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Monitoring Broiler Breeder Flocks for *Mycoplasma gallisepticum* Infection after Vaccination with ts-11

by

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SUMMARY

Mycoplasma control in any poultry company requires an integrated approach involving diligent biosecurity, animal husbandry and disease surveillance.

Mycoplasma gallisepticum (MG) infection is very costly to the broiler industry as it predisposes birds to a variety of primary and secondary respiratory diseases thus reducing production efficiency and profitability. Since the primary disease is rather insidious, relatively difficult to confirm (especially in vaccinated flocks), prone to becoming quiescent and vertically transmitted, control measures must begin at the breeder level and involve conscientious flock surveillance. While disease eradication is the best approach to MG control the economic pressures of modern broiler production often preclude such drastic measures. Vaccination programmes are often introduced to reduce the economic impact of the disease in breeder flocks and to minimize, or hopefully prevent vertical transmission.

To prevent vertical transmission and lateral spread of field strain MG infection, early diagnosis is critical. Flock testing needs to be done every 2-4 weeks (depending on prevailing risk), on 90 birds (for an average flock of 7 000 birds) to satisfy statistical requirements for the detection of a 5% infection-rate with 99% confidence.

The RSPA test provides an inexpensive, reliable and rapid means of evaluating the serological response to ts-11 strain vaccination during the rearing phase. A floor pen trial confirmed that, three to ten weeks after ts-11 vaccination at 10 weeks of age, the RSPA test reactor rate is between 30% and 60%. PCR was used to confirm the absence of field challenge.

A retrospective analysis of 4 years of RSPA test data from broiler breeder flocks immunized with the live ts-11 strain MG vaccine indicated that traditional RSPA test monitoring protocols were unreliable as a means of differentiating ts-11 vaccination from field strain MG infection. Non-infected (PCR negative) vaccinated flocks reached sero-

positive agglutination rates of 100% making the differentiation of vaccine response and field infection impossible during the lay cycle. RSPA monitoring of broiler breeders during the pullet rearing stage (0-20 weeks) was in contrast still very effective.

While previously reported trials indicated that the introduction and subsequent serological monitoring of in-contact non-vaccinated sentinels may enhance the efficacy of the RSPA monitoring procedure this trial indicated that it does not. The ts-11 strain MG spread to in-contact sentinels so rapidly under field conditions that the serum agglutination pattern of these birds mimics that of the vaccinated pullets.

The potential for ts-11 strain MG to spread from bird to bird is a reality and even spread from pen to pen (within the same house) may be possible if biosecurity is inadequate. The decision to vaccinate should include consideration as to the consequence of ts-11 strain MG spread to surrounding susceptible flocks.

The use of molecular diagnostic techniques on pooled tracheal swabs taken from representative flock birds is a potentially cost effective and reliable means of differentiating ts-11 vaccine strain from field strain MG. PCR amplification of DNA from tracheal swab samples and strain identification based on amplicon size was shown to be a reliable and sensitive means of detecting ts-11 strain following vaccination. The proprietary PCR primer used in this trial was specifically designed to identify the ts-11 strain by amplifying a 229 bp fragment that is characteristic and distinguishable from all other MG field strain isolates based on amplicon size. This technique provides the opportunity to differentiate field strain infection from vaccine strain MG, provided strain specific PCR primers are available.

It is recommended that the RSPA assay is used to differentiate effective vaccination from field exposure during pullet rearing and PCR assay is used to monitor broiler breeder flocks for MG challenge during the laying cycle and confirm that point-of-lay broiler breeder pullets are free of field strain MG infection. Where possible flocks with a confirmed field strain challenge should be eliminated and all hatching eggs removed from the hatchery and destroyed.

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TABLE OF CONTENTS

CHAPTER 1 - INTRODUCTION 1

Defining the Problem 4

Identifying the options 6

1. Continue using the RSPA test to monitor breeding flocks. 6
2. Use the RSPA to monitor non-vaccinated sentinels. 7
3. Use ts-11 specific PCR primers to confirm the presence of vaccine strain MG. 7

Experiment Objectives 8

CHAPTER 2 - LITERATURE REVIEW 9

Controlling *Mycoplasma gallisepticum* in Broiler Breeder Flocks. 9

Mycoplasma gallisepticum Infection 9

Bio-Security 17

Immunization 20

Inactivated Vaccines 20

Live Vaccines 21

Connecticut F-strain 21

Temperature sensitive mutants 22

Monitoring 25

Indirect 27

Direct 30

Medication 34

CHAPTER 3 - MATERIALS AND METHODS 36

1. A Floor Pen Study to Evaluate the Serological response of Broiler Breeders After Vaccination with ts-11 Strain *Mycoplasma gallisepticum* Vaccine 36

2. A Retrospective Study on the use of the RSPA Test in ts-11 Vaccinated Flocks. 39

3. Evaluation of Sentinels to Monitor MG Status of ts-11 Vaccinated Breeder Flocks 39

CHAPTER 4 - RESULTS 41

1. A Floor Pen Study to Evaluate the Serological response of Broiler Breeders After Vaccination with ts-11 Strain *Mycoplasma gallisepticum* Vaccine 41

2. Retrospective Study on the use of the RSPA Test in ts-11 Vaccinated Flocks 44

3. Evaluation of Sentinels to Monitor MG Status of ts-11 Vaccinated Breeder Flocks 47

CHAPTER 5 - DISCUSSION 51

1. A Floor Pen Study to Evaluate the Serological response of Broiler Breeders After Vaccination with ts-11 Strain *Mycoplasma gallisepticum* Vaccine 51

2. Retrospective Study on the use of the RSPA Test in ts-11 Vaccinated Flocks...... 56

3. Evaluation of Sentinels to Monitor MG Status of ts-11 Vaccinated Breeder Flocks 58

CHAPTER 6 - CONCLUSIONS AND RECOMMENDATIONS.............................. 59

REFERENCES.. 62

LIST OF TABLES

Table	Title	Page
1.1.	RSPA status of vaccinated and non-vaccinated birds kept together in an isolator for 8 weeks	**6**
2.1.	Survival of MG under various conditions(Shimizu *et al*., 1990)	**10**
2.2.	Survival of MG on Various Substances (Christensen et al., 1994; Chandiramani et al., 1966)	**10**
2.3.	The rate of spread of Mycoplasma gallisepticum in a flock of susceptible chickens	**12**
2.4.	Comparison of *Mycoplasma gallisepticum* vaccine strains (adapted from Kleven 2002).	**24**
2.5.	Comparison of the indirect *Mycoplasma gallisepticum* assay techniques.	**30**
2.6.	Comparison of direct assay techniques for Mycoplasma gallisepticum	**34**
3.1.	Pullet vaccination history.	**37**
4.1.	Antibody response as measured by the Rapid Serum Plate Agglutination (RSPA) Test	**42**
4.2.	Proportion of pullets yielding MG ts-11 by PCR assay	**43**
4.3.	The average *Mycoplasma gallisepticum* RSPA test results taken over a 4 year period and representing 5 million broiler breeders from 112 farms.	**45**
4.4.	The percentage positive Rapid Serum Plate Agglutination (RSPA) tests performed on sera from 25 non-vaccinated commingled sentinels in 18 flocks comprising approximately 7 500 broiler breeders each	**48**
4.5.	The mean positive result of Rapid Serum Plate Agglutination (RSPA) tests performed on sera from 25 non-vaccinated commingled sentinels in 18 flocks comprising approximately 7500 broiler breeders each.	**49**

LIST OF FIGURES

Figure	Title	Page
2.1.	Effect of *E. coli* Lipopolysaccharide challenge on average daily gain of 26 day old broilers (adapted from Klasing *et al.,*1987)	**14**
2.2.	The influence of stocking density on the characteristics of the epidemiological curve for MG. (adapted from McMartin et al., 1987).	**17**
3.1	Experimental pen and treatment layout.	**36**
4.1.	The average RSPA status during rear of 43 flocks in region 1 after vaccination with ts-11 at 10 weeks of age.	**46**
4.2.	The average RSPA status during lay of 43 flocks in region 1 after vaccination with ts-11 at 10 weeks of age.	**46**
4.3.	The average RSPA status during rear of 40 flocks in region 2 after vaccination with ts-11 at 10 weeks of age.	**46**
4.4.	The average RSPA status during lay of 40 flocks in region 2 after vaccination with ts-11 at 10 weeks of age.	**46**
4.5.	The average RSPA status during rear of 21 flocks in region 3 after vaccination with ts-11 at 10 weeks of age.	**46**
4.6.	The average RSPA status during lay of 21 flocks in region 3 after vaccination with ts-11 at 10 weeks of age.	**46**
4.7.	The average RSPA status during rear of all 104 flocks after vaccination with ts-11 at 10 weeks of age.	**46**
4.8.	The average RSPA status during lay of all 104 flocks in region 1 after vaccination with ts-11 at 10 weeks of age	**46**
4.9.	The percentage of unvaccinated commingled sentinels showing a positive RSPA test result during rearing, after flock vaccination at 10 weeks of age.	**48**
4.10.	The percentage of unvaccinated commingled sentinels showing a positive RSPA test result during lay, after flock vaccination at 10 weeks of age.	**48**
4.11.	The mean positive RSPA test result of non-vaccinated commingled sentinels during rearing after flock vaccination at 10 weeks of age.	**49**
4.12.	The mean positive RSPA test result of non-vaccinated commingled sentinels during lay, after flock vaccination at 10 weeks of age.	**50**
4.13.	Comparison of % positive RSPA tests from vaccinated flock birds and non-vaccinated in-contact sentinels.	**50**
5.1	A comparison of *Mycoplasma gallisepticum* rapid serum plate agglutination (RSPA) test results after ts-11 vaccination as recorded by various authors.	**53**

CHAPTER 1 - INTRODUCTION

Although there are many different mycoplasma species only four are known to be economically important pathogens of domestic poultry. In South Africa *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are of significance to the chicken industry. Although both MG and MS are known to predispose birds to respiratory disease, MG has traditionally been associated with chronic respiratory disease (CRD) and MS with infections synovitis (Ley, 2003).

The Mycoplasmas belong to a unique group of prokaryotes without a cell wall, which makes them fastidious, fairly difficult to culture (specific nutrient requirements) and generally slow growing (Kleven, 2003). The avian mycoplasmas are usually isolated on Frey's or Bradbury's media which contain animal serum and are high in protein (10-15%). Although these organisms are easily overgrown by contaminants because they are slow growing, their inherent resistance to penicillin (no cell wall) and the toxic effects of thallium acetate, means that these two ingredients can be used to create a selective growth media (Kleven, 2003).

