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The Prevalence of *Mycoplasma gallisepticum* Infection in Chickens from Peninsular Malaysia

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Abstract: The detection of *Mycoplasma gallisepticum* (MG) infection in chickens in selected commercial farms (breeder, broiler and layer), Progeny-pipped Embryos (PE) and day old chicks Poor Quality Chicks (PQC) and Normal Chicks (NC) obtained from Peninsular, Malaysia was determined. These farms have the history of vaccination with mild live and killed MG vaccine or antimicrobial prophylactic or treatment. To conduct this study, a total of 3056 samples were collected and conventional PCR test was performed using specific gene target sequence, encoding the surface protein for detection of MG directly from the clinical samples without prior isolation of the target MG. The primer was designed to bind to the adherence protein a (GAPA) and amplify a 505 bp DNA fragment and there are no secondary copies of this gene. Results revealed that five hundred seventy one positive samples of MG out of 3056 samples were obtained and the overall prevalence was 18.68%. This study shows high detection of MG infection through vertical and horizontal transmission from many geographically distinct areas of the country although, these farms had vaccination and treatment history. So, these results demonstrated that MG eradication program is not successful, despite the use of live and killed MG vaccines, an extensive medication program and strict biosecurity. It is recommended that PCR assay is used to monitor broiler breeder flocks for MG challenge during the laying cycle using progenies and confirm that point of lay broiler-breeder pullets are free of field MG strain. In addition to the importance of flock health monitoring in progenies, especially those pipped embryos and poor quality chicks and especially breeders in order to provide MG-free progeny.

Key words: Prevalence, *Mycoplasma gallisepticum*, chickens, progenies, PCR, gap, gene, Malaysia

INTRODUCTION

Mycoplasma gallisepticum has resulted in important problems of flock health in chicken and turkey industry worldwide (Kleven and Levisohn, 1996; Ferguson *et al*., 2004; Buim *et al*., 2009). The infection causes direct and indirect losses in breeder and layer production. The losses occurred as a result of decreased hatchability and egg production, reduced quality of day old chicks, increased mortality of chicks, reduced growth rate, increased costs of eradication procedures which involved site cleaning and depopulation, costly monitoring programs which involved serology and PCR and increased costs of control procedures which involved medication and vaccination (Ley, 2003; Raviv *et al*., 2008). Although, flock health programs are practiced such as usage of antibiotics and vaccination, the incidence of MG infection is still prevalent in Malaysia and worldwide (Ling, 2005). According to Tan (2004), the prevalence of MG infection in chicken flocks was high and ranging from 42-73%, although isolation of MG ranges from 10-70%. The prevalence of MG in commercial and village chickens in Penang, Malaysia was 85% based on Enzyme-Linked Immunosorbent Assay (ELISA) (Tan, 2004). The overall prevalence of MG in pipped embryos from Penang, Perak, Selangor, Melaka and Johor was 1.14% (Ling, 2005). The prevalence of MG in pipped embryos from vaccinated and non-vaccinated parent stocks was 2.05 in Malaysia (Ling, 2005).

The Polymerase Chain Reaction method (PCR) proved as a valuable tool under practical diagnostic conditions and might provide the same or higher sensitivity than the isolation and the serological methods (Kempf *et al*., 1993; Ewing *et al*., 1996; Salisch *et al*., 1998; Mekkes and Feberwee, 2005). Accordingly, the nucleic acid detection methods are preferred as attractive alternative methods (Kempf, 1997; Feberwee *et al*., 2005). The use of molecular diagnostic methods on pooled swab samples from birds

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flock was a costly but useful and dependable way of MG strain identification. It is useful when trying to differentiate Mycoplasmas field strain from vaccine strain. The cost of the PCR test could be reduced by pooling samples (Tyrrell and Andersen, 1994; East, 2008). PCR test was used to examine the flocks of broiler breeder for MG challenge through, the laying cycle and confirmation that broiler breeder pullets are free of MG field strain. The propriety detection PCR primer was specifically designed to identify the *gapA* gene, the functional characterization of this gene highlights its significant role as an important conservation *cytadhesion* gene. There is still lack of information and reports in Malaysia about MG infection in different sources of eggs from breeder farms vaccinated with live, killed vaccines or treated with antibiotics. Therefore, the objectives of this study were to determine:

