# Monitoring reveals a high prevalence of *Mycoplasma synoviae* in layer flocks in Croatia

## Željko Gottstein[1*], Liča Lozica[1], Maja Lukač[1], Dušanka Vidas[2], Ana Hell Kurevija[2], and Danijela Horvatek Tomić[1]

*[1]Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*
*[2]Žito d.o.o., Osijek, Croatia*

### ABSTRACT

*Mycoplasma synoviae* (MS), despite its lower pathogenicity, has the ability to cause significant losses in poultry production but is usually underdiagnosed. In egg layer production, losses could be significant because of the drop in egg production and poor eggshell quality. Problems with colibacillosis and other infections secondary to MS have been continuously reported on Croatian egg layer farms. As a result, regular monitoring of flocks also included screening of the seroprevalence and molecular detection of MS using ELISA and qPCR tests, respectively. During monitoring, altogether 1135 samples were tested, including 1067 serum samples and 68 tracheal swabs, in a total of 126 flocks and 83 longitudinally merged flocks on 15 farms during the period from 2017 to 2021. The results showed a high general prevalence of MS with 86.6 % positive layer farms, while grouped flock seroprevalence and prevalence were 98.6% and 85.7%, respectively. With age, seroprevalence and ELISA titers rise significantly compared to the rearing period, with a significant mutual correlation over the entire production period. Additionally, there is a significant correlation between ELISA titers and age in weeks. Several flocks covered longitudinally from the first week of age, over the rearing period to the end of production, showed low prevalence during the rearing period, with a later significant rise in titer and prevalence, which indicates the dominance of horizontal transmission during production. Overall results indicate the need for a prompt reaction regarding preventive measures, such as better flock management, biosecurity and vaccination, which would reduce the losses and improve production.

**Key words**: *Mycoplasma synoviae;* layer flocks; seroprevalence; ELISA; molecular detection; qPCR

## Introduction

Mycoplasmosis in poultry is a group of diseases caused by four pathogenic *Mycoplasma* species that can be transmitted vertically or horizontally. The species *M. gallisepticum* (MG)*, M. synoviae* (MS)*, M. iowe* (MI) and *M. meleagridis* (MM), can cause significant respiratory and synovial problems, with reduced egg quality and production, neurological problems, as well as skeletal problems and embryo mortality (FERGUSON–NOEL et al., 2020). The *Mycoplasma* species that still causes worldwide problems, regardless of poultry production development level, is MS (LANDMAN, 2014).

Despite the control of other *Mycoplasma* species based on eradication and strict control of parent flocks, outbreaks are possible (MICHIELS et al., 2016). Although MS is less pathogenic and more difficult to isolate, it often goes under the diagnostic radar and can cause significant losses later during production (STIPKOVITS and KEMPF, 1996). Both MS and MG can cause mucosal lesions, and thus open the door to more profound and serious localized and systematic infections (FABERWEE et al., 2008). MS can cause respiratory and synovial problems with varying severity, but it is usually the initiator of multicausal respiratory and systemic problems, most often together with an infectious bronchitis virus, *Escherichia coli*, *Gallibacterium anatis* etc. (SID et al., 2016; FERGUSON–NOEL and NOORMOHAMMADI, 2020). Moreover, MS is recognized as a cause of lower egg shell quality and eggshell apex abnormalities, with a drop in egg production (FABERWEE et al., 2009). Recent results of metagenomics research into broilers' respiratory problems' etiology showed that MS is one of the most frequently isolated pathogens besides MG, *Avibacterium paragallinarum*, *Ornithobacterium rhinotracheale* and *Escherichia coli* (PATEL et al., 2018). Previous results regarding monitoring of MS in Europe showed that in some parts MS is endemic and has 25 to 95 % prevalence in the commercial layer parent and layer farms (FABERWEE et al., 2008; KURSA et al., 2019; CORTES et al., 2020), while our previous short monitoring showed that it has seroprevalence of over 80% in Croatia (HORVATEK TOMIĆ et al., 2018.). The MG is under official control in parent poultry flocks in the majority of countries, including Croatia (ANONN, 2018; ANONN, 2011), so thorough monitoring of this pathogen has resulted in low prevalence over the years in Croatia (ZELENIKA et al., 1999).

Vertical transmission seems to be most important for initial infection with MS, although later, horizontal transmission within a farm is more dominant, especially on multi-age farms, where the rearing period usually has a lower prevalence, on average 55%, and without clinical symptoms (ter VEEN et al., 2020). However, once infected, the flock stays infected for life, thanks to the specific surface glycoprotein gene expression and continuous escape of the immune system (KLEVEN, 2008). In addition, therapy is reduced to several antimicrobials, due to the specific cell structure, which is further complicated by the development of multidrug resistance (MORROW et al., 2020).

Clinical symptoms and impaired production on layer farms in Croatia indicated that MS could be the cause of the problem. Croatia does not have a parent layer flock, so vertical control is based on eradication measures in foreign countries. So, the aim of this study was detailed serological and molecular monitoring in several integrated egg layer companies and several smaller farms, to identify the general prevalence of MS on the farms, and its transmission dynamics within the farm after longitudinal flock screening.