Mycoplasmas are among the most important egg-borne diseases in the South African poultry industry. The consequence of a widespread infection in a layer or breeder operation can be devastating as a result of both direct and indirect losses occurring throughout the production cycle (Ley, 2003). Loses can occur as a result of:

1. decreased egg production
2. decreased hatchability
3. reduced egg selection pressure because of the reduced hatching egg availability
4. reduced day old chick quality
5. increased chick mortality because of the exacerbated consequence of concurrent infection
6. increased medication cost
7. reduced growth rate and feed conversion
8. costly control measures involving biosecurity, vaccination and medication

9. costly eradication measures involving depopulation and site cleaning
10. costly monitoring programs involving serology and PCR

Mycoplasmosis can spread by both lateral and vertical routes so it is imperative that any control programme is based on maintaining a disease free breeding flock. It is particularly difficult to keep multi-age sites MG free. Lateral transmission occurs through direct contact and indirectly through mechanical means by way of fomites and mechanical vectors (Ley, 2003). Vertical transmission can occur (Lin and Kleven, 1982a) despite vaccination (Glisson and Kleven, 1984; Glisson and Kleven, 1985), or treatment (Ortiz, Froyman and Kleven, 1995) and infected birds remain carriers (Bencina and Dorrer, 1984) for extended periods of time. Early detection of infection is essential to prevent disease spread.

Total eradication is the best approach to mycoplasma control and needs to begin at the breeding farms. Where MG is detected in a breeding flock, depopulation of the infected sites followed by thorough site cleaning and an extended bird free period of 4 to 6 weeks is necessary. Re-stocking with mycoplasma negative birds, strict biosecurity measures to prevent the re-introduction of the disease and an effective monitoring program, must follow this to achieve success. Eradication programmes are not only expensive but also very difficult to implement, especially on multi-age sites.

In many areas of the world, including South Africa, mycoplasma-free breeding stock is placed on mycoplasma-contaminated, multi-aged farms and these disease free birds rapidly become infected. Depopulation of infected flocks is impractical and consequently many of these farms remain MG positive. Similarly multi-age commercial layer complexes house many thousands of birds of varying ages and the cost of depopulation has prohibited the elimination of MG from most of these sites. In many layer and breeder complexes the emphasis has shifted from eradication to control.

Differentiating between immunized and infected broiler breeder flocks, is pivotal to the control and eradication of MG under practical conditions. Serological monitoring

procedures have been used extensively for early detection of *Mycoplasma gallisepticum* flock infection thus forming the foundation for MG control and eradication programmes (Ley, 2003).

During the 1960s the rapid serum plate agglutination test (RSPA) displaced the tube agglutination test as the most frequently used procedure for monitoring flocks for the presence of MG antibodies. Although non-specific reactors and false positives occur, the RSPA test was selected as the primary procedure for screening flocks because of its high sensitivity and low cost (Glisson *et al.,* 1984; Kleven, 1997; Yoder, 1989). Although serum dilution can be used to eliminate false positive RSPA reactors, since MG and MS antigens agglutinate red blood cells the haemagglutination inhibition (HI) test (higher specificity) is commonly used as a "confirmatory" test (Ross, T., Slavik, M., Bayyari, G., and Skeeles, J., 1990; Ryan, 1973). The need for a reproducible test with enhanced specificity, sensitivity, and the potential for automation have stimulated the wide use of the enzyme linked immunosorbent assay (ELISA) procedure (Ley, 2003).

The introduction of vaccination as a method for MG control has complicated the evaluation of antibody assay procedures used to monitor flocks. Inactivated oil based vaccines and the live Connecticut F-strain vaccine stimulate a humoral antibody response, which is indistinguishable from field exposure based on the RSPA, HI and ELISA titers (Abd-El-Motelib, T.Y. and Kleven, S.H., 1993; Kleven, 1981). In contrast the non-virulent live vaccines, including ts-11 (Select Laboratories, Inc., Gainesville, Ga) and 6/85 (Nobilis MG 6/85, Intervet®) induce a relatively low level of circulating antibodies under experimental conditions suggesting that it may be possible to differentiate between antibody reactions induced by either vaccination or field challenge (Abd-El-Motelib *et al.*, 1993; Ross *et al*., 1990).

At present, isolation and molecular identification provide the only effective and accurate method to distinguish a vaccine reaction from field exposure to MG. Such tests are expensive and time consuming so there would be a tremendous advantage to being able to use the simple and rapid agglutination techniques to distinguish vaccine reaction from

field exposure. This dissertation investigates the serological response of broiler breeders to vaccination with ts-11 under experimental conditions; includes the analysis of 4 years of serological data from ts-11 vaccinated broiler breeder flocks and explores the possibility of using in-contact sentinels to enhance the efficacy of serological monitoring to distinguish infected from non-infected flocks.

DEFINING THE PROBLEM

In 1994 three out of six breeder units in an integrated broiler breeder company in South Africa were experiencing problems with low-grade *Mycoplasma gallisepticum* (MG) infection. These units were jointly responsible for producing approximately 3 million hatching eggs per week, each unit was situated in a geographically distinct area of the country and three different breeds were represented. Each area was very heavily populated with chicken and other avian species including broiler breeders, broilers, commercial layers, backyard scratch fowls and feral birds.

Although parent pullets were being transferred to the laying-farms free of MG, many of these flocks were being challenged during lay and a fairly high percentage of the broilers derived from these infected flocks were considered MG positive (RSPA performed at slaughter). Although lateral infection of broiler flocks was an obvious possibility, progeny from non-infected parent flocks were commonly sero-negative at slaughter, indicating that vertical transmission was likely. Chronic respiratory disease was adversely affecting broiler performance and the impact of MG infection was exacerbated by an aggressive Newcastle disease vaccination program (La Sota strain vaccine applied by spray at one day of age and again at 16-18 days).

An MG eradication program (elimination of positive flocks) had failed despite the concomitant use of an inactivated MG vaccine, an extensive medication program and strict biosecurity. A vaccination program utilizing the live ts-11 MG vaccine was implemented to prevent vertical transmission (short-term objective), to displace the field strain MG from the breeder units (medium term objective), and to eradicate MG from the

breeder flocks (long-term objective). The use of this live vaccine as part of the control program made flock monitoring with the traditional RSPA test difficult.

Since the accurate and timely identification of field-strain MG in the breeder flocks was key to disease containment and/or eradication a great deal of time and money was being spent on intensive monitoring programmes. Although ts-11 vaccination had been shown to induce a relatively low level of seroconversion in experimental groups (Ley, McLaren, Miles, Barnes, Miller, and Franz, 1997; Abd-El-Motelib et al., 1993) and the vaccine manufacturer claimed it was possible to differentiate vaccinated from infected with the RSPA test, a small pilot study was initiated to confirm the feasibility of using the RSPA testing to distinguish vaccinated from non-vaccinated birds. Ten specific pathogen free (SPF) white leghorn pullets (Lohman, Cuxhaven) were reared in a filtered air positive pressure (FAPP) house and confirmed MG negative at 10-weeks of age by PCR. Five of these birds were vaccinated by eye drop with MG ts-11 as directed by the manufacturer and the other five were used as non-vaccinated controls. All ten birds were placed in an isolator for the 8 week experiment. All birds were bled at the onset of the trial and weekly thereafter for eight weeks. Sera were tested for the presence of antibodies with a *Mycoplasma galisepticum* RSPA test using the commercial NOBILIS® MG antigen (Intervet). At the end of the trial, an in-house MG specific PCR probe was performed on tracheal swabs from all the birds to track the spread of the ts-11.

The RSPA test results of birds kept in an isolator for 8 weeks (the pilot trial) are presented in Table 1.1. By 2 weeks post vaccination 2/5 vaccinated birds became RSPA positive and by 3 weeks 3/5 were positive. Although all 5 vaccinates were MG probe positive at 8 weeks two vaccinated birds remained RSPA negative for the entire 8 weeks.

Despite being in direct contact with the vaccinates for 8 weeks all of the non-vaccinated pullets were MG probe negative at 8 weeks and none showed any sign of seroconversion as measured by RSPA.

Table 1.1. RSPA status of vaccinated and non-vaccinated birds kept together in an isolator for 8 weeks

Treat	RSPA	Weeks Post Vaccination								
		0	1	2	3	4	5	6	7	8
	Pos	0	0	2	3	3	3	3	3	3
Vacc	Neg	5	5	3	2	2	2	2	2	2
	% Pos	0	0	40	60	60	60	60	60	60
	Pos	0	0	0	0	0	0	0	0	0
Non-vacc	Neg	5	5	5	5	5	5	5	5	5
	% Pos	0	0	0	0	0	0	0	0	0

This pilot study was sufficient confirmation of the manufacturer's claims/research findings, for management to agree to proceed with field vaccination with ts-11 strain MG.

Once ts-11 vaccination was introduced on a large scale it was very difficult to differentiate between a normal ts-11 vaccine reaction and a field challenge using RSPA test. Flocks seldom exceeded 30% positive rates during rearing and frequently became 100% positive within 10-30 weeks of transfer to the laying house despite being PCR negative for field strain MG. This was in contrast to the previously reported pattern of RSPA test seroconversion (100% seroconversion within weeks of vaccination) following vaccination of breeder with ts-11 strain MG vaccine (Abd-El-Motelib et al., 1993, Whithear, K.G., Soeripto, Harrigan, K.E. and Ghiocas, E., 1990a). *These findings indicated the need to verify the flock seroconversion pattern to ts-11 strain MG vaccination of broiler breeders in the Republic of South Africa, under both experimental and field conditions.*

IDENTIFYING THE OPTIONS

1. Continue using the RSPA test to monitor breeding flocks.

The RSPA test had the advantage of being quick, simple and inexpensive. It had, in the past been used very effectively to indirectly determine flock exposure to MG and data supplied by the vaccine manufacturer (Select Laboratories, Inc., Gainesville, Georgia USA) and published data (Abd-El-Motelib *et al.*, 1993, Whithear *et al.*, 1990a) suggested that it *was* possible to distinguish between poorly vaccinated, well vaccinated and field

strain challenged flocks based on the RSPA test reactor rate of sera from sample birds representing the flock.