- The common pathogenic MG in different farms in Malaysia using PCR amplification methods
- The prevalence of MG infection in selected commercial chickens which include breeder, broiler and layer farms and selected progenies which include pipped embryos, day old poor quality and normal chicks obtained from Peninsular, Malaysia

MATERIALS AND METHODS

Samples collected from 2005-2006 from commercial chicken farms and progeny from breeder farms consisted of Pipped Embryo (PE), day old Poor Quality Chicks (PQC) and Normal Chicks (NC) from Peninsular Malaysia and farms with different CRD control measures) were obtained from Dr. Tan Ching Giap (Biologics Lab, UPM). A total of 3056 swab samples were collected and immediately stored in PPLO broth after culture in agar and kept in -4°C. Choanal cleft, tracheal and air sac swabs were obtained from each chicken, chick and embryo were pooled into one sample. The total numbers of 1831 samples from progenies Pipped Embryo (PE), day old Poor Quality Chicks (PQC) and day old Normal Chicks (NC). Samples were obtained from different geographically distinct areas of Peninsular Malaysia. Progenies were collected from breeder farms where the age of breeder was from 25 (peak of production) to 45 weeks and these farms have vaccination or no vaccination history.

The samples size of Pipped Embryo (PE) was 1243 samples, day old Poor Quality Chicks (PQC) was 248 samples and day old Normal Chicks (NC) was 340 samples. The total numbers of 1225 samples were obtained from commercial breeder, broiler and layer farms from different geographically distinct areas of Peninsular Malaysia and these chickens have vaccinations or no vaccination history. The swabs were removed under sterile condition and the broth was preceded for DNA extraction. Genomic DNA was extracted using a conventional salt based method with some modifications (OIE, 2004).

The 1st step of total nucleic acid extraction was lysis of cell and tissue sample. A volume of 70 μL 10% SDS as a tissue cell lysis solution was added into each tube followed by adding a volume of 1 μL of 50 μg μL^{-1} proteinase K into each tube. The mixtures of mycoplasma broth, SDS solution and proteinase K were vortexed to resuspend the cell pellet in all the tubes samples. The mixtures were incubated at 65°C for 30 min and shaken thoroughly every 5 min.

The lysed samples were then placed on ice for 5 min or in the refrigerator at 4°C for 30 min. Precipitation of protein and peptide was achieved by adding 300 μL ammonium acetate to each of the lysed sample and vortex mixed vigorously for 10 sec. The debris was pelleted by centrifugation for 10 min at 14,000 rpm. A volume of 850 μL of the supernatant was transferred to a clean microcentrifuge eppendorf tube and the pellet was discarded. Total nucleic acid was precipitated by adding 550 μL of isopropanol to the recovered supernatant and the tube was inverted 40 times. Then the total nucleic acids were pelleted by centrifugation at 14,000 rpm at 4°C for 10 min in a microcentrifuge. The isopropanol was then carefully poured off without dislodging the nucleic acids pellet. The total nucleic acid pellet was then centrifuged twice with 1 mL 75% ethanol for 10 min at 14,000 rpm.

The ethanol was carefully poured off without dislodging the nucleic acid or the pellet. The nucleic acid samples were then placed in the laminar air flow chamber overnight until the ethanol had evaporated. The DNA samples were re-suspended in 50 μL of double-distilled water and used for polymerase chain reaction. The conventional PCR procedure was performed according to the method described by Marois *et al.* (2000) with some modifications. The primer used in this study was designed to bind to the Adherenc Protein A (GAPA) and amplify a 505 bp DNA fragment. The forward primer sequences was MG gapA 5F- TCARCGTTTCTAAGATTCCTTTTG and the reverse primer MG gapA 6R-GCATCAAAACCAGT AAATTCTTGG. There was no secondary copy of this gene and it was considered as one of the important conservation cytadhesion gene.

