

Investigations of Pathological Immunohistochemical and Immunocytochemical Findings in Natural Infection with *Mycoplasma gallisepticum* in Laying Hens

Özgür Özdemir¹, Orhan Yavuz², Hüdaverdi Erer¹ & Zafer Sayın³

ABSTRACT

Background: Mycoplasmosis is an infectious disease caused by *Mycoplasma gallisepticum* (MG), usually seen in the respiratory system of chickens, chick and turkeys, that causing great economic loss. The disease is characterized by respiratory system lesions such as sinusitis, tracheitis, airsacculitis, pneumonia and other symptoms such as loss of yield, arthritis, tenosynovitis. In this study, it was aimed to investigate diagnose of the disease by pathologic and molecular techniques in hens that naturally infected with MG as well as the usability of immunocytochemical (ICC) method in diagnose of the disease.

Materials, Methods & Results: For this purpose, 98 hens were collected from 10 different coops that serologically positive. After necropsy, routine pathological procedures were performed to samples taken from nose, sinus, larynx, trachea, lung and air sacs. Scraping samples taken from lungs and tracheas were evaluated by ICC. Immunohistochemical (IHC) staining was performed to samples taken from nose, sinus, larynx, trachea, lung and air sacs. Indirect immunoperoxidase method was applied in the both IHC and ICC staining. Rabbit polyclonal anti MG antibody was used as primer antibody in the IHC and ICC staining. Additionally, culture and PCR techniques were applied to tracheas of all hens for MG. The GPO3 and MGSO genes were made for PCR analysis. In the tracheal examinations, 23 cases were positive for PCR, 17 cases ICC positive, 16 cases IHC positive and 10 culture samples found positive. All of culture positive cases were also positive for other three methods. When findings in all organs were evaluated, in 37 cases were detected positive by IHC (38%) and 23 cases were positive by ICC (23.5%). In the IHC positive cases, the first order was trachea in 16 cases followed by in 11 cases in sinus, in 8 cases in lung, in 6 cases air sac and 4 cases in nose, respectively. In 8 cases, IHC positivity was found in at least two organs. IHC positivity was detected in the nose, sinus and tracheal epithelia as well as in the macrophages within subepithelial lymphoid infiltration, vascular walls and endothelium. As the disease became chronic, it was found that the agents were seen more in the lymphoid tissue than the epithelium. In ICC staining positivity was found in 17 cases in the trachea and 11 cases in the lung. There were only 5 cases positive by ICC in both organs.

Discussion: Clinical and pathological findings as well as serological, microbiological, molecular techniques and immunohistochemical methods are to be important methods in the diagnosis of the disease. While the culture results are shown as the gold standard in diagnosis of the disease, it is possible to obtain the results in the earliest 7-10 days in cultures and at least 20 days must be passed in order to say a cultural negative. In addition, in the field studies, it mentioned the use of vaccines, antibiotics and protective drugs affected the results of microbiology and serology; the importance of using techniques such as IHC and PCR for the diagnosis of the causative agents. The results of the present study indicate that the most important organ in the diagnosis of the disease is the trachea, and the most effective method is PCR followed by IHC and ICC methods. It was concluded that the results of ICC staining close to IHC staining, and ICC could be used for diagnostic purposes in positive reactions obtained from the tracheas or the other organs.

Keywords: Fast diagnosis, immunocytochemistry, immunohistochemistry, pathology, PCR.

DOI: 10.22456/1679-9216.91349

Received: 20 December 2018

Accepted: 29 March 2019

Published: 26 April 2019

¹Department of Pathology & ³Department of Microbiology, Faculty of Veterinary Medicine, Selçuk University, Konya, Turkey. ²Department of Pathology, Faculty of Veterinary Medicine, Aksaray University, Aksaray, Turkey. CORRESPONDENCE: O. Yavuz [vetorhanyavuz@gmail.com - Tel: +90 5053556928]. Aksaray University, Faculty of Veterinary Medicine. TR-68100 Aksaray, Turkey.

INTRODUCTION

The agent can be obtained from lesions in infected birds, sero-positive cases and some cases characterized by typical *Chronic Respiratory Disease* (CRD) [14]. In field research into the prevalence of the disease in Turkey, *Mycoplasma gallisepticum* (MG) infection were found as 8.3% [26], 12% [7], 16.3% [1] and 8.5% [5] in observations made by serological and molecular techniques.

Positive reactions were detected in endothelium, lumen and walls of the brain and kidney arteries after intravenous injection of MG in chickens [4]. In a previous study, the researches have found positive staining in lungs with pneumonia by IHC [29]. In another study, scientists detected positive reactions in the infraorbital sinus, lung and tracheal epithelia by avidin-biotin-immunoperoxidase method in turkeys [20]. It was pointed out that the immunopositive staining were pronounced in trachea epithelia after anti-*Mycoplasma* drug administrations in broiler chicks [2]. Some researchers demonstrated positive staining on the surface of peripheral blood cells by florescent antibody technique [13].

