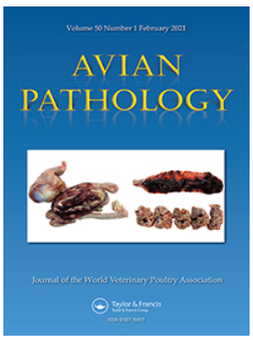

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ORIGINAL ARTICLE

Decrease of *Mycoplasma gallisepticum* seroprevalence and introduction of new genotypes in Dutch commercial poultry during the years 2001–2018

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ABSTRACT

Almost two decades ago, in addition to a compulsory *M. gallisepticum* (Mg) monitoring programme of breeding stock based on European Union regulations, the Dutch poultry industry added national regulations to further reduce the Mg prevalence in Dutch commercial poultry. Currently, all commercial chicken and turkey flocks except broilers are monitored for Mg. All breeding flocks on a farm where one or more flocks tested Mg positive are culled. Mg positive layer pullets are channelled and layer pullets placed on Mg positive multi-age farms are vaccinated. The monitoring data obtained were analysed covering a period of 17 years. Moreover, 31 Dutch Mg isolates from the same period were analysed by multilocus sequence typing (MLST) and compared to available PubMLST data. The results show that in breeding stock the seroprevalence decreased from 1.6% to 0.0%, in commercial layers from 6.3% to 1.9%, and in meat turkeys from 17.6% to 2.4%. The MLST results showed the presence of closely related and identical sequence types (STs) within the different Dutch poultry types. Similar STs were found in Northern and Southern Europe only. The results show a fast decline in the Mg prevalence since 2001, although in layers the Mg prevalence has stabilized and suggests backyard poultry might pose a risk for commercial poultry. The need for Mg control across poultry sectors and in trade was confirmed by the similarity in STs found in different types of poultry and regions. These results from the Dutch poultry industry can be extrapolated to Mg control in general.

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KEYWORDS
Control; epidemiology; monitoring; multilocus sequence typing (MLST); *Mycoplasma gallisepticum*; poultry

Introduction

Mycoplasma gallisepticum (Mg) has been regarded as the most important mycoplasma species in commercial poultry from the clinical and economical point of view. It may cause chronic respiratory disease and significant downgrading of carcasses, but also decreased growth, egg production drops, and impaired hatchability (Mohammed *et al.*, 1987; Stipkovits & Kempf, 1996; Raviv & Ley, 2013). The severity of clinical signs may vary between strains and is also dependent on the occurrence of other respiratory agents and adverse environmental conditions (Stipkovits & Kempf, 1996; Raviv & Ley, 2013). The economic importance of Mg is underlined by its inclusion as an OIE-listed disease (World Organisation for Animal Health, 2020).

In the Netherlands, until two decades ago Mg control was only focussed on breeding stock as prescribed by European Union (EU) regulations. By EU regulations (The Council of the European Union, 1990, 2009) frequent monitoring of breeding stock is compulsory and the trade of hatching eggs or progeny of Mg positive breeding flocks is prohibited within the EU. Hatching eggs from outside the EU may be imported from Mg negative flocks only (The Council of the European Union, 1990, 2009). Mg infected meat turkeys and layers can pose a risk for breeding stock. However, there is no EU regulation for the control of Mg in meat turkeys and layers.

The Dutch poultry industry extended the EU regulations with national regulations (PPE, 1993, 2012; Kamp, 2014), in order to further decrease Mg prevalence in Dutch commercial poultry in general and, as a consequence, reduce the risk of Mg infections in breeding stock. Before 2001, only breeding stock was monitored and Mg positive flocks were culled while Mg negative flocks in other houses of the farm were not. In 2001, the culling of Mg positive breeder flocks was expanded to all flocks on a farm on which one or more flocks tested Mg positive, including flocks which tested negative. Also that year, an additional compulsory monitoring programme was added for replacement breeding stock, commercial layers and layer pullets, and meat turkeys. In 2005–2006, a compulsory Mg vaccination programme for flocks reared for Mg infected multi-age layer farms was introduced as several studies showed that, although Mg vaccination did not prevent colonization of the field strain, it could contribute to the reduction of shedding and transmission and displacement of the

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Mg field strain on a farm (Levisohn & Kleven, 1981; Kleven *et al.*, 1998; Feberwee, Landman *et al.*, 2006; Feberwee, von Banniseht-Wysmuller *et al.*, 2006). At the same time, a compulsory channelling of Mg positive rearing layer flocks to single-age farms was introduced in national regulations. Moreover, information campaigns focusing on the improvement of biosecurity were launched. The data obtained from the Mg seromonitoring after implementation of the different measures were analysed. The yearly prevalence from 2001 up to and including 2018 of Mg in breeding farms, layer farms and meat turkey farms is reported here. Additionally, *M. gallisepticum* isolates collected during the same time period have been used for multilocus sequence typing (MLST) (Bekö *et al.*, 2019) to investigate the genetic diversity and the relatedness of isolates obtained during this period from Mg outbreaks in different poultry types, including backyard poultry and isolates from other geographical areas.

