conjunctivas of the hens which were infected beforehand. These researchers stated that the identification power of mgc 2 primers was less than 14 copies of *M. gallisepticum* per reaction for plasmid DNA standards and less than 10 copies for genomic DNA standards.

The purpose of this study was to determine *M. gallisepticum* by RT-PCR and to evaluate pathologic findings in lungs, air sacs, trachea, hearth, liver and kidney of chicks with mycoplasmosis.

▸ Materials and Methods

The chicks were supplied from 3 different breeders' flocks and ten chicks from each flock were used in the study. Conjunctival and tracheal swab samples and tissue samples from trachea, air sac, lung, liver, kidney and hearth were collected from chicks. Half of the collected tissues were kept for RT-PCR and microbiological examinations and the other halves were transferred to 10% buffered formalin solution for pathological examinations.

• *Pathological examination*

Fixed tissues in 10% buffered formalin solution were routinely processed and embedded in paraffin blocks. These blocks were sectioned at 5 µm thickness and stained with hematoxyline and eosine (H-E). The stained sections were investigated using a light microscope.

• *Bacteriological examination*

The method of Türkaslan and Salihoğlu (1998) was used for *Mycoplasma* spp. isolation and identification. Each tissue samples were homogenized in 1 mL liquid medium by MagNA Lyser (Roche Diagnostics, Germany). Tissue samples were inoculated by 1/10 of Freys medium broth and incubated for 72 hours in 37 ^{0}C. Color changes were observed during the incubation. One mL of sample was taken from the broths in which turbidity was detected and passages are made to Freys medium agar and Freys medium broth. These passages were examined by stereo microscope (Magnüs Analitics, India) for 10 days, and colonies that looked like fried egg were accepted as positive findings of *Mycoplasma* spp.

• *DNA isolations from swab samples*

High Pure PCR Template DNA Extraction Kit (Roche, Germany, Katolog #11796828001) was used for DNA isolation. Swab samples are vortexed by soaking into 1-2 mL sterilized physiological salt water. One mL of supernatant was taken for DNA isolations and then 380 µL Bacteria Lysis Buffer and 20 µL proteinase-K were added. After incubation at 65 ^{0}C for 10 min and 95 ^{0}C for 10 min, 100 µL isopropanol was added to the suspension and mixed. The suspension was put in filtered tubes and centrifuged for 1 minute at 8000 rpm and supernatant was removed. The suspension was put in clear collection tube and 500 µL Wash Buffer Inhibitor was added and the suspension was centrifuged for 1 minute at 8000 rpm. Then the suspension

was transferred in clear collection tube and treated twice with 500 µL Wash Buffer was addition and following centrifugation at 8000 rpm for 1 min. In the last step, filter tubes were placed in an eppendorf tube and DNA was eluted by adding 100 µL preheated elution buffer (70 ^{0}C) and was centrifuged for one minute 8000 rpm. The isolated DNA's were kept at -20 ^{0}C until PCR analysis.

• *DNA isolations from tissue samples*

High Pure PCR Template DNA Extraction Kit (Roche, Germany) was also used for DNA extraction from tissue samples. About 5 mg of tissue samples were taken and 200 µL of Tissue Lysis Buffer and 40 µL proteinase-K were added. Having incubated for 1 hour at 55 ^{0}C, 100 µL isopropanol was added to the suspension and mixed. The suspension was put in filtered tubes and centrifuged for 1 min at 8000 rpm and supernatant was removed. The suspension was put in clear collection tube and 500 µL Inhibitor Removal Buffer was added and the suspension was centrifuged for 1 min at 8000 rpm. Then the suspension was transferred in a clear collection tube and treated twice with 500 µL Inhibitor Removal Buffer and DNA was eluted from columns as described above. DNA was quantified 260 and 280 nm UV using spectrophotometer (NanoDrop ND-2000, Germany).

• *Real Time PCR Analyses*

Grodio et al (2008)'s method was used by same minor modifications. In RT-PCR analysis, Light Cycler Taqman Master Kit (Roche, Germany, Katalog # 04535286001) was used. In amplification, mgc2-F (5'-GGTCCTAATCCCCAACAAAGAAT-3') and mgc2-R (5'-CTTGGTTGGTTCATATTAGGCATTT-3') primers and Taqman probe (5'-6-FAM-CCACAGGGCTTTGGTGGCCCA-TAMRA) were used. These primers were previously reported (Garcia et al 2005) and specifically developed from a repeated region of *M. gallisepticum* genome.

