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Seroprevalence of *Mycoplasma synoviae* in Dutch commercial poultry farms

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Running title: *M. synoviae* seroprevalence

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Abstract

Before the year 2000, *Mycoplasma synoviae* was associated mainly with subclinical respiratory infections in broilers in the Netherlands and was considered to have low clinical and economic impact. The subsequent occurrence of *M. synoviae* arthritis and amyloid arthropathy, and eggshell apex abnormalities has resulted in an increasing demand for *M. synoviae* free poultry. Therefore, a cross-sectional seroprevalence study was carried out over a 12 month period during 2005 and 2006. Ten blood samples per farm were generally used because *M. synoviae* was expected to spread quickly. However for grandparent and layer breeder stock 24-60 blood samples per house were available from a voluntary *M. synoviae* monitoring programme. Sera were tested by means of rapid plate agglutination (RPA) test (agglutination at dilution $\geq 1:8$ was considered positive). The numbers of farms sampled out of the national total were: broiler grandparent 53/53, broiler parent rearing 34/150, broiler parent 114/300, broiler 185/800, layer grandparent 13/13, layer parent 40/50, layer 173/1250 and meat turkey 50/75. The seroprevalence of *M. synoviae* in commercial poultry was high, especially in commercial layers where it was 73% (95% C.I. (67-80)); in layer and broiler grandparent stock it was 0% and 10% respectively, based on sample sizes equal to the population size. In layer and broiler parent farms it was 25% (95% C.I. (19-31) and 35% (95% C.I. (28-44)), respectively; while in both broiler parent rearing and broiler farms it was 6% (95% C.I. (0-13) and (3-9), respectively); in meat turkey it was 16% (95% C.I. (10-22)).

Introduction

The economic significance of *M. synoviae* has been a subject of debate for many years but the increasing occurrence in the Netherlands of arthropathic and amyloidogenic *M. synoviae* strains in chickens and turkeys as well as strains that induce eggshell apex abnormalities and egg production losses (Landman & Feberwee, 2001, 2004; van Beek *et al.*, 2002; Feberwee *et al.*, 2007) has increased awareness of the clinical and economic impact of infection with this mycoplasma.

Layer and commercial turkey flocks infected with arthropathic *M. synoviae* strains may suffer losses due to growth retardation and culling of lame birds, while layer flocks infected with *M. synoviae* strains with oviduct tropism may suffer egg production losses directly and indirectly due to deficient eggshell quality. Earlier reports described the occurrence of *M. synoviae*-associated airsacculitis in broilers resulting in increasing condemnations at slaughter (Kleven *et al.*, 1972; Hopkins & Yoder, 1982), while other studies reported a decrease in egg production in layer flocks associated with *M. synoviae* infection (Mohammed *et al.*, 1987; Morrow *et al.*, 1990).

The occurrence of arthropathic *M. synoviae* strains and strains associated with eggshell pathology in the Netherlands has fuelled discussions about an organized control programme for this mycoplasma. However, before starting such a programme it was necessary to determine the prevalence of *M. synoviae* seropositive farms in different poultry categories and to determine the epidemiological risks between the different subsectors in the poultry industry. The seroprevalence of *M. synoviae* in Dutch commercial poultry was therefore determined by means of a rapid plate agglutination (RPA) test on a representative number of blood samples and active commercial poultry farms during a twelve month period.

Materials and Methods

Categories of commercial poultry. The categories of commercial poultry included in this survey were grandparents (layer and meat type production), broiler breeders (rearing and production), layer breeders (production), commercial layers, broilers and meat turkeys. The survey was carried out in the years 2005-2006.

Farm sample size. The number of farms to be investigated in order to assess the prevalence of *M. synoviae* serologically positive farms per poultry category was determined based on an accepted error between 5 and 10 percent. Exceptions were layer breeder stock and grandparent stock as most of the former and all of the latter farms were sampled following a voluntary *M. synoviae* control programme. Therefore, the sample size of these categories of poultry was close or equal to the population size.

The 95% confidence interval (C.I.) for finite population numbers and the difference in 95% C.I. between the different subtypes as well as sample sizes were calculated with Winepiscopo 2.0 (Thrusfield *et al.*, 2001).

Serum samples from grandparent and layer breeder flocks. The serological data of layer breeder and grandparent stock (production layer and meat type) were derived from the voluntary *M. synoviae* organized disease control programme. The aim of this programme was to detect *M. synoviae* positive flocks soon after introduction of the infection. Therefore, grandparent stock was monitored every 4 weeks (starting at 20 to 22 weeks of age until the end of the production period) by collecting 30 to 60 serum samples per house and layer breeder stock was tested at intervals of 4 to 8 weeks (starting at 20 to 22 weeks of age until the end of production) by collecting 24 to 60 samples per house. Both regimes aimed at detecting a prevalence of *M. synoviae* antibodies of between 5 to 15% with 95% C.I. (Cannon

& Roe, 1982). The data were derived from one year of monitoring and included multiple submissions from the same farms.