In this study, the usefulness of immunocytochemistry (ICC) staining method in tissue scraping and touching samples in the rapid diagnosis of the disease, which can result in shorter time, has been discussed. The results of ICC staining were compared with culture, PCR and immunohistochemistry (IHC) staining results. By early and rapid diagnosis, necessary precautions will be taken in poultry houses and the yield losses caused by the disease will be prevented.

MATERIALS AND METHODS

Material of the study

The study material consisted of 98 hens from 10 different coops which were not vaccinated against MG, serologically positive and clinically demonstrating CRD. The necropsies of the hens were performed and samples were taken for microbiologic, histopathologic and cytological examinations.

Serological examination

Clinically, the blood samples taken from the coops shows CRD findings were checked for serum agglutination test positivity. When at least half of the samples were positive, the coops were included in the study.

Cytological examination

After the necropsy, the touch and scraping preparations were collected from the trachea and lung and fixed with acetone solution. Then these slides were stained with ICC techniques.

Histopathologic examination

Following necropsy, respiratory tract organs (The nasal cavity, Infra-orbital sinuses, trachea, larynx, lungs and air sacs) were examined grossly and fixed in 10% formalin solution. After dehydration and clarification, paraffin blocks were prepared. The paraffin blocks were cut at 5 µm thick by microtome and examined under light microscope, after stained with Haematoxylin-Eosin (HE)

The changes observed in histopathological examinations of HE-stained sections were scored as before [18]. According to this, scoring the histopathological changes were evaluated as mild (+1), moderate (+2) and severe (+3).

IHC and ICC examinations

For this purpose, sections of 5 µm thick poly-lysinized slides were stained according to the IHC Polymer kit procedure (NovoLink Max Polymer Detection System RE7280-K)¹ after deparaffinization and rehydration. According to this, 15 min of antigen retrieval was performed by Proteinase K at room temperature. After washing with deionized water, endogenous peroxidase activity was removed by 3% H₂O₂. Between each step, 5 min washes were done 3 times by TBS. the protein block was dropped and incubated for 10 min. Following this procedure, Rabbit polyclonal anti MG antibody² (orb10563) was instilled and incubated for 1 h at room temperature. Then, post-primer block solution was added to the slides and incubated for 30 min and the Polymer solution was dropped for 30 min. Slides stained by DAB (3,3'-diaminobenzidine tetrahydrochloride) for 5 min. After counter-staining with hematoxylin, slides were closed by coverslips and evaluated under a light microscope.

Cytological preparations were stained by the same procedure, but antigen retrieval was not performed. The negative control slides of both staining methods were also stained according to the same procedure. However, TBS was used instead of the primer antibody.

PCR analysis

DNA extraction from the samples was done by the DNA purification. The PCR analysis of the GPO3 and MGSO genes were performed using the primers F- 5'-TGGGGAGCAAACAGGATTAGATACC-3' and R- 5'-TGCACCATCTGTCACTCTG TTAACCTC-3'. These primers showed a single amplification, the size of which was 280 bp.

The extracted DNA was amplified in a total volume of 50 µL (5 µL 10 × PCR buffer, 750 mM Tris HCl, pH 8.8, 200 mM, (NH₄)₂SO₄, 0.1% Tween 20); (5 µL 25 mM MgCl₂; 250 µM each deoxynucleotide triphosphate); (1.25 U Taq DNA Polymerase; 20 pmol each primer and 25 ng target DNA).

The cycling conditions with the thermal cycler were the initiation step at 94°C for 2 min, followed by 35 cycles coupling 94°C for 15 s, 53°C for 15 s and 72°C for 15 s and a final extension at 72°C for 5 min.

The PCR products were analyzed by electrophoresis on 1.5% agarose gel, and the gel was stained with ethidium bromide (0.5 µg/mL) and photographed.

Microbiologic examination

Samples were incubated in *Mycoplasma* Agar Base³ supplemented with *Mycoplasma* selective

supplement-G³ for 7 days at 37°C oven with 5% CO₂. The cultures were examined on a light microscope and *Mycoplasma* suspect “cooked egg”-looked colonies were passed into the *Mycoplasma* Agar without antibiotics to distinguish the bacteria from the L-form. Isolated *Mycoplasma* were cultured in *Mycoplasma* Broth Base³ supplemented with *Mycoplasma* selective supplement G, stored at -80°C and validated by PCR.

RESULTS

Histopathological

Lesions and scores seen in histopathological examinations are given in the Table 1. According to these results trachea and larynx were most affected organs in terms of changes in the glands, epithelial degenerations and cell infiltrations in the propria layer (Figure 1A-C). Hyperemia and lymphoid around the parabronchi in the lungs (Figure 1D), and thickening due to lymphoid infiltration at varying degrees with edema in air sacs were prominent in these organs than the others (Figure 1E). In addition, amyloid deposits were found in the intercellular area around the lamina propria of the trachea and the parabronchus in the lungs (Figure 1F).

