Epidemiological survey on Mycoplasma synoviae *infection in Portuguese broiler breeder flocks*

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Broiler breeder, ELISA, Epidemiology, *Mycoplasma synoviae*, Mycoplasmosis, PCR.

Summary
Since modernization and expansion of the poultry industry, infections with *Mycoplasma* spp. bacteria have been reported as a cause of considerable economic losses. The prevalence of *Mycoplasma synoviae* infection in 974,000 Portuguese broiler breeders, belonging to 36 flocks, was investigated from December 2008 to March 2012. This study was conducted using a commercial indirect enzyme-linked immunosorbent assay (ELISA) for the analysis of serum antibodies, and a polymerase chain reaction (PCR) for the tracheal tissue. Twenty-four flocks were simultaneously found positive by ELISA and PCR [66.7%, 95% confidence interval (CI): 43.5-76.9%]. The *M. synoviae* prevalence among chickens averaged 40.3% (483/1,200), with values ranging from 0.0 to 83.3% per flock. The prevalence of farms where *M. synoviae* positive birds have been found was determined in different poultry categories such as density, biosecurity, strains, offspring quality, premises'age, and others husbandry factors. Prevalence values were significantly higher among birds housed in new facilities (less than 3 years old) and were also significantly higher in the production period. The high prevalence of *M. synoviae* infection detected in the present study suggests the need to adopt appropriate control measures.

Indagine epidemiologica sulle infezioni da Mycoplasma synoviae *in allevamenti di polli da riproduzione in Portogallo*

Parole chiave
ELISA, Epidemiologia, *Mycoplasma synoviae,* Micoplasmosi, Polli da riproduzione, PCR.

Riassunto
Le infezioni da *Mycoplasma* spp. sono causa di notevoli perdite economiche per il settore avicolo. Questo studio, condotto nel periodo dicembre 2008 e marzo 2012, ha riguardato la prevalenza di infezione da *Mycoplasma synoviae* in 974.000 allevamenti di polli da riproduzione in Portogallo, appartenenti a 36 allevamenti. Lo studio è stato condotto utilizzando un'ELISA indiretta (kit commerciale), per l'analisi degli anticorpi e un saggio PCR (reazione a catena della polimerasi), per l'analisi del tessuto tracheale. Ventiquattro allevamenti sono risultati simultaneamente positivi all'ELISA e al test PCR [66,7%, intervallo di confidenza 95% (CI): 43,5-76,9%]. La prevalenza di *M. synoviae* tra gli animali è risultata in media pari al 40,3% (483/1.200), con valori tra 0,0% e 83,3% per allevamento. La prevalenza di allevamenti in cui sono stati trovati animali positivi a *M. synoviae* è stata determinata valutando i seguenti fattori di allevamento: densità dei polli, biosicurezza, ceppi del microrganismo, qualità dei pulcini, età dei locali ecc. I valori di prevalenza sono risultati significativamente più alti tra gli animali alloggiati in nuove strutture (meno di 3 anni) e nel periodo di produzione. L'alta prevalenza di infezione da *M. synoviae* rilevata in questo studio suggerisce la necessità di adottare adeguate misure di controllo.