Serum samples from other poultry categories. Ten serum samples per farm were randomly collected from submissions to obligatory monitoring programmes (e.g. salmonella, *M. gallisepticum*). In contrast to grandparent and layer breeder stock only one submission per farm was analyzed. Another difference was that sampling was limited to one poultry house per farm, aiming at detection of seroprevalence at farm level of 25 to 30% with 95% C.I. It was assumed that *M. synoviae* would spread quickly after introduction to a farm.

The broiler breeder rearing and the commercial laying flocks were sampled at the end of rearing or production period, respectively while broilers and turkeys were sampled at slaughter age. Samples from meat type breeder flocks were taken during the production period. *M. synoviae* was expected to have enough time to spread at the farm level. The data were obtained in the same time period as for grandparent and layer breeder stock; however the samples were collected during two monitoring periods of 2 to 3 months each.

Serological testing. The *M. synoviae* RPA test (Intervet International, Boxmeer, the Netherlands) as described by Feberwee *et al.* (2005) was used to assess the presence or absence of *M. synoviae* antibodies. A sample was regarded as *M. synoviae* positive if agglutination at dilution 1:8 or higher was observed (Feberwee *et al.*, 2005).

All samples originating from grandparent and layer breeder stock were first tested at a dilution of 1:2. Subsequently, if present, 10 clearly positive samples were serially diluted from 1:4 to 1:32 in 0.5 M phosphate buffered saline pH 7.2 (PBS) and re-tested. Serum samples from the other categories of poultry were tested at a dilution of 1:8. The flock was considered to be positive for *M. synoviae* if two or more serum samples showed agglutination at a dilution of 1:8 or higher.

Results and Discussion

The choice of the RPA test to study the seroprevalence of *M. synoviae* in Dutch commercial poultry was based on previous research in which the specificity and sensitivity of this laboratory test was compared to that of culture, PCR and various commercial ELISA kits and found to perform equally as well as ELISA kits (Feberwee *et al.*, 2005).

The seroprevalence of *M. synoviae* positive farms was significantly lower in layer type grandparent farms than that in the meat type grandparent stock (layer type: 0/13 (= 0%) and meat type: 5/53 (= 10%)), with sample sizes equal to the population size. However, for meat grandparent stock from the total submissions investigated (1072) a low number of *M. synoviae* positive submissions were found (15 (1.4 %)). Most *M. synoviae* seropositive submissions originated from meat type grandparent flocks of ≥ 51 weeks of age (66% of positive flocks).

M. synoviae can be transmitted vertically and horizontally (Kleven, 2003; Stipkovits & Kempf, 1996). The high level of biosecurity, stringent control of contact routes and geographical isolation of grandparent farms minimize the risk of introducing *M. synoviae* to these farms through direct and indirect contact explaining the low prevalence found. Furthermore, culling of *M. synoviae* positive flocks reduces the risk of vertical transmission of this mycoplasma to the offspring, which is reflected in the low seroprevalence of *M. synoviae* in rearing breeder stock (2/34 (= 6% (95% C.I. (0-13))). As the risk of vertical transmission is minimized by culling *M. synoviae* infected grandparent stock, horizontal transmission is expected to be the most important transmission route for infected rearing breeder stock.

The prevalence of *M. synoviae* serologically positive farms was significantly higher in meat production breeder stock (40/114 (= 35% (95% C.I. (28-44))) than in meat rearing breeder stock. Here horizontal transmission is also regarded as the most important infection

route. The prevalence of *M. synoviae* positive layer breeder farms was significantly lower (10/40, 25% (95% C.I. (19-31))) than that of broiler breeder stock, possibly due to the voluntary *M. synoviae* monitoring programme aimed at detecting *M. synoviae* infection as early as possible. The number of positive submissions 23/538 (4.2%) was low. Most *M. synoviae* seropositive submissions originated from layer type breeder flocks of ≥ 51 weeks of age (60% of positive flocks).

The prevalence of *M. synoviae* serologically positive farms in commercial layer stock was high and significantly higher than in all other poultry categories (127/173 (= 73% (95% C.I. (67-80))) (Table 1). This finding is in agreement with data of other research groups. The prevalence study of Hagan *et al.* (2004), which was based on the detection of *M. synoviae* antibodies in eggs, reported a prevalence of 78.6% in commercial layer flocks in East England. In another study (Mohammed *et al.*, 1986) a *M. synoviae* prevalence of 87% was found in commercial layer flocks in Southern California. The infection was associated with older flocks that had been moulted or frequently medicated. The high prevalence and persistence of *M. synoviae* infections in layer stock can be explained by the frequent occurrence of multiple age housing and lower biosecurity standards in this sector (Stipkovits & Kempf, 1996; Kleven, 2003). *M. synoviae* infected commercial layer stock therefore pose a significant epidemiological risk for other categories of poultry.