**Materials and methods**

*Farms under monitoring.* Monitoring was conducted in 15 egg layer companies in the period from 2017 to 2021. During monitoring, the flocks were of different ages, and some flocks were monitored longitudinally, during rearing and the production period. In total 126 longitudinally separate flocks and 83 longitudinally merged flocks were sampled, with in total 1135 samples tested (supplementary table). Only one flock was vaccinated using the MS-H vaccine.

*Serum samples and ELISA monitoring.* On average, 10 (4-20) blood samples per flock were taken for ELISA monitoring, and sera were separated and stored at –20 ºC until testing. Altogether 1067 serum samples from 112 flocks in total on 9 farms were tested using a commercial MS ELISA kit (BioChek, Reeuwijk, The Netherlands) according to the manufacturer's instructions. Absorbance was measured using a µQuant spectrophotometer reader (Bio-Tek Instruments, Winooski, VT, USA) at 405 nm, and the specific serum antibody titer was calculated according to manufacturer's instructions, where a titer ≥594 was considered positive.

*Molecular monitoring.* In 14 flocks, 68 tracheal swabs (5 per flock) were taken on 7 farms and frozen immediately at -20 ºC until further analysis.

DNA was isolated individually from each swab using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Co., St Louis, MO, USA). From the isolated DNA, the concentration was detected using a BioDrop device (Cambridge, UK) and the samples were stored at -20 ºC until the qPCR analysis. The MS specific qPCR was performed using the previously described protocol, using primers and a probe (RAVIV and KLEVEN, 2009) targeting the 16S–23S rDNA ISR region: forward- ctaaatacaatagcccaaggcaa, reverse- cctcctttcttacggagtaca and probe- 5'FAM-agcgatacacaaccgcttttagaat-BHK1-3'. The reaction was performed on an Mx3005P device (Stratagene, La Jolla, CA, USA) using 7.5 µL of 2x GoTaq® Probe qPCR Master Mix kit (Promega, Madison, WI, USA), 3 µL of DNA, and primers in concentrations as published (RAVIV and KLEVEN, 2009) in a total of 15 µL reaction volume. All reactions of tracheal swabs were performed in duplicate over 40 cycles, with serial dilution of positive controls of known concentrations, performed in triplicate and run in parallel to form a standard curve, for MS quantitation in the tracheal swabs. The Ct values and positive qPCR data were analyzed using MxPro qPCR Software (Stratagene, La Jolla, CA, USA).

*Statistical analyses.* The statistical analyses were performed using Statistica 13.5.0.17. (TIBCO Software Inc., USA) software. Normality of data spread was tested using the Kolmogorov-Smirnov test. The significance of differences in the average flock positive rate and ELISA titer between four age timeframes (0-20, 21-40, 41-60 and >60) was analyzed using the Kruskal-Wallis test, with statistical significance set at P≤0.05. In addition, correlation matrices were used to test the correlation between ELISA titer and positive rate per flock, as well as ELISA titer and age in weeks.

### Results

The analyses of seroprevalence and molecular detection of MS in samples delivered for diagnostics included layers from 1 week to 3.5 years of age (supplementary table). On 15 different farms, with 83 longitudinally merged and 126 sampled flocks in total, 1135 samples were taken, of which

1067 and 68 were analyzed by ELISA and qPCR, respectively (Table 1). Of all 15 tested farms, 13 farms or 86.6% were positive, as well as 96.4% and 83.3% of the longitudinally merged and the total number of flocks, respectively, irrespective of the method used. Of all 1135 individual samples, 69.5% were MS positive.

*Seroprevalence results.* The results of ELISA serum tests showed an extremely high average prevalence of MS in layer flocks in Croatia, with 98.6% positive longitudinally merged flocks (Table 1). Regarding the total number of flocks, 93 out of 112 flocks tested were positive, or 83.03%. From 1067 tested serum samples, 744 samples were positive, or 69.7%. The results also showed higher titers as the layers become older, with a significant rise (p<0.05) in titer after 20 weeks of age (Fig. 1). Regarding the positive rate of tested samples, it also rose significantly (P<0.05) after 20 weeks of age (Fig. 2). The results of the correlation test between ELISA titer and positive rate in groups by week (Fig. 3) also showed high significance (r=0.74192, P<0.001).

The results of the ELISA tests in the flocks kept in the same poultry house showed 100% prevalence in the flocks kept there before, at the end of previous production cycle, with high ELISA titers (Fig. 4A and B). Then, there were usually low, negative titers during the rearing period (until 20 weeks of age), with 0% prevalence, and then a rise in titers after 24 weeks (Fig. 4A-C). In some flocks (Fig. 4A) there was already a slight rise in titer and prevalence to 33.3 % during the rearing period at 13 weeks of age. The results of correlation between age (in weeks) and average flock ELISA titer showed high significance (r=0.86718, P<0.001) (Fig. 5).The results also showed a positive titer and higher positive rate (30 %) in the imported flock just after arrival at the end of the rearing period (Table 1, Farm 6, Flock IMPORT).

*Molecular monitoring results.* The results of the qPCR analyses also showed a high prevalence of MS in the tested flocks, with 12 out of 14, or 85.7% positive flocks (Table 1). On the sample level, of 68 samples, 45 or 66.2 % were positive.