The primer of this gene target sequence was used specifically to detect MG and there were no gene size polymorphisms on this gene (Tan, 2008). Emphasis on development of this method was on the need for typing of MG directly from the clinical samples without prior isolation of the target MG. Optimization was carried out

Table 1: Reagents used in conventional PCR master mixture reaction

Reagents	Quantity
10×PCR buffer	2.5 μL (10×) (Vivantis®, Malaysia)
$MgCl_2$	2.5 μL (50 mM, Vivantis®, Malaysia)
dNTPs	1 μL (10 mM each, Vivantis®, Malaysia)
Primer MG-14F	0.5 μL (20 pmole)
Primer MG-13R	0.5 μL (20 pmole)
Taq polymerase	0.2 μL (15 mM, Vivantis®, Malaysia)
DNA template	2 μL (656 ng)
Distilled water	Add to a final volume of 25 μL
Total volume $tube^{-1}$	25 μL

by varying the PCR conditions, e.g., DNA concentration, amplification cycle number and primer annealing temperature. The amplification of fragment DNA was performed in an automatic thermal cycler (MyCycler, BioRad, USA). The reaction volume was set up in a 25 μL reaction mixture as stated in Table 1. The reaction procedure consisted of an initial denaturation step at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and extension at 72°C for 1.5 min and ended with 1 cycle of final extension at 72°C for 10 min. PCR amplified DNA fragments were detected in 1.5% agarose gel electrophoresis in TAE (1x) performed at 130v/30 min, stained with ethidium bromide (10 mg mL^{-1}) and bands visualized on UV light, the expected positive samples showed as bands matched with band size 505 bp. The size of the amplified product was compared using a 100 bp plus DNA ladder (Vivantis®, Malaysia).

RESULTS AND DISCUSSION

The conventional PCR test in this study was performed using specific gene target sequence, encoding the surface protein for detection of MG directly from the clinical samples without prior isolation of the target MG. The target MG primer was designed to bind to the adherence protein A (*gapA*) gene and amplify a 505 bp DNA. All field isolates from different sources, vaccine and reference strains showed identical bands patterns as presented on (Fig. 1). There were no secondary copies of this gene and it was considered as one of the important conservation *cytadhesion* gene. The primer of this gene target sequence was used specifically to detect MG and there were no gene size polymorphisms on this gene (Tan, 2008). The advantage of this study method is that direct detection of MG from swabs samples in the field condition can be done without prior isolation of the target MG.

The conventional PCR assay using gapA partial gene sequence in this study showed the limit of MG detection directly from swab samples therefore it counterpart the limit of MG detection in pure culture. The results agree with previous investigation that demonstrated the highest

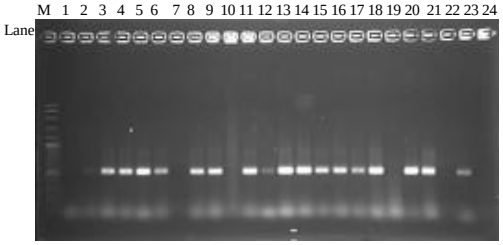

Fig. 1: PCR product of 505 bp of positive MG from Pipped Eembryo (PE) samples amplified using the GAP A primer set. M = VC 100 bp plus DNA Ladder, Lanes 3-6, 8, 9, 11, 13-18, 21 positive samples tested, Lane 20 = positive control MG S6, Lane 24 = negative control

MG detection by using the conventional PCR method and the lowest by using the commercial kit A. The comparison of the four PCR tests targeted 16S rRNA, mgc2, gapA and lipoprotein discovered that the most sensitive was gapA gene detection method (Domanska-Blicharz *et al*., 2008). The PCR results of pipped embryos samples showed that 160 out of 1243 samples were positive for MG obtained from 27 batches of pipped embryos and the total detection rate was 13.7% (Table 2). The PCR results of day old Poor Quality Chicks (PQC) showed that 42 out of 248 samples were positive for MG obtained from 13 batches of poor quality chicks and the total detection rate was 16.9% (Table 3). The PCR results of day old Normal Chicks (NC) showed that 43 out of 340 samples were positive for MG obtained from 15 batches of normal chicks and the total prevalence was (12.6%) (Table 4).

This indicated that the high presence may be due to vertical transmission, although these farms had vaccination and treatment history. All the samples were collected from the breeder group at crest age production curve that was in the late term of the eggs production curve (breeder age 52 weeks onward or >28 weeks egg production). Vertical transmission is very important as MG can be passed to the embryo. Vertical transmission is found lowest at periods represented by crests on the production curve and highest at periods represented by troughs on the egg production curve. The highest infection in the young chickens is due to the vertical transmission of the organisms.

