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Two strains of *Mycoplasma synoviae* from chicken flocks on the same layer farm differ in their ability to produce eggshell apex abnormality

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ABSTRACT

Mycoplasma synoviae (Ms) is considered to be an economically important poultry pathogen. Although the full economic costs of infection in layer chickens are still under debate, the prevalence of Ms is known to be high in some countries and earlier reports have shown a correlation between infection and Eggshell Apex Abnormality (EAA). This work is a continuation of an earlier study of a clinical case of EAA on a layer hen farm where the presence of two different strains of Ms, based on the sequence of the 5′ end of the *vlhA* gene, was demonstrated. Both strains could be detected in the trachea but only one (designated strain PASC8) appeared able to colonize the oviduct, while the other (designated TRACH) was not found in the oviduct and has not been related to EAA. The PASC8 partial *vlhA* gene sequence differs from that of the TRACH in having a 39 nucleotide deletion in the proline rich region and three point mutations in the RIII region. Based on this information an experimental infection was performed in SPF chickens using groups infected with either the PASC8 or the TRACH strain and a non-infected control group. Both Ms strains were detected in the trachea of infected birds, but only the PASC8 strain was found in the oviduct. Furthermore, EAA developed only in the group infected with PASC8 strain. Compared to the control group, both strains produced an adverse impact on egg production: a decrease in the numbers laid and in their average weight ($P < 0.05$) This work demonstrates a difference in oviduct tropism between two Ms strains and a possible relationship to the production of EAA in experimental conditions.

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1. Introduction

Mycoplasma synoviae (Ms), together with *Mycoplasma gallisepticum* (Mg), are considered to represent the most economically important avian *Mycoplasma species* in the poultry industry. Ms has been related to respiratory syndrome and articular disease (Kleven, 1997). Its role in chicken meat breeds is well established and largely correlated with productivity and economic losses (Mallinson, 1985). It is also known that Ms is transmitted vertically through eggs (Jordan, 1975) in breeding birds but in the layer sector the role of Ms has emerged relatively recently. Few studies have been conducted on Ms in layers but its involvement in eggshell apex abnormality (EAA) was first reported in the Netherlands (Feberwee et al., 2009) and soon afterwards in other countries around the world (Catania et al., 2010; Strugnell et al., 2011; Gole et al., 2012; Brandão et al., 2014; Jeon et al., 2014; Landman, 2014). This type of shell abnormality has been linked to decreased production, decreased egg quality and increased operating costs (Catania et al., 2010; Landman, 2014).

EAA is thought to be associated with the presence of Ms in the oviduct (Feberwee et al., 2009) but not all Ms strains appear capable of producing this condition (Catania et al., 2010). There are differences in the prevalence of EAA (Catania et al., 2010) which may be explained by the different tropisms of the infecting Ms strains, although this has yet to be fully confirmed. Earlier studies indicated that the prevalence of Ms in the yolk of abnormal eggs was over 50% while normal eggs were negative (Catania et al., 2010). Experimental studies in layers showed synergistic effects of Ms with Infectious Bronchitis Virus (IBV) with an increase in abnormal eggs (Feberwee et al., 2009), and these data are in

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accordance with earlier reports of the increase of pathogenic respiratory and articular effects of Ms in the presence of IBV (Kleven et al., 1972; Landman and Feberwee, 2004).

Epidemiological studies performed in different European countries have shown the high prevalence of Ms in the layer sector (Hagan et al., 2004; Dufour-Gesbert et al., 2006; Feberwee et al., 2008; Gole et al., 2012). However despite the high prevalence of Ms, the incidence of EAA does not appear high (Catania et al., 2010). Bradbury et al. (2003) reported that Ms seropositive layer flocks produced higher numbers of second class eggs but their study did not pinpoint the particular cause of the downgrading.

The *vlh*A protein, which is found in Ms and is an abundant immunodominant surface lipoprotein, is encoded by the *vlh*A gene (Noormohammadi et al., 2000). Various authors have described differentiation of Ms strains based on the sequences of the *vlhA* gene (Benčina et al., 2001; Hammond et al., 2009; El-Gazzar et al., 2012). The N-terminal conserved region of this gene exists in a single copy and the sequence shows high variability among strains. It contains tandem repeats that encode a proline-rich repeats region (PRR) and also a region that is highly polymorphic (RIII) (Benčina et al., 2001).

This paper is the continuation of a previous description of Ms on a layer farm in Italy (Catania et al., 2010) in which Ms was detected in birds from two EAA-affected flocks, but not in an adjacent flock where no eggshell abnormalities were seen. Furthermore, a difference in the prevalence of EAA between the two affected flocks was noted. It was thought that these observations might be explained by the involvement of more than one Ms strain (Catania et al., 2010). In order to investigate this hypothesis, a follow-up study of the farm was carried out. The *vlh*A genes of Ms isolates were analysed and an experimental infection was conducted in SPF chickens using two different isolates in order to see if either or both were able to produce EAA lesions and to evaluate the oviduct tropism under experimental conditions.