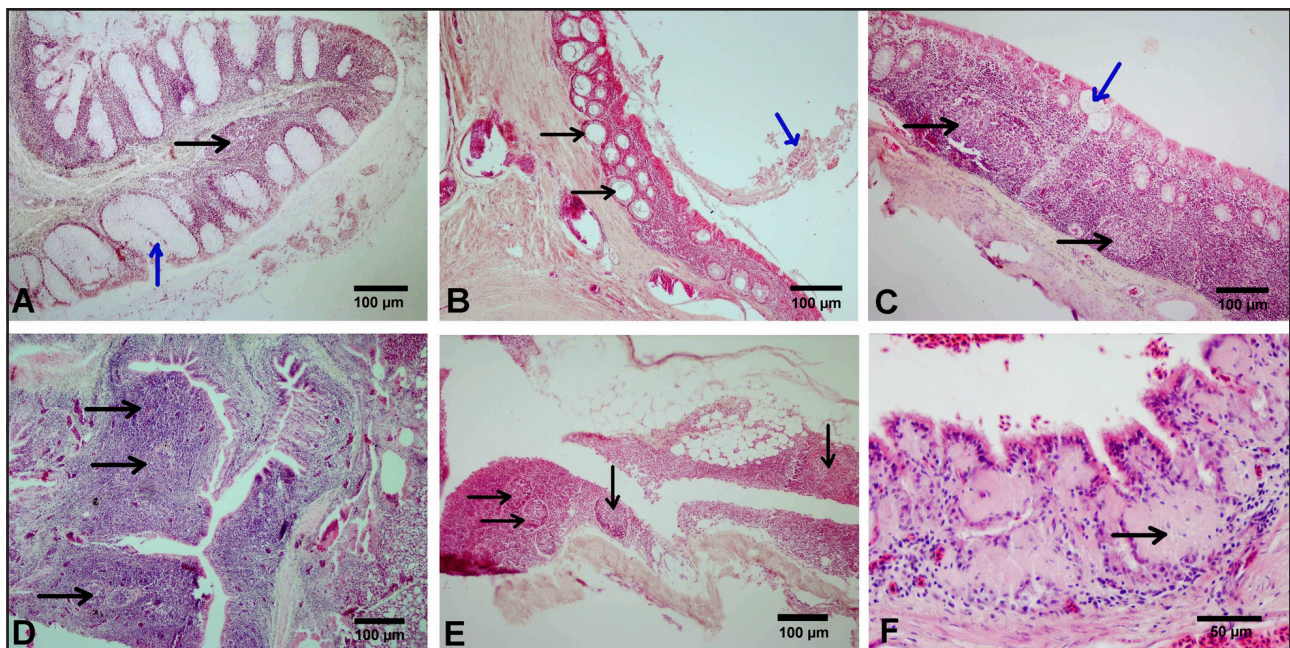


Figure 1. Histopathological changes, H&E [A-E x100, Fx400]. A- Degeneration in the epithelium, lymphoid cell infiltration in the propria (+2) (black arrow), hypertrophy of the gland (blue arrow) in nose. B- Hypertrophy of the gland (black arrows) and mucopurulent exudate in lumen of larynx. C- Severe lymphoid cell infiltration (+3) and germinal center (black arrows) and hypertrophy of the gland (blue arrow) in trachea. D- Lymphoid hyperplasia (arrows) (+3) around the parabronchi in lung. E- Lymphoid cell infiltration in air sac (arrows) (+3). F- Amyloidosis in bronchus wall in lung.

Table 1. Distribution of histopathological lesions according to organs (98 samples).

Organ	Lesion scores*	Degeneration and Necrosis	hyperplasia	Hyperemia	Heterophile Infiltration	Lymphoid Cell Infiltration	Hypertrophy in Gland
Nose	0	95	98	94	98	88	98
	+1	3	0	4	0	8	0
	+2	0	0	0	0	2	0
	+3	0	0	0	0	0	0
Sinus	0	77	97	93	94	26	94
	+1	18	1	5	3	52	4
	+2	3	0	0	1	18	0
	+3	0	0	0	0	2	0
Larynx	0	66	97	93	95	6	65
	+1	28	1	5	3	40	31
	+2	3	0	0	0	39	1
	+3	1	0	0	0	12	0
Trachea	0	74	96	94	96	23	78
	+1	19	1	4	2	47	17
	+2	4	0	0	0	24	2
	+3	1	0	0	0	4	0
Lung	0	93	97	58	97	72	
	+1	3	1	40	0	19	
	+2	2	0	0	1	6	
	+3	0	0	0	0	1	
Air sac	0	90	98	86	97	83	
	+1	6	0	10	1	8	
	+2	2	0	0	0	4	
	+3	0	0	0	0	3	

*No lesion (0); mild (+1); moderate (+2) and severe (+3).

ICC and IHC

When the findings in all organs are evaluated, in 37 cases IHC positive (38%) and in 23 cases ICC positive (23.5%) reactions were detected (Figure 2-4). In IHC positive cases; Larynx and trachea (Figure 5) were the organs that showed most positivity in 16 cases (Figure 6C-D). In 11 cases in the infraorbital sinus (Figure 6B), in 8 cases in lungs (Figure 6E), in 6 cases in air sacs (Figure 6F-G), and in 4 cases in the noses positive immunoreactivities were observed (Figure 6A). In 8 cases, IHC positivity was found in at least two organs.

