Seroprevalence and prevalence of *Mycoplasma synoviae* in laying hens and broiler breeders in Spain

Verónica Cortés,[*,1] Sandra Sevilla-Navarro,[*,†] Cristina García,[*] Ana Tudón,[*] Clara Marín,[†] and Pablo Catalá-Gregori[*,†]

[*]*Centro de Calidad Avícola y Alimentación Animal de la Comunidad Valenciana (CECAV), Castellón, Spain; and* [†]*Universidad Cardenal Herrera-CEU, CEU Universities, Valencia, Spain*

ABSTRACT *Mycoplasma* species are worldwide recognized poultry pathogens, with *Mycoplasma synoviae* being the second most important species from the clinical point of view that causes considerable economic losses in the poultry industry. The aim of this study was to assess the seroprevalence, prevalence, and phylogenetic variants of *M. synoviae* present in layers and broiler breeders' farms of *Gallus gallus* species located in eastern Spain. Thus, 19 and 23 flocks of layers and broiler breeders, respectively, were analyzed at 3 different ages. To assess seroprevalence, sera samples were analyzed by ELISA. Tracheal swabs were tested by PCR to assess the prevalence. A *M. synoviae* seroprevalence of 95 and 74% was detected in layers and broiler breeders, respectively. Regarding age-wise analysis, the positive rates obtained seemed to be higher as the age of sampling increased. As per PCR results, a prevalence of 95% in layers and 35% in broiler breeders was obtained. The genetic analysis showed that the strains present in broilers breeders were vaccine strains (MS H strain). In contrast, 6 different field strains were detected in layer hens. In conclusion, this study carried out in eastern Spain showed a higher seroprevalence and prevalence of *M. synoviae* field strains in layer flocks regarding broiler breeders, highlighting the usefulness of monitoring flocks to control this poultry pathogen. Moreover, our findings suggest *M. synoviae* vaccination in broiler breeders could be an effective prevention strategy.

Key words: *Mycoplasma synoviae,*, prevalence, seroprevalence, ELISA, PCR

2021 Poultry Science 100:100911
https://doi.org/10.1016/j.psj.2020.11.076

INTRODUCTION

Mycoplasma species are recognized as poultry pathogens, with *Mycoplasma synoviae* being the second most important species from the clinical and economical point of view (Feberwee et al., 2009). This pathogen is the responsible of significant economic losses in the poultry industry (Lierz et al., 2007) because it can cause respiratory syndrome, articular disease, and growth retardation in chickens and turkeys Kleven and Bradbury, 2008). In addition, in layers, *M. synoviae* can induce shell abnormalities, causing a decrease in egg quality and production, increasing the operating costs (Catania et al., 2010; Landman, 2014). Avian mycoplasmosis may be transmitted either vertically by broiler breeders through eggs (Xue et al., 2017) or horizontally, often by direct contact between ill and unaffected carriers (Marois et al., 2000). Moreover, *Mycoplasma* can remain in the flock constantly as forms (Nascimiento et al., 2005).

M. synoviae is mainly treated with antibiotics, such as oxytetracyclines (Landman et al., 2008). Nevertheless, owing to the temporary effect of antibiotic treatment, the emergence of resistance, and the risk of residues in eggs for consumption, alternative strategies such as surveillance and vaccination should be considered for *M. synoviae* control (Feberwee et al., 2009). Vaccination is a commonly used tool to control this infection in commercial flocks in many countries with significant commercial poultry industries (Zhu et al., 2018), but the success of these control programs depends on the accurate and timely diagnosis of infected flocks (Gharibi et al., 2018). *Mycoplasma* isolation by traditional culture method is difficult, expensive, time consuming, and inconclusive (Ewing et al., 1996). For this reason, nowadays, some different techniques have been used to look for the presence of *M. synoviae*, such as antibodies detection in sera samples with ELISA (Gole et al., 2012) and DNA detection through PCR (Sun et al., 2017). These

Received October 22, 2020.
Accepted November 23, 2020.