This option would require *verification of the seroconversion characteristics of a commercial broiler breeder flock in the Republic of South Africa, under both experimental and field conditions.*

2. Use the RSPA to monitor non-vaccinated sentinels.

To improve the specificity of the monitoring procedure while maintaining the advantage of using the quick, simple and inexpensive RSPA test as a means of indirectly monitoring breeding flocks, it might be possible to use non-vaccinated commingled, in-contact or separately penned sentinels for monitoring purposes. Lateral spread of ts-11 strain MG to non-vaccinated commingled pen mates and birds in adjacent pens, a short distance from the vaccinated birds was shown to be limited (Ley et al., 1997b; Abd-El-Motelib and Kleven, 1993). This limited seroconversion may be a means of accentuating the difference between RSPA response of vaccinated and exposed birds.

This option would require *verification of the seroconversion characteristics of non-vaccinated commingled or in-contact pen mates and those in adjacent pens, a short distance from a vaccinated commercial broiler breeder flock in the Republic of South Africa, under both experimental and field conditions.*

3. Use ts-11 specific PCR primers to confirm the presence of vaccine strain MG.

Although the PCR test is a lot more expensive than the RSPA assay the specificity and sensitivity of this technique makes it an attractive alternative. It is especially useful when trying to distinguish field strain from vaccine strain mycoplasmas. The cost could be reduced significantly by pooling samples (Tyrrell and Anderson, 1994).

This option would require *verification of the effectiveness of a practical flock sampling technique, pooling of samples and a locally available ts-11 specific PCR probe test.*

EXPERIMENT OBJECTIVES

A floor pen study was designed to determine whether:

1. the previously reported pattern of RSPA test seroconversion following vaccination with ts-11 strain (Abd-El-Motelib *et al.*, 1993, Whithear *et al.*, 1990a) applied to broiler breeders raised in the Republic of South Africa.
2. lateral spread of ts-11 strain MG to non-vaccinated commingled pen mates and birds raised in adjacent pens, a short distance from the vaccinated birds, would occur and how it would affect the RSPA test results.
3. using a ts-11 specific PCR for determining flock MG infection status warranted further investigation.

Four years of RSPA test results from ts-11 vaccinated commercial broiler breeder flocks were reviewed to establish

1. the pattern of flock seroconversion under field conditions
2. the accuracy of the RSPA seroconversion characteristics as a means of distinguishing vaccination from infection using the PCR offered by a local diagnostic laboratory as a confirmatory test.

Finally, individually tagged, non-vaccinated, commingled sentinels were introduced into a vaccinated commercial broiler breeder flock and monitored serologically and by PCR to establish the feasibility of this technique of MG monitoring.

CHAPTER 2 - LITERATURE REVIEW

CONTROLLING *MYCOPLASMA GALLISEPTICUM* IN BROILER BREEDER FLOCKS.

The focus of an MG control programme is to minimize the economic impact of the disease. The four main components of such a control programme are bio-security, disease monitoring, medication and immunization. Control measures need to be focused on reducing the frequency of challenge; enhancing host resistance; reducing organism virulence; and reducing the challenge dose. Strict bio-security practices limit the risk of lateral and vertical transmission while immunization and medication are directed more specifically at minimizing the risk of trans-ovarian transmission. The specific focus of these interventions is guided by the implementation of an intensive disease-monitoring programme.

Mycoplasma gallisepticum Infection

Mycoplasma gallisepticum (MG) infection can cause primary disease in young chickens typified by coughing, rales, airsacculitis and poor growth. More frequently however the disease is characterized by severe secondary infection (usually *Escherichia coli*) resulting in a high level of condemnations at processing. MG infection of broiler flocks exacerbates the effect of any factor compromising the respiratory system, including the administration of viral vaccines, poor environmental management and concomitant infection with other respiratory pathogens.

Adult birds seldom show clinical signs of infection but mild change in egg quality and drops in egg production may be noticed. More importantly transovarial transmission of MG does occur and offspring from infected flocks perform notoriously badly.

MG is a very fragile (no cell wall), host adapted (avian only), fastidious (specialized growth requirements) organism (Kleven, 2003). Since it is thought not to be able to survive outside the host for more than a few days (Ley, 2003) direct contact between

susceptible and clinically or sub-clinically infected birds is the most important means of lateral transmission.

Table 2.1. Survival of MG under various conditions(Shimizu *et al*., 1990)

Conditions	Survival
Sunlight	<15-120 min
UV light	30-90 min
Well water with 1% serum	7 days
Well water	4-5 days
50% soil extract	1-3 days
Dry at 4°C	61 days
Dry at 20°C	10-14 days

Table 2.2. Survival of MG on Various Substances (Christensen et al., 1994; Chandiramani et al., 1966)

Substance	Survival
Cotton	4 days
Rubber	2 days
Straw	2 days
Shavings	8 hours
Wood	1 day
Feed	4 hours
Feathers	4 days
Hair	3 days
Ear	4 hours
Nose	1 day
Skin	<4 hours
Buffer	1 day
Chicken faeces	1-3 days
Muslin cloth	3 days @20°C, 1 day @ 37°C
Egg yolk	6 weeks @20°C, 18 weeks @ 37°C

Backyard flocks (McBride, Hird, Carpenter, Snipes, Danaye-Elmi and Utterback, 1991), wild birds (Fischer, Stallknecht, Luttrell, Dohndt and Converse, 1997; Ley, Berkhoff and McLaren, 1996; Mikaelian, Ley, Claveau, and Lemieux, 2001, Stallknecht, Luttrell,

Fischer and Kleven, 1998), commercial layer flocks (Mohammed, Carpenter and Yamamoto, 1987) and previously infected convalescent birds whether treated or not (Glisson *et al.*, 1984; Glisson *et al.*, 1985; Lin and Kleven 1982a; Ortiz, A., Froyman, R. and Kleven S.H., 1995) have been identified as important carriers so direct contact with such birds should be avoided.

Three published studies have shown that *Mycoplasma gallisepticum* may survive outside the host a lot longer than commonly accepted (Chandiramani, Van Roekel and Olesiuk, 1966; Christensen, Yavari, McBain and Bradbury, 1994; Shimizu, Nagatomo and Naghama, 1990). This research implies that lateral transmission of MG through indirect contact is a risk which is probably one of the more important reasons why outbreaks continue to occur despite increased efforts to control the disease.

The frequency with which exposure to MG occurs through both direct and indirect contact can be controlled through bio-security

Strain variance in pathogenicity or virulence has been well established (Yoder Jr., 1986; Kleven, Khan and Yamamoto, 1990; Whithear, Soeripto and Ghiocas, 1990b). Less virulent (F) strains have been used in the field to displace more virulent field strains from multi age complexes (Kleven *et al.*, 1990).

Mycoplasma gallisepticum appears to have the capacity to alter the expression of surface antigens to evade the host immune response (Kleven, 2002; Boguslavsky, Menaker, Lysnyansky, Liu and Levisohn, 2000; Liu, Garcia, Levisohn, Yogev and Kleven, 2001). This variable surface antigen expression could explain the phenomenon of chronic infection and carrier state despite the initiation of a strong immune response (Ley, 2003).

The source-case of an outbreak is the result of vertical transmission or direct contact with infected birds. Both vertical and lateral transmission of *Mycoplasma gallisepticum* occurs most frequently during the acute phase of the infection when organism levels in the respiratory tract peak (Glisson, *et al.*, 1984).

Vertical transmission was found to peak at 25-50% approximately 4 weeks after infection and then decline to 3-5% (Sasipreeyajan, Halvorson and Newman, 1987, Lin, *et al*., 1982b, Glisson *et al*., 1984; Glisson, *et al.,* 1985; Roberts and McDaniel, 1967). Although under field conditions egg transmission rates are generally very low these infected birds invariably cause entire flocks to become MG positive through lateral spread (Levisohn and Kleven, 2000).

The mucous membranes of the upper respiratory tract and eyes are the portals of entry for lateral infection (Bradbury and Levisohn, 1996). Spread within a flock initially occurs via contaminated droplets projected from one bird to another during coughing and sneezing (Bradbury, 2003). As the infection rate within the flock increases so too does the level of environmental contamination and the possibility for indirect spread via contaminated fomites (Ley, 2003).

Spread within a flock occurs in 4 phases (according to antibody response) as depicted in Table 2.3. (McMartin, Khan, Farver and Christie, 1987):

1. **Latent phase** when the source cases incubate the disease and develop an immune response.
2. **Early phase** when lateral spread is initiated.
3. **Late phase** when lateral spread is rapid.
4. **Terminal phase** where the remainder of the flock become antibody positive.

Table2. 3. The rate of spread of Mycoplasma gallisepticum in a flock of susceptible chickens (McMartin *et al*., 1987):

Phase	Days post inoculation	% antibody positive
1	12 - 21	0
2	13 - 42	5 - 10
3	20 - 74	90 - 95
4	23 - 93	100

Host resistance to infection and hence rate of spread is dependent on several factors. The immune system is probably the most important host factor which determines the consequence of exposure to MG.

Mycoplasma infection has been shown to have a direct effect on both B and T lymphocyte proliferation, cytokine release and antibody production, which indicates a combined antibody and cell mediated response (Avakian and Ley, 1993; Gaunson, Philip, Whithear and Browning, 2000; Razin, 1985). The relative efficiency of a production manager to minimize host, agent and environment dependent stress is directly related to the bird's state of well-being and hence performance.

Stress has been defined as a non-specific response of the body to any demand made upon it which can be interpreted in poultry production to mean the metabolic response of the body to external factors that negatively impact well-being.

Stress of any kind stimulates a cascade of physiological and biochemical changes which have an immune-suppressive effect (Siegel, 1994). Activation of the neuro-endocrine axis stimulates corticosteroid secretion, which modulates immune cell activity. Lymphocytes stimulated by corticotropin releasing hormone are capable of ACTH secretion thus creating a lymphocyte-neuro-endocrine feedback axis.

Corticosteroids released in response to stress form a complex with receptors on lymphoid cells and then pass into the nucleus to influence nucleic acid organization. Although this stimulates an increase in circulating heterophils and granulocytes the number of circulating lymphocytes is reduced and antibody and cell mediated immunity depressed (Siegel, 1994). Corticosteroids also reduce inflammation by reducing macrophage migration and inhibiting phagocytosis.

Stress is cumulative and impacts animal performance measurably once the aggregate of each individual stress exceeds the animals coping mechanisms (Klasing, Laurin, Peng, and Fry, 1987). Growth rate is negatively correlated with disease stress so it is reasonable to assume that small birds in a flock have a higher disease stress load than the large birds

in the flock (environmental conditions and genetics are constant). Since the negative impact that stress has on performance is directly proportional to the existing stress load the capacity to cope with additional stress varies with each individual in a population. This has been demonstrated by comparing the effect of induced inflammatory stress (injected antigen) on growth rate of birds under different stress loads (large vs small birds) in a flock. The growth retarding effect of the injected antigen was significantly more obvious in the smaller (stressed) birds than the larger (non-stressed) birds in the group, as shown in Figure 2.1 (Klasing *et al,* 1987).