Materials and methods

Categories of commercial poultry

Farms with breeding (grandparent and parent) layer and meat stock, commercial layers and meat turkey flocks were included in the monitoring programme. An Mg seropositive farm is a farm with one or more Mg positive flocks in that year. In layers, Mg positive farms are categorized as vaccinated or non-vaccinated; the vaccination status was only determined for Mg positive flocks. A farm was categorized as non-vaccinated if at least one non-vaccinated flock was Mg positive, regardless of the presence of Mg vaccinated layer flocks on that farm. Farms were categorized as vaccinated when all Mg positive flocks on the farm were vaccinated. Inactivated vaccine (Poulvac® MG, Zoetis Belgium, Zaventhem, Belgium) was used in over 90% of the Mg positive and Mg vaccinated flocks, while live vaccine 6/85 (Nobilis® MG 6/85, Intervet International B.V., Boxmeer, the Netherlands) was used in the remainder of these flocks. The data were obtained in the years 2001 up to and including the year 2018.

Sampled flock, sample size, monitoring frequency and monitoring period

Breeding stock

As monitoring was based on a compulsory programme, the number of breeding farms that were monitored yearly was close or equal to the total number of breeding farms that year. The flocks were monitored at 16 weeks of age before movement to the production house. Breeder flocks were then monitored at 20–22 weeks of age, 30 weeks of age and every 12 weeks thereafter. Grandparent flocks were monitored every 4 weeks. Sampling consisted of 1% of the flock with a minimum of 30 and maximum of 60 serum samples per house aiming at detecting a seroprevalence of 5–10% at a confidence level of 95% (95% C.L.) (Thrusfield *et al.*, 2001). Mg positive serological results were verified by testing 60 trachea samples per house in the Mg polymerase chain reaction (PCR), aiming at detecting a prevalence of 5% or more (95% C.L.), regardless of flock size (Thrusfield *et al.*, 2001).

Layers

Commercial layer pullets, both Mg vaccinated as well as Mg non-vaccinated, were monitored at 15–16 weeks of age, before movement to the production house. Twenty-four serum samples per house were taken aiming at detecting a seroprevalence of 12% or more (95% C.L.). On the layer farm serum samples were taken at the end of the production period by collecting 10 samples per house aiming to detect a seroprevalence of at least 25% (95% C.L.) (Thrusfield *et al.*, 2001). Mg positive serological results on layer farms were not confirmed by serology/or PCR and were regarded as positive based on serological results. Mg vaccinated and seropositive flocks were included as Mg positive farms in the overall prevalence.

Meat turkeys

Meat turkey flocks, defined as birds of the same date of hatch and gender, were sampled at slaughter age with 24 samples aiming to detect a seroprevalence of about 12% (95% C.L.). Mg positive serological results were not confirmed by serology/and or PCR.

Serological tests

From 2001 to 2012, the Mg rapid plate agglutination (RPA) test (Intervet International) and Mg haemagglutination inhibition (HI) test (Royal GD, Deventer, the Netherlands) were performed (Feberwee *et al.*, 2005). Samples were screened using the Mg RPA test at a 1:2 dilution. If agglutination was present, the 10 samples with the strongest agglutination from the submission were serially diluted twofold from 1:4 to 1:32 in 0.5 M phosphate buffered saline pH 7.2 (PBS) and re-tested in both the Mg RPA and the HI-tests. A flock was regarded as Mg seropositive if at least 15% of the total number of samples showed agglutination at a serum dilution of 1:2 and at least 3% were positive in both the RPA test and the HI test at a serum dilution 1:8 or higher (Feberwee *et al.*, 2005). From the years 2013 onwards, the RPA test (Soleil, Cantenay-Épinard, France) was used as a screening test whereas the Mg enzyme-linked immunosorbent assay (ELISA) (IDEXX Laboratories, Inc., Westbrook, ME, USA) was used as a confirmation test (Landman, 2014). In the case of at least three Mg RPA test positive

samples at a dilution of a 1:2, all positive samples with a maximum of 10 were retested using the ELISA. In cases where more than 10 samples were RPA positive, the 10 strongest reacting samples were tested. A flock was considered Mg seropositive when at least three samples were RPA positive and ELISA positive. The combination of tests and criteria for a positive result was previously determined by our laboratory to result in a flock specificity of 99.7% and a flock sensitivity of 99.9% using data of 2108 flocks with known status based on the combination of serology, PCR and flock history (unpublished data Royal GD).