Introduction

Several mycoplasmas (genus *Mycoplasma*) are pathogens of mammals, birds, reptiles, fish, and arthropods, they cause a wide variety of diseases and mainly affect respiratory and genital tracts, as well as joints (Vogl *et al.* 2008). Infections with *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are considered endemic in the poultry industry in several countries, where they cause considerable economic losses to heavy breeders, broilers, and layers (Kleven 2008, Noormohammadi 2007). The failure to eradicate *M. gallisepticum* and *M. synoviae* from commercial poultry flocks has been largely due to the ability of these organisms to establish lifelong infections and to spread both by horizontal and vertical transmission among their hosts (McAuliffe *et al.* 2006, Butcher and Jacob 2009). *Mycoplasma synoviae* most frequently occurs as a subclinical or inaparent infection of the upper respiratory tract. Nevertheless, this agent can also cause an infectious synovitis. In both cases, infection with *M. synoviae* might result in a decrease of egg production rate, growth and hatchability rates, and in a downgrading of carcasses at slaughter due to airsacculitis and arthritis (Fiorentin *et al.* 2003, Kleven 2003b, Peebles *et al.* 2011). In recent years, the occurrence of arthropathic and amyloidogenic strains of *M. synoviae*, as well as strains that induce eggshell apex abnormalities and egg production losses, has increased the economic impact of this pathogen (Feberwee *et al.* 2008). *Mycoplasma synoviae* can be found in eggs laid by infected breeders. Although this vertical transmission route is regarded as not very efficient, a higher shed of the organism may occur if immunosuppression factors are present (Behbahan *et al.* 2005, Dhondt *et al.* 2007). Diagnosis is based on epidemiological data, clinical signs, analysis of macroscopic lesions, specific serology, and isolation and molecular characterization of *Mycoplasma* spp. Monitoring must be part of control programs performed in breeder flocks and is mostly feasible by routine serology and polymerase chain reaction (PCR) (Feberwee *et al.* 2005, Luciano *et al.* 2011).

As data are lacking in Portugal, the aim of this study was to assess the prevalence of *M. synoviae* in different production systems of commercial poultry breeder flocks, by means of specific serology and PCR.

Materials and methods

Flocks and birds

A cross-sectional investigation was conducted between December 2008 and March 2012 to determine positivity to *M. synoviae* among non-vaccinated broiler breeder flocks. Among the 36 poultry farms under assessment in the present study, 8 were located in the North, 24 in the Center and 4 in South Portugal. The study area comprises more than 75% of the poultry business in the country. The number of flocks studied from each poultry farm varied between 2 and 4. The study was carried out in a total of 974,000 birds: 13 flocks were Ross 308 breeder (n = 385,000 birds), 20 were Cobb 500 breeder (n = 535,000) and 3 were Hubbard breeder (n = 54,000). Flock size ranged from 15,000 to 30,000 birds (average per flock: 27,000). A total of 1,200 serum samples were collected from the 36 breeder flocks. The sampled animals aged from 1 to 60 weeks. Serum samples were obtained in the rearing and production sites by collecting 30 to 70 samples per flock. This sample size has been based on an expected prevalence ranging from 5 to 15%, an accepted absolute error of 8-12% and a confidence level of 95% (Thrusfield 2007).

Flocks found seropositive were subject to PCR analysis. Samples of tracheal tissue were collected for PCR from freshly dead hens. Samples from at least 6 birds were pooled for analysis. Data on the health management and risk factors were recorded from all flocks.

Serology

Blood samples (2 ml per bird) were asseptically collected from the wing veins. Serum samples were tested for antibodies to *M. synoviae* by an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (BioChek© MS Antibody Test Kit, Gouda, Holland). This serological kit uses a highly purified recombinant antigen protein, which is present in all known *M. synoviae* strains. The BioChek© MS Antibody Test Kit is highly sensitive for early detection of antibodies to *M. synoviae* (>98% sensitivity). Briefly, 100 µl of each serum sample diluted at 1:500 was tested. An undiluted and diluted control was distributed in duplicate in each plate. Samples are incubated for 30 minutes at 18-26 °C. Antibodies to *M. synoviae* will bind and form an antigen-antibody complex. Non-specific antibodies and other serum proteins are then washed away with 350 µl of distilled water (4 repeats). Anti-chicken immunoglobulin (Ig) G labelled with the enzyme alkaline phosphatase was then added to the wells and incubated for 30 minutes at 18-26 °C. An additional wash was carried out to remove unreacted conjugate and then substrate added in the form of p-Nitrophenyl Phosphate chromogen followed by incubation for 15 minutes at 18-26 °C. Reaction was quenched with 100 µl of stopping solution, a yellow colour developed in presence of antibodies to *M. synoviae*. Furthermore, the colour intensity directly relates to the amount of

specific antibodies present. Absorbance or optical density values were measured at 650 nM.

