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Effect of Mentofin application on the clearance of *Mycoplasma gallisepticum* (MG) from naturally infected layer chickens' trachea

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Summary: Aim of this study was to determine if Mentofin would have any effect on *Mycoplasma gallisepticum* (MG) clearance from the tracheal epithelium of chickens in commercial layer flocks, which were naturally infected with MG. Results indicated that, compared to the control group, there was a significant and continuous decline in MG infection in chickens of Mentofin group determined by culture and Real-Time Polymerase Chain Reaction (MGrPCR) ($P<0,05$). Serology results in the control group indicated an increase in MG positivity from 25% to 40% ($P>0,05$), while there was no change in the Mentofin group ($P>0,05$). Culture results for MG positivity decreased from 85% to 5% in the Mentofin group, while this decrease was from 80% to 35% in the control group ($P<0,05$). There was a prominent decrease from 100% to 20% in MGrPCR positives in the Mentofin group ($P<0,05$) compared to a non-significant change observed from 95% to 80% in the control group ($P>0,05$). Results of this study indicate that Mentofin clearly had an effect on MG clearance from the tracheal epithelium, supported by detection of decline in MG infection in layers.

Keywords: Chicken, Mentofin, *Mycoplasma gallisepticum.*

Mentofin uygulamasının *Mycoplasma gallisepticum* (MG) ile doğal infekte yumurtacı tavukların trakeasından arınması üzerine etkisi

Özet: Bu çalışmanın amacı Mentofin'in *Mycoplasma gallisepticum* (MG) ile doğal infekte ticari yumurtacı sürülerin trakeal epitellerinden MG'un arınması üzerine etkisinin belirlenmesidir. Sonuçlar, kontrol grubu ile karşılaştırıldığında Mentofin grubundaki tavuklarda MG infeksiyonunda kültür ve Real-Time Polymerase Chain Reaction (MGrPCR) ile belirlenen belirgin ve sürekli bir düşüş olduğunu göstermiştir ($P<0,05$). Seroloji sonuçları kontrol grubunda MG pozitiflik %25'den %40'a yükselirken ($P>0,05$), Mentofin grubunda bir değişiklik olmamıştır ($P>0,05$). Kültür sonuçlarındaki MG pozitiflik Mentofin grubunda %85'den %5'e düşerken, kontrol grubunda bu düşüş %80'den %35'e olmuştur ($P<0,05$). Mentofin grubundaki MGrPCR pozitifliğinde belirgin şekilde olan %100'den %20'ye düşüş ($P<0,05$), kontrol grubunda %95'den %80'e ($P>0,05$) olan hafif bir düşme olarak gözlenmiştir. Çalışma sonuçları Mentofin'in trakeal epitelden MG arınmasında belirgin bir etkisinin olduğunu yumurtacılarda MG infeksiyonunda düşmenin belirlenmesi ile desteklenen şekilde göstermiştir.

Anahtar sözcükler: Mentofin, *Mycoplasma gallisepticum*, tavuk.

Introduction

Mycoplasma gallisepticum (MG) causes Chronic Respiratory Disease (CRD) in chickens and infectious synovitis in turkeys (11, 19, 28, 30). Main economical problems of poultry companies in MG infections are loss in carcass weight, reduction in feed consumption and egg production, and increase in treatment costs (12). MG-infected chicken breeder flocks transfer the agent to their progeny via their eggs leading to airsacculitis in broilers, respiratory problems and reduction in egg production in layers (8, 12, 16, 20). Another MG-infection related problem in poultry production is embryonic deaths in hatcheries. Since it is almost impossible to eliminate MG-infection in a poultry flock entirely with antibiotics, care should be taken to grow MG-free breeders (22). Additionally, subclinical MG-infections in flocks should regularly be tested by serological tests (such as Enzyme Linked Immunosorbent Assay - ELISA), culture (3, 14, 15, 26, 27) and Real-time Polymerase Chain Reaction (rPCR) (4, 7, 10, 13).

Mentofin, a natural product consisting of some essential fatty acids and natural herbal essences (10% eucalyptus oil, 10% menthol, 33% liquid builders, and 47% saponins) has been safely used in broiler and layer chicken production (5, 6). Previous field trials with poultry indicated that Mentofin was able to help

preventing respiratory problems, increasing performance and strengthening the immune system (5, 6, 9).

MG infections in chickens have been an ongoing problem for many years in Turkey (18). The persistence of the disease despite many control measures by the poultry producers made us think of using alternative approaches for prevention of birds from this infection. Therefore, we conducted a preliminary study to test Mentofin by determining its effect on MG clearance from the tracheal epithelium of MG-infected commercial layer chickens with serology, culture and rPCR.