Table 1. Results of the seroprevalence and molecular monitoring of *Mycoplasma synoviae*
in the individual layers' samples, flocks and farms.

| | SAMPLES | | | FLOCKS | | | | | | FARMS |
| | | | | TOTAL No. | | | LONGITUDINALLY MERGED | | | |
| | $\sum$ | ELISA | qPCR | $\sum$ | ELISA | qPCR | $\sum$ | ELISA | qPCR | $\sum$ |
| Total | 1135 | 1067 | 68 | 126 | 112 | 14 | 83 | 69 | 14 | 15 |
| Positive | 789 | 744 | 45 | 105 | 93 | 12 | 80 | 68 | 12 | 13 |
| Positive rate (%) | 69,5 | 69,7 | 66,2 | 83,30 | 83,03 | 85,7 | 96,4 | 98,6 | 85,7 | 86,66 |
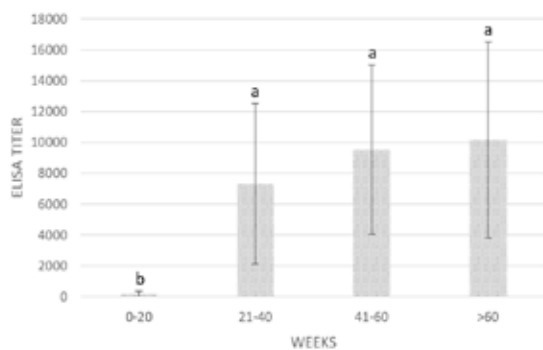


Fig. 1. Average flock ELISA titers in different groups by week. Significantly different ELISA titer results between weeks are marked with different lower case letters (a, b).
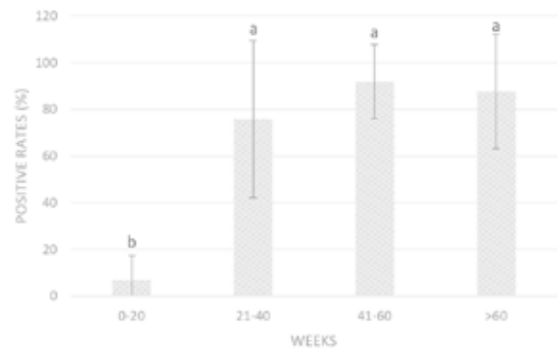


Fig. 2. Average flock positive rates (%) in different week groups. Significantly different positive rate results between weeks are marked with different lower case letters (a, b).
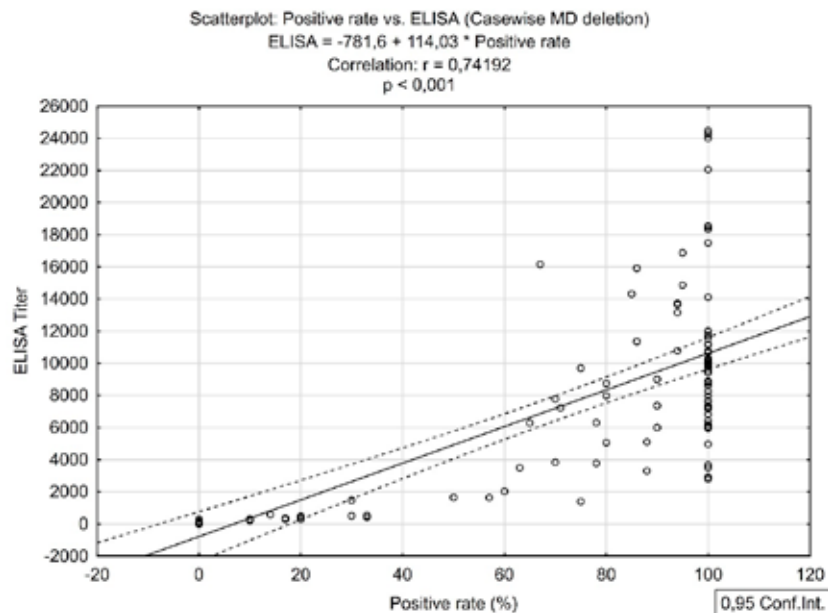


Fig. 3. Correlation between ELISA titers and positive rates (%) in all flocks.

A)



B)