Polymerace chain reaction

Field samples were randomly collected from hens not more than 8 hours after natural dead and submitted to a commercial laboratory (Controlvet, Tondela, Portugal). Samples of tracheae (6 trachea portions from 6 birds per seropositive flock) were prepared for PCR as described by Ramírez (Ramírez *et al*. 2006). The amount of the macerate used was 25 mg. Afterwards, the macerate was transferred into the kit lysis buffer. Briefly, 900 ml of initial cell suspensions were centrifuged (12,000 × g, 4 °C, 20 minutes) and the pellets were washed once in 500 µl of phosphate buffer saline (PBS) and resuspended in 20 µl PBS. Samples were heated at 95 °C for 2 minutes. A forward primer (Ms2FF 5'-TAA AAG CGG TTG TGT ATC GC-3') was used with a reverse primer (23SR 5'-CGC AGG TTT GCA CGT CCT TCA TCG-3') targeting the 23S rRNA gene. The concentration of primers was 20 µM. Reaction mixtures contained 2.5 U of *Taq* DNA polymerase (AB Gene), 0.2 µM of Ms2FF primer, 1 x reaction buffer, 1.75 mM MgCl2, 0.2 mM dNTPs, and water up to a volume of 50 µl. One microliter of the template was added to the reaction. Amplification was achieved with a first step at 80 °C for 30 seconds, 5 denaturation cycles at 94 °C for 15 seconds, renaturation at 55 °C for 30 seconds, and elongation at 72 °C for 1 minute, followed by 30 cycles as previously described, but with an extension of 2 seconds per cycle in the elongation step. A 5µl amount of each amplified product was separated by electrophoresis on a 1.5% agarose gel.

Gels were stained with ethidium bromide (3 µg ml^{-1}) and observed under UV light. The positive control was DNA from a *M. synoviae* reference material (*M. synoviae* field isolate) and the negative control was water free from DNases and RNases, instead of the DNA sample. The size of the expected amplicon was 312 bp, which corresponds to the expected band visualized on agarose gel size.

Variables

Number of birds: house capacity was between 10,000 and 25,000 or between 25,000 and 30,000 birds. The offspring quality was good, no sanitary problems were reported in the broiler houses nor was noted any bad offspring quality when sanitary problems were reported in the broiler houses. The broiler breeder strains considered in this study were: Ross, Cobb and Hubbard. As for the region, the poultry houses were located in the North, Center and South Portugal. All the considered premises were new or not older than 3 years and light control was either natural or artificial. The level of biosecurity varied depending on the respect of the relevant rules.

Medication (respiratory signs) were also considered, in cases in which during the flock life at least 1 medication focusing on respiratory signs had been reported and/or if at least 1 medication focuses on enteric or septicemic signs had been reported.

Other variables included in this study are:

- mortality: flock total mortality less than 8% or equal/superior to 8%;
- egg production: total egg production per hen less than the strain standard, equal to the strain standard or more than the strain standard;
- hatchability: flock average hatchability at 60 weeks less than the strain standard, equal to the strain standard or more than the strain standard;
- misshapen eggs: presence of misshapen eggs during flock life more than normal or normal;
- site: rearing houses, period between 0 and 20 weeks, or production houses, period between 20 and 60 weeks.

As for the data analysis, Chi square and Fischer exact tests were used to compare percent results according to independent variables. Analyses were done with SPSS 19.0 software for Windows considering a probability (p) < 0.05 as statistically significant. Whenever appropriate, the exact binomial test established confidence intervals (CI) for the proportions with a 95% confidence level.

Results and discussion

Four hundred and eighty-three birds (40.3%, 95% CI: 8.4-43.1) had antibodies to *M. synoviae*. Out of the 36, both ELISA and PCR found 24 positive flocks (66.7%, 95% CI: 43.5-76.9). Twelve flocks were considered negative, i.e. 5 flocks had seropositivity less than 15% and were PCR-negative, and in seven flocks all tested animals were seronegative. All the 24 flocks with seropositivity higher than 15% were PCR-positive. Table I summarizes these results.