2. Materials and methods

2.1. History of the layer flocks

A previous Ms infection was described by Catania et al. (2010) in a layer farm of Hy-Line Brown layers of different ages kept in Northern Italy, where a standard virus but not mycoplasma vaccination programme for layers had been applied. All birds came from Italy but the flocks were from different suppliers. At the onset of the occurrence of EAA only three houses, containing flocks designated 1 (24 weeks of age), 2 (69 weeks) and 3 (37 weeks), were occupied and only flocks 2 and 3 showed abnormal eggs with typical lesions of EAA. Ms was detected by serological, cultural, and molecular techniques only in birds from the two affected flocks 2 and 3, but not in flock 1, where no eggshell abnormalities were seen. There was no evidence of recent infectious bronchitis virus infection by PCR or serological tests. The incidence of EAA was 0.1% in flock 2 and 1.3-1.8% in flock 3, having only a significant effect on production in the latter. Ms was isolated from tracheal samples in both the EAA-affected flocks but was isolated only from oviduct samples of birds from flock 3. In addition, Ms DNA was detected in several yolks of abnormal eggs from the same flock. No gross or microscopic lesions were observed in the oviducts (Catania et al., 2010).

2.2. Sampling procedures for the follow-up study

After the confirmation of MS positive results in the farm (flock 2 and 3), ten bloods and 10 tracheal swabs were collected every two weeks from birds selected at random in flock 1, in order to pinpoint the appearance, if any, of Ms and to carry out a follow-up study. Birds in flock 2 were not sampled as they were at the end of the laying cycle and had been taken for slaughter at 77 weeks, even though some isolates from this flock were from our previous study (Catania et al., 2010) were examined further in this follow-up study (see section PCR and sequencing below).

As soon as we detected MS positives by PCR in flock 1 (by 32 weeks of age), 20 blood samples and 20 tracheal swabs were taken from randomly-selected birds and the same numbers of samples were collected from flock 3 (week 45). In addition, five birds in each flock were blood sampled and selected for necropsy (see below). In flock 3 they were taken from a cage producing abnormal eggs, while in flock 1 the five were taken randomly because no abnormal eggs had been produced. The farmer was stimulated to improve the surveillance to detect the abnormal eggs, in order to estimate the time required to produce abnormal eggs under field conditions. Five more birds were sampled from flock 1 at week 73. During this time 30 abnormal eggs were collected from flock 3, in addition to 30 normal eggs from each of flocks 1 and 3.

2.3. Serology

Sera from blood samples from flocks 1 and 3, were tested by haemagglutination inhibition test (HAI) for IBV using different virus variants (793B, IT02, QX, M41) (Gelb and Jackwood, 1998) and EDS'76). The ELISA (IDEXX®, Westbrook, MA, USA) was used to examine sera for Mg and Ms and the rapid serum agglutination test (RSA) (IDVET, Montpellier, France) was used as an additional test for Ms. (Kleven, 1998)

2.4. Necropsy

The selected birds from flocks 1 and 3 were euthanized for further pathological and microbiological examination. The carcasses from each flock were kept separated and each bird was also examined separately using aseptic technique and a standard protocol was used to examine for macroscopic lesions and for collection of swab samples from the trachea and oviduct for mycoplasma testing and for routine bacteriological examination. Portions of kidney, oviduct, trachea, and lung tissue were submitted to the Virology Department of the Istituto Zooprofilatico della Venezie, Italy, for IBV testing by real-time PCR (Callison et al., 2006). Oviduct tissues were also taken for histopathological examination. Some abnormal hock joints contained excess fluid, which was taken (with a swab) for analysing the presence of Ms by PCR.

2.5. Routine bacteriology, mycoplasma culture and identification

For routine bacteriology, samples from trachea and oviduct were inoculated onto Blood agar (Biolife, Milan, Italy), MacConkey agar (CONDA, Madrid, Spain) and Brain Heart Infusion broth (CONDA, Madrid, Spain), and cultivated at 37 °C in an aerobic, anaerobic and 5% CO_2 environment for 48 h. For mycoplasma culture, tracheal and oviduct swabs were inoculated using a selective broth medium for avian mycoplasmas, modified Frey broth (Frey et al., 1968), and incubated at 37 °C in 5% CO2 conditions for at least 15 days. During this time the cultures were checked daily and when a colour change or turbidity was seen, the broth was inoculated onto a semi-solid medium, if no change was seen after 15 days, an aliquot of the broth was inoculated onto a Frey semi-solid medium. The inoculated agar plates were checked for mycoplasmas daily for 15 days, after this period samples were considered negative. In order to identify the Mycoplasma species, DNAs were extracted from broths of suspect samples, and a 16S-rDNA PCR and denaturing gradient gel electrophoresis (DGGE) method was performed (McAuliffe et al., 2005), whereas the

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