C)
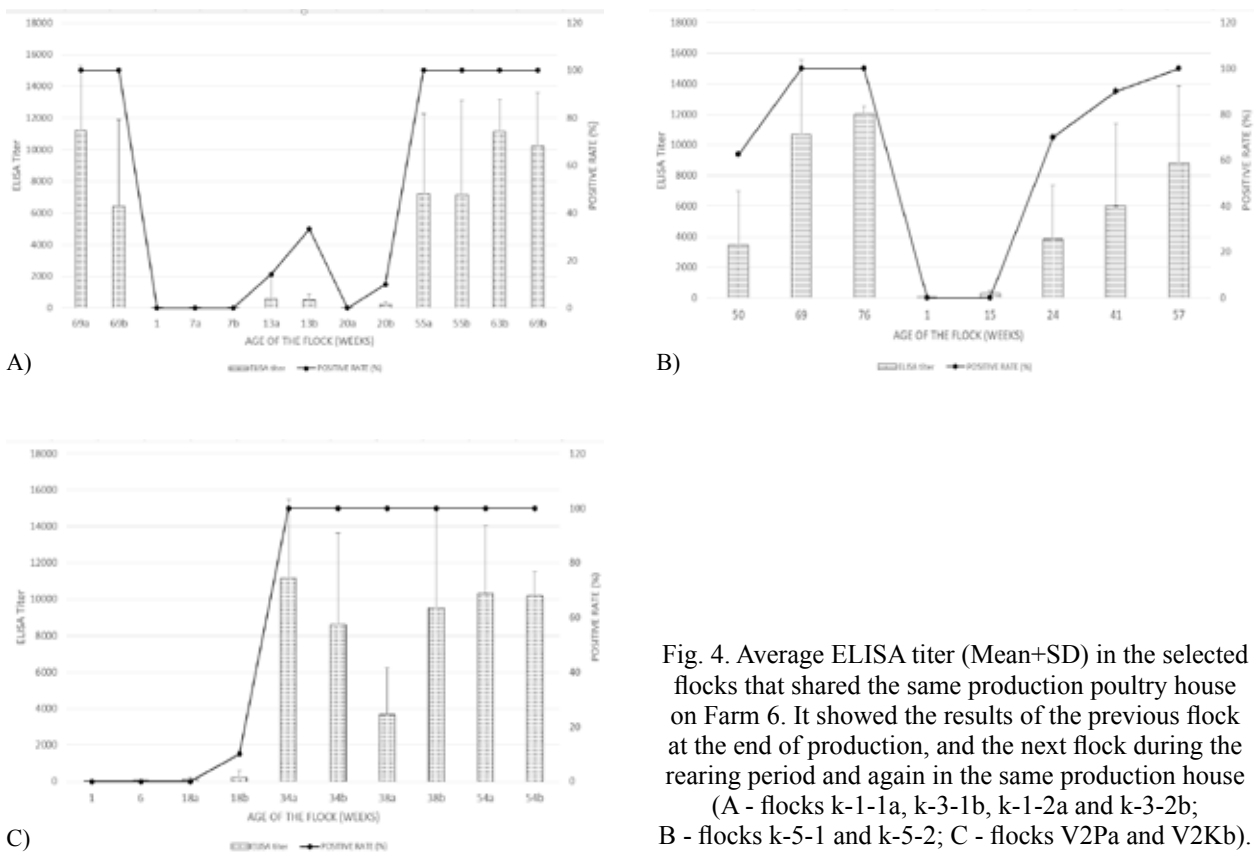
Fig. 4. Average ELISA titer (Mean+SD) in the selected flocks that shared the same production poultry house on Farm 6. It showed the results of the previous flock at the end of production, and the next flock during the rearing period and again in the same production house (A - flocks k-1-1a, k-3-1b, k-1-2a and k-3-2b; B - flocks k-5-1 and k-5-2; C - flocks V2Pa and V2Kb).



Fig. 5. Correlation between ELISA titers and age (weeks) in flocks selected in Figure 4.

Table 2. Supplementary - Detailed results on farms under monitoring with average ELISA titers and molecular detection results in each flock