In ICC staining, positivity was found in 17 cases in trachea and 11 cases in lung (Figure 7 A-C), and both organs in 5 cases. In 22 cases both IHC and ICC were

found positively whereas in 1 case ICC was positive and IHC was negative. In this case, PCR was found positive.

Culture and PCR

PCR was positive in 23 cases (Figure 8) at 280 bp and bacterial isolation was done in 10 cases (Figure 2). In all culture positive cases, PCR also gave a positive result. IHC positive was found in 21 of the 23 cases with PCR positive. ICC positivity was found in one of the 2 IHC negative cases. In other words, 22 of the 23 positive cases of PCR were positively stained with IHC or ICC.

When we looked at coop-based positivity rate, 70% of coops were positive by culture, 90% of coops were positive by PCR and all of coops were positive by IHC and ICC (Figure 8).

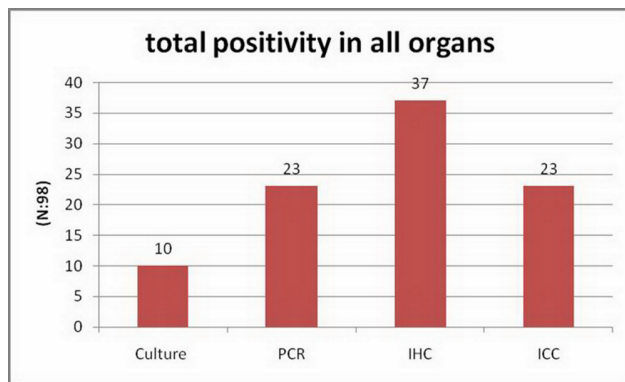


Figure 2. Culture, PCR and immunological staining results according to all organs.

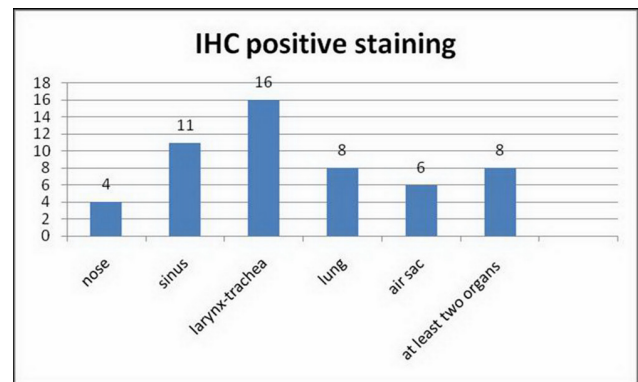


Figure 3. Distribution of IHC staining results according to organs.

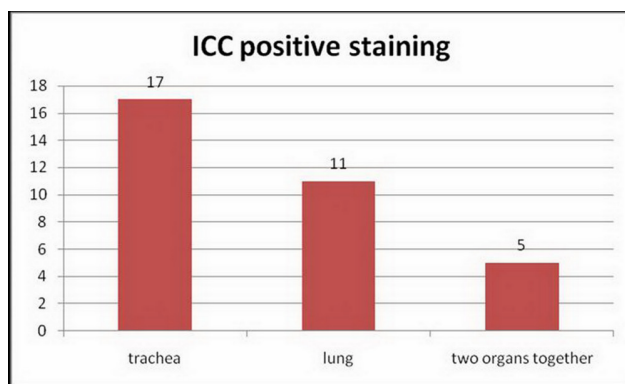


Figure 4. ICC staining results in the trachea and lung.

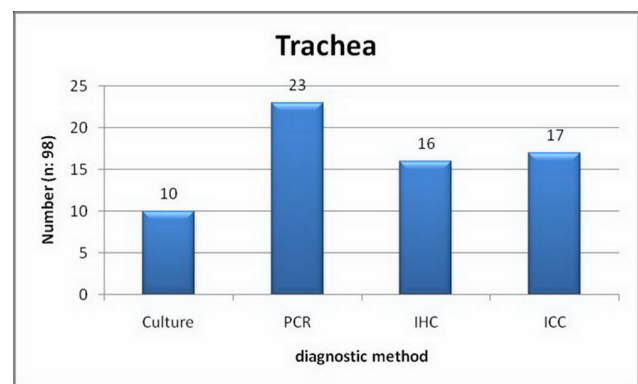


Figure 5. Culture, PCR and immunological staining results in the trachea.