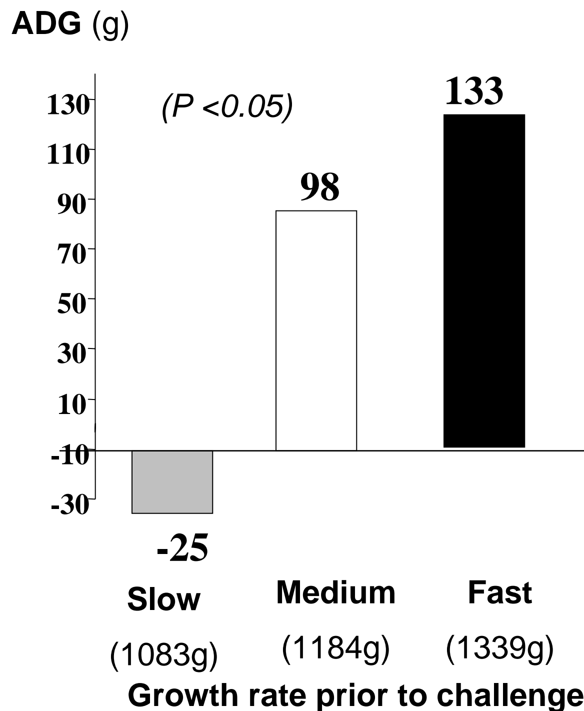


Figure 2.1. Effect of *E. coli* Lipopolysaccharide challenge on average daily gain of 26 day old broilers (adapted from Klasing *et al.*,1987)

Although stress level has to exceed threshold to affect performance directly any amount of stress is immunosuppressive and erodes host resistance. Stress lowers the minimum dose required for infection and increases the risk of disease developing subsequent to exposure.

The risk of disease spread within the population is increased by the presence of susceptible individuals. Infected birds act as amplifiers for the infectious organisms and thus increase the challenge dose to which the pen mates are exposed. Disease control strategy would not be complete without measures to reduce stress.

In an intensive production system where birds are housed in a controlled environment bird performance is dependant on the manager's ability to satisfy bird requirement.

Genetic potential (genotype), of broiler breeds exceeds the production parameters (phenotype), obtained under commercial conditions. Environmental constraints to production efficiency include variability in nutrition, temperature, air quality, lighting, litter condition, stocking density, exposure to disease and inappropriate management of feeding, drinking and ventilation systems. Disharmony between the flock and the environment will result in diversion of protein and energy into non-productive metabolic activities, which detract from potential production standards and frequently add to the cost of salable product (Beck, 1996; Humphry, Koutsos and Klasing, 2003.).

Environmentally induced physiological and psychological stress of any kind has an immune suppressive effect (Siegel, 1994). Tracheal epithelium is damaged as a result of vaccine virus replication, formalin fumigation, ammonia, dust and inflammatory response (Anderson, Wolfe, Cherms and Roper, 1968; Carlile, 1984). The cilliary escalator clearance system of the upper respiratory tract plays a critical role in preventing respiratory tract infection with respiratory pathogens (Bradbury, 1998).

Cold air paralyzes the cilliary cells, reducing the efficacy of the innate protective mechanisms and increasing susceptibility to lower respiratory tract infection. Heating of the house environment lowers relative humidity, increases evaporation from the mucous membrane surface, increases mucus viscosity, and reduces the efficacy of the cilliary clearance mechanisms. The narrow trachea and bronchi of young chicks are more likely to become blocked with inhaled and inflammatory response debris.

There is a strong association between respiratory disease and air quality in animal confinement units (Maghirang, Manbeck and Puri, 1991). Birds are continuously exposed to aerial pollutants in the form of organic and inorganic dust, microbes, endotoxins, and noxious gasses (Sander, 1994). Most dust particles in the poultry house are <1μm (90% are between 0.1 and 0.5μm) and consist of feather barbules, skin debris, feed particles, litter components and microorganisms. Particles >10μm deposit in the nasal passages and cause nasal blockage, those between 5 and 10μm are retained in the upper respiratory tract and particles <5μm are able to reach the air sacs and lungs.

Ammonia, the product of bacterial deamination or reduction of the nitrogenous fraction of poultry manure, is a highly irritant gas that accumulates to high levels with increasing litter moisture (Sander, 1994). Although the damage resulting from low-level exposure to ammonia is not detectable, continuous exposure to levels of 10ppm causes excessive mucous production, matted cillia and areas of decilliation (Nagaraja, Emery, Newman and Pomeroy, 1983). Increased mucous secretion causes thickening and increased viscosity of the mucous blanket, which compromises the mechanical efficiency of the mucocilliary clearance system (Sander, 1994).

Stocking density is an important epidemiological variable affecting the characteristics of an MG epidemic curve as shown in Figure 2.2 (McMartin *et al.,* 1987). At the stocking densities used in South Africa (22 birds/m^2) the ascending part of the epidemic cure is near vertical, indicating extremely rapid spread of MG within flocks.

Poultry production has grown rapidly globally and economic pressure has driven producers to maximize return on assets managed. The traditional poultry producing areas have become more densely populated creating the equivalent of huge multi-age sites. The effective epidemiological unit for infectious disease has expanded from house to farm to region. The frequency and dose of exposure to MG has increased and the risk of infection is consequently significantly higher thus emphasising the need for good biosecurity (Kleven, 2002).

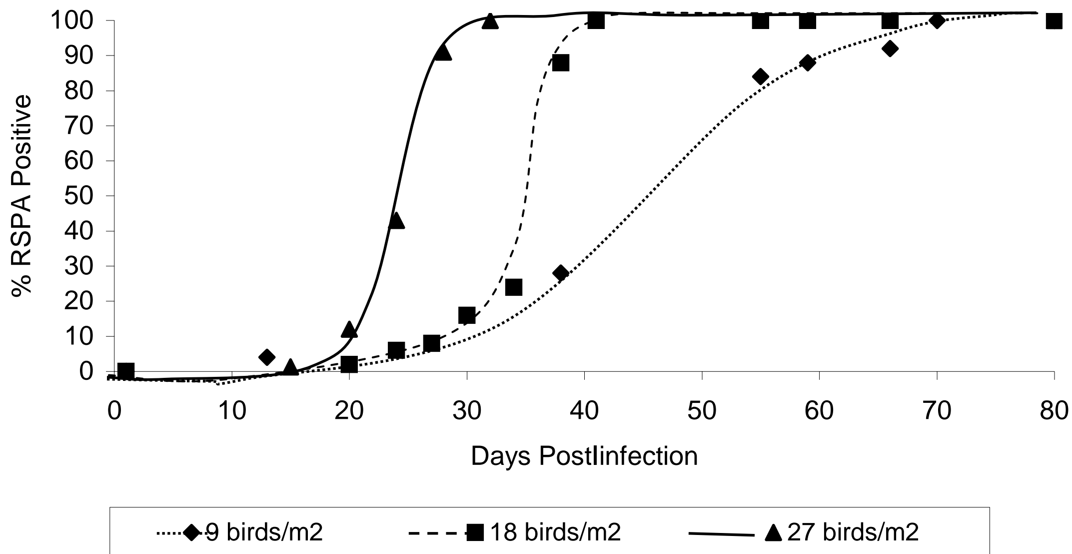


Figure 2.2. The influence of stocking density on the characteristics of the epidemiological curve for MG. (adapted from McMartin et al., 1987).

BIO-SECURITY

MG infection can only occur when the organism comes into contact with the host.

Risk = chance x frequency

Every event in the production process that involves transgression of the house/ site/ farm/complex boundary needs to be evaluated as to necessity. Each time the bio-security line is crossed there is risk of contact between an infectious organism and the host. Avoidance is the best form of prevention but where the event is unavoidable bio-security measures need to be implemented to alleviate risk.

Successful Mycoplasma control begins with a Mycoplasma-free breeding flock because vertical transmission is difficult to prevent (Bradbury, 1998). Strict bio-security and the implementation of all-in all-out production systems are invariably adequate to avoid lateral infection and spread.

Despite the increased need for bio-security under the high-risk conditions generated by industry growth, control programs are frequently compromised by cost saving initiatives. For short-term return, investment in bio-security is difficult to justify. In the commercial layer industry where economy of scale has spurned the development of enormous multi-age sites effective MG control is difficult (Mohammed *et al.,* 1987).

MG has been shown to survive for as long as 2 weeks at room temperature, and for as long as 8 weeks at 4°C (Shimizu *et al.,* 1990). Moisture and organic matter significantly increase the ability for survival in the environment and egg debris appears to be very protective (Chandiramani *et al.,* 1966).

The persistence of MG in the environment creates the opportunity for horizontal transmission of infection within and between flocks (within a house, from house to house and from farm to farm). All-in-all-out management systems with extreme emphasis on thorough cleaning and disinfection are crucial to any eradication or control programme. Houses should be left bird free for 4-6 weeks between grow-outs to avoid cycle-to-cycle transmission.

MG can survive long enough in feed to be transmitted with feed movement (Christensen *et al.,* 1994). Good manufacturing practice, especially heat treatment during feed preconditioning and pelleting will ensure that feed leaving the feed mill is uncontaminated. On farm contamination of feed is a possibility so although the chance of infection via this route is fairly low (low challenge dose) the frequency of exposure (number of feedings per day) is high. Feed constitutes a risk for infection and should not be moved from one house to another.

House dust is a good medium for the transfer of respiratory pathogens and MG is no exception. Good ventilation practice will reduce the dust and pathogen load and reduce the opportunity for lateral transmission within the house (Sander, 1994). Dust and feathers exiting the house do however provide a means of MG spread from house to house and site to site.

Water is often overlooked as a source of infection, coming possibly from a contaminated water source but more likely through contamination by infected birds within the house. Nipple drinkers reduce the possibility of lateral spread but sufficient chlorination of water is also very effective.

People are the biggest risk with respect to contamination since they have opportunity for contact with other potentially infected birds and MG can be transferred on the skin, hair, and clothes or as a transient nasal infection (Christensen *et al.,* 1994). By necessity personnel working on site have to cross the bio-security line on a daily basis making avoidance impossible.

As with humans, all animals can act as mechanical vectors and transfer MG from one poultry house to another. Animal access, including rodents, to the breeder house constitutes a serious break in bio-security.