The estimated prevalence of *M. synoviae* positive flocks in broilers and turkey stock was unexpectedly low, being 11/185 (= 6% (95% C.I. (3-8))) and 8/50 (= 16% (95% C.I. (10-22))) respectively. However, the *M. synoviae* prevalence was probably underestimated in these poultry subtypes. The frequent use of antibiotic treatments in both turkeys and broilers, and the short life span of broilers may have influenced the prevalence found. Additionally, in turkeys serology is less sensitive than culture and PCR, as the antibody response to *M. synoviae* in these birds may be weak. This was illustrated previously in a study where 89% of

the *M. synoviae* infected turkeys examined were positive by culture, while only 58% developed a detectable antibody response (Ortiz & Kleven, 1992). More recently van Beek *et al.* (2004) showed in a longitudinal field study of meat type turkeys that the serological response against *M. synoviae* occurred 6 to 8 weeks after the first *M. synoviae* PCR positive test result was obtained from tracheal swabs. However, culture and PCR are more laborious and costly to use in a monitoring programme.

The accuracy of the estimated *M. synoviae* seropositive farms is dependent on the number of farms sampled, the number of birds sampled per farm and the sampling frequency at a given flock seroprevalence. Moreover, test characteristics such as specificity and sensitivity will also determine the accuracy of the seroprevalence found.

As for the number of farms sampled, most layer breeder and all grandparent farms were monitored. Regarding the other farm subtypes, the number of farms to be monitored (sample size with an accepted error of 5 to 10%) was calculated with Winepiscopes 2.0 based on an estimation *a priori* of the prevalence of seropositive farms out of total (Table 1). The advance estimation was made based on personal field experience and previously obtained monitoring results from voluntary *M. synoviae* control programmes.

As mentioned earlier, the number of birds sampled per farm and the sample frequency per flock may also influence the accuracy of the detected seroprevalence. In this study there was a difference in sample size and sampling frequency between different poultry categories. The larger number of samples and higher sample frequency that was used for grandparent and layer breeder stock could have influenced the flock specificity of the test. It is well known that the predictive value of an individual positive test result is reduced (number of false positives increases) by using a greater number of samples while retaining the same cut off (agglutination at dilution 1:8 or higher) to classify a flock as *M. synoviae* positive (Martin *et al.*, 1992). Due to this effect, the calculated seroprevalence may have been overestimated in

those poultry categories which were monitored using a larger number of samples and at a higher frequency. A field study carried out in 2001 on 22,393 Dutch field sera using *M. synoviae* RPA with undiluted sera and dilutions of 1:2, 1:4 and 1:8 showed a test specificity of 97.6%, 99.0%, 99.7% and 99.9% respectively (Feberwee, 2006). Subsequently, using HERDACC 3.0 (Jordan, 1995), the specificity expected in a herd of 1000 individuals using a test with a specificity of 99.9% would be 99.0% if 10 animals are sampled and 94.0% if 60 birds are sampled. However, HERDACC 3.0 calculates herd specificity using one positive sample, while in our study two or more positive samples were used as the criterion. This means that the herd specificity in our study is higher than that calculated with HERDACC 3.0. Furthermore, the herd sensitivity will be dependent on seroprevalence at a flock level and the number of samples taken. Using HERDACC 3.0 including a known *M. synoviae* RPA test with a sensitivity of 80% and a cut off of 1:8 or higher (Feberwee *et al.*, 2005), the expected sensitivity in a herd of 1000 individuals will be 34% if 10 animals are sampled and 93% if 60 birds are sampled at a seroprevalence of 5%. If the seroprevalence is much higher (50% or higher) as expected in fast spreading infections, which is the normal for *M. synoviae*, the expected herd sensitivity will be 99% or 100% if 10 or 60 birds are sampled, respectively.

It was assumed that *M. synoviae* would spread quickly after introduction to a farm. This was based on field experience and the knowledge that lateral spread of *M. synoviae* is rapid by both direct contact and between cages in the same room (Kleven, 2003). However, there are also studies describing the occurrence of slow spreading (atypical) strains or strains producing low RPA titres (Weinack *et al.*, 1983; Kleven, *et al.*, 2007). Using HERDACC 3.0 and including a known *M. synoviae* RPA test sensitivity of 52 % at cut off of 1:8 or higher for a Dutch slow spreading *M. synoviae* strain (Feberwee *et al.*, 2005), the expected sensitivity in a herd of 1000 individuals would be 24% if 10 animals are sampled and 80% if 60 birds are

sampled at a seroprevalence of 5%, while the herd sensitivity would be 95% and 100%, respectively in case of 50% seroprevalence.