[1]Corresponding author: v.cortes@cecav.org

techniques arise as a very interesting alternative for diagnosis because of their sensitivity, specificity, capability of examination accomplishment on a large scale and economic viability (Nascimiento et al., 1991).

Before starting a control programme, it is suitable to know the microorganisms' prevalence at the region in the different poultry species involved. Epidemiologic studies performed in different European countries have shown the high prevalence of *M. synoviae* in poultry (Hagan et al., 2004; Dufour-Gesbert et al., 2006; Gole et al., 2012). However, the presence and distribution of *M. synoviae* is unknown in farms located in eastern Spain. In this context, the aim of this study was to assess the seroprevalence, prevalence, and phylogenetic variants of *M. synoviae* in layers and broiler breeders' farms of *Gallus gallus* species, located in this region.

MATERIALS AND METHODS

This study was carried out on 19 laying and 23 broiler breeders' farms located in eastern Spain over a period of 18 mo. The productive categories of commercial poultry included in this study were layer hens and broiler breeders. The main regional poultry companies were involved in the study. All the samples were analyzed at the Centro de Calidad Avícola y Alimentación Animal de la Comunidad Valenciana, located in Spain.

Selection of Target Population

First, to find the target population, all the active farms and flocks located in eastern Spain during previous 12 mo were identified. During this period, 56 active farms with 134 flocks were found for laying hens and 16 active farms with 66 flocks for broiler breeders. A sample size of 26 and 23 flocks of layers and broiler breeders, respectively, was calculated (Table 1). The flock's selection was performed by random simple sampling using a number generator without repetition (http://nosetup.org/), giving a correlative number to each unit.

To calculate the sample size for the study, the active flock was taken as an epidemiologic unit. A 95% of CI was considered, and a prevalence of 10% was expected. The active flocks previously described were taken as population size. The sample size was calculated as follows:

$$n = \left(1 - \ \propto^{1/d}\right) \times \left(N - \frac{d-1}{2}\right)$$

where n = sample size; $\propto$ = type I error = 1 − 95% CI; d = expected prevalence; and N = population size.

Sampling Procedures

The flocks were sampled 3 times at different ages, when it was possible. The first sampling was carried out at 26–28 wk for layers and at 30–32 wk for broiler breeders, coinciding with the laying peak. The second sampling was made 10 wk later for both productive orientations to observe potential antibodies oscillation. And, the last sampling was performed at 60 wk for layers and at 50 wk in broiler breeders, to increase the probability of finding a field strain (Dufour-Gesbert et al., 2006; Seifi and Shirzad, 2012). At each sampling time, 14 serum samples and 10 tracheal swabs with sterile aluminum swabs (Deltalab, Barcelona, Spain) were collected from live birds selected randomly at each flock (Dufour-Gesbert et al., 2006). All collected samples were transported to the laboratory under refrigeration conditions at 0°C to 4°C. The farmer checked the animals daily for symptoms related to *M. synoviae*.

Serology Analysis

Serum samples were processed according to Garcia et al. (2016). During the analysis, the sera samples were maintained under refrigeration conditions at 0°C to 4°C. The serologic analysis was performed by ELISA using a commercial test (BioChek MS ELISA kit; BioChek, ER Reeuwijk, The Netherlands), which is an assay designed to detect *M. synoviae* antibodies in serum. Titers were calculated as described by the manufacturer. Each test sample was diluted (1:500) in sample diluent reagent as per the manufacturer's instructions. Briefly, 100 µL of diluted sample (1:500) was added to the appropriate well. Each sample was run in a single well. The plated was covered, and samples were incubated at room temperature 22°C to 27°C for 30 min. Each well was then washed with 350 µL of wash buffer (4 washing times). Then, 100 µL of conjugate reagent was added into the appropriate wells. The wells were covered and incubated at room temperature 22°C to 27°C for 30 min. Each well was then washed as previously described, and 100 µL of substrate reagent was added to each well. The plate was again covered and incubated for 15 min. Each reaction was quenched with 100 µL of stop solution. Absorbance was measured at 405 nm. Sera with s/*P*-values higher than the cutoff level of 0.5 (titer ≥ 594) were considered positive.