Since MG is an avian specific pathogen, wild birds can harbour an active infection or develop a carrier state so are a potential hazard (Ley *et al.,* 1996; Fischer *et al.,* 1997; Stallknecht *et al.,* 1998; Mikaelian, *et al*., 2001). Where infected wild birds are allowed access to the poultry house, feed and/or water, both infection and cross contamination can occur.

Exposure to MG does not necessarily mean infection. Host resistance and organism virulence and challenge dose are important variables.

$$\textit{Chance} = \frac{1}{\textit{Host resistance}} \times \textit{virulence} \times \textit{challenge dose}$$

While effective bio-security measures reduce the challenge dose, the manager relies on effective immunization to improve host resistance. Vaccine strains have also been used to displace virulent field strains from multi-age complexes thus reducing challenge strain virulence (Kleven *et al*., 1990).

University of Pretoria etd – Collett, S R (2005)

IMMUNIZATION

Birds infected with virulent strains of MG early in life have a lower incidence of vertical transmission when compared to birds infected during lay. Although a degree of resistance develops subsequent to infection many birds remain sub-clinical carriers after recovery (Bencina *et al.*, 1984).

Controlled exposure to field strain MG before the onset of lay was initially considered as a means to reduce the impact of natural infection occurring during lay. This would however increase susceptibility to other respiratory pathogens, induce a carrier state and maintain instead of eradicate the disease.

Inactivated Vaccines

Inactivated MG vaccine became popular in the early 1980s. Although originally used in commercial layer flocks to prevent egg production loss, these bacterins were later used in broiler breeder flocks to reduce the vertical transmission rate.

Although bacterin immunization cannot be used alone to eradicate MG (Levisohn *et al*., 2000) it is an important part of a control program. Challenged vaccinates are readily infected with pathogenic MG, so egg transmission and lateral spread still occurs (Levisohn *et al*., 2000). The level of colonization and vertical transmission rate are however reduced (Kleven, 1985; Yagihashi, Nunoya, Sannai and Tajima, 1992) and birds are reasonably well protected against clinical signs (Yoder, Hopkins and Mitchell, 1984; Karaca and Lam, 1987), lesions and production losses (Hildebrand, Page and Berg, 1983; Yoder and Hopkins, 1985).

Several adjuvant enhanced bacterin vaccines are commercially available but they are expensive and administration is difficult because they need to be injected twice with a 4-6 week interval (Ley, 2003). Subunit vaccines containing MG surface proteins have revived interest in the inactivated vaccines (McLaren, Ley, Berkhoff and Avakian, 1996; Czifra, Sundquist, Hellman and Stipkovits, 2000).

Live Vaccines

The search for a more effective means of eradication led to the development of live vaccines. The challenge was to develop a live vaccine that was sufficiently virulent to stimulate a protective immune response but lacked the ability to spread.

Connecticut F-strain

For many years the Connecticut F-strain MG vaccine was used to reduce the economic impact of the disease. In 1980 it was shown that there was a definite benefit from the vaccination of birds between ages of 8 and 18 weeks of age with the F- strain via the drinking water (Carpenter, Mallinson, Miller, Gentry and Schwartz, 1981). Immunized birds did not however produce as well as non-vaccinated, non-infected birds. It was concluded that immunization with the F-strain should only be undertaken when the risk of infection exceeded 60% (Carpenter *et al.*, 1981).

Although the F-strain vaccine is produced commercially and used in the USA it is not available outside the country. Vaccine safety is questionable since lateral and vertical transmission of the F-strain can occur and it is highly pathogenic in turkeys (Lin *et al.*, 1982b). Direct contact is necessary for F-strain spread in chicken flocks so the risk of spread to adjacent farms is limited (Kleven, 1981). The capacity for lateral spread of the F-strain within chicken flocks is an advantage because inexpensive mass vaccination techniques can be used. In addition, long-term vaccination in multi-age sites results in the replacement of the field strain by the F-strain (Kleven *et al.*, 1990; Cummings and Kleven, 1986).

Although eye-drop vaccination with the F-strain at six weeks of age reduced the colonization rate of the upper respiratory tract it failed to prevent infection after field-strain challenge (Cummings *et al.,* 1986; Levisohn and Dykstra, 1987). Immunization with the F-strain does not prevent transovarial transmission (Glisson *et al.*, 1984).

Despite the advantages of the F-strain vaccine it also has many of the disadvantages of the inactivated vaccines:

1. MG free chickens tend to lay better than F-strain immunized ones.
2. F-strain is too virulent for young chicks and broilers.
3. F-strain is fully virulent for turkeys.
4. F-strain is capable of lateral spread.
5. F-strain does not completely block transovarial transmission when birds are challenged with virulent MG.

Temperature sensitive mutants

Although temperature sensitive mutant strains are able to colonize the upper respiratory tract they are not able to survive at the temperature of the lower tract and air sacs. Colonization of the upper respiratory tract induces a strong protective immune response while lower tract damage and vertical transmission of MG is avoided.

Live attenuated strain vaccines were originally thought to prevent field infection by blocking and competing for attachment sites but protection is more likely the result of an appropriate immune response induced by vaccine strain colonization of the upper respiratory tract (Levisohn, *et al.*, 1987).

Ts-11 and 6/85 are the only live Mycoplasma vaccines registered in South Africa. ts-11 (Select Laboratories, Inc., Gainesville, Georgia USA) is a frozen vaccine stored at –70° C which is thawed at room temperature immediately before use and administered by eye-drop to birds that are at least 9 weeks of age (Ley, 2003). The 6/85-vaccine is freeze-dried (Nobilis MG 6/85, Intervet®) and is applied by aerosol spray to birds 6 weeks or older (Ley, 2003).

Only one application of ts-11 or 6/85 is necessary and birds should be vaccinated at least 3 weeks before expected exposure. It is safe to inoculate birds simultaneously with other respiratory vaccines. There is reportedly no colonization of the oviduct, or vertical transmission (Evans and Hafez, 1992).

As with any live vaccine there are concerns as to their safety. There is always the possibility of reversion to virulence and spread to non-target flocks. Closely related derivatives of all three live vaccine strains have been detected in non-target flocks (Ley, 2003; Ley, Avakian and Berkhoff, 1993).

ts-11

ts-11 is a live chemically induced mutant strain developed in Australia for its temperature sensitivity (Whithear *et al*., 1990a).

The ts-11 mutant strain persists in the upper respiratory tract for the life of the flock and ensures life long immunity (Whithear, 1996). Although continual stimulation of the immune system is necessary for protection against field strain challenge it also generates a positive response on plate agglutination test.

Several conclusions can be drawn from a comparative study involving three live vaccines (F-strain, ts-11 and 6/85) and one inactivated oil based vaccine (MG-bac) (Abd-El-Motelib *et al.,* 1993).

In general ts-11 and 6/85 strains induced milder post vaccination reactions than the F-strain, weaker immunological responses and did not persist in the respiratory tract for the trial period. The F-strain vaccinated chickens had the fewest and mildest air sac lesions after challenge with a virulent R-strain.

There is a complex relationship between infectivity, pathogenicity and immunogenicity of MG strains. The level of protection induced with a live MG vaccine is correlated to the virulence of the vaccine (Lin *et al*., 1982b). The more virulent F-strain vaccines induce higher levels of circulating antibodies than the less virulent ts-11 and 6/85 vaccine strains. Protection does however not correlate with circulating antibody titre (Lam and Lin, 1984; Talkington and Kleven, 1985; Whithear *et al*., 1990a; Abd-El-Motelib *et al*., 1993). Local production of Ig A by respiratory epithelial cells may play an important part in the protection against MG infection (Avakian *et al*., 1993).

Table 2.4. Comparison of *Mycoplasma gallisepticum* vaccine strains (adapted from Kleven 2002).

Vaccine Characteristic	F-strain	6/85	TS-11
Form	Lyophilized	Lyophilized	Frozen
Route	Various	Spray	Eye-drop
Virulence: after vaccination			
• **Clinical signs**	mild	none	none
• **Egg production loss**	moderate	mild	mild
Persistence			
• **Culture**	Excellent	Poor	Good
• **PCR**			
Transmission			
• **Lateral**			
Within flocks	Moderate[1]	Poor	slow
Between flocks	Numerous– turkeys[2]	Turkeys and chickens	Chickens
• **Vertical**	Occurs[3]	None	None
Serum antibody response	Moderate	None	Slow
Protection. After field-strain challenge:			
• **Clinical signs**	absent	absent	absent
• **Egg production drops**	mild	moderate	moderate
• **Vertical transmission**	occurs	occurs	occurs
Displacement of field strain	Excellent	???	Good

1 (Cummings and Kleven, 1986, Kleven et al., 1990) 2. (Ley et al., 1993) 3. (Lin and Kleven, 1982a)

Nobilus 6/85

In the early 1990s an MG strain, Intervet 6/85 was created by in vitro passage modification and tested as a vaccine candidate for the prevention and control of MG (Evans *et al.*, 1992).

Colonization of the trachea was detectable from 4 to 8 weeks after vaccination (Ley, *et al.*, 1997). Vaccinates were protected against airsacculitis and although egg production dropped acutely after challenge with field strain MG, recovery was rapid. Challenge

studies at Intervet have shown 6/85-strain immunized birds produce more eggs than the F-strain immunized birds after virulent MG challenge.

It is possible to differentiate vaccine reaction from field challenge serologically. In contrast to field challenge 6/85-strain vaccinates do not produce sufficient antibodies to cause agglutination on RSPA tests (Ley *et al.*, 1997a; Abd-El-Motelib *et al.*, 1993)

The use of 6/85 strain vaccine poses less of a risk to surrounding complexes than the F-strain vaccine. There is little if any lateral spread of infection and low virulence, even with repeated passage in either turkeys or chickens (Ley *et al*., 1997; Evans *et al*., 1992). There have been instances where a virulent 6/85 strain MG was recovered from a non-vaccinated turkey flock (Kleven, 2002).

MONITORING

Disease surveillance involves the continuous observation of a flock for the purpose of early disease detection and control. The aim of a broiler breeder MG surveillance program is to prevent vertical transmission of the disease. Early disease detection through monitoring is pivotal in preventing vertical transmission of MG.

Breeder flocks must be monitored for MG at regular intervals for early detection of changes in prevalence. A change in prevalence over time indicates a change in incidence which signals the need for corrective action to prevent disease spread. Unless monitoring includes true random sampling, results cannot be taken to be absolute measures of disease incidence and prevalence, but may serve as adequate indicators for intervention.

Vertical transmission rates vary from 50% in the acute phase of infection to less than 3% in the chronic phase and yet whole flocks derived from infected parents invariably become MG positive (Levisohn *et al*., 2000). Vertical transmission begins as early as 17 days after exposure in non-immunized birds and as late as 82 days in birds receiving 2 inactivated oil based bacterin vaccines (Glisson *et al*., 1984).