In conclusion, although the occurrence of over- and underestimates of *M. synoviae* seroprevalence cannot be excluded the effect of overestimation due to higher sampling frequency and larger number of samples will have been limited, while underestimation for poultry categories monitored with a lower number of samples will have occurred only if slow spreading *M. synoviae* strains are present or are at low prevalence (start of infection).

M. synoviae control programmes are based on the detection of infection and elimination of *M. synoviae* positive flocks. However, such an approach is only sustainable if there is a low *M. synoviae* prevalence (as found in grandparent stock). Where freedom of *M. synoviae* is not economically sustainable by elimination of infected flocks (as in layers), medication and vaccination may be alternatives. Both can contribute to reduction of clinical signs, vertical transmission and the economic impact of *M. synoviae* infections. The major shortcomings of medication are the inability to completely eliminate *M. synoviae* from infected birds (Whithear, 1996; Kleven, 2003) and the fact that it should be based on MIC profiles, which are costly and laborious to determine and can be performed only in specialized laboratories. Although both, live and dead *M. synoviae* vaccines are commercially available and have been shown to reduce lesions, unlike *M. gallisepticum* no studies have been published on the quantitative effect of these vaccines on the horizontal spread and reduction of shedding of *M. synoviae* (Whithear, 1996; Kleven 2003; Feberwee *et al.*, 2005, 2006a,b). Furthermore, the use of *M. synoviae* vaccines in the field has been limited because until recently the clinical and economic impact of *M. synoviae* has been controversial. However, there are increasing numbers of reports documenting economic losses due to respiratory *M. synoviae* strains (Morrow *et al.*, 1990; Lockaby *et al.*, 1998; Kang *et al.*, 2002) and arthropathic strains (Landman & Feberwee, 2001; van Beek *et al.*, 2002; Kleven, 2003). The

recent novel eggshell pathology and concomitant egg production losses due to *M. synoviae* (Feberwee *et al.*, 2007) should be added to this list. These observations have increased awareness that *M. synoviae* can severely affect poultry health and support calls for an organized *M. synoviae* control programme and its maintenance as an OIE listed disease.

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Table 1. M. synoviae seroprevalence and comparison of 95 % confidence intervals (C.I.) of production meat grandparent (MGP), production layer grandparent (LGP), rearing meat parent (MP), production meat parent (MP), production layer parent (LP), commercial layers (CL), broiler and turkey farms.

	A priori expected prevalence (%) ^a	Estimated sample size 5-10% error ^b (n farms)	Active Dutch farms	Sample size used (n farms)	M. synoviae serological positive ^c	Prevalence (%) of seropositive farms (95% C.I.)	Comparison of 95 C.I. between poultry categories ^d
Farm^c							
Rearing MP	5	50-17	150	34	2	6 (0-13)	A, D, F, G
Production MP	50	169-73	300	114	40	35 (28-44)	B
Production CL	50	294-90	1250	173	127	73 (67-80)	C
Broilers	50	260-86	800	185	11	6 (3-9)	D
Turkeys	40	63-42	75	50	8	16 (10-22)	E
Farms^{f,g}							
Production MGP	-	-	53	53 ^h	5	10	E, F
Production LGP	-	-	13	13 ^h	0	0	G
Production LP	5	30-14	50	40	10	25 (19-31)	H
Submissions							
Production MGP	-	-	-	1072	15 ⁱ	1.4	-
Production LGP	-	-	-	215	0	0.0	-
Production LP	-	-	-	538	23 ^j	4.2	-

^aA priori expected prevalence used to calculate the sample size with Winepiscope 2.0.

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^bNumber of farms to be investigated based on the *a priori* expected prevalence and an accepted error of 5 to 10%.

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^cTwo or more samples showing agglutination in the *M. synoviae* RPA test at dilution $\geq 1:8$.

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^dRows with a different upper case letter are significantly different from each other based on their C.I.

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^e10 blood samples per farm and two monitoring periods of 3 months within one year.

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^f24 to 60 blood samples per house, every 4 weeks for grandparent stock and every 4 to 8 weeks for layer breeder stock collected during one year.

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^gMore sampling moments per flock during the same period.

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^hSample size equals population size.

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ⁱMost *M. synoviae* seropositive submissions originated from meat-type grand parent flocks of ≥ 51 weeks of age (66% of positive flocks).

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^jMost *M. synoviae* seropositive submissions originated from layer-type breeder flocks of ≥ 51 weeks of age (60% of positive flocks).

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