RT-PCR protocol was 95 ^{0}C for 10 min and 45 cycles of 95 ^{0}C 30 sec, 60 ^{0}C' 30 sec and 72 ^{0}C 1 min using a Roche Light Cycler 2.0. In all steps of RT-PCR analysis, DNA of *M. gallisepticum* S6 strain was used as positive control which was supplied from Pendik Veterinary Control and Research Institute, Mycoplasma Laboratory and distilled water was used as negative control.

• *Standard curve*

Based on Macfarland method, genomic DNA isolated from 3x10^8 CFU/mL *M. gallisepticum* S6 and decimally diluted to make up standard curve of copy numbers from 3x10^0 to 3x10^8. The productivity of *M. gallisepticum* genomic DNA standard curve between the works was calculated from the mean CP values triplicates.

►Results

Pathological findings and their ratios were given in Table 1. Nasal and conjunctiva discharge, coughing and wheezing were among the clinical findings of the chicks examined in this study. The most significant necropsy finding was yellow-gray colored exudates found in trachea and bronches. Goblet and epithelial hyperplasia and mononuclear cellular infiltration composed of macrophages, lymphocytes and plasma cells were detected in the lamina propria of trachea and bronchia (Figure 1A-1B). In the mucosa of air sacs heterophyl, lymphocyte and macrophage infiltrations were seen. Also degeneration and necrosis were present in the epithelia.

Table 1. Pathological findings obtained in the study and their observation rates.

Macroscopic Findings	1th Coop	2nd Coop	3rd Coop
Mucous lacrima flow	5/10	5/10	3/10
Mucous flow in nose	4/10	5/10	3/10
Catarrhal exudate in trachea	5/10	7/10	6/10
Pneumonic areas in lungs	4/10	6/10	6/10
Catarrhal exudate in air sacs	2/10	3/10	1/10
Fibrine in pericard	1/10	-	-
Fibrine in liver	1/10	-	1/10
Microscopic Findings			
Goblet cells hyperplasia in trachea	5/10	7/10	6/10
Macrophage lymphocyte and plasma cell infiltrations in lamina propria of trachea	4/10	6/10	6/10
Hyperplasia in epithelium of bronches	5/10	7/10	6/10
Peribronchiolar macrophage, lymphocyte and plasma cell infiltrations	5/10	7/10	6/10
Desquamation and necrosis in epithelia of air sacs	3/10	3/10	1/10
Pericarditis fibrinosa	1/10	-	-
Perihepatitis fibrinosa	1/10	1/10	1/10
Microbiologic Findings	2/10	2/10	4/10
RT-PCR Findings	8/10	6/10	8/10

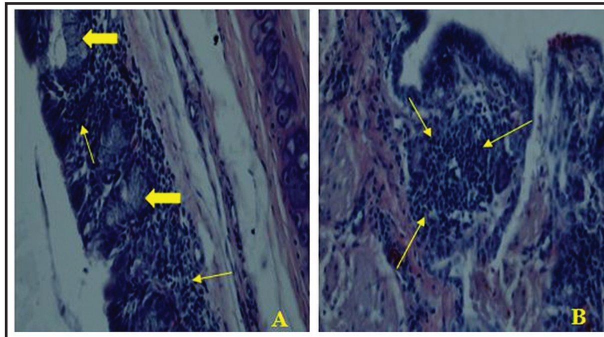

Figure 1. Hyperplasia in goblet cells, in trachea (thick arrows) and mononuclear cell infiltrations (thin arrows), H-E, x 260 (A), Peribronchiolar, infiltration of plasma cell and lymphocyte, H-E, x 260 (B).

Mycoplasma spp. were successfully isolated in only tracheal tissues of 8 broiler chicks. In two cases (20%) in the first and second flocks and 4 cases (40%) in the third lock, *Mycoplasma* spp. were isolated. *M. gallisepticum* DNA was detected from samples of 8 broilers (80%) from the first and third flocks and 6 chicks from the second flocks by RT-PCR. The ratios of the microbiological and RT-PCR methods were given in Table 1. Copy-numbers of *M. gallisepticum* genomic DNA and representative mean CP values in tissue and swap samples chicks that were *M. gallisepticum* positive were given in Table 2.