The impact of the disease influences the embryo's ability in completing the process of hatching. If the infected progenies are introduced into the flock, they later might serve as a source of horizontal transmission. The failure of the yolk to incorporate the organism into the egg in some cases, the status of the maternal immunity, stress and level of infection are the reasons for the variability of

Table 2: Numbers and percentages of pipped embryos from various farms with positive MG by PCR

No.	States	Control/treatment	Samples no.	Positive	Percent
1	Melaka	Live vaccine	105	28.0	26.6
2	Melaka	Antibiotic	224	8.0	3.6
3	Selangor	Live vaccine	235	31.0	13.2
4	Selangor	Antibiotic	61	2.0	3.3
5	Negeri Sembilan	Killed vaccine	243	48.0	19.7
6	Negeri Sembilan	Live vaccine	55	0.0	0.0
7	Penang	Live vaccine	101	22.0	21.8
8	Penang	Antibiotic	47	11.0	23.4
9	Perak	Antibiotic	96	20.0	20.8
10	Johor	Antibiotic	76	0.0	0.0
Total			1243	170.0	13.7

Table 3: Numbers and percentages of day old poor quality chicks from various farms with positive MG by PCR

No.	States	Control/treatment	Samples no.	Positive	Percent
1	Selangor	Live vaccine	145	15.0	10.3
2	Johor	Antibiotic	18	1.0	5.6
3	Penang	Live vaccine	6	4.0	66.7
4	Negeri Sembilan	Live vaccine	20	0.0	0.0
5	Negeri Sembilan	Killed vaccine	59	22.0	37.3
Total			248	42.0	16.9

Table 4: Numbers and percentages of day old normal chicks from various farms with positive MG by PCR

No.	States	Control/Treatment	Samples no.	Positive	Percent
1	Selangor	Live vaccine	87	8.0	9.2
2	Selangor	Antibiotic	101	2.0	2.0
3	Negeri Sembilan	Killed vaccine	128	28.0	21.9
4	Negeri Sembilan	Live vaccine	10	0.0	0.0
5	Penang	Live vaccine	14	5.0	35.7
Total			340	43.0	12.6

organism shedding through vertical transmission. The detection rate of MG vertical transmission is lower in birds infected with MG virulent strains early in life when compared to higher incidence of the MG vertical transmission when the birds are infected during lay. The rate of the vertical transmission differ from 50% in the acute phase to <3% in the chronic phase of infection and always the entire flocks derived from the infected parents become MG positive (Levisohn and Kleven, 2000). Initially, controlled exposure to the field MG strain before the lay was considered a way to decrease the natural infection impact occurring during lay (Bencina and Dorrer, 1984).

The PCR results of commercial breeder, broiler and layer farms showed that 316 out of 1225 samples were positive for MG obtained from 17 batches of commercial farms and the total prevalence was (25.8%) (Table 5).This indicated that the high presence may be due to horizontal transmission although, these farms had vaccination and treatment history. Probably the MG organism was transmitted through infected birds, eggs and wild birds, vehicles or fomites (Jordan, 1985). There were other probable factors that lead to the MG infection in the flock such as the type of management and other stressors such as cold during the rainy season and health programmes of the flock which includes vaccination could be the stressful conditions to the chickens and lead to reduced immunity against MG infection. Total 81 positive samples of MG out of 496 samples using PCR were obtained from pipped embryos farms and the prevalence was 16.33%. These breeder farms were vaccinated with mild live vaccine. Total 48 positive samples of MG out of 243 samples using PCR were obtained from pipped embryos farms and the prevalence was 19.75%. These breeder farms were vaccinated with killed vaccine. Tatal 41 positive samples of MG out of 504 samples using PCR were obtained from pipped embryos farms and the prevalence was 8.14%. These breeder farms were treated with antibiotic (Table 6). About 19 positive samples of MG out of 171 samples using PCR were obtained from day old poor quality chicks farms and the prevalence was 11.11%. These breeder farms were vaccinated with mild live vaccine.