The incubation period for MG varies considerably with the degree of stress, the virulence of the challenge strain and complicating respiratory infection but can be as short as 6 days. In most outbreaks birds become serologically positive before showing symptoms (Ley, 2003). Conversely susceptible flocks exposed to virulent MG challenge may show clinical signs before becoming serologically positive.

The procedure used to monitor breeder flocks must be sufficiently specific and sensitive to detect infection within a flock before vertical transmission occurs or at least before potentially infected eggs hatch.

The index case could produce infected eggs within 17 days but peak shedding occurs when colonization peaks at 3-6 weeks after flock exposure (Glisson *et al*., 1984; Glisson *et al*., 1985; Sasipreeyajan *et al*., 1987). After flock exposure to MG, there is a latent phase of 12 to 21 days in which less than 5% of the flock has a detectable antibody response (McMartin *et al*., 1987). To prevent vertical transmission the monitoring system must be capable of detecting infection at the 5% level with 99% confidence. The sample size (n) that must be tested to have 99% confidence in determining whether MG is present at a prevalence of 5% in a flock of (N) birds can be estimated by calculation (Cannon and Roe 1982).

$$n = [1-(1-0.99)^{1/d}] \times [N-d/2] +1$$

For a flock of 7,000 birds with a prevalence of 5% (350/7,000) the required sample size is:

$$n = [1-(1-0.99)^{1/350}] \times (7{,}000-350/2) + 1$$

$$n = [1-0.9869] \times 6825 +1$$

$$n = 0.0131 \times 6825 +1$$

$$n= 90.4075$$

$$\mathbf{n \sim 90}$$

To detect a 5% infection-rate with 99% confidence in a flock of 7000 birds the minimum sample size is 90 (Laughlin, and Lundy, 1976).

To prevent infected eggs from entering the hatchery it would be necessary to sample flocks every 2 weeks (assuming 100% sensitivity for the test system). The testing interval can be extended by two weeks where hatchery tracking systems allow infected egg removal from the setters. This extension also allows sample size to be reduced since disease prevalence increases to approximately 10% by 13-42 days after exposure (McMartin *et al*., 1987).

For a flock of 7,000 birds it is only necessary to sample 45 birds every two weeks (DiGiacomo, 1986.).

$$n = [1-(1-0.99)^{1/700}]x\ (7{,}000-700/2) + 1$$

$$n = [1-0.9934] \times 6650 + 1$$

$$n = 0.0066 \times 6650 + 1$$

$$n = 44.89$$

$$\mathbf{n \sim 45}$$

Monitoring of flocks for early disease detection can be achieved by direct or indirect methods.

Indirect

Indirect monitoring involves the detection of serum antibodies generated in response to infection. Although tube agglutination was the original test used for flock monitoring this has given way to the Rapid Serum Plate Agglutination (RSPA) or the Haemagglutination Inhibition test (HI). More recently the indirect ELISA test has become the method of choice (Ley, 2003).

Rapid Serum Plate Agglutination (RSPA)

Agglutination refers to the clumping of particulate matter by antibodies called agglutinins. The reaction takes place on the surface of particles such as erythrocytes,

bacteria or latex with surface antigens arranged in such a manner that specific binding sites are exposed to the agglutinins. The reaction takes place in two phases, beginning with specific antibody-antigen binding, followed by visible aggregation of the particles.

Particles are ordinarily held in suspension by repulsive force generated by their negative surface charge (*zeta* potential). Clumping is initiated when the attraction between the agglutinin and the surface antigen exceeds the *zeta* potential. An extensive lattice of antigen-antibody complex develops causing the aggregate to precipitate (Nichols and Nakamura, 1980). The concentration of hydrogen ions in solution will affect the test sensitivity by altering the zeta potential. Dirty glassware, certain chemicals and autoagglutinating viruses can cause false positives.

The test procedure involves mixing a drop of serum (0.02ml) with a drop of antigen (0.03ml) on a glass plate, gently agitating the plate for 2 minutes and then evaluating the degree of agglutination. It is preferably to work on a plain white background and although it is important to be consistent in terms of how long after mixing the plate is read it is useful to evaluate the speed with which agglutination occurs. The higher the antibody levels the more rapid and pronounced the agglutination.

The degree of agglutination is graded from 0 - no agglutination, 1 – fine particles (<1mm), 2 – moderate clumping (1-2mm), 3 - rapid agglutination forming large (>2 mm) clumps.

The RSPA test provides an inexpensive means of very rapidly detecting early antibody response (IgM) within 7-10 days after infection with MG (Kleven *et al.*, 2000). False positives are the most frequent errors encountered with the RSPA test, thus compromising test specificity. False positives may occur for 4-8 weeks following oil emulsion vaccine administration (serum contamination) and in the presence of cross-reacting *Mycoplasma synoviae* antigens (Glisson *et al*., 1984; Kleven *et al*., 2000). Antigen is available commercially from various sources and the sensitive and specificity of this test can be compromised by source and batch variation (Avakian, Kleven and Glisson, 1988).

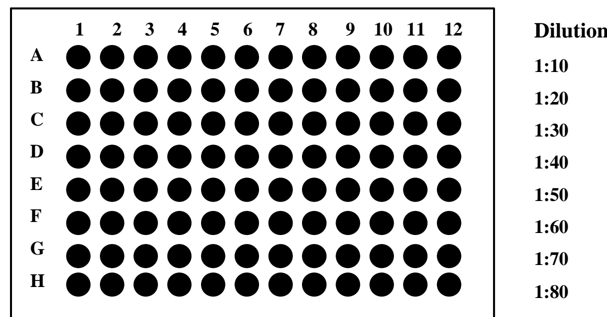
Although it is common practice in the USA to confirm RSPA positives with an HI test this is seldom done in South Africa. Instead RSPA test are conducted on two-fold serial dilutions of sera using phosphate buffered saline. Sera that agglutinates with a 1:8 dilution is considered positive. This method does however reduce sensitivity and may not eliminate false positives (Kleven *et al.*, 2000).

Haemagglutination Inhibition (HI)

Being derived from the Greek word *hamia* for blood and *agglutination* meaning rapid clumping, haemagglutination by definition means the rapid clumping of red blood cells. Haemagglutination occurs when MG surface antigens form cross linkages between red blood cells causing them to adhere to each other and clump.

The principle of the haemagglutination inhibition test is that immune serum contains immunoglobulins that are able to specifically inhibit homologous MG haemagglutinin from causing erythrocytes to clump.

Mycoplasma HI tests are traditionally done in microtitre plates using 4 haemaglutinating (HA) units of antigen per test and the constant antigen - diluting serum (β) technique. A serum control is usually included by performing the first dilution (1:10) with buffered saline and then subsequent dilutions in antigen. Generally titres of 1:40 to 1:80 are considered positive (Kleven, 1998).



Although widely used in the USA, the HI test is seldom used in South Africa because antigen is not readily available. The test is very specific, inexpensive and relatively easy to perform. Being IgG dependent, the HI test is not as sensitive as the RSPA test but sera becomes strongly positive 10 days after challenge (Glisson *et al.*, 1984; Kleven, 1975;

University of Pretoria etd – Collett, S R (2005)

Kleven and Levisohn, 1996; 1972; Kleven, Jordan and Bradbury, 2000). There are several opportunities for interference so it is important to perform the test under constant conditions to ensure reliable results.

Table 2.5. Comparison of the indirect *Mycoplasma gallisepticum* assay techniques.

	Agglutination	**Haemagglutination Inhibition**	**ELISA**
Cost	Low	Low	medium
Speed	rapid	moderate	moderate
Automation	poor	poor	good
Stage of infect.	Early (7-10 days)	Intermediate (>10d)	intermediate(>10d)
Antibody	IgM	IgG	IgG
Sensitivity	High (early)	Medium(early)//High	Medium
Specificity	Medium	High	Medium(95%)
Antigen			
• **Availability**	Good	Poor	Good
• **Quality**	variable	variable	good

ELISA

MG specific indirect enzyme-linked immunosorbent assay (ELISA) kits are commercially available from several companies. The ELISA test is easily automated so although relatively expensive it is becoming the test of choice in many laboratories. As with the HI test the ELISA is IgG dependent so there is around a 10-day lag phase between infection and antibody detection thus compromising sensitivity in the early stages of infection. Specificity is around 95% being compromised by non-specific background reactivity. The HI test is frequently used as a confirmatory test in such instances (Kleven *et al.*, 2000) although direct methods of detection like isolation and identification or PCR remain the only definitive confirmatory methods (Kleven, 1998).

Direct

Direct monitoring methods are based on detection of the *Mycoplasma gallisepticum* organism or part thereof. Although these methods are classed as the standard for MG

diagnosis they are seldom used to monitor flocks in South Africa because they take time and are generally expensive.

Isolation and Identification

Isolation and identification remains the gold standard for confirmation of infection. *Mycoplasma gallisepticum* is a fastidious organism requiring a protein based media enriched with 10-15% heat inactivated horse or swine serum (or serum factors), glucose and yeast factors (Kleven, 1998). Since MG is relatively resistant to penicillin (no cell wall) and thallium these antimicrobial agents are usually added to the media to suppress bacterial and fungal growth.

Samples from clinical cases should preferably be inoculated into the growth media at the time of collection but certainly within 24 hours. Samples require protection during storage and transport and although freezing with dry ice is preferable wet ice is adequate for short periods of time (Kleven, 1998).

In the acute stages of infection, 5-10 tracheal or choanal cleft cultures are adequate since MG spreads rapidly in susceptible flocks (DiGiacomo, 1986; McMartin *et al*., 1987). Within the first 2-3 months after infection disease prevalence reaches 100% and the upper respiratory tract is densely populated. In the first 10-20 days and during the chronic phases of infection 40-100 samples per flock are required (DiGiacomo, 1986; McMartin *et al*., 1987; Kleven, 1998).

MG colonies which are small (0.1-1 mm diameter) and smooth with a dense raised centre (Kleven, 1998) usually appear after 4-5 days of incubation at 37° C. This is in contrast to the nonpathogenic mycoplasmas which may appear within 24 hours of inoculation. Mixed cultures are common and species identification is usually established by using hyperimmune serum inhibition, but direct and indirect immuno flouresence is also frequently used (Kleven, 1998).

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a process whereby in vitro enzymatic synthesis is used to amplify specific DNA or RNA sequences to facilitate organism identification (Janeway, and Travers, 1997; King, 1988). This system of amplification enables organism identification from extremely small samples and has revolutionized research and diagnostic pathology.