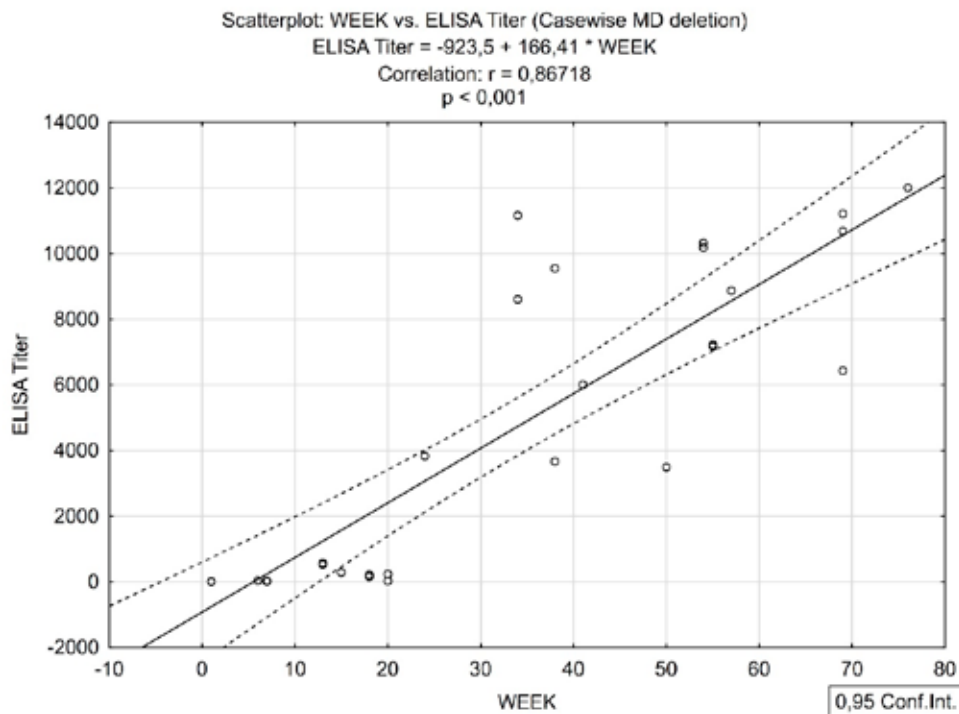| RESULTS OF SEROPREVALENCE | | | | | | |
|---|---|---|---|---|---|---|
| FARM | FLOCKS | | Total No. | YEAR | AGE (weeks) | ELISA TITER (Av. ± SD) | No. of positive/ tested |
| | Longitudinally merged | | | | | | |
| | No. | Flock name | | | | | |
| 1 | 1 | A | 1 | 2017 | 67 | 0±0 | 0/8 |
| | 2 | B | 2 | 2017 | 12 | 0±0 | 0/10 |
| | 3 | C-1 | 3 | 2017 | 26 | 0±0 | 0/9 |
| | 4 | 3/4 | 4 | 2017 | 48 | 501±876 | 3/10 |
| | 5 | C-2 | 5 | 2019 | 63 | 7623±3146 | 9/9 |
| | 6 | D-1-3 | 6 | 2019 | 76 | 6304±4009 | 7/9 |
| 2 | 7 | 2 | 7 | 2017 | 87 | 7773±1653 | 7/10 |
| | 8 | 3 | 8 | 2017 | 45 | 5055±5811 | 8/10 |
| | 9 | 5 | 9 | 2017 | 28 | 1401±2277 | 6/8 |
| | 10 | 5 | 10 | 2019 | 67 | 10256±1353 | 9/9 |
| | 11 | 6 | 11 | 2019 | 48 | 7353±3505 | 10/10 |
| 3 | 12 | 4 | 12 | 2018 | 25 | 8731±7162 | 8/10 |
| | 13 | 3 | 13 | 2018 | 69 | 1651±3161 | 5/10 |
| | 14 | 1 | 14 | 2018 | 20 | 349±527 | 2/12 |
| | 15 | 2 | 15 | 2018 | 20 | 290±348 | 2/12 |
| 4 | 16 | 1 | 16 | 2018 | 31 | 2925±2678 | 5/5 |
| 5 | 17 | p-1-1 | 17 | 2018 | 64 | 3490±2646 | 6/6 |
| | 18 | p-1-2 | 18 | 2018 | 17 | 45±32 | 0/10 |
| | | | 19 | 2018 | 22 | 27±20 | 0/10 |
| | | | 20 | 2018 | 49 | 9396±2110 | 10/10 |
| | 19 | p-2-1 | 21 | 2018 | 51 | 2040±2105 | 3/5 |
| | | | 22 | 2018 | 70 | 6040±3521 | 10/10 |
| | 20 | p-2-2 | 23 | 2018 | 27 | 9922±1573 | 9/9 |
| | 21 | p-3-1 | 24 | 2018 | 44 | 8890±4221 | 10/10 |
| | | | 25 | 2018 | 53 | 5954±3562 | 10/10 |
| | 22 | p-4-1 | 26 | 2018 | 44 | 2796±994 | 10/10 |
| | | | 27 | 2018 | 53 | 7661±2841 | 10/10 |

Table 2. Supplementary - Detailed results on farms under monitoring with average ELISA titers and molecular detection results in each flock (continued)

| RESULTS OF SEROPREVALENCE | | | | | | | |
|---|---|---|---|---|---|---|---|
| FARM | FLOCKS | | | YEAR | AGE (weeks) | ELISA TITER (Av. ± SD) | No. of positive/ tested |
| | Longitudinally merged | | Total No. | | | | |
| | No. | Flock name | | | | | |
| 5 | 23 | p-5-1 | 28 | 2018 | 64 | 4967±2774 | 10/10 |
| | 24 | p-5-2 | 29 | 2018 | 17 | 71±36 | 0/10 |
| | | | 30 | 2018 | 22 | 7971±2687 | 9/9 |
| | | | 31 | 2018 | 49 | 3773±4217 | 7/9 |
| | 25 | P1 | 32 | 2019 | 22 | 1485±3389 | 3/10 |
| | 26 | P2 | 33 | 2019 | 62 | 9985±1518 | 10/10 |
| | 27 | P3 | 34 | 2019 | 38 | 8253±2700 | 10/10 |
| | 28 | P4 | 35 | 2019 | 38 | 6821±3217 | 10/10 |
| | 29 | P5 | 36 | 2019 | 22 | 471±915 | 2/10 |
| | 30 | P1 | 37 | 2020 | 23 | 7219±8235 | 5/7 |
| | 31 | P2 | 38 | 2020 | 71 | 18494±7991 | 5/5 |
| | 32 | P3 | 39 | 2020 | 58 | 16160±10733 | 4/6 |
| | 33 | P4 | 40 | 2020 | 58 | 17493±6961 | 7/7 |
| | 34 | P5 | 41 | 2020 | 23 | 15910±9564 | 6/7 |
| 6 | 35 | V-p | 42 | 2018 | 49 | 9703±6955 | 6/8 |
| | | | 43 | 2018 | 58 | 10291±4163 | 7/7 |
| | 36 | V-k | 44 | 2018 | 30 | 3297±3048 | 7/8 |
| | | | 45 | 2018 | 39 | 1645±1619 | 4/7 |
| | 37 | V2Pa | 46 | 2018 | 1 | 14±16 | 0/10 |
| | | | 47 | 2018 | 6 | 36±17 | 0/10 |
| | | | 48 | 2018 | 18 | 158±89 | 0/10 |
| | | | 49 | 2019 | 34 | 11159±4350 | 9/9 |
| | | | 50 | 2019 | 38 | 3666±2587 | 10/10 |
| | | | 51 | 2019 | 54 | 10311±3734 | 10/10 |
| | 38 | V2Kb | 52 | 2018 | 18 | 214±390 | 1/10 |
| | | | 53 | 2019 | 34 | 8600±5035 | 9/9 |
| | | | 54 | 2019 | 38 | 9543±5349 | 10/10 |