Detection of M. synoviae With PCR Method

Sample Processing All the swabs were stored at −20°C until the PCR analysis. The 10 swabs taken in each flock were divided into 2 pools of 5 swabs. The flock

Table 1. Flock sampling size in layers and breeders according to *Mycoplasma synoviae* expected prevalence.

	Expected prevalence (%)	Active farms	Active flocks	Estimated sample size (number of flocks)	Sample size used[1] (number of flocks)
Laying hens	10	56	134	26	19
Broiler breeders	10	16	66	23	23

[1]Owing to production and biosecurity issues, some layers farms did not participate in the study.

Table 2. Percentage of *Mycoplasma synoviae* positive samples detected by ELISA and PCR.

	Sample, n_T	ELISA, n (%)	PCR, n (%)
Laying hens	19	18 (95)	18 (95)
Broiler Breeders	23	17 (74)	8 (35)

Abbreviations: n, positive samples; n_T, sample size.

was considered positive if at least 1 of the 2 pools tested were positive.

DNA Extraction DNA was extracted from each pool using the QIAmpcadorPathogen Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. First, pools of 5 swabs were diluted in 200 µL of PBS (OXOID, Hampshire, England), which were mixed by pulse vortexing with 20 µL of proteinase K, 1 µL of carrier, and 100 µL of lysis buffer. After incubation for 15 min at 20°C–25°C, the samples were briefly centrifuged to remove drops. Then, 350 µL of buffer ACB were added and mixed thoroughly by pulse-vortexing to adjust the binding conditions for DNA purification. The mixture was put on a 2-mL collection column and centrifuged at 6,000 × *g* for 1 min. The collection tube was washed with 600 µL wash buffer (AW1) and centrifuged as described previously. After a second wash step with 600 µL wash buffer (AW2) and centrifugation at 20,000 × *g* for 2 min, the DNA was eluted from the column by addition of 100 µL elution buffer (AVE) and incubated at room temperature for 1 min. After incubation, the DNA was centrifuged at 20,000 × *g* for 1 min. The DNA extracted was then collected in sterile microtube and preserved at −20°C after PCR analysis.

Real-Time PCR All the PCR were performed with the INgene q DMS VIhA SYBR Detection Real Time-PCR kit (INGENASA, Madrid, Spain), following the manufacturer's recommendations. The PCR was performed in 7,300 Thermocycler (Applied Biosystems) in a total volume of 22 µL reaction mix containing 10 µL of mixture A, 10 µL of mixture B, and 2 µL of extracted DNA. PCR reactions included an initial hot step at 95°C for 5 min and 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s.

DNA Sequence Analysis To confirm the identity of the isolates obtained in this study, 26 PCR-positive *M. synoviae* products were purified with AccuPrep PCR/Gel Purification Kit (Bioneer [Ref: K-3038]) and sequenced. The sequencing step was performed with a BigDye Terminator v3.1.at ABI PRISM H3130 DNA automatic sequencer (Applied Biosystems) and VhlA dir and VhlA rev as primers.

RESULTS

Owing to production and biosecurity issues, some layers selected farms did not participate in the study, so the final number of layer flocks analyzed was 19. All animals were handled as per the principles of animal care published by Spanish Royal Decree 53/2013 (Spain, 2013).