PCR

In the case of a positive Mg serological test result in breeding and grandparent flocks, 60 trachea swabs per Mg seropositive house were taken and tested in pools of six swabs in the Mg PCR. From the years 2001–2005, the PCR was performed as described by Lauerman (1998) (PCR-1) and from 2006–2011 according to Mekkes & Feberwee (2005) (PCR-2). From 2011 onwards the Mg PCR was performed as described by Raviv & Kleven (2009) (PCR-3).

MLST of Mg isolates

Genotyping of Mg was performed using the MLST method described by Bekö *et al.* (2019). The genetic diversity of the 31 available Dutch isolates from the 2001–2018 period was determined based on the analysis of six different loci (*atpG*, *dnaA*, *fusA*, *rpoB*, *ruvB*, *uvrA*). The samples were obtained from Mg field outbreaks of different poultry types, including backyard poultry, from the years 2001 to and including 2018, and included strains from broiler breeders ($n = 14$), layer breeders ($n = 1$), commercial layers ($n = 6$), meat turkeys ($n = 1$), broilers ($n = 1$) and backyard poultry ($n = 8$). The MLST data of 23 Mg isolates of other origins and data available in PubMLST were used to investigate whether the dominant Dutch sequence types (STs) also occurred in other geographical areas. PubMLST included data from Northern Europe, Southern Europe, Eastern of Europe, United States, Australia and the Middle East. The background of all samples and PubMLST data used is outlined in detail in the supplementary data. BioNumerics v7.6.3 (Applied Maths, Sint-Martens-Latem, Belgium) was used for sequence analysis, to determine STs and clonal complexes (CCs). A Minimum Spanning Tree (MST) was constructed using allelic profiles of the six loci.

Statistical analysis

Because the production period of layer flocks is often longer than a year, and thus the number of sampled farms is lower than the number of farms with flocks in production, a 95% confidence interval (95% C.I.) for the yearly prevalence in layers was calculated. The following formula for finite populations was used:

$$p \pm 1.96 \times \sqrt{\frac{p(1-p)}{n} \times \frac{N-n}{N-1}}$$

where p = proportion of positive farms, N = number of farms in the Netherlands, n = number of farms sampled.

For breeding poultry and turkeys no confidence intervals were calculated because the number of monitored farms practically equalled the number of farms with flocks in production.

The trend of Mg infection per poultry category was calculated using logistic regression (Stata 15.1, Stata Corp, College Station, TX, USA) and expressed as odds for 2001 and odds ratio for the successive years. Lower odds reflect a lower number of Mg positive farms, while an odds of 1 reflects an equal number of positive and negative farms. The odds ratio reflects the change in odds over successive years. An odds ratio between 0 and 1 reflects a decrease in the odds, while an odds ratio of 1 reflects stable odds, and an odds ratio higher than 1 an increase in the odds in the year in question compared to the previous year. For the odds ratios 95% C.I.s are given; if an odds ratio of 1 is not included in the confidence interval it is significant ($P < 0.05$).

Results and discussion

Mg control programmes in general are based on regular monitoring by serology. Regular serological monitoring of commercial poultry is a useful and cost-effective way to monitor Mg prevalence, provided that sample sizes and tests with appropriate sensitivity and specificity are used. The seromonitoring of Mg in Dutch poultry is done using Mg serological tests as prescribed by the OIE Terrestrial Manual (Kleven & Bradbury, 2018) which is in agreement with the results of previous studies in which the specificity and sensitivity of these serological tests were compared with those of culture and PCR (Feberwee *et al.*, 2005). The serial use of Mg RPA and HI tests or RPA and ELISA tests has shown comparable results (Feberwee *et al.*, 2005, Royal GD unpublished data).

An overview of the Mg prevalence in the different poultry types, alongside the measures taken and diagnostic tests used, is given in Figure 1. The analysis of the seromonitoring data showed that the prevalence of Mg seropositive farms decreased significantly in all poultry types in the Netherlands over the last 17 years. In breeding stock, the Mg prevalence significantly decreased from 1.6% to 0.0% (Table 1). The odds for breeding stock to become Mg positive were 0.01 in 2001 and the odds ratio (95% C.I.) for each