Table 2. Supplementary - Detailed results on farms under monitoring with average ELISA titers
and molecular detection results in each flock (continued)

| FARM | FLOCKS | | | YEAR | AGE (weeks) | ELISA TITER (Av. ± SD) | No. of positive/ tested |
|---|---|---|---|---|---|---|---|
| | Longitudinally merged | | Total No. | | | | |
| | No. | Flock name | | | | | |
| 6 | 38 | V2Kb | 55 | 2019 | 54 | 10180±1334 | 10/10 |
| | 39 | k-4-1 | 56 | 2018 | 50 | 6172±2006 | 8/8 |
| | | | 57 | 2018 | 69 | 11343±6334 | 6/7 |
| | | | 58 | 2018 | 76 | 11755±666 | 10/10 |
| | 40 | k-5-1 | 59 | 2018 | 50 | 3488±3472 | 5/8 |
| | | | 60 | 2018 | 69 | 10680±4882 | 7/7 |
| | | | 61 | 2018 | 76 | 12005±505 | 8/8 |
| | 41 | k-5-2 | 62 | 2018 | 1 | 2±1 | 0/7 |
| | | | 63 | 2018 | 15 | 288±160 | 0/10 |
| | | | 64 | 2018 | 24 | 3833±3538 | 7/10 |
| | | | 65 | 2019 | 41 | 5997±5362 | 9/10 |
| | | | 66 | 2019 | 57 | 8863±5011 | 3/3 |
| | 42 | k-1-1 | 67 | 2018 | 69 | 11208±4061 | 10/10 |
| | 43 | k-3-1 | 68 | 2018 | 69 | 6433±5469 | 10/10 |
| | 44 | k-1-2a | 69 | 2018 | 1 | 4±3 | 0/10 |
| | | | 70 | 2018 | 7 | 33±18 | 0/5 |
| | | | 71 | 2018 | 13 | 576±1389 | 1/7 |
| | | | 72 | 2018 | 20 | 24±24 | 0/10 |
| | | | 73 | 2019 | 55 | 7222±5045 | 10/10 |
| | 45 | k-2-2 | 74 | 2018 | 7 | 41±16 | 0/5 |
| | | | 75 | 2018 | 13 | 74±21 | 0/7 |
| | | | 76 | 2018 | 20 | 79±71 | 0/10 |
| | | | 77 | 2019 | 55 | 8776±5939 | 10/10 |
| | 46 | k-3-2b | 78 | 2018 | 7 | 11±13 | 0/5 |
| | | | 79 | 2018 | 13 | 527±401 | 2/6 |
| | | | 80 | 2018 | 20 | 233±181 | 1/10 |
| | | | 81 | 2019 | 55 | 7168±3511 | 10/10 |

Table 2. Supplementary - Detailed results on farms under monitoring with average ELISA titers
and molecular detection results in each flock (continued)

| FARM | FLOCKS | | Total No. | YEAR | AGE (weeks) | ELISA TITER (Av. ± SD) | No. of positive/ tested |
|------|--------|--|-----------|------|-------------|------------------------|-------------------------|
| | Longitudinally merged | | | | | | |
| | No. | Flock name | | | | | |
| 6 | 46 | k-3-2b | 82 | 2019 | 63 | 11154±2010 | 10/10 |
| | | | 83 | 2019 | 69 | 10237±3366 | 10/10 |
| | 47 | k-4 | 84 | 2019 | 26 | 291±326 | 2/10 |
| | | | 85 | 2019 | 34 | 9913±3759 | 10/10 |
| | 48 | k-1 | 86 | 2019 | 20 | 425±391 | 2/10 |
| | | | 87 | 2020 | 75 | 14307±17116 | 17/20 |
| | 49 | k-2 | 88 | 2019 | 20 | 304±283 | 1/10 |
| | | | 89 | 2020 | 75 | 6284±7996 | 13/20 |
| | 50 | V3P | 90 | 2019 | 30 | 9793±5158 | 10/10 |
| | | | 91 | 2019 | 46 | 8989±3633 | 9/10 |
| | 51 | V3K | 92 | 2019 | 30 | 5099±4943 | 7/8 |
| | | | 93 | 2019 | 38 | 10076±3594 | 10/10 |
| | | | 94 | 2019 | 46 | 10771±866 | 10/10 |
| | 52 | V1P | 95 | 2019 | 38 | 11568±2395 | 10/10 |
| | 53 | V1K | 96 | 2019 | 38 | 7357±4536 | 9/10 |
| | 54 | k-3 | 97 | 2020 | 51 | 14861±8626 | 19/20 |
| | 55 | k-4 | 98 | 2020 | 74 | 10780±8357 | 17/18 |
| | 56 | k-5 | 99 | 2020 | 41 | 16869±7955 | 19/20 |
| | 57 | V1P | 100 | 2020 | 78 | 18540±6872 | 5/5 |
| | 58 | V1K | 101 | 2020 | 78 | 18324±8885 | 5/5 |
| | 59 | V2P | 102 | 2020 | 45 | 24468±154 | 5/5 |
| | 60 | V2K | 103 | 2020 | 45 | 24262±344 | 5/5 |
| | 61 | V3P | 104 | 2020 | 82 | 24014±1045 | 5/5 |
| | 62 | V3K | 105 | 2020 | 82 | 22073±5519 | 5/5 |
| | 63 | IMPORT | 106 | 2021 | 20 | 408±757 | 4/12 |
| 7 | 64 | 1 | 107 | 2019 | 28 | 1±2 | 0/4 |