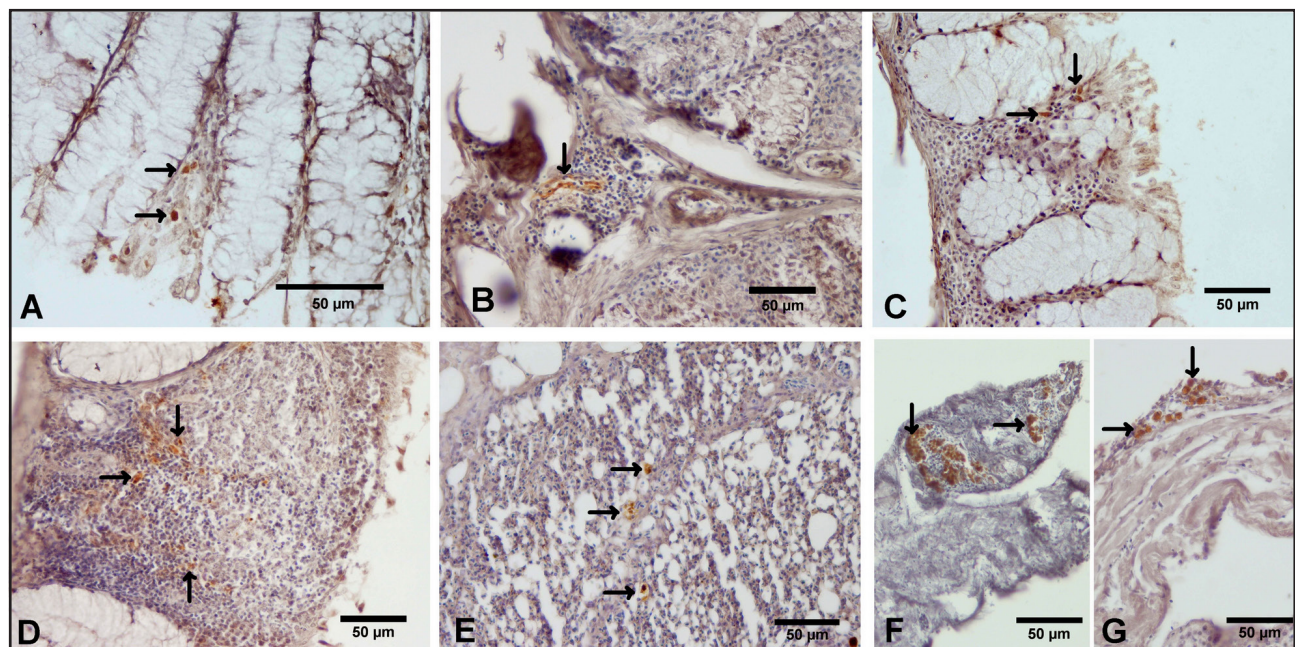


Figure 6. IHC positive staining (DAB) [Ax630, B-Gx400]. A- IHC positive staining in macrophages between glands, nose. B- IHC positive staining in endothelium and wall of vessel, sinus. C- IHC positive staining in propria of larynx. D- IHC positive staining in macrophages in propria in trachea. E- IHC positive staining in air capillaries in lung. F&G- IHC positive staining in lamina propria and epithelium of air sacs.

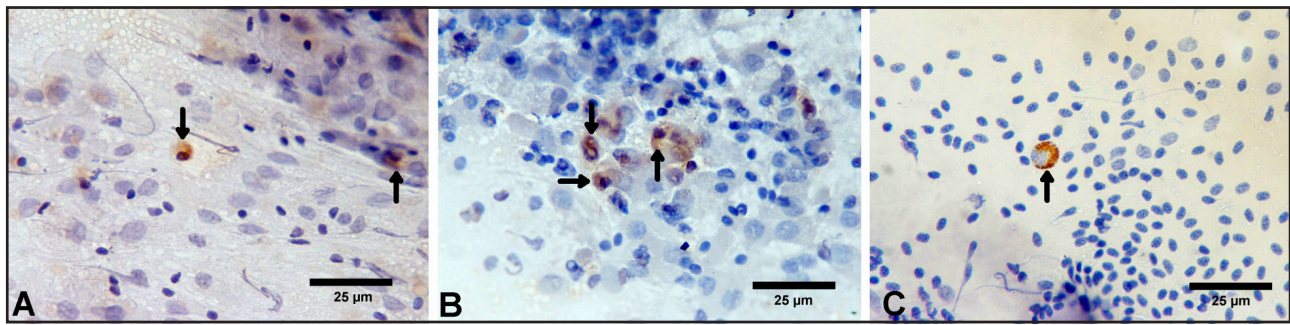


Figure 7. ICC positive staining (DAB) [1000x]. A and C- Lung. B- Trachea.

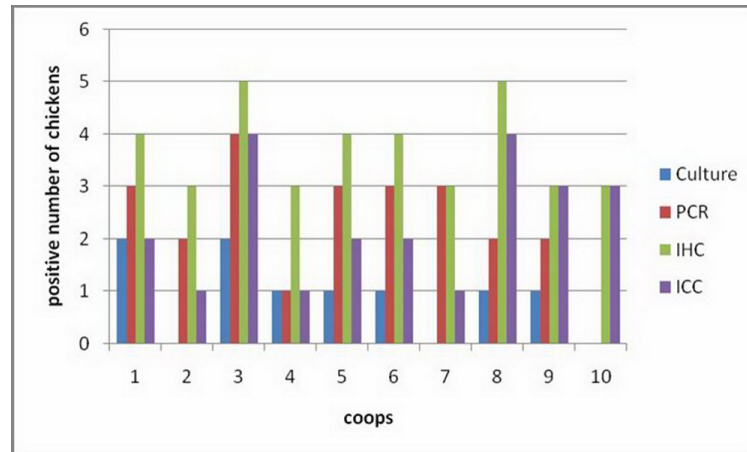


Figure 8. The distribution of diagnostic methods according to chicken coops.