Prevalence values were significantly higher among birds housed in new facilities, less than 3 years old (77.3%). Prevalence was also significantly higher in the production period (multi-age farms) (Table I). This survey reveals a high prevalence of *M. synoviae* in broiler breeder commercial flocks. Positive birds, flocks and farms were found in all the assessed geographical regions of Portugal, which represent the core of the poultry industry in the country. At best of the author's knowledge, no other studies on the prevalence of *M. synoviae* positive poultry farms or regarding different poultry categories in Portugal are currently available.

Table I. *Prevalence of* Mycoplasma synoviae *infection in Portuguese broiler breeder flocks between December 2008 and March 2012.*

Variable/category		Flocks tested (n)	Relative distribution (%)	Positive (n)	Flock prevalence (%)	95% CI (%)
No. of birds	10,000-25,000	8	22.2	7	87.5	47.3-99.7
	25,000-30,000	28	77.8	17	60.7	40.6-78.5
Offspring quality	Good	13	36.1	7	53.8	25.0-80.8
	Bad	23	63.9	17	73.9	51.6-89.8
Strains	Ross	13	36.1	9	69.2	38.6-90.9
	Cobb	20	55.6	13	65.0	40.8-84.6
	Hubbard	3	8.3	2	66.7	9.4-99.2
Region	North	8	22.2	7	87.5	47.3-99.7
	Center/South	28	77.8	17	60.7	40.6-78.5
Premises' age (years)	0-3	22	61.1	17	77.3*	46.5-85.1
	> 3	14	38.9	5	35.7*	12.8-64.9
Light	Natural	18	50	11	61.1	35.7-82.7
	Artificial	18	50	13	72.2	46.5-90.3
Biosecurity	Good	19	52.8	15	78.9	54.4-93.9
	Bad	17	47.2	9	52.9	27.8-77.0
Medication (respiratory signs)	Yes	16	44.4	9	56.3	29.9-80.2
	No	20	55.6	15	75.0	50.9-91.3
Medication (other signs)	Yes	30	83.3	20	66.7	47.2-82.7
	No	6	16.7	4	66.7	22.3-95.7
Mortality	< 8 %	19	52.8	12	63.2	38.4-83.7
	≥ 8 %	17	47.2	12	70.6	44.0-89.7
Egg production	< STD	10	27.8	6	60.0	26.2-87.8
	STD	18	50	13	72.2	46.5-90.3
	> STD	8	22.2	5	62.5	24.5-91.5
Hatchability	< STD	11	30.6	9	81.8	48.2-97.7
	STD	20	55.6	11	55.0	31.5-76.9
	> STD	5	13.9	4	80.0	28.4-99.5
Misshapen eggs	Yes	17	47.2	13	76.5	50.1-93.2
	No	19	52.8	11	57.9	33.5-79.7
Site	Rearing	36	50.0	3	8.3***	1.8-22.5
	Production	36	50.0	24	66.7***	49.0-81.4

* $p = 0.013$; *** $p < 0.001$ (only statistically significant differences are shown).

Mycoplasmas are important avian pathogens, which cause large economic losses in Portugal and worldwide. This investigation, regarding *M. synoviae*, one of the main species of mycoplasmas, was carried out in one of the most important stages of the poultry industry, *i.e.* broiler breeders (Kleven 2008). This type of birds stays long periods in the rearing and production sites. This means that they are exposed to several agents that interfere with their defense system and predispose them to infection. Very often, infections with *M. synoviae* are subclinical, but they still induce damage in the infected hosts and may cause immunosupression (Feberwee *et al.* 2008). If the vertical transmission characteristic of this pathogen is considered, the detected high prevalence might imply a continuous dissemination within the broiler farms. This fact potentially amplifies the prevalence of infection and disease, with an impact on their effects on economical losses (Cobb 2011, Kleven 2003a, Stipkovits *et al.* 2012).