Materials and Methods

Samples and sampling plan: Two commercial Nick-Brown MG-infected layer flocks, diagnosed by serology, culture, and rPCR prior trial, were selected from Balikesir, Marmara region/Turkey. Mentofin and control groups, with flock sizes of 12.690 and 12.105 birds, were 57 and 61 weeks old, respectively. Twenty chickens from each group were randomly selected, marked, and sampled for blood (in the 1st, 3rd and 8th week for the detection of serum antibodies against MG for Rapid Slide Agglutination - RSA test and by ELISA) and for tracheal swabs (in the 1st, 2nd, 4th, 5th, 6th, 7th and 8th weeks of the trial for culture and rPCR) throughout the 8 week trial period.

Mentofin application: Mentofin (Ewabo Co. Ltd., Germany) was applied to the Mentofin group at the 2nd and 5th weeks of the trial. Mentofin application was carried out as spray to flock in the first day, and then administered by adding it to drinking water in the 2nd, 3rd and the 4th days of the trial in the dose of 200 ml/1000 liter drinking water.

Serology: RSA (Nobilis® MG Antigen, Intervet International Co., Holland, Cat. No: A-650) and ELISA (Biocheck, Holland, Cat. No: CK 114) tests were used for the detection of specific antibodies according to manufacturers' instructions.

Culture: A validated MG-culture detection described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013 of World Organization for Animal Health (OIE) was adapted (29). Tracheal swabs were streaked onto Frey's Agar plates (BBL, Becton-Dickinson, No. 211456) and incubated in humid and microaerobic environment (partial 5% CO_2) at 37 °C for 5 days. Each MG-suspect colony observed under stereomicroscope was transferred into Frey's Broth (BBL, Becton-Dickinson, No. 212346) and after 3 consecutive transfers, pure culture of each isolate was used for identification tests (25) and rPCR.

MGrPCR: A validated MG-specific PCR described in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013 of OIE (29) was adapted to LightCycler 2.0 as MG realtime PCR (MGrPCR) system (Roche, Germany) and used for the detection of MG-DNA from tracheal swab samples. After performing the DNA isolation procedure as addressed in OIE, rPCR was applied by using the forward and reverse PCR primers MG1 (GAACGGGGTGCTTGCTTGCACCCA) and MG2 (TTCAAAGGATACCGTCACAC), which were selected from a region within the sequence of MG lipoprotein gene partial codons, with previously determined sensitivity and specificity for MG and an expected amplicon size of 400 bp as follows (29): Each reaction had a volume of 20 µl including 18 µl of reaction mixture containing 1 × LC FastStart DNA SYBR Green I Master Mix (Roche, Germany), $MgCl_2$ (4 mM), and 0.5 µM concentration of each primer and 2 µl of template DNA. Cycling parameters used were: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 50°C for 10 sec, and extension at 72°C for 20 sec. Melting curve analysis was automatically performed by LightCycler 2.0 software (Version 3) and the melting peaks were expected to have melting temperature (T_m) of 82°C.

Statistical analysis: Data were analyzed by Chi-Square Test. Binomial Test was applied for between group comparison positive and negative data separately and exact test was chosen asymptotic only. McNemar Test was used for inside group comparison. Differences were considered significant at a probability level of $P<0.05$ in all analyses. All statistical analysis was performed with SPSS software (version 20.0, SPSS Inc, USA).

Results

Serology: During the study period, there was no change in the RSA results as numbers of MG-antibody positive birds of Mentofin group. There was a slight insignificant increase in the numbers of MG-antibody positive chickens 4 days after Mentofin application, where this number decreased to the initial numbers at the end of the study ($P<0,05$). In the control group, the decrease in the number of MG-antibody positive birds by RSA results, and the slight decrease and then an increase in the numbers of MG-antibody positive chickens at day 4 and at day 18, respectively was found insignificant ($P>0,01$) (Table 1).

Culture and *MGrPCR:* There was a significant and continuous decline in the number of MG-infected birds in the Mentofin group after Mentofin application ($P<0,05$), while a comparably small and a fluctuant decline in the number of MG-infected birds was determined in the control group ($P>0,05$). There was a significant decline in MG positive birds detected by rPCR after 1st and 2nd mentofin application ($P<0,05$), however this decline was found insignificant in both groups' culture results ($P<0,05$) (Table 2).

Table 1. Serological test results for MG-antibody levels in Mentofin and control groups
Tablo 1. Mentofin ve kontrol gruplarının MG antikor düzeyini gösteren serolojik test sonuçları

Application No	Day No	Mentofin (n=20)				Control (n=20)			
		RSA		ELISA		RSA		ELISA	
		Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)
1st	5 d before	19 (95) [a]	1 (5) [x]	12 (60) [ab]	8 (40) [y]	18 (90) [a] A	2 (10) [x]	5 (25) [b]	15 (75) [y]
1st	4 d after	19 (95) [a]	1 (5) [x]	17 (85) [a]	3 (15) [x]	19 (95) [a] A	1 (5) [x]	3 (15) [b]	17 (85) [y]
2nd	18 d	19 (95) [a]	1 (5) [x]	12 (60) [ab]	8 (40) [y]	12 (60) [ab] B	8 (40) [y]	8 (40) [b]	12 (60) [y]