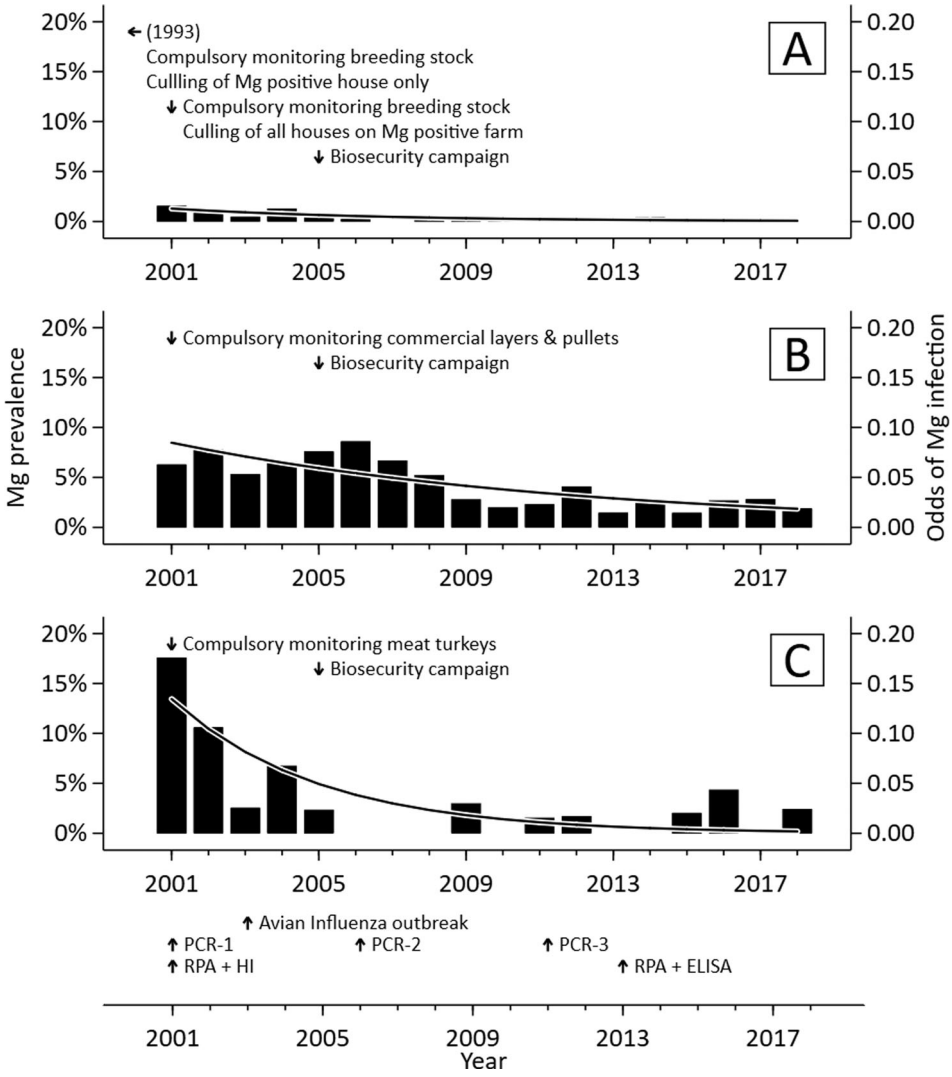

Figure 1. Mg prevalence (bars), odds of Mg infection (line), Mg related measures, diagnostic Mg tests used and important events related to Mg for breeding poultry (A), commercial layers (B) and meat turkeys (C) in the Netherlands between 2001 and 2018. PCR-1: Lauerman (1998), PCR-2: Mekkes & Feberwee (2005), PCR-3: Raviv & Kleven (2009).

of the successive years was 0.84 (0.78–0.91) (Figure 1 (A)), showing that the odds of infection were very low at the start of this period and declined significantly by year. In commercial layers, the Mg prevalence dropped from 6.3% to 1.9% (Table 2), while the odds for an Mg infection were 0.08 in 2001 with an odds ratio of 0.91 (0.90–0.92) for each successive year, which results in a slow but still significant decline of the odds (Figure 1(B)). In meat turkeys, the Mg prevalence decreased from 17.6% to 2.4% in this period (Table 3) with an odds of Mg infection of 0.13 in 2001 and an odds ratio of 0.78 (0.71–0.85) for each successive year, which is a strong and significant reduction of the odds (Figure 1(C)).

From the same tables, it can also be deduced that the number of poultry farms per poultry type is decreasing. However, the decrease in the number of farms is in time accompanied by an increase in the number of birds per farm (Statistics Netherlands, 2020), and therefore also the economic impact of an Mg infection.