Despite the good level of biosecurity and stringent control of contact routes of Portuguese breeder farms, a considerably high prevalence of *M. synoviae* was found in this study. Therefore, probably culling *M. synoviae*-positive flocks is not a solution to reduce the risk of this *Mycoplasma* transmission, contradicting the conclusions of Buim and colleagues (Buim *et al.* 2009), such an approach is only sustainable if there is a low prevalence of

M. synoviae. So, medication and vaccination may be good alternatives. In this survey, flocks not treated to clinical respiratory signs or other signs had the highest values of prevalence, but this difference was not statistically significant.

Prevalence results (66.7%) in the present study were higher in comparison with those from other studies. In the Netherlands, Feberwee and colleagues (Feberwee *et al.* 2008) found a flock prevalence of 35%. A study in South America found a prevalence of 15% (Buim *et al.* 2009) and, in Middle East, (Amer *et al.* 2012) reported a prevalence rate at 27%. These values suggest that there is an increased prevalence of *M. synoviae* in breeder flocks.

According to Feberwee and colleagues (Feberwee *et al.* 2005) and Luciano and colleagues (Luciano *et al.* 2011), mycoplasmosis diagnosis based only on seroconversion may be inadequate. These authors suggest the adoption of other techniques to confirm the presence of the agent, PCR costs have been decreasing and made it attractive to be established as a routine confirmatory technique (Hammond *et al.* 2009).

The prevalence of *M. synoviae* positive farms was significantly higher in the production site than in rearing site. This fact suggests multi-age farms as the most important variable regarding infection. All the rearing and old production sites were single-age systems and new production sites were all multi-age farms. The failure to eradicate *M. synoviae* in commercial poultry flocks is in part due to the ability of this organism to establish lifelong infections in their hosts and due to the physical design of the modern poultry premises (Kleven 2003b). Another plausible reason for these high values in the production site, when compared with the rearing one, is the fact that the immune system may be down-regulated due to stress factors in this phase. In fact, stress factors in the production site, including male aggressions, intensive egg production and nutricional imbalance, can depress the immune system of the hen predisposing to infections such as that with *M. synoviae* (Peebles *et al.* 2011, Stanley *et al.* 2001, Xavier *et al.* 2011).

In the present study, the prevalence value was significantly higher among birds housed in new facilities (less than 3 year old). The construction of multi-age farms (farms with birds of different ages) and the pathogen's ability to cause lifelong infections and spread by horizontal transmission may also have contributed to the high prevalence of infection. It is known that the distance between flocks can influence positivity to *Mycoplasma* among birds (Feberwee *et al.* 2005). The older facilities with lower biosecurity standards reveal better results, probably because of farm isolation. It has been assumed that *M. synoviae* would spread quickly after introduction on a farm (Hartput *et al.* 1998, Landman and Feberwee 2008). These results suggest that the multi-age housing seen in the newest facilities is more important than facilities' age or biosecurity. Therefore, multi-age farms pose a significant epidemiological risk.

Taking into account that breeders must be free of pathogenic agents, the high prevalence of *M. synoviae* is quite worrying. Vaccines are available, but vaccination is not yet common in Portugal.

Although very strict hygiene rules are being implemented, poultry farms built in the latest years are designed considering mainly an economical perspective and very rarely is disease prevention a primary consideration. The consequence is that farms have grown in size and density, and an ideal environment was created for agents as *M. synoviae* to thrive (Marois *et al.* 2005). Under these circumstances, it is necessary to determine new and more effective strategies to reduce losses due to *Mycoplasma* infections (Kleven 2003b).

Until recently, the economic impact of *M. synoviae* has been considered controversial. Economic loss reports increase every day, including eggshell pathology reports, and this fact develops awareness of poultry community all over the world (Catania *et al.* 2010, Landman and Feberwee 2008).

In conclusion, infections with *M. synoviae* are endemic in broiler breeder flocks in Portuguese poultry farms. This fact should alert animal health authorities to the economical impact of *M. synoviae*. Strategies for the planning and construction of new poultry premises and a raising awareness should be put into practice among the poultry industry.

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