Gene nucleic acid base pair sequence is unique to strain for each organism. PCR technology enables particular pieces of genetic material (DNA or RNA) to be identified and replicated. The chain reaction is an automated three-step process of denaturation, annealing and extension, which is repeated several times.

A test tube containing the necessary ingredients (thermostable polymerase, the two primers, the four pyrimidine bases with phosphate and sugar residues) is placed into a thermal-cycler, which then takes the mixture through a predetermined temperature program. It heats up to 94°C for denaturation, cools to 40-60°C for annealing and then heats up to 72°C for extension and double helix formation. One cycle lasts about 3 minutes and a billion copies of the target DNA are made in less than 2 hours.

The concentration and source of thermostable DNA-polymerase, concentration of Magnesium or Manganese, concentration and purity of target DNA and primers, denaturing temperatures, annealing time and temperature, extension time and temperature and the number of cycles all affect the specificity of the PCR (Podzorski and Persing, 1994). The most critical temperatures are those during denaturation and annealing.

Inadequate heating leads to incomplete denaturation and therefore poor amplification.

Conditions during annealing affect the specificity of the PCR. Stringent conditions tend to reduce the efficiency of DNA amplification by hindering primer annealing. In contrast non-stringent conditions result in mispriming and amplification of non-target DNA (Podzorski *et al.*, 1994).

PCR techniques can be used to identify MG directly from clinical material/samples or following isolation. The direct PCR test is itself very sensitive and specific but the reliability of the result is dependent on the sampling procedure. Tracheal and choanal swabs are most commonly used. The same principles apply with regard to sampling for isolation and direct PCR identification.

Although the PCR test is a lot more expensive than the serological assays the specificity and sensitivity of this technique makes it an attractive alternative. It is especially useful when trying to distinguish field strain from vaccine strain Mycoplasmas. The cost can be reduced significantly by pooling samples (Tyrrell *et al.*, 1994).

Restriction Fragment Length Polymorphism (RFLP)

Specific pieces of genetic material (double stranded DNA) are identified and separated by restriction enzyme digestion at recognition sites (Jackwood, and Jackwood, 1998). Electrophoresis is used to separate the short strands of DNA created in this process and organisms identified by comparing the banding pattern with reference standards (Lauerman, Chilian, Closser and Johansen, 1995).

Although useful for MG strain differentiation RFLP is time consuming and laborious (Kleven, 2002).

Random Amplified Polymorphic DNA (RAPD)

This technique, also known as the arbitrarily primed polymerase chain reaction (AP-PCR) was developed in the early 1990s and has been comprehensively reviewed (Welsh and McClelland, 1991; Williams, Kubelik, Livak, Rafaiski and Tingey, 1990; Williams, Hanafey, Fafaiski and Tingey, 1993).

RAPD is quick and simple and has been used very successfully for MG strain identification (Ley *et al.*, 1997; Kleven, 1998; Fan, Kleven, Jackwood, Johansson, Pettersson and Levisohn, 1995). Although the test requires a pure isolate for accurate strain identification and repeatability and is influenced by reaction conditions it is a very

useful routine test that can give meaningful results within a day (Ley, 2000; Kleven, 2002; Jackwood *et al*., 1998).

Multiplex PCR

With this technique two or more unique target sets of sequences are amplified simultaneously. This is particularly useful for the purpose of running an internal control to verify the integrity of the PCR system and conditions.

Table 2.6. Comparison of direct assay techniques for Mycoplasma gallisepticum

	Culture and Identification	Direct PCR
Cost • ingredients • equipment • labour	Medium Medium Low + high High	Medium Medium High Medium
Speed	5-7 days	1 day
Automation	Poor + good	Good
Stage of infect.	Sample dependent	Sample dependent
Sensitivity	Excellent	Good
Specificity	Excellent	Excellent
Availability	Limited	Limited

MEDICATION

Medication itself has failed as a means of eradication because of the inability of even modern highly effective antibiotics to sterilize the infection. Several medication strategies have however been used successfully to limit vertical transmission, prevent flock infection and avert the full effect of MG infection on bird performance.

Although the Mycoplasmas are resistant to antimicrobials like penicillin that affect cell wall synthesis, several classes of antibiotics including the Macrolides, Tetracyclines and Flouroquinolones have *in vitro* and *in vivo* activity (Ley, 2003; Levisohn, 1981; Wang, Ewing and Aarabi, 2001).

In vitro sensitivity testing is complicated by the unique characteristics and growth requirements of these organisms (Bradbury *et al.*, 1994) but resistance to commonly used antibiotics has been demonstrated (Zanella, Martino, Pratelli and Stonfer, 1998; Migaki, Avakian, Barnes, Ley, Tanner and Magonigle, 1993). Antibiotic resistance can develop rapidly in the field during mass medication programs and has also been shown to occur *in vitro* (Zanella *et al*., 1998). I*n vivo* response to medication does not necessarily parallel *in vitro* sensitivity/resistance because during flock medication it is not possible to separate the effect of the antimicrobial on MG itself and secondary invaders.

It is possible to halve the egg production loss caused by MG infection of layers by adding Tylosin to the feed at 50g per tonne in the first layer feed through to peak production and 25g per tonne for the rest of the production cycle (Ose, Wellenreiter and Tonkinson, 1979). In-feed medication of broiler breeders with Streptomycin, Dihydrostreptomycin, Oxytetracycline, Chlortetracycline, Flouroquinolone, Erythromycin or Tylosin has also been shown to reduce the frequency of vertical transmission of MG (Ley, 2003).

Differential-pressure egg-dipping in a suitable antibiotic solution and egg injection at 18 days either alone or in combination can be used to reduce vertical transmission (Hodgetts, 1992; Ghazikhanian, Yamamoto, McCapes, Dungan, Larsen and Ortmayer, 1980). These processes adversely affect hatchability and may not totally eliminate vertical transmission.

When vertical transmission is likely, broilers can be treated with antibiotics prophylactically for the first three to five days of life then again for three to five days at the time of re-vaccination (14-21 days) for Newcastle disease (Ley *et* al., 1997).

Mycoplasma gallisepticum control or eradication requires an integrated approach involving biosecurity, vaccination and in some cases judicious use of antibiotics.

CHAPTER 3 - MATERIALS AND METHODS

1. A FLOOR PEN STUDY TO EVALUATE THE SEROLOGICAL RESPONSE OF BROILER BREEDERS AFTER VACCINATION WITH TS-11 STRAIN *MYCOPLASMA GALLISEPTICUM* VACCINE

Two adjoining isolation rooms with separate ventilation systems were set up as depicted in Figure 3.1 for this experiment. Two pens, set 2m apart in room 1 housed 35 and 15 birds respectively.

Figure 3.1. Experimental pen and treatment layout.

Room 1			Room 2
Pen 1	2 meter passageway	Pen 2	Pen 3
Treatment A Vaccinated. *Commingled* n= 25 **Treatment B** Non-vaccinated *Commingled* n=10		**Treatment C** Non-vaccinated separate n= 15	**Treatment D** Vaccinated n=5

Pen 1 contained 25 birds vaccinated with ts-11 strain MG at 8 weeks of age (treatment A) commingled with 10 non-vaccinated birds (treatment B). The second pen in room 1 housed 15 non-vaccinated birds (treatment C). A single pen in room 2 housed 5 birds vaccinated with ts-11 strain MG at 8 weeks of age (treatment D).

The concrete floor in each pen was covered with fresh pine shavings. Water was supplied *ad libitum* by automatic, suspended bell-drinkers and feed was replenished daily in suspended tube feeders.

Fifty-five Cobb broiler breeder pullets aged five weeks, and derived from non-vaccinated, MG- negative broiler breeder grandparents, (RSPA procedure) were individually

identified with wing bands and randomly allocated to one of the three pens. Previous vaccination history is detailed in Table 3.1. Pullets were reared according to the recommendations of the primary breeder with respect to feed issue and lighting. The birds were serviced daily by a caretaker who, other than moving only from non-vaccinated to vaccinated groups, took no special precautions in respect of possible transmission of the vaccine mycoplasma between pens or houses so as to simulate field conditions.

At 8 weeks of age, 25 birds from pen 1 and all 5 birds in pen 3 were vaccinated with ts-11 (Merial, Inc., Gainesville, Ga.). The vaccine was administered by placing one drop onto the exposed cornea of the birds.

Table 3.1. Pullet vaccination history.

Age in days	Vaccine	Route
0	Mareks - HVT + Rispens	Sub-cutaneous injection
	Newcastle disease – VH, ABIC, Teva	Aerosol - coarse spray
6	Coccidiosis - Paracox™, Shering Plough AH	Oral – drinking water
21	Newcastle - VH, ABIC, Teva	Aerosol – fine spray
	Infectious Bronchitis – Nobilis IB H120, Intervet SA	

Serum samples were obtained from each pullet on the day of vaccination, at 7-day intervals to 6 weeks post vaccination and then at 14-day intervals to 12 weeks post vaccination.

Serum was individually tested for the presence of MG specific antibodies by RSPA using NOBILIS® MG antigen (Intervet) and the results recorded by individual wing tag number. The procedure was carried out according to the recommendations detailed in the USDA National Poultry Improvement Plan. Agglutination reactions were classified as “fine” (1+), “moderate” (2+) or “heavy” (3+) after a 2-minute period of agitation.

Dry choanal swabs were collected from 5 birds in each pen 2-weeks post vaccination and from all birds at 3 and 12 weeks post-vaccination. Dry tracheal swabs were collected from all birds at 12-weeks post vaccination. The swabs were allocated code numbers and submitted to a diagnostic laboratory (Molecular Diagnostic Services).

The dry samples were tested for the presence of MG and ts-11 using a proprietary PCR primer that was specifically designed to identify the ts-11 strain. This assay amplifies a 229 bp fragment that is characteristic of ts-11. All other MG field strain isolates that are amplified with this assay produce a different sized amplicon and can therefore be distinguished from the vaccine.

There has been one field strain isolate, from the USA, that has been found to amplify with a similar sized amplicon to that of ts-11. The ts-11 specific assay does amplify the 6/85 vaccine but the two strains are easily distinguishable from each other since the amplicons differ in size (Kleven, 2002).