The low odds for Mg infection in breeding stock shows that Mg infections in these flocks were relatively rare. During the monitoring period, there was a significant decline in Mg infections and the prevalence decreased to 0.0% which means that the risk of vertical transmission has been minimized (Raviv & Ley, 2013). In layers, the Mg prevalence dropped significantly since 2001; however, the majority of the decline occurred before 2010 and the Mg prevalence in layers has since then stabilized at 1.5–2.8%. The percentage of Mg vaccinated layer farms has remained stable from the year 2010 onwards as well. Although meat turkeys had the highest odds of infection in 2001, they had the lowest odds ratio for successive years. This reflects the rapid decline in prevalence over the study period. In some years, Mg was not detected on meat turkey farms (Table 3). As multi-age farm systems are very common in the Dutch meat turkey industry and no registered vaccine was available for use in turkeys, the decrease in prevalence has been achieved by other measures like increasing

Table 1. Prevalence of Mg[a] on breeding chicken farms from 2001 to 2018.

	Sampled farms[b,c]			Seropositive farms[d,e]			Prevalence (%)		
Year	LR	MR	Total	LR	MR	Total	LR	MR	Total
2001	75	439	514	1	7	8	1.3	1.6	1.6
2002	63	444	507	3	3	6	4.8	0.7	1.2
2003[f]	49	365	414	0	2	2	0.0	0.5	0.5
2004	45	343	388	0	5	5	0.0	1.5	1.3
2005	45	350	395	1	2	3	2.2	0.6	0.8
2006	47	382	429	0	1	1	0.0	0.3	0.2
2007	48	382	430	0	0	0	0.0	0.0	0.0
2008	54	395	449	0	1	1	0.0	0.3	0.2
2009	55	412	467	0	1	1	0.0	0.2	0.2
2010	57	395	452	0	1	1	0.0	0.3	0.2
2011	77	387	464	0	2[g]	2	0.0	0.5	0.4
2012	69	380	449	0	1[h]	1	0.0	0.3	0.2
2013	64	373	437	0	0	0	0.0	0.0	0.0
2014	69	362	431	1	1	2	1.4	0.3	0.5
2015	71	379	450	0	1	1	0.0	0.3	0.2
2016	63	380	443	0	0	0	0.0	0.0	0.0
2017	63	367	430	1	0	1	1.6	0.0	0.2
2018	74	334	408	0	0	0	0.0	0.0	0.0

LR = layer (grandparent and parent) breeding farms, MR = meat (grandparent and parent) breeding farms (including rearing farms). Flocks were monitored at 16 weeks of age and then every 4 (grandparent) to 8 weeks (parent), starting at 20–22 weeks of age by collecting 30–60 serum samples per house.

[a]Farms that were found Mg positive based on serology & Mg PCR.

[b]Number of sampled farms ≈ actual number of producing farms, therefore the 95% C.I. ≈ calculated prevalence.

[c]At the farm 1% of the flock was sampled with a minimum sample size of 30 and a maximum of 60 samples per house. A flock is defined as a group of chickens or turkeys (≥250 birds) of the same age and housed together in the same house, and the same gender for turkeys.

[d]From 2001 up to and including the year 2012, 15% of the samples show agglutination at a serum dilution of 1:2 and 3% are positive in both the RPA test and HI test at a serum dilution 1:8 or higher and confirmed by Mg PCR. From 2013 up to and including 2018 Mg RPA positive test results in combination with two or more samples positive in the Mg ELISA was regarded as specific positive and confirmed by Mg PCR.

[e]From the year 2001 onwards the farm was considered the epidemiological unit for *M. gallisepticum* (all birds on a farm were culled, even if only the birds in one house were infected).

[f]Year of avian influenza outbreak in the Netherlands.

[g]One of these farms was found positive by clinical signs and PCR in-between serological samplings.

[h]Meat type replacement breeding flock.

biosecurity, practical channelling and antibiotic treatments (Raviv & Ley, 2013). After the Dutch avian influenza outbreak in 2003, an increase in Mg infected breeding farms, layer farms and meat turkey farms was observed (Figure 1). During the avian influenza outbreak, 30.7 million birds from 255 farms were culled (Landman & Schrier, 2004) and an increase in poultry imports was seen to replenish the stock after the outbreak was controlled. Import may have increased the risk of introduction of Mg infected poultry flocks in general, and subsequently could have contributed to an increased risk of horizontal transmission within and between the different Dutch poultry categories.

The molecular characterization of 31 Mg isolates obtained from different poultry types shows a high diversity in sequence types (ST) (Figure 2). The 31 isolates represented 21 different STs. Of these 21 STs, one ST was a known ST available in PubMLST and 14 were related to known STs available in PubMLST (14, 19, 29, 30, 33, 34, 35, 68, 74 and 79). Six STs were new and unrelated to any known ST. The Dutch isolates from the year 2001 up to and including 2018 are presented in Figure 3 according to poultry type, year and CC. From 2001 to 2003, CC 5 was the most dominant CC. After the 2003 avian influenza outbreak, CC 5 disappeared and three other dominant (CCs 1, 2 and 7) appeared (Figure 3). Strains belonging to these three CCs also appear in Northern and Southern Europe but not in other parts of the world. The Dutch MLST data, as well as the additional data of Northern and Southern Europe, showed the presence of isolates belonging to the same CC (1, 5 and 11 and 1, 2 respectively) in different poultry types (including backyard poultry) indicating that there is a risk of transmission between poultry types or a common source and that this should be accounted for in organized Mg control programmes in general.