Total 22 positive samples of MG out of 59 samples using PCR were obtained from day old poor quality chicks farms and the prevalence was 37.29%. These breeder farms were vaccinated with killed vaccine. One positive sample of MG out of 18 samples using PCR were obtained from day old poor quality chicks farms and the prevalence was 5.60%. These breeder farms were treated with antibiotic (Table 6). Total 13 positive samples of MG out of 111 samples using PCR were obtained from day old normal chicks farms and the prevalence was 11.71%. These breeder farms were vaccinated with mild live

Table 5: Numbers and percentages of commercial chicken samples from various farms with positive MG by PCR

No.	Type/breed	States	Control/treatment	Samples no.	Positive	Percent
1	Village	Penang	Antibiotic	397 .0	137	34.5
2	Village	Kedah	Antibiotic	135.0	33	24.4
3	Breeder	Melaka	Live vaccine	122.0	0	0.0
4	Layer	Melaka	Antibiotic	120.0	62	51.7
5	Broiler	Selangor	Antibiotic	190.0	17	8.9
6	Layer	Negeri Sembilan	Killed vaccine	96.0	29	30.2
7	Broiler	Negeri Sembilan	Antibiotic	134.0	11	8.2
8	Broiler	Perak	Antibiotic	31.0	27	87.1
Total				1225.0	316	25.8

Table 6: Numbers and percentages of pipped embryos, poor quality and normal chicks from various farms with positive MG by PCR

	Live vaccine		Killed vaccine		Antibiotics	
Treatment/samples	Positive/total	Detection rate	Positive /total	Detection rate	Positive/total	Detection rate
Pipped embryos	81/496	16.33	48/243	19.75	41/504	8.14
Poor quality chicks	19/171	11.11	22/59	37.29	1/18	5.60
Normal chicks	13/111	11.71	28/128	21.86	2/101	2.00

*Incidence rate within rows with different superscripts differed significantly at $p<0.05$

vaccine. About 28 positive samples of MG out of 128 samples using PCR were obtained from day old normal chicks farms and the prevalence was 21.86%. These breeder farms were vaccinated with killed vaccine. Two positive samples of MG out of 101 samples using PCR were obtained from day old normal chicks farms and the prevalence was 2%, these breeder farms were treated with antibiotic (Table 6).

The statistical analysis was done and all datasets were expressed as percentage incidence for the farms. Comparisons were made across vaccination, types and antibiotic treatment for the incidence rates using one way analysis of variance. Significantly, different incidence rates were elucidated using the Tukey HSD test. All statistical procedure was conducted at 95% confidence level.

This study showed high detection rate of MG infection from many geographically distinct areas of the country. In progenies coming from breeder farms that have been vaccinated with mild live vaccine the total detection rate was 16.33% in pipped embryos, 11.11% in day old poor quality chicks and 11.71% in day old normal chicks. This might be due to the mild vaccine strains ts-11 and 6/85 used in the present study induced milder post vaccination reactions than the F strain. They did not persist in the respiratory tract during the trial period and give weaker immunological response. However, the F strain vaccinated chickens had the mildest air sac lesions after challenge with a virulent R strain (Evans and Hafez, 1992). The correlation between infectivity, immunogenicity and pathogenicity of MG strains is complicated. The protection level of live MG vaccine is associated to the virulence of the vaccine (Lin and Kleven, 1982). Higher levels of circulating antibodies was induced by the more virulent F strain as compared to low levels of circulating antibodies that was induced by the less virulent ts-11 and 6/85 vaccine strains but the protection does not correlate with the circulating antibody titer (Lam and Lin, 1984; Talkington and Kleven, 1985; Whithear *et al*., 1990). In progenies coming from breeder farms that have been vaccinated with killed vaccine, the total detection rate was 19.75% in pipped embryos, 37.29% in day old poor quality chicks and 21.86% in day old normal chicks. This finding agree with previous study which reported that killed bacterin immunization is an important part of a control program but it cannot be used alone to eradicate MG and provide minimal protection from the disease and the economic losees (Kleven, 2008). Challenged vaccinates may be infected with pathogenic MG thus, egg transmission and lateral spread still occurs (Levisohn and Kleven, 2000).