Seroprevalence

Based on ELISA results, the flocks were divided into 3 groups: negatives, positives without vaccination, and positives with vaccination (vaccinated flocks with a suspect titer of infection [Biocheck Interpretation and Application of Results Manual, Biocheck, 2018]). The flocks without vaccination with 10% or more positive reactions were considered positive serologically (Kleven and Bradbury, 2008). Veterinary services of vaccinated flocks reported that vaccination was carried out with Vaxsafe MS H vaccine (Bioproperties Ltd., Ringwood, Victoria, Australia) and was applied intraocularly at 1 d of life. The vaccination reported for broiler breeders' flocks was 7 of 23 (30%). No vaccination was reported in laying hens.

The ELISA applied on sera samples from the analyzed flocks demonstrated a high presence of antibodies against to *M. synoviae*. Table 2 shows the results obtained from samples collected without considering vaccination, showing 18 of 19 (95%) and 17 of 23 (74%) *M. synoviae* seroprevalence in laying hens and broiler breeders, respectively. Regarding age-wise analysis, the positive flocks' rates seemed to be higher as the age of sampling increased. In addition, the positive sera rates seemed to be also higher in laying hens than in broiler breeders (Table 3).

Prevalence

The *M. synoviae* detection was made by PCR from samples of the positive groups as per ELISA results (Table 2). From 35 positive flocks screened in this study by ELISA, *M. synoviae* was detected in 18 of 18 (100%) flocks of laying hens, and in 8 of 17 (47%) broiler breeders. Considering all the flocks, the prevalence was

Table 3. *Mycoplasma synoviae* seroprevalence as per sampling time.

	Layers						Broiler breeders					
Sampling time[1]	Tested sera	Positive sera	Positive rates (%)	Flocks	Positive flocks	Positive rates (%)	Tested sera	Positive sera	Positive rates (%)	Flocks	Positive flocks	Positive rates (%)
First	168	105	63	13	10	77	294	100	34	21	8	38
Second	196	179	91	17	16	94	308	111	36	22	12	55
Third	98	97	98	10	10	100	322	118	37	23	13	57

[1]The first, second, and third sampling time was 26–28, 36–38, and ≥ 60 wk of age for layers hens and 30–32, 40–42, and ≥ 50 wk of age for broiler breeders, respectively.