DISCUSSION

In this study, we compared the results of culture, PCR, IHC and ICC staining with clinical and pathological findings in natural infected hens, and investigated the possibility of using ICC staining method in the diagnosing of the disease.

Histopathologically; hydropic degeneration, loss of cilia and desquamation on respiratory tract epithelium, lymphoid cell infiltrates and lymphofollicular reaction in lamina propria, hypertrophy and morphological changes (distortion) in mucous glands, catarrhal exudate accumulation in the larynx and trachea can observe [12,14,15,27]. Similar to previous field surveys [18,25], changes were observed in the nasal, sinus, larynx, trachea, lung, and air sacs in this study. Amyloid deposits in the trachea and lungs were thought to be related to chronic inflammation, and a similar change has been reported in the trachea in MG-positive animals in a previous study [18].

Culture is still declared as a gold standard to diagnose of the disease [17,22]. However, it has some deficiencies such as it need much more effort, the possibil-

ity of contamination, inadequate live agent in sampling during culture. It has been reported that tests such as serologic tests and cultures used in the diagnosis of MG infection as well as highly sensitive PCR can be used [16]. Kempf *et al.* [11] reported a 67% positivity by culture and 97% positivity by PCR in tracheal swap specimens after SPF chickens infected with MG. In another study, it was reported that isolation studies in CRD require a long period of time and require a lot of work, only serologic testing is inadequate, and that PCR technique is a quick and reliable method to detection of infection [8]. In a field study, PCR, IHC and ICC methods showed higher positivity than cultures and this situation was evaluated as affecting culture results of protective antibiotic applications [18]. These methods have been found to be faster and more reliable than culture.

The positivity rates are changed by PCR in field studies performed in natural infected chickens in Turkey. The positivity rate was 2.46% by PCR and, 73%, 18.7%, and 29.1% by rt-PCR, respectively [8,10,19,25]. In this study, PCR positivity was found to be 23.4% and it is among the values in others.

In the diagnosis of MG tracheal specimens are frequently used. Although the tissue specimens taken from the tracheas are used in culture and molecular methods, in recent years it has been replaced by tracheal swabs and rinses [4,8,14,18,23,25]. Processing of these specimens is easier than tracheal tissue parts. In this study, ICC method was used to determine the disease agent from tracheal cytology, which is easier to obtain and process. It has been shown that the results are as useful as the IHC and PCR, as well as being faster than the culture, and a highly accurate method. The researchers tried to determine natural infections by culture and PCR technique from tracheal swap specimens [8]. It has been reported that there is a relation between the severity of the disease and transmission route in poultry, and the aerosolized infections are more severe than the oral route. It has been shown that the causative agent mostly localized in the tracheal tissues with aerosol infection route by IHC [21]. In the case of experimental infections caused by aerosol route, it was reported that cultured reisolation rate was 100% and PCR positivity was 75% in the tracheal swaps in 3 weeks, 40% in culture and 60% in PCR positivity observed in 5 weeks [23]. In the present study, PCR positivity was detected in 23 of 98 hens and culture positivity was found in 10 of 98 hens, although all of the poultry were serologically positive. This situation shows that as the disease becomes chronic, the detection rate of causative agents decreases. Tracheal specimens showed positive correlation between IHC and ICC staining and close to PCR, suggesting that ICC is an alternative method that can be easily used for diagnosis.

Immunoperoxidase method has been reported to be more advantageous than the fluorescence antibody method [17]. It was emphasized that the use of antibiotics, vaccinations and the use of preventive drugs in field trials affected the results of microbiology and serology, and the necessity of using techniques such as IHC and PCR to diagnose the agents. In the same study, CRD was found to be positive by IHC in 20 of 33 coops, and the disease rate in coops was expressed as 60.6% [18]. Agents were studied by avidin-biotin-immunoperoxidase method in turkeys, and positive staining was found more intense in the infraorbital sinus epithelia than in the pulmonary and tracheal respiratory epithelia [20]. Yılmaz *et al.* [30] reported that IHC and PCR were found