Table 2. M. gallisepticum genomic DNA copies and their crossing points (CP).

	M. gallisepticum Genomic DNA copies		CP Values
	Copy/mL	Log 10	
Trachea	$5.99 \pm 3.73 \times 10^5$	4.76	25.44
Air Sacs	$8.19 \pm 1.20 \times 10^2$	2.62	32.84
Lung	$6.50 \pm 2.17 \times 10^2$	2.44	33.74
Liver	-	-	-
Hearth	-	-	-
Kidney	-	-	-
Trachea (Swab)	$5.83 \pm 2.73 \times 10^4$	3.90	28.74
Conjunctiva (Swab)	$3.86 \pm 1.07 \times 10^4$	3.58	31.22

► Discussion

We have detected pathological findings of catarrhal exudates in bronchus and trachea, thickening of air sacs mucosa of chicks. Degenerations and necrosis of air sacs and hyperplasia of epithelia of trachea and bronchus, goblet cells were seen. Mononuclear cell infiltrations were also determined in the lamina propria of these organs. These findings were previously were reported in *M. gallisepticum* infection in chicks (Nunoya et al 1987, Levisohn and Kleven 2000, Gaunson et al 2000). However; similar pathological findings can also be observed in other diseases such as haemophilus infection, infectious bronchitis and infectious laryngotracheitis (Ley 2003, Zanella 2007). Other than this possibility which hampers pathological diagnosis of the disease might be difficult if *M. gallisepticum* infection is complicated by secondary factors like Escherichia coli (Ley 2003).

The high detection rate of pathological findings in trachea, lung, air sacs comparing with other organs corresponds to the high diagnosis ratio of nucleic acids quantitatively in tissue and swap samples of trachea, lung and air sacs and by real time PCR. Since primary target organs of the disease are respiratory system organs, pathologic lesions' severity will be proportional with the number of bacteria. Again in this study, macroscopic and microscopic lesions were observed in three and four cases respectively. However, no *M. gallisepticum* DNA was detected in heart and liver samples by RT-PCR, which suggested that the lesion in these organs possibly caused by secondary factors. Since the pathological findings are not solely enough for diagnosis of mycoplasmosis, in most of the cases pathologic diagnosis must be supported by microbiological methods. However the microbiological methods require a long period of time, and sometimes can be difficult due to use of antibiotics and the requirement of certain amount of bacteria in the material. Although positive results can generally be obtained in 4-7 days, 30 days are needed to make a descriptive diagnosis. However, this time table is rather a long period of time for poultry breeding (Türkaslan and Salihoğlu 1989, Özdemir and Erer 2008).

Different PCR protocols were reported for diagnosis of *M. gallisepticum* infection (Çarlı and Eyigör 2003, Garcia et al 2005, Callison et al 2006). PCR can be an alternative for microbiological, pathological and serological diagnosis methods (Levisohn and Kleven 2000, Garcia et al 2005, Mekkes and Feberwee 2005, Grodio et al 2008). However, PCR and especially RT-PCR based techniques require a good deal of groundwork for devices and expensive consumable materials contrary to other methods. As well as providing reliable diagnosis in a short time, the advantage of molecular methods based on PCR is to diagnosis of disease in flocks which treated with antibiotics beforehand (Çarlı and Eyigör 2003). Because, PCR based techniques can amplify both DNAs of alive and death M. gallisepticum. In this study, nucleic acids belongs to *M. gallisepticum* were detected in 22 of the chickens by RT-PCR. The fact that in all of the cases in which *M. gallisepticum* infection was identified, bacterial nucleic acids were remarkably amplified in tissue and swap samples of trachea. Accordingly, we were able to isolate and identify the agent in samples taken only from the trachea suggests that there must be a large number of agents present in these organs. Moreover, it can increase the rate of success in the agent isolation of the swabs taken from trachea and conjunctiva during microbiological examinations.

► Conclusions

The sensitivity of RT-PCR and pathologic methods were compared in the diagnosis of mycoplasmosis in chicken. It was shown that RT-PCR can be possible in the diagnosis of mycoplasmosis in chicken when the agent cannot be isolated by microbiologic mean.

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