The progenies coming from breeder farms that were treated with antibiotic, the total detection rate was 8.14% in pipped embryos, 5.60% in day old poor quality chicks and 2% in day old normal chicks. This result indicated that the medication can not be used as a method for eradication of MG. However, several medication approaches have been used successfully to limit vertical transmission, avoid the MG infection effect on performance of the birds and prevent flock infection. Several antibiotics including the macrolides, tetracyclines and flouroquinolones have *in vitro* and *in vivo* activities, although the mycoplasmas are resistant to antimicrobials like penicillin which affects cell wall synthesis. During the flock medication, it is impossible to separate the antimicrobial effect on MG and the secondary invaders, therefore *in vivo* response to medication did not essentially match the *in vitro* resistance (Levisohn, 1981; Wang *et al*., 2001; Ley, 2003). In the breeder farms, the highest MG was detected in the progenies collected from

breeders that were vaccinated with killed MG vaccine and the lower MG was detected in progenies collected from breeders that were vaccinated with live MG vaccine. However, the lowest MG was detected in the progenies collected from breeders that were treated with antibiotics. This might indicate that the pattern of MG detection rate through vertical transmission varies according to different control measures. These findings have the same opinion and confirmed the previous study in Malaysia that showed the pattern of caseous airsac varies according to different control measures for MG in the breeder farm. Also, most caseous airsac lesions were observed in embryos of eggs that were collected throughout the eggs production curve for breeders that were vaccinated with killed MG vaccine and in embryos of eggs that were collected when the egg production were at crest production curve for breeders that were vaccinated with live MG vaccine (Tan, 2008).

In this study, the overall prevalence of MG from progenies and commercial chickens farms was 18.68%. This rate seems high consent with the previous reports that mentioned the high detection rate of MG infection in poultry farms in Malaysia (Ganapathy *et al*., 2001; Mutalib *et al*., 2001). Based on serological test, the prevalence of MG in commercial and village chickens in Penang, Malaysia was found to be 85% (Tan, 2004). Another investigation demonstrated that the MG prevalence in pipped embryo from vaccinated and non vaccinated parent stock was 2.05%, however a further report mentioned that the overall prevalence of MG in pipped embryos from five states in Malaysia was 1.14% (Ling, 2005). The highest detection finding in this study was also recorded previously in Malaysia by Shah-Majid (1996) who conducted a seroprevalence study on village chickens using ELISA and found that 26% samples were positive to MG. Observations of MG in village chickens from other countries include Benin (Chrysostome *et al*., 1995), Botswana (Mushi *et al*., 1999), India (Chakraborty *et al*., 2001) and Zambia (Pandey and Hasegawa, 1998). The overall prevalence in these investigations ranged from 26 (Malaysia) to 62% (Benin). Another study recorded, 13-32% seroprevalence of MG infection in some selected poultry farms in the southern part of Bangladesh (Amin *et al*., 1992). The highest MG detection rate might be due to the replacement of breeding stock with the same flock progeny. However, concentrated nature of poultry production give the opportunity for recycling of the pathogens due to population production density and generating the huge multi age sites (Pradhan, 2002). The additional factors that contribute MG infection are poor ventilation, litters contamination, lack of movement restriction of the technical personnel, visitors and such other persons as well as other bio-security measures (Dulali, 2003). Another factor which relate to MG infection are tracheal epithelium damage as a result of vaccine virus replication, formalin fumigation, ammonia, dust and inflammatory response (Anderson *et al*., 1968; Carlile, 1984). The immune system plays important role on the epidemic MG infection. Previous studies on the immunodominant surfaces proteins of MG demonstrated high rate of expression variation in these proteins, switching and modulation of the immunity that suggested the variable expression in these proteins might be the essential adaptive mechanism which enables the MG organism to escape from the host, adaptation to the environments of the host resulting to the tissue invasion and producing the chronic infection (Bencina *et al*., 1994; Garcia *et al*., 1994; Glew *et al*., 2000; Levisohn and Kleven, 2000).

CONCLUSION

A sensitive PCR test was developed to detect MG at the level of the *gapA* gene. Total 561 positive samples of MG out of 3056 samples with overall prevalence 18.68% were detected in this study using PCR test which indicated that the PCR assay was very sensitive and specific technique used to monitor the breeder flocks and progenies coming from breeder farms for MG challenge during the laying cycle and confirm that point of lay broiler breeder pullets are free of MG field strain. The early detection of the disease through monitoring is essential in preventing MG vertical transmission.

There is high prevalence of MG infection in commercial chickens and progenies originally from breeder farms. This shows that MG eradication program (elimination of the positive flocks) had failed despite the practice of using mild live vaccine, killed MG vaccine, an extensive medication program and strict biosecurity measures. It is recommended that vaccination program utilizing the live MG vaccine be implemented in the farm to prevent vertical transmission to displace the MG field strain from the breeder flocks and to eradicate MG from the breeder flocks.

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