Table 2. Supplementary - Detailed results on farms under monitoring with average ELISA titers and molecular detection results in each flock (continued)

| FARM | FLOCKS | | Total No. | YEAR | AGE (weeks) | ELISA TITER (Av. ± SD) | No. of positive/ tested |
|---|---|---|---|---|---|---|---|
| | Longitudinally merged | | | | | | |
| | No. | Flock name | | | | | |
| **RESULTS OF SEROPREVALENCE** | | | | | | | |
| 8 | 65 | 1 | 108 | 2019 | 37 | 13154±4668 | 17/18 |
| | 66 | 3 | 109 | 2019 | 62 | 13728±5136 | 17/18 |
| | 67 | 4 | 110 | 2019 | 67 | 14104±4382 | 19/19 |
| | 68 | 6[1] | 111 | 2019 | 55 | 13642±5314 | 17/18 |
| 9 | 69 | 1 | 112 | 2018 | 20-180 | 7983±6516 | 8/10 |

| FARM | FLOCKS | | | YEAR | AGE (weeks) | qPCR (Av. no. of DNA copies per swab ± SD) | No. of positive/ tested |
|---|---|---|---|---|---|---|---|
| **RESULTS OF MOLECULAR MONITORING** | | | | | | | |
| 1 | 70 | C-1 | 113 | 2020 | 60 | 1175±1114 | 5/5 |
| | 71 | D-1 | 114 | 2020 | 41 | 7645±5166 | 5/5 |
| | 72 | D-2 | 115 | 2020 | 41 | 3628±2441 | 5/5 |
| | 73 | D-3 | 116 | 2020 | 49 | 3±3 | 3/5 |
| | 74 | D-4 | 117 | 2020 | 49 | 1±3 | 1/5 |
| 10 | 75 | 1 | 118 | 2020 | 60 | 73 ± 127 | 4/5 |
| | 76 | 2 | 119 | 2020 | 19 | 191 ± 177 | 4/5 |
| 11 | 77 | 1 | 120 | 2020 | 66 | 51 ± 98 | 2/5 |
| 12 | 78 | 1 | 121 | 2020 | 33 | 0 ± 0 | 0/5 |
| 13 | 79 | 1 | 122 | 2020 | 29 | 400 ± 363 | 3/5 |
| | 80 | 2 | 123 | 2020 | 36 | 3615 ± 2991 | 5/5 |
| 14 | 81 | 1 | 124 | 2020 | 38 | 3578 ± 3914 | 5/5 |
| 15 | 82 | 1 | 125 | 2020 | 26 | 0 ± 0 | 0/4 |
| | 83 | 2 | 126 | 2020 | 42 | 1313 ± 2599 | 3/4 |

[1]Flock vaccinated using MS-H vaccine

**Discussion**

Infection with MS is a chronic disease, easily transmitted vertically, with a usual horizontal spread in multi-age egg layer production (ter VEEN et al., 2020). In layer flocks in Croatia, there are continuous clinical and production problems that imply MS infection. There are no recent seroprevalence and prevalence studies of MS in Croatia, except our short preliminary monitoring in 2017 (HORVATEK TOMIĆ et al., 2018). In tested layer flocks, vaccination was only performed on Farm 8, in Flock 6, using an MS-H strain, but with unusually high titers. On the other farms, the titers are probably result of an infection with wild strains.

Our results confirm the report of the previous preliminary monitoring (HORVATEK TOMIĆ et al., 2018), with a slightly higher prevalence per farm of 86.66%. On the level of grouped flocks, average prevalence, regardless of the method, was 96.6%, and for seroprevalence and prevalence it was 98.6% and 84.6%, respectively. These results are in congruence with recently published comparative monitoring in Spain (CORTÉS et al., 2020), reporting 95% seroprevalence and prevalence. Earlier reports (KAPETANOV et al., 2010; BUIM et al., 2009; SUZUKI et al., 2009) showed a lower seroprevalence in commercial layer flocks, ranging from 53 to 90%, while recent results of MS prevalence reported by KURSA et al. (2019) during seven-year monitoring in the Polish layer flocks ranged from 19 to 44%.