[a, b]: Different small letters indicate statistical significance at the same line for positive data (P < 0,05)
[x, y]: Different small letters indicate statistical significance at the same line for negative data (P < 0,05)
A, B: Different capital letters indicate statistical significance at the same row for inside group comparison positive and negative data together (P < 0,05)
[a, b]: Farklı küçük harfler aynı sıradaki pozitif verilerin istatistiksel farklılığını belirtir (P < 0,05)
[x, y]: Farklı küçük harfler aynı sıradaki negatif verilerin istatistiksel farklılığını belirtir (P < 0,05)
A, B: Farklı büyük harfler aynı satırdaki pozitif ve negatif verilerin birlikte ve grup içi karşılaştırmasındaki istatistiksel farklılığını belirtir (P < 0,05)

Table 2. Numbers of MG-infected birds detected from Mentofin group by culture and rPCR before and after Mentofin applications in comparison with those of the control group
Tablo 2. Mentofin ve kontrol grubunun Mentofin uygulamasından önce ve sonra MG kültür ve rPCR sonuçlarının karşılaştırılması

Application No	Day No	Mentofin (n=20)				Control (n=20)			
		Culture		rPCR		Culture		rPCR	
		Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)
	5 d before	17 (85) A	3 (15)	20 (100) A	0 (0)	16 (80) AB	4 (20)	19 (95) A	1 (5)
1st	1 d after	14 (70) A	6 (30)	12 (60) B	8 (40)	18 (90) A	2 (10)	16 (80) A	4 (20)
	13 d after	6 (30) [a] B	14 (70)	7 (35) [a] BC	13 (65)	11 (55) [ab] BD	9 (45)	15 (75) [b] A	5 (25)
	1 d after	6 (30) [a] B	14 (70)	6 (30) [a] C	14 (70)	12 (60) [ab] BD	8 (40)	15 (75) [b] A	5 (25)
2nd	10 d after	3 (15) B	17 (85)	8 (40) BC	12 (60)	4 (20) C	16 (80)	6 (30) B	14 (70)
	17 d after	1 (5) [a] B	19 (95) [x]	4 (20) [a] C	16 (80) [x]	7 (35) [ab] CD	13 (65) [x]	16 (80) [b] A	4 (20) [y]

[a, b]: Different small letters indicate statistical significance at the same line for positive data (P < 0,05)
[x, y]: Different small letters indicate statistical significance at the same line for negative data (P < 0,05)
A-D: Different capital letters indicate statistical significance at the same row for inside group comparison, positive and negative data together (P < 0,05)
[a, b]: Farklı küçük harfler aynı sıradaki pozitif verilerin istatistiksel farklılığını belirtir (P < 0,05)
[x, y]: Farklı küçük harfler aynı sıradaki negatif verilerin istatistiksel farklılığını belirtir (P < 0,05)
A-D: Farklı büyük harfler aynı satırdaki pozitif ve negatif verilerin birlikte ve grup içi karşılaştırmasındaki istatistiksel farklılığını belirtir (P < 0,05)

Discussion and Conclusion

In this study, effect of Mentofin application on levels of MG-infected birds in naturally infected flocks was observed. This study was conducted in flocks selected as typical representatives for Turkish layer chicken production, which do not have good management practices. Houses had poor ventilation and were unclean with non-hygienic cages. Additionally, the environment around the houses was not properly managed for cleaning and pest control. There was no proper structural and organizational biosecurity action taken in the organization.

There was no substantial change in the number of MG-antibody positive birds after Mentofin applications in the Mentofin flock, as expected. This was probably due to the stable MG-antibody levels produced against the MG antigen for a long period of time in the serum, despite the possible elimination of the MG. The positivity in our ELISA (which detects IgG - the dominant antibody in chronic infections) test results with a slight fluctuation in the previously chronically- MG infected flock, can be related to this. Contrary to the serology results, there was a significant decline in MGrPCR results, where MG positive numbers reduced to less than half of the group. All these findings indicate that MG-antibody levels were still high due to the continued persistence of the antibodies in the serum after chronic infection, but MG was eliminated up to a level,

as shown by reduction in positive birds tested by MGrPCR.

Antibacterial properties of eucalyptus derivates have been previously reported in studies by Babayi et al. (1), Barbour et al. (2), Jain et al. (17), Mohamed and Ibrahim (21), Nair et al. (23), and Navarro et al. (24). In this study we used culture and rPCR methods to detect MG-infected birds and found that MG-infected bird numbers had significantly and continuously decreased in the Mentofin group, despite no considerable change in the control group. This dramatic decrease was found significant only in MGrPCR results, indicating its superiority over culture. Therefore, we recommend the use of rPCR in MG detection in the flocks, since it is not uncommon to experience difficulties in MG isolation, leading to false negative results in culture compared to PCR.

In conclusion, results of this study indicate that Mentofin clearly had an effect on MG clearance from the tracheal epithelium, supported by detection of decline in MG infection in layers. The actual action mechanism of Mentofin on MG clearance from naturally infected chicken trachea is still unknown, and requires further detailed investigations.

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