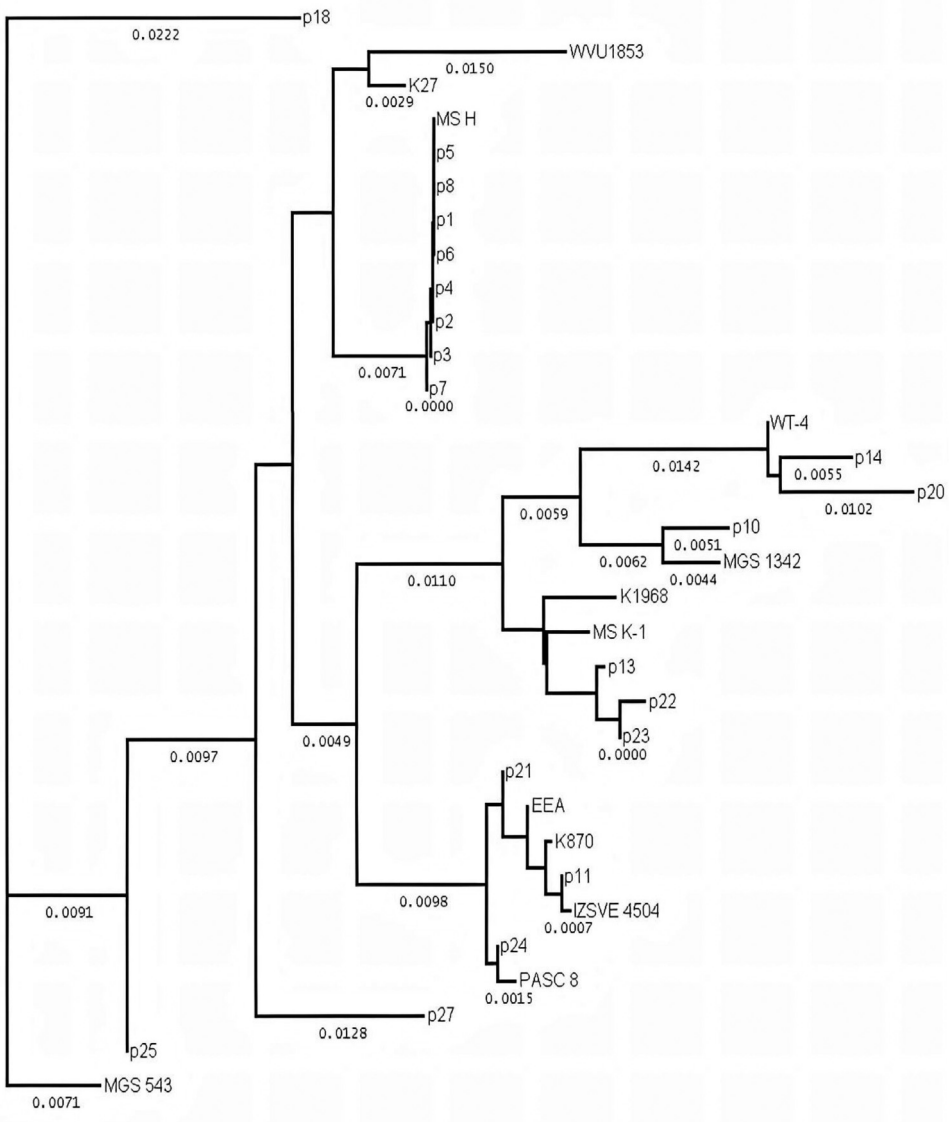

Figure 1. Dendrogram of 20 *Mycoplasma synoviae* PCR positive samples. The field strains isolated were 11 (IZSVE/4504 [1 of 11], MSK-1 [4 of 11], MGS 1342 [1 of 11], MGS 543 [1 of 11], PASC 8 [3 of 11], WT4 [1 of 11]). The vaccine strains MS H were 9.

observed in 18 of 19 (95%) flocks in layers and 8 of 23 (35%) flocks in broiler breeders.

Genotypes

The sequencing of the vlhA gene was carried out from the 26 *M. synoviae* PCR positive samples (18 from layers hens; 8 from broiler breeders), including samples from vaccinated flocks (Table 2). Different genotypes were found in laying hens, 11 of 18 (61%) being field strains, 1 of 18 (5%) being vaccine strain (MS H), and the rest of samples (6 of 18 [33%]) could not be completely sequenced owing to a low quantity and/or quality of DNA obtained (Figure 1). The field strains isolated were IZSVE/4504 (1 of 11), MSK-1 (4 of 11), MGS 1342 (1 of 11), MGS 543 (1 of 11), PASC 8 (3 of 11), and WT4 (1 of 11). Only 1 genotype was found in broiler breeders, all being (8 of 8 [100%]) vaccine strain (MS H).

DISCUSSION

M. synoviae infection was first reported in America (Sun et al., 2017), after that, researchers of different countries with an importance in the poultry industry came to confirm the presence of this pathogen. In eastern Spain, there were no previous studies about the seroprevalence and prevalence of *M. synoviae*. Our findings showed a high seroprevalence of *M. synoviae*, being 95% in laying hens and 74% in broiler breeders. Similar results were reported by Kapetanov et al. (2010), who found *M. synoviae* high seroprevalence rates in adult flocks (90%) and in flocks during the rearing period (40%) in 2009 in Serbia. On the other hand, another previous survey reported lower seroprevalences in commercial layers of 69% (Buim et al., 2009) and 53% (Suzuki et al., 2009) measured by ELISA. Likewise, Feberwee et al. (2008) too reported, in the Netherlands, a lower seroprevalence in broiler breeders (35%). These heterogenic results could be explained by the different age of

sampling, the disease pressure or the vaccination seroprevalence differentiation. In our study, the differences found between both productions systems are probably owing to the fact that vaccination against *M. synoviae* is more commonly used in broiler breeder hens (Gharibi et al., 2018) than in laying hens. This way, none of the studied laying hens' farms reported vaccination.