The age-wise analyses showed an increase in seroprevalence as well as titers within groups according to age in weeks, with a significant correlation between them, as well as a highly significant correlation between titers and weeks. The same was confirmed by several other studies (SAÂDIA et al., 2014; XUE et al., 2017), and with the results of HAGAN et al. (2004), who confirmed age as a risk factor for higher seropositivity. However, our results in flocks during the rearing period showed low or negative titers, and low or no seroprevalence compared to the previously reported 29-84% (KAPETANOV et al., 2010; ter VEEN et al., 2020). The only higher prevalence in the rearing flocks was detected in a recently imported flock, just before the production cycle at the age of

20 weeks, and in one flock at the age of 13 weeks, with prevalence of 30 and 33.3%, respectively, both on Farm 6, with low titers and non-vaccinated. As could be seen from the supplement table, chicks in the first week came with no titer, usually from abroad. On this basis, we can presume that layer breeder flocks should be MS negative or maybe vaccinated with strains that stimulate higher local, but lower systemic immunity, such as the MS-H strain. Figure 4 shows that infection during the rearing period is rare, but possible. Due to the high biosecurity measures undertaken during the early period of life, and probably the lower MS load on the rearing farm, signs of seroconversion appear near the end of rearing, since seroconversion after *Mycoplasma* infection is rather slow (MICHIELS et al., 2016). If we compare this with the analyses performed by ter VEEN et al. (2020), it is probable that vertical transmission does not occur and that horizontal transmission is probably low during the rearing period, as both serocoversion and seroprevalence are low at the end of rearing, compared to theirs, which was 78%. However, Figures 4A and B also show high titers and seroprevalence in the previous flocks approaching the end of production, probably resulting in high contamination of the poultry houses, and the possible lateral spread of MS to the neighboring ones. Later, we can see a significant rise in titer and prevalence after the rearing period, and during the following production cycle in the next flock occupying the same poultry house. This indicates high lateral contamination between production houses, and then evenly within the flock during the production period. A similar pattern could be seen on Farm 5 within Flocks p-1-1 and 2, and p-5-1 and 2, which could be expected from this pathogen with easy entrance via the respiratory system, and the high pressure mentioned from surrounding houses, despite its relatively short survival period (CHRISTENSEN et al., 1994). So, the extremely high titers and seroprevalence detected on the majority of farms could be the result of multi-age farms, lower biosecurity and the close proximity of houses on the farm during production, compared to the rearing period, which are the main risk factors for MS transmission (ter VEEN et al., 2020). On

Farm 1, seroprevalence did not exist in 2017, even in older flocks, but by 2020 it was present in the majority of tested flocks, probably as a result of the less strict biosecurity measures.

Some companies, such as company 8 mentioned above, began vaccinating using the MS-H strain after confirmation of MS infection, as clinical signs and problems with colibacillosis and other secondary infections were continuous, and this improved the production results. The only vaccinated flock was Flock 6, but the titer was higher than expected for only MS-H strain vaccination (MORONATO et al., 2018), probably because of the later vaccination at the end of the rearing period and placement into production houses with significant contamination.

The results of this monitoring indicate that MS is present on the majority of farms, probably causing significant and continuous problems, and proves that monitoring, regardless of method, is a useful diagnostic procedures to help cope with this infection. Since vaccination showed the ability to control the infection, even reducing the load of wild strains in poultry houses, this is certainly significant information for owners with contaminated farms to reconsider vaccination, besides upgrading biosecurity, as an important preventive measure of choice to improve production.

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### SAŽETAK

*Mycoplasma synoviae* (MS), unatoč nižoj patogenosti, može uzrokovati značajne gubitke u proizvodnji peradi, no obično je poddijagnosticirana. Gubici u proizvodnji konzumnih jaja mogu biti značajni zbog pada proizvodnje i loše kvalitete ljuske jajeta. Problemi s kolibacilozom i drugim zarazama koje se javljaju kao sekundarne MS-u kontinuirano se prijavljuju na farmama nesilica konzumnih jaja u Hrvatskoj. Stoga je u praćenje zdravlja na farmama uključeno i otkrivanje seroprevalencije i molekularnog dokaza MS-a primjenom ELISA i qPCR testova. Tijekom praćenja testirano je ukupno 1135 uzoraka, 1067 seruma i 68 obrisaka dušnika, u ukupno 126 jata, odnosno 83 longitudinalno grupiranih jata na 15 farmi od 2017. do 2021. godine. Rezultati su pokazali visoku opću prevalenciju od 86,6% pozitivnih farmi na MS. Kod longitudinalno grupiranih jata seroprevalencija je iznosila 98,6%, dok je molekularnim monitoringom prevalencija bila 85,7%. S dobi, seroprevalencija i ELISA titar značajno rastu, uz značajnu međusobnu korelaciju tijekom cijelog razdoblja proizvodnje. Također, postoji značajna korelacija između ELISA titra i dobi u tjednima. Jata praćena longitudinalno od dobi jednog tjedna do kraja proizvodnje, pokazuju nisku prevalenciju tijekom uzgojnog razdoblja. Kasnije, u razdoblju proizvodnje, dolazi do značajnog porasta titra i prevalencije, što ukazuje na dominantan horizontalni prijenos tijekom proizvodnje. Rezultati monitoringa skreću pozornost na potrebu brze reakcije na farmama s ciljem poboljšanja preventivnih mjera, naročito boljeg upravljanja jatima, biosigurnosnih mjera i cijepljenja, što bi značajno smanjilo gubitke i poboljšalo proizvodnju.

**Ključne riječi:** *Mycoplasma synoviae*; kokoši nesilice; seroprevalencija; ELISA; prevalencija; qPCR