positive in 4 of 27 cases and culture was detected positive in 3 of 27 cases. They also noticed IHC is as useful as culture and PCR in the diagnosis of *Mycoplasmosis*. The agent is shown by the Fluorescent antibody technique, which adheres to the surface of peripheral blood cells. Thus, damage in erythrocytes and reduction in the chemotactic response of HD-11 cells (chicken tumor cells) were detected [13]. After administration of anti-*Mycoplasma* drugs in infected broilers, agents were demonstrated in the tracheal epithelium by immunoperoxidase method [2]. It has reported that, *Mycoplasma* antigens found on the arterial wall, lumen and endothelial surfaces by immunofluorescence staining [4]. In vitro studies have noticed that MG colonizes both surface and cytoplasm of sheep and poultry erythrocytes [28]. In this study, IHC positivity was detected in the nose, sinus and tracheal epithelium as well as vessel walls and endothelium and macrophages and subepithelial lymphoid infiltration. (Figure 6A-D). As the disease became chronic, it was found that the agents were seen more in the lymphoid tissue than the epithelium. Similarly, in another study, they reported that the identified agents by IHC in lymphoid cell infiltrates on the nose, sinus, trachea and air sacs in their experimental *Mycoplasma* infection [6]. Toplu *et al.* [24] reported that positive staining by IHC in the chukar partridge was in macrophages in the lymphoid tissue, as well as in the tracheal epithelium, whereas immunopositive staining in vascular endothelium was only in the paranasal sinuses. They have interpreted that the disease is localized in the upper respiratory tract. In the present study, vessel staining were densely on the trachea and also found on the nose, sinus and air sacs. This can be interpreted as the spread of the disease and bacteremia may occur after aerosol transmission.

Between 2001 and 2004 in Brazil, *Mycoplasma* positivity was found in 72.7% of the poultry houses by multiplex PCR [3]. In Pakistan, natural infection in broiler was found to be 46% by PCR [9]. In Turkey, CRD was found positive to be 60.6% by IHC in chicken coops [18]. In this study, MG was detected by serologically CRD positive chicken coops as in 70% by culture, in 90% by PCR and in all cases by IHC and ICC. This situation shows that the disease is widespread in Turkey. In addition, PCR made only from tracheal samples can be interpreted as the reason why the PCR results are less positive than IHC and ICC results.

CONCLUSIONS

In conclusion, in this field study involving natural infections with MG, the most important organ in diagnosis of the disease is the trachea and the most effective method is PCR, followed by IHC and ICC methods. It was concluded that ICC showed a similar staining to IHC and could be used for diagnostic purposes as a result of staining of trachea and other organs. In order to increase the diagnostic rate in field studies of the disease, it is recommended that both IHC, ICC and PCR should be sampled from all

respiratory organs and tissues, rather than a single tissue type.

MANUFACTURERS

¹Novocastra, Leica Biosystems, GmbH, Wetzlar, Germany.

²Biorbyt Ltd. Cambridge, UK.

³Oxoid Ltd. Basingstoke, Hampshire, UK.

Funding. This research was supported by Selçuk University Scientific Research Committee Project Number: 10401131.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

- 1 **Akan M. 2008.** *Mycoplasma* infections in chickens: Protection and Control. *Mektup Ankara*. 6: 21-24.
- 2 **Amer M.M., Hanafei A.E., El-Bayomi K.M. & Zohair G.A. 2009.** Comparative study on the efficacy of some anti-*Mycoplasma* drugs on the performance of commercial broiler flocks from infected breeders. *Global Veterinaria*. 3: 69-74.
- 3 **Buim M.R., Mettifogo E., Timenetsky J., Kleven S. & Ferreira A.J.P. 2009.** Epidemiological survey on *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by multiplex PZR in commercial poultry. *Pesquisa Veterinária Brasileira*. 29: 552-556.
- 4 **Clyde W.A. & Thomas L. 1973.** Tropism of *Mycoplasma gallisepticum* for arterial walls. *Proceedings of the National Academy of Sciences of the United States of America*. 70: 1545-1549.
- 5 **Dakman A., Günaydın E., Türkyılmaz M.A., Güleç M., Coşar M. & Özdemir Ü. 2009.** *Mycoplasma* infections in breeder chicken establishments. *Etlik Vetereiner Mikrobiyoloji Dergisi*. 20: 27-34.
- 6 **Gharaibeh S. & Hailat A. 2011.** *Mycoplasma gallisepticum* experimental infection and tissue distribution in chickens, sparrows and pigeon. *Avian Pathology*. 40: 349-354.
- 7 **Güler L. 1992.** Konya bölgesindeki kümes hayvanlarında serolojik yoklamalarla müspet bulunan CRD vakalarından etken izolasyon çalışmaları. 60p. Konya, TR. Thesis. (MSc in Veterinary Science) - Institute of Konya Animal Diseases Research Ministry of Agriculture and Forestry, Republic of Turkey.
- 8 **Gürbüz E. 2008.** The use of PCR for the diagnosis of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in chickens. 76p. Konya, TR. Dissertation (PhD in Veterinary Science) - Institute of Health Sciences, University of Selçuk.
- 9 **Islam A., Aslam A., Chaudhry Z.I., Ahmed M.U.D., Rehman H.U., Saeed K. & Ahmad I. 2011.** Pathology of *Mycoplasma gallisepticum* in naturally infected broilers and its diagnosis through PCR. *International Journal of Agriculture and Biology*. 13: 835-837.
- 10 **Kahya S., Yılmaz Ö., Eyigör A., Temelli S. & Çarlı T. 2015.** Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by Real-Time PCRs and *Mycoplasma gallisepticum*-antibody detection by an ELISA in chicken breeder flocks. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*. 213: 361-366.
- 11 **Kempf I., Blanchard A., Gesbert F., Guittet M. & Bennejean G. 1993.** The polymerase chain reaction for *Mycoplasma gallisepticum* detection. *Avian Pathology*. 22: 739-750.
- 12 **Kerr K.M. & Olson N.O. 1967.** Pathology in chickens experimentally inoculated or contact-infected with *Mycoplasma gallisepticum*. *Avian Diseases*. 11: 559-578.
- 13 **Lam K.M. 2004.** Morphological changes in chicken after *in vivo* exposure to *Mycoplasma gallisepticum*. *Avian Diseases*. 48: 488-493.
- 14 **Mayor O.Y. 1968.** Histopathological aids to the diagnosis of certain poultry disease. *The Veterinary Bulletin*. 38: 273-285.
- 15 **Mayor O.Y. 1974.** The histopathology of certain common poultry diseases, Food and Agriculture Organization of the United Nations, Rome: Via delle Terme di Caracalla, pp.1-49.
- 16 **Nascimento E.R., Yamamoto R., Herrick K.R. & Tait R.C. 1991.** Polymerase Chain reaction for detection of *Mycoplasma gallisepticum*. *Avian Diseases*. 14: 62-69.