Besides the test characteristics, the accuracy of the yearly estimated prevalence of Mg seropositive farms will be determined by the number of farms, sample size per house and the sampling frequency. As for the number of farms sampled, all breeding and turkey farms and the majority of layer farms were monitored yearly. However, for each poultry category, there was a different sample size and sample frequency. For breeding stock, due to large sample size, high sample frequency and low prevalence herd specificity may decrease (HERDACC 3.0, (Jordan, 1995)) which could lead to more false positive results and to an overestimation of the prevalence of Mg seropositive farms. This effect, however, is minimized by introducing a threshold in positive reactions, serial testing and using an Mg PCR as a confirmatory test. This confirmatory test also prevents the culling of false positive flocks. The lower sample frequency and sample size for the other poultry categories could have led to an underestimation of the prevalence of Mg seropositive farms. Antibiotic prophylaxis against Mg is not practiced in the Netherlands; the effects of therapeutic treatment with antibiotics on the detection of Mg are considered negligible with the current monitoring programme.

Vaccination against Mg has been shown to be a useful tool in the control of Mg in commercial layers, especially on Mg infected multi-age commercial layer farms where it is difficult to control Mg. Several studies showed that Mg vaccines reduce signs of clinical disease, and contribute to the reduction of vertical transmission (Glisson & Kleven, 1984; Kleven *et al.*, 1984; Kleven, 1985; Whithear, 1996; Barbour *et al.*, 2000; Kleven, 2008; Noormohammadi & Whithear, 2019). Other studies showed that inactivated and live vaccines could contribute to the reduction of Mg shedding and transmission within flocks (Kleven, 1985; Feberwee *et al.*, 2006a, b). Moreover, for live vaccination programmes there is evidence from experimental and field studies that the vaccine strain may replace the field strain when multiple successive flocks are

Table 2. Prevalence of Mg on commercial layer farms from 2001 to 2018.

Year	Producing farms	Sampled farms	Seropositive farms[a,b]			
			Number	Prevalence (%)	95% C.I.	Percentage of positive farms vaccinated[c]
2001	1325	1285	81	6.3	6.1–6.5	51
2002	1161	1054	82	7.8	7.3–8.3	54
2003[d]	1047	695	37	5.3	4.4–6.3	59
2004	1311	734	48	6.5	5.4–7.7	54
2005	1255	1000	76	7.6	6.9–9.3	50
2006	1199	999	86	8.6	7.9–9.3	59
2007	1135	1000	67	6.7	6.2–7.2	63
2008	1104	958	50	5.2	4.7–5.7	60
2009	1110	930	26	2.8	2.4–3.2	42
2010	1122	954	19	2.0	1.6–2.3	63
2011	1065	899	21	2.3	1.9–2.7	62
2012	1035	783	32	4.1	3.4–4.8	69
2013	986	751	11	1.5	1.0–1.9	64
2014	943	747	18	2.4	1.9–2.9	67
2015	917	757	11	1.5	1.1–1.8	64
2016	894	709	19	2.7	2.1–3.2	43
2017	881	640	18	2.8	2.1–3.5	83
2018	874	680	13	1.9	1.4–2.4	46

[a]Every layer flock was monitored at 16 weeks of age with 24 samples per house and 10 samples per house that were collected 9 weeks before slaughter. The monitoring data only include data at the end of the layer period.
[b]From 2001 up to and including the year 2012, 15% of the samples showed agglutination at serum dilution 1:2 and 3% of the samples are positive in both the RPA test and HI test at serum dilution 1:8 or higher. From 2013 up to and including the year 2018, Mg RPA positive test results in combination with three samples or more positive in the Mg ELISA were regarded as specific positive.
[c]Farms where the Mg seropositive flocks at the end of lay were all Mg vaccinated.
[d]Year of avian influenza outbreak in the Netherlands.

vaccinated (Whithear, 1996; Turner & Kleven, 1998; Barbour *et al.*, 2000; Feberwee, von Banniseht-Wysmuller *et al.*, 2006; Kleven, 2008). For this reason, Mg vaccination was mandated on Mg positive multi-age layer farms in the Netherlands. However, these studies showed that Mg vaccination could not prevent colonization with Mg (Kleven, 1985; Feberwee, Landman *et al.*, 2006; Feberwee, von Banniseht-Wysmuller *et al.*, 2006) and, therefore, farms with Mg vaccinated and seropositive flocks were included as Mg positive in the monitoring data because they still can be regarded as a risk for other commercial poultry flocks.