Standard molecular techniques were used to extract and amplify segments of the organism. Briefly, DNA was extracted from each of the dried swabs. Four µl of the extract was amplified using two separate PCR assays, one that used ts-11 specific primers and a second that used a primer pair that amplify all MG field strains (Molecular Diagnostic Services South Africa, Bioproperties Australia.). The products were analyzed for the presence of the appropriate sized amplicon on ethidium bromide stained 2% agarose gels. The specificity and sensitivity of the assays were controlled by the inclusion of internal standards in each run. A poultry housekeeping gene was also targeted to ensure that sufficient material was collected, that the sample had been correctly transported and extracted and that there were no assay inhibitors. The presence of the 229 bp amplicon, using the ts-11 specific primers/assay, was the criteria used to interpret the sample as being ts-11 vaccine positive.

The trial was terminated at 12 weeks post vaccination when the birds were 20 weeks of age. Euthanasia was by cervical dislocation.

2. A RETROSPECTIVE STUDY ON THE USE OF THE RSPA TEST IN TS-11 VACCINATED FLOCKS

Following the pilot study (outlined in the introduction) breeder flocks were vaccinated by eye drop at 10 weeks of age with ts-11 strain MG. Sera from fifty birds per house (7 500 birds) were collected and tested for agglutination with the commercial MG antigen, every 5 weeks. The results were recorded separately for each individual house (4 rearing houses per rearing farm transferred to 6 laying houses per laying farm) as percentage RSPA positive. The flock status was determined by averaging these results such that n=200 (4 houses x 50 samples from each) from 0-20 weeks and n=300 (6 houses x 50 samples from each) from 20 weeks on. This sample size facilitated the detection of seroconversion with 99% confidence to a 2% level within the flock (farm) and a 10% level within the house.

Since vaccination of a flock took 4-5 days to complete and the date of bleeding was not rigidly controlled the recorded age of bleeding is only accurate to the nearest week.

During the 4-year monitoring period 43, 40 and 21 flocks were monitored in each region respectively. These results thus represent the MG status of approximately 5-million broiler breeders as measured by RSPA.

3. EVALUATION OF SENTINELS TO MONITOR MG STATUS OF TS-11 VACCINATED BREEDER FLOCKS

Broiler breeder rearing flocks were vaccinated at 10 weeks of age with ts-11 strain MG by eye drop. On the day of vaccination 25 birds were randomly selected as non-vaccinated sentinels from each flock of approximately 7 500 birds. These sentinels were individually identified with wing tags and suitably marked, to facilitate easy identification, prior to being returned to the flock. Sera from the sentinels and 25 randomly selected vaccinated flock birds were collected and tested for agglutination with the commercial MG antigen. Sera was collected and tested on the day of selection and then every 5 weeks thereafter. Sampling ceased once the flock RSPA results were in excess of 80% positive for two consecutive tests.

The agglutination results were recorded against the individual bird's wing tag number as being negative (0), fine (+), moderate (2+), or heavy (3+), after 2 minutes during which the plates were agitated. To establish the degree of seroconversion the RSPA results were summarized for each group of sentinels in terms of the percentage positive reactors and the strength of the agglutination reaction. The mean strength of agglutination* for positive reactors was calculated (as previously reported) in order to help differentiate between non-specific or vaccine reactions and field challenge (Abd-El-Motelib *et al.*, 1993).

$$^{*}\text{Mean} = \frac{\text{Sum of positive scores}}{\text{Number of positive reactors}}$$

It was postulated, from trial data (Ley *et al.*, 1997b) that a high rate of seroconversion in this sentinel group would indicate field challenge.

Five weeks after vaccination of the flock, dry choanal swabs were taken from five of the sentinels in each group (pooled into groups of 5). Fifteen weeks after vaccination dry choanal swabs were taken from ten of the sentinels in each group (pooled into groups of 5). The dried samples were tested for the presence of field strain MG using a PCR that was specifically designed to distinguish between the field and vaccine strains.

Since vaccination of a flock took 4-5 days to complete and the date of bleeding was not rigidly controlled the recorded age of bleeding is only accurate to the nearest week.

During the monitoring period the results from 18 rearing flocks (0-18 weeks) and 13 laying flocks (19-65 weeks) were recorded and analyzed.

CHAPTER 4 - RESULTS

1. A FLOOR PEN STUDY TO EVALUATE THE SEROLOGICAL RESPONSE OF BROILER BREEDERS AFTER VACCINATION WITH TS-11 STRAIN *MYCOPLASMA GALLISEPTICUM* VACCINE

RSPA

The RSPA test results are presented in Table 4.1. MG antibodies were detectable in the sera of vaccinated birds at 2 weeks (treatment A) and 3 weeks (treatment D) after vaccination. This initial positive RSPA reaction was mild with only 16% of the serum samples in treatment A showing fine reactions (1+). Both the proportion of reactors and the intensity of the agglutination reaction increased in the vaccinated group over the 12-week trial period and stabilized at a 40% to 60% reactor rate. Reactors had a mean RSPA score of 1 (treatment D) to 1.5 (treatment C) at 20 weeks of age.

The antibody response in the commingled non-vaccinates in pen 1(treatment B) was first detected at 5 weeks after vaccination of the pen-mates (Treatment A). Seroconversion in this group was detected 3 weeks later than in the vaccinated group. At the termination of the experiment, 30% of commingled non-vaccinates (treatment B) were positive to the RSPA test with fine (1+) agglutination reactions noted.

None of the non-vaccinated pullets in treatment C, that were penned 2 meters away from the vaccinated group (treatment A) showed positive RSPA reactions throughout the 12 week period.

The serum from vaccinated pullets, housed separately but with a common caretaker (Treatment D) yielded fine (1+) agglutination reactions from 2 weeks after administration through to the termination of the experiment. As with the vaccinated birds in pen 1, (treatment A) the proportion of positive reactors to the RSPA test ranged from 20% to 60% during this period.

Table 4.1. Antibody response as measured by the Rapid Serum Plate Agglutination (RSPA) Test

Treatment A – Vaccinated (n=25)

RSPA Test Results		Weeks Post Vaccination 0	1	2	3	4	5	6	8	10	12
Negative	0	25	25	21	19	21	12	14	13	12	10
Positive	1+	0	0	4	6	4	11	8	9	9	9
	2+	0	0	0	0	0	2	2	2	3	3
	3+	0	0	0	0	0	0	0	0	0	2
	Mean Score	0	0	1	1	1	1.1	1.2	1.2	1.3	1.5
	% Reactors	0	0	16	24	16	54	42	46	50	58

Treatment B – Non-Vaccinated Commingled Sentinels (n=10)

RSPA Test Results		Weeks Post Vaccination 0	1	2	3	4	5	6	8	10	12
Negative	0	10	10	10	10	10	9	9	8	7	7
Positive	1+	0	0	0	0	0	1	1	2	3	3
	2+	0	0	0	0	0	0	0	0	0	0
	3+	0	0	0	0	0	0	0	0	0	0
	Mean Score	0	0	0	0	0	1	1	1	1	1
	% Reactors	0	0	0	0	0	10	10	20	30	30

Treatment C – Non-Vaccinated, Separate Pen (n=15)

RSPA Test Results	Weeks Post Vaccination 0	1	2	3	4	5	6	8	10	12
Negative	15	14	13	13	13	13	12	12	12	12
Positive	0	0	0	0	0	0	0	0	0	0

Treatment D – Vaccinated Separate Room (n=5)

RSPA Test Results		Weeks Post Vaccination 0	1	2	3	4	5	6	8	10	12
Negative	0	5	5	5	4	4	4	3	2	3	3
Positive	1+	0	0	0	1	1	1	2	3	2	2
	2+	0	0	0	0	0	0	0	0	0	0
	3+	0	0	0	0	0	0	0	0	0	0
	mean	0	0	0	1	1	1	1	1	1	1
	%	0	0	0	20	20	20	40	60	40	40

PCR assay results

The results of the PCR assays are presented in Table 4.2. All of the choanal swabs taken at 2 and 3 weeks post vaccination from the commingled vaccinates (Treatment A) were ts-11 positive. In contrast none of the non-vaccinated commingled sentinels in pen 1 (Treatment B) were PCR positive for ts-11 at 2 weeks but 50% were positive 3 weeks after vaccination of pen mates.

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Table 4.2. Proportion of pullets yielding MG ts-11 by PCR assay

Period Post-Vaccination	% positive on ts-11 PCR probe							
	Treatments				Treatments			
	A		B		C		D	
	Vaccinated		Non-vaccinated		Non-vaccinated		Vaccinated	
	Choanal swabs	Tracheal swabs	Choanal swabs	Tracheal swabs	Choanal swabs	Tracheal swabs	Choanal swabs	Tracheal swabs
2 Week	100		0		0		80	
3 Week	100		50		0		60	
12 Week	20	48	0	30	0	6.6	40	60

When the trial was terminated, 12 weeks after vaccination, 20% of the choanal swabs derived from vaccinated birds in Treatment A were ts-11 positive on PCR assay in contrast to 48% positive for tracheal swabs.

None of the choanal swabs from commingled non-vaccinated pullets (Treatment B) were ts-11 positive at 12 weeks, while tracheal swabs were at this stage 30% positive.

One bird in Treatment C yielded a positive result for the ts-11 specific PCR done on the tracheal swab taken at the termination of the experiment.

PCR assay for ts-11 on choanal swabs derived from separately housed vaccinates (Treatment D) were 80% positive at 2 weeks and 60% positive at 3 weeks post vaccination. At 12 weeks post vaccination 40% of the choanal swabs were PCR positive compared to 60% of the tracheal swabs.

None of the pullets yielded non-ts-11 strain MG on PCR assay at the completion of the trial.

2. RETROSPECTIVE STUDY ON THE USE OF THE RSPA TEST IN TS-11 VACCINATED FLOCKS

A summary of the field results accumulated over a 4-year period following implementation of a ts-11 vaccination program in three separate breeder units is presented in Table 4.3. The data is displayed graphically in Figures 4.1 through to 4.8.

The results from twelve flocks that showed signs of RSPA seroconversion prior to vaccination have been omitted from this analysis. These flocks were assumed to have been exposed to field strain MG prior to vaccination.

At transfer, the point-of-lay broiler breeder pullet groups are compromised and pen-mates are in many cases moved to different houses. Clearly this marks a disruption of statistical groups and hence the data accumulated from the rearing farms is presented and analyzed separately to that collected from the laying farms.

The number of RSPA reactors in the flock increased gradually after vaccination to peak at 40% to 65% positive, 8 to 10 weeks after vaccination and this was followed by a slight decline before transfer. This pattern of flock seroconversion during the rearing phase was consistent in all three of the regions. The strength of this trend is emphasized by the very high R^2 values of 0.92 to 1 for the best-fit polynomial.

The trend during the laying phase was somewhat different. The number of birds RSPA positive increased to 100% within 15 to 