- 17 OIE. 2015. Avian Mycoplasmosis, chapter 2.3.5, 10p. Available in <http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.05_%20AVIAN_MYCO.pdf> [Accessed online in December 2015].
- 18 Özdemir Ö. & Erer H. 2008. Pathological and microbiological investigations on the lesions of the respiratory systems of laying hens. *Eurasian Journal of Veterinary Sciences*. 24: 55-68.
- 19 Özmen M. 2011. Real-time PCR techniques for detection *Mycoplasma gallisepticum* in chicken trachea and statistical analysis. 69p. Adana, TR. Dissertação (Mestrado em Bioengenharia) - Department of Biotechnology Institute of Natural and Applied Sciences, Çukurova University.
- 20 Radi Z.A., Trampel D.W., Smith B.S., Rosenbusch R.F. & Goll F. 2000. Immunohistochemical detection of *Mycoplasma gallisepticum* antigens in turkey respiratory tissues. *Avian Diseases*. 44: 399-407.
- 21 Rauf M., Chaudhary Z.I., Younas M., Anjum A.A., Khan H.M. & Ali M.A. 2014. Detection of *Mycoplasma gallisepticum* in experimentally inoculated layer birds by immunohistochemistry. *The Journal of Animal & Plant Sciences*. 24: 385-391.
- 22 Salisch H., Hinz K.H., Graack H. & Ryll M. 1998. A comparison of a common PCR-based test to culture methods for detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in currently infected chickens. *Avian Pathology*. 27: 142-147.
- 23 Slavik M.F., Wang R.F. & Cao W.W. 1993. Development and evaluation of the polymerase chain reaction method for diagnosis of *Mycoplasma gallisepticum* infection in chickens. *Molecular & Cellular Proteomics*. 7: 459-463.
- 24 Toplu N., Avcı H. & Epikmen E.T. 2012. Pathomorphological and immunohistochemical examinations in naturally infected partridges (*Alectoris chukar*) and Japanese quails (*Coturnix coturnix japonica*) with *Mycoplasma gallisepticum*. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*. 59: 29-34.
- 25 Tuzcu M., Özmen M., Karakoç S.R., Tuzcu N. & Yoldaş A. 2012. Diagnosis of Mycoplasmosis in chicks by pathological and Real Time-PCR methods. *Eurasian Journal of Veterinary Sciences*. 28: 82-86.
- 26 Ülgen M. 1991. Comparative bacteriological and serological studies on Chronic Respiratory Disease of poultry. 81p. Bursa, TR. Dissertation (PhD in Veterinary Science). *Institute of Health Sciences, Uludağ University*.
- 27 Ülgen M., Şen A. & Çarlı T. 1998. Investigation of pathogenicity of *Mycoplasma* strains from chickens in tracheal organ culture. *Veterinarium*. 9: 52-55.
- 28 Vogl G., Plaickner A., Szathmary S., Stipkovits L., Rosengarten R. & Szostak M.P. 2008. *Mycoplasma gallisepticum* invades chicken erythrocytes during infection. *Infection and Immunity*. 76: 71-77.
- 29 Yılmaz F. & Timurkaan N. 2011. Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* Antigens by immunohistochemical method in pneumonic broiler chicken lungs. *Journal of Animal and Veterinary Advances*. 10: 2557-2560.
- 30 Yılmaz F., Timurkaan N., Kılıç A., Kalender H. & Kılınç Ü. 2011. Detection of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* in chickens by immunohistochemical, PCR and culture methods. *Revue de Médecine Vétérinaire*. 16: 79-86.