Table 3. Prevalence of Mg on meat turkey farms from 2001 to 2018.

	Sampled farms[a]	Seropositive farms[b,c]	Prevalence (%)
2001	125	22	17.6
2002	113	12	10.6
2003[d]	78	2	2.6
2004	89	6	6.7
2005	85	2	2.4
2006	82	0	0.0
2007	76	0	0.0
2008	69	0	0.0
2009	67	2	3.0
2010	67	0	0.0
2011	65	1	1.5
2012	58	1	1.7
2013	56	0	0.0
2014	52	0	0.0
2015	49	1	2.0
2016	46	2	4.3
2017	41	0	0.0
2018	41	1	2.4

[a]Number of sampled farms ≈ number of farms with flocks in production, therefore the 95% C.I. ≈ calculated prevalence.
[b]Based on 24 samples per flock (same hatch date and gender) at slaughter age.
[c]From 2001 up to and including the year 2012, 15% of the samples showed agglutination at serum dilution 1:2 and 3% were positive in both the RPA and HI test at serum dilution 1:8 or higher. From 2014 up to and including the year 2018 Mg RPA positive test results in combination with three samples or more positive in the Mg ELISA were regarded as specific positive.
[d]Year of avian Influenza outbreak in the Netherlands.

On the basis of the results of this 17-year seromonitoring it can be concluded that the Mg monitoring and control programme contributed to a significant decrease in the seroprevalence of Mg in the Netherlands during the past 17 years. Although the contribution of the different measures to the reduction of the prevalence of Mg seropositive farms cannot be quantified, they possibly have contributed to the control of Mg. Over time the structure of the Dutch poultry industry also changed, the number of poultry farms decreased while the number of birds per farm increased. However, multi-age housing is still common on layer farms as well as meat turkey farms. Because Mg infections in breeding poultry are rare, as at most two positive flocks a year are found and these flocks are culled immediately, the contribution of vertical transmission to introduction of Mg in commercial poultry in the Netherlands has minimized over the years. Currently, horizontal transmission can be regarded as the most important risk for the introduction of Mg into commercial poultry in the Netherlands. As similar STs were found in different commercial poultry types and backyard poultry, it cannot be ruled out that these poultry types can be a reservoir for breeding poultry. This confirms the need for extension of the Dutch Mg control programme to layers and meat turkeys, and the importance of isolation of commercial poultry from backyard poultry. The measures taken in the Dutch poultry industry and the use of molecular epidemiological techniques such as MLST to find reservoirs of Mg and add specific measures, can be extrapolated to Mg control in general. Quantifying of risk factors related