Regarding age-wise analysis, the seroprevalence was numerically higher as the age of sampling increased. In addition, the positive flock rates and seroprevalences were also numerically superior in laying hens with respect to broiler breeders. This finding is in agreement with data of other research groups, who reported seroprevalences of 47.8% in breeder flocks older than 60 wk of age (Seifi and Shirzad, 2012). In line with our findings, Saâdia et al. (2014) demonstrated the increased seroprevalence of *M. synoviae* with the age, 16th wk 64%, 32th wk 82%, and 56th wk 100%. In the study by Hagan et al. (2004), seropositivity was associated with age as a risk factor. Moreover Xue et al. (2017), reported increasing rates regarding age-wise, with 1- to 3-day-old chicks that reported seroprevalence from 29 to 54%, comparing with the rates of 35-wk-old chicks that reached 71 to 83%.

Concerning prevalence, few studies reported prevalence in laying and breeding hens by PCR. We found a higher one (95%) in layers (identical to seroprevalence) and a lower one (35%) in broiler breeders. Those prevalence results overcome the expected prevalence and thus validate the sampling size calculation. In this regard, Gharibi et al. (2018) reported 72% of prevalence in breeders' flocks in Iran; Buim et al., 2009, isolated rates of 72.7% of *Mycoplasma* spp. with the predominance of *M. synoviae*. Similarly, in a study conducted by Köhn et al. (2009), 84% of commercial layer flocks during laying period were positive for *Mycoplasma* in PCR test, 75% being *M. synoviae*.

The genetic analysis showed that the prevalence detected in broiler breeders was owing to vaccination (MS H strain), so no field strain was present in these flocks. This fact is in line with the high prevalence of vaccine strain that has been recently reported (Zhu et al., 2018). In contrast, most of strains detected in layer hens were classified as field strains, and only 1 strain was from vaccine origin, although no vaccination in layer was reported on the survey. Possibly, cross contamination occurred at this farm or vaccination reporting was omitted to this study. The wide variation of *M. synoviae* genotypes has also been observed by other authors (Felice et al., 2020). Kursa et al. (2019) described common strains to those found in our study as MSK-1 in Poland, whose first description was in the United Kingdom. The PASC8 strain has also been described by Catania et al. (2016) in Italy, where correlation between the eggshell apex abnormality and the presence of this strain in the oviducts was observed. However, in our study, this farm did not report symptoms or eggshell apex abnormalitycaused by this strain. These opposite results between layers and broiler breeders could be originated because of the different biosecurity measures and vaccination programmes. Among field strains, 6 different genotypes were detected, suggesting a wide variation in strains within the same *Mycoplasma* species. The phylogenetic analysis helps to determine the origin of the strain but not to predict its pathogenicity (Sun et al., 2017).

In conclusion, this study is the first to describe seroprevalence, prevalence and phylogenetic analysis of *M. synoviae* in layers and broiler breeders reared in eastern Spain. Our findings demonstrate the usefulness of monitoring flocks to help to control and prevent the presence of *M. synoviae* in the poultry industry. Likewise, vaccination in broiler breeders could be an effective prevention strategy that could be extended to layer hens.

ACKNOWLEDGMENTS

This work was supported by Centro de Calidad Avícola y Alimentación Animal de la Comunidad Valenciana (CECAV) and Asociación Avícola Valenciana (ASAV). We thank Joaquín Girón (Merck Sharp & Dohme, MSD) and Jose Luis Balaguer (CEVA) for their technical and economic assistance.

DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

Biocheck, Biocheck Interpretation and application of results manual, Accessed Mar. 2019. https://www.biochek.com/wp-content/uploads/2018/01/BIOCHEK-INTERPRETATION-Manual.pdf?x14571

Buim, M. J., E. Mettifogo, J. Timenetsky, S. Kleven, and A. J. Piantino Ferreira. 2009. Epidemiological survey on Mycoplasma gallisepticum and *M. synoviae* by multiplex PCR in commercial poultry. Pesq. Vet. Bras. 29:552–556.

Catania, S., D. Bilato, F. Gobbo, A. Granato, C. Terregino, L. Iob, and R. A. J. Nicholas. 2010. Treatment of eggshell abnormalities and reduced egg production caused by Mycoplasma synoviae infection. Avian Dis. 54:961–964.

Catania, S., F. Gobbo, D. Bilato, L. Gagliazzo, M. L. Moronato, C. Terregino, J. M. Bradbury, and A. S. Ramírez. 2016. Two strains of Mycoplasma synoviae from chicken flocks on the same layer farm differ in their ability to produce eggshell apex abnormality. Vet. Microbiol. 193:60–66.

Dufour-Gesbert, F., A. Dheilly, C. Marois, and I. Kempf. 2006. Epidemiological study on Mycoplasma synoviae infection in layers. Vet. Microbiol. 114:148–154.

Ewing, M. L., L. H. Lauerman, S. H. Kleven, and M. B. Brown. 1996. Evaluation of diagnostic procedures to detect Mycoplasma synoviae in commercial multiplier-breeder farms and commercial hatcheries in Florida. Avian Dis. 40:798–806.

Feberwee, A., T. S de Vries, and W. J. Landman. 2008. Seroprevalence of Mycoplasma synoviae in Dutch commercial poultry farms. Avian Pathol. 37:629–633.

Feberwee, A., C. J. Morrow, S. A. Ghorashi, A. H. Noormohammadi, and W. J. Landman. 2009. Effect of a live Mycoplasma synoviae vaccine on the production of eggshell apex abnormalities induced by a *M. synoviae* infection preceded by an infection with infectious bronchitis virus D1466. Avian Pathol. 38:333–340.

Felice, V., C. Lupini, G. Mescolini, F. Silveira, A. Guerrini, E. Catelli, and A. Di Francesco. 2020. Molecular detection and characterization of Mycoplasma gallisepticum and Mycoplasma synoviae strains in backyard poultry in Italy. Poult. Sci. 99:719–724.

Garcia, C., J. M. Soriano, and P. Catala-Gregori. 2016. Development of a seroprevalence map for Mycoplasma gallisepticum in broilers and its application to broilers from Comunidad Valenciana (Spain) over the course of two years (2009–2010). Vet. Med. 61:136–140.

Gharibi, D., R. Ghadimipour, and M. Mayahi. 2018. Detection of Mycoplasma Gallisepticum and Mycoplasma Synoviae Among Commercial Poultry in Khouzestan Province, Iran. Arch. Razi Inst. 73:139–146.

Gole, V. C., K. K. Chousalkar, and J. R. Roberts. 2012. Prevalence of antibodies to Mycoplasma synoviae in laying hens and possible effects on egg shell quality. Prev.Vet. Med. 106:75–78.

Hagan, J. C., N. J. Ashton, J. M. Bradbury, and K. L. Morgan. 2004. Evaluation of an egg yolk enzyme-linked immunosorbent assay antibody test and its use to assess the prevalence of Mycoplasma synoviae in UK laying hens. Avian Pathol. 33:93–97.

Kapetanov, M., D. Orlic, D. Potkonjak, and M. Velhner. 2010. Mycoplasma in poultry flocks in the year 2009 compared to the year 2000 and significance of the control measures in Serbia. Lucrari Sci. Med. Vet. 43:249–253.

Köhn, S., J. Spergse, C. Ahlers, M. Voss, T. Bartels, R. Rosengarten, and M. E. Krautwald-Junghanns. 2009. Prevalence of Mycoplasmas in commercial layer flocks during laying period. Berl. Munch. Tierarztl. Wochenschr. 122:186–192.

Kleven, S. H., and J. M. Bradbury. 2008. Avian mycoplasmosis (*Mycoplasma gallisepticum, M. synoviae*). Pages 482–496 in OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees). OIE Standards Commission. ed. Office International des Epizooties, Paris, France.

Kursa, O., G. Tomczyk, and A. Sawicka. 2019. Prevalence and phylogenetic analysis of mycoplasma synoviae strains isolated from Polish chicken layer flocks. J. Vet. Res. 63:41–49.

Landman, W. J. M., D. J. Mevius, K. T. Veldman, and A. Feberwee. 2008. In vitro antibiotic susceptibility of Dutch Mycoplasma synoviae field isolates originating from joint lesions and the respiratory tract of commercial poultry. Avian Pathol. 37:415–420.

Landman, W. J. 2014. Is Mycoplasma synoviae outrunning Mycoplasma gallisepticum? A viewpoint from The Netherlands. Avian Pathol. 43:2–8.

Lierz, M., N. Hagen, N. Harcourt-Brown, S. J. Hernandez-Divers, D. Luschow, and H. M. Hafez. 2007. Prevalence of mycoplasmas in eggs from birds of prey using culture and a genus-specific mycoplasma polymerase chain reaction. Avian Pathol. 36:145–150.

Marois, C., F. Oufour-Gesbert, and I. Kempf. 2000. Detection of Mycoplasma synoviae in poultry environment samples by culture and polymerase chain reaction. Vet. Microbiol. 73:311–318.

Nascimiento, E., R. Yamamoto, K. Herrick, and R. Tait. 1991. Polymerase chain reaction for detection of mycoplasma gallisepticum. Avian Dis. 35:62–69.

Nascimiento, E., V. L. A. Pereira, M. G. F. Nascimiento, and M. L. Barreto. 2005. Avian micoplasmosis update. Braz. J. Poult. Sci. 7:1–9.

Saâdia, N., A. Rachid, A. Falak, R. Naoufal, L. A. Idriss, F. F. Ouafaa, and E. H. Mohammed. 2014. Detection of mycoplasma synoviae infection in broiler breeder farms of Morocco using serological assays and real time PCR. J. Life Sci. 8:815–821.

Seifi, S., and M. R. Shirzad. 2012. Incidence and risk factors of Mycplasma synoviae infection in broiler breeder farms of Iran. Vet. World 5:265–268.

Spain Royal Degree 53/2013, 1st of Febrary, por el que se Establecen las Normas Básicas Aplicables Para la Protección de los Animales Utilizados en Experimentación y Otros Fines Científicos, Incluyendo la Docencia. 2013. Boletín Oficial del Estado, Madrid, Spain.

Sun, S., X. Lin, J. Liu, Z. Tian, F. Chen, Y. Cao, J. Qin, and T. Luo. 2017. Phylogenetic and pathogenic analysis of Mycoplasma Synoviae isolated from native chicken breeds in China. Poult. Sci. 96:2057–2063.

Suzuki, K., J. Origlia, F. Alvaez, M. Faccioli, M. Silva, J. Caballero, L. Nunrez, and L. Castro. 2009. Relative risk estimation for Mycoplasma synoviae in backyard chickens in Paraguay. Int. J. Poult. Sci. 8:842–847.

Xue, J., M. Y. Xu, Z. J. Ma, J. Zhao, N. Jin, and G. Z. Zhang. 2017. Serological investigation of Mycoplasma synoviae infection in China from 2010 to 2015. Poultry Science. 96:3109–3112.

Zhu, L., M. A. Shahid, J. Markham, G. F. Browning, A. H. Noormohammadi, and M. S. Marenda1. 2018. Phylogenetic and pathogenic analysis of Mycoplasma Synoviae isolated from native chicken breeds in China. BMC Genom. 19:117.