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

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**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

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**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 (Nascimento 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
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 (Nascimento 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 included in this study were layer hens and broiler breeders. 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*.

**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:

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

where $n =$ sample size; $\alpha =$ 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 kit (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 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 $\geq 594$) were considered positive.

**Detection of *M. synoviae* With PCR Method**

**Sample Processing** All the swabs were stored at $-20\degree$C until the PCR analysis. The 10 swabs taken in each flock were divided into 2 pools of 5 swabs. The flock

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**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 useda (number of flocks) |
|-----------|-------------------------|--------------|---------------|------------------------------------------|---------------------------------------|
| Laying hens | 10                      | 16           | 66            | 23                                       | 19                                    |
| Broiler breeders | 10                    | 56           | 134           | 26                                       | 19                                    |

---aOwing to production and biosecurity issues, some layers farms did not participate in the study.
was considered positive if at least 1 of the 2 pools tested were positive.

**DNA Extraction** DNA was extracted from each pool using the QIAamp 

dotPathogen 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 VhA 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 VhA dir and VhA 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

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

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

**Abbreviations:** n, positive samples; nT, sample size.

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

| Sampling time | Layers | Broiler breeders |
|---------------|--------|-----------------|
|               | Tested sera | Positive sera | Positive rates (%) | Flocks | Positive flocks | 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 |

1The 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.
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), 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 heterogeneous results could be explained by the different age of...
sampling, the disease pressure or the vaccination sero-prevalence 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, Saadía 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), sero-positivity 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 Köhm 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 PASCs 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 abnormalities caused 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.

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**DISCLOSURES**

The authors declare no conflicts of interest.

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