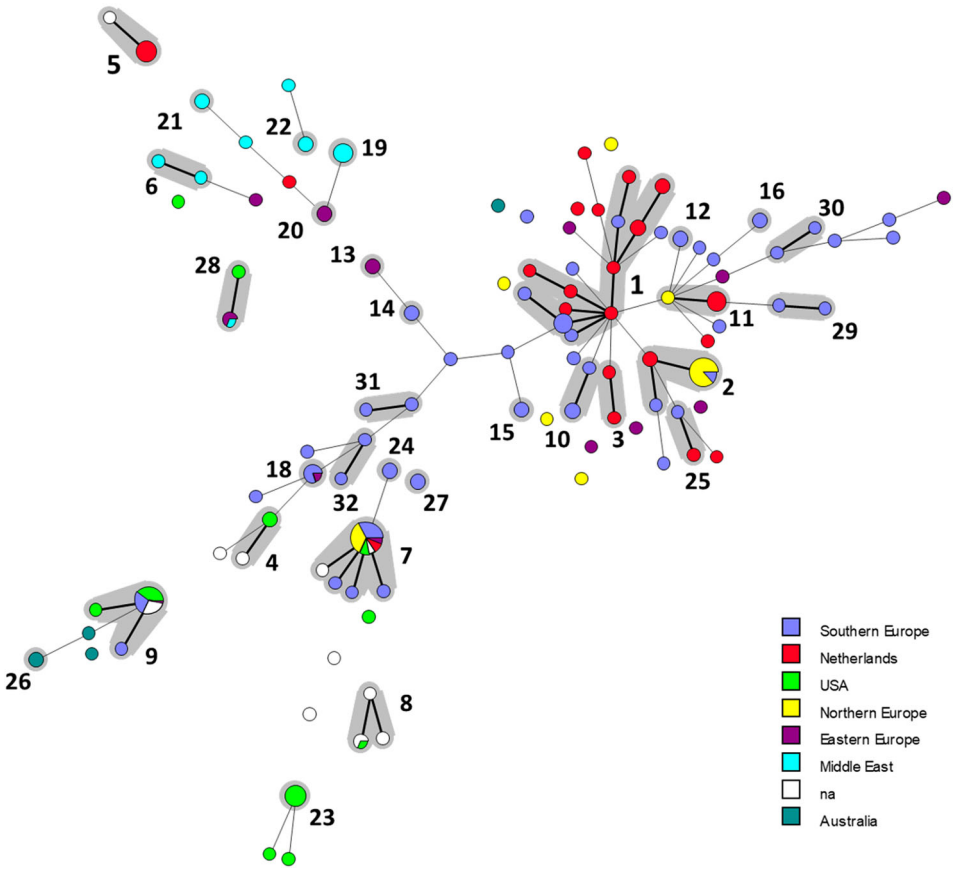

Figure 2. Relatedness of 84 sequence types (STs) of 191 Mg samples represented in a Minimum Spanning Tree (MST). Data include MLST data of 141 strains available in PubMLST and 31 Dutch isolates. In the MST, each circle represents a different ST, the size of the circle and the partition lines within a circle represent the relative and absolute number of samples represented by the ST, respectively (na = not applicable, these concern vaccine and type strains). The colour indicates the geographical origin of the isolate. The length and thickness of lines reflects the allelic differences between two STs, wherein heavy lines connect single locus variants and thin lines connect double locus variants. Unconnected circles represent singletons. Numbers identify CCs.

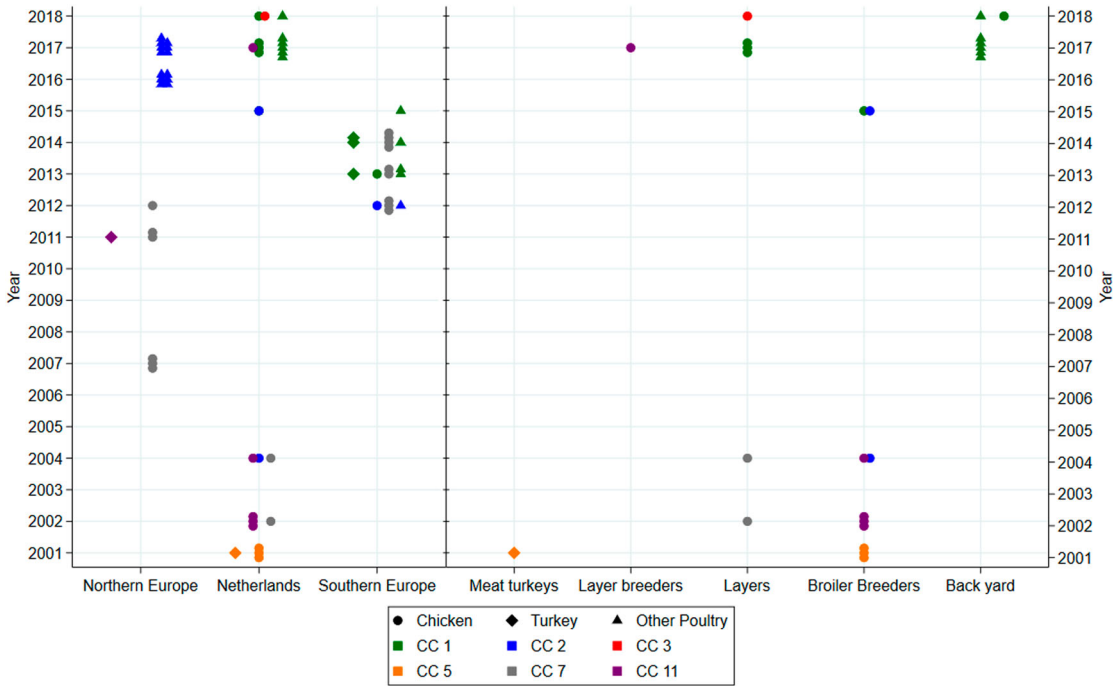

Figure 3. Dominant Mg CCs in the Netherlands from 2001 until 2018 compared to Mg CCs from other geographical regions (left) and to different poultry types within the Netherlands (right). CC is indicated by colour; poultry species is indicated by shape. Other poultry: pheasant, peacock, goose, guinea fowl, partridge, pheasant or quail.

to horizontal transmission of Mg, as was done for Ms in layer pullets by Ter Veen *et al.* (2020), could also help to further decrease Mg prevalence in the Netherlands.

Disclosure statement

No potential conflict of interest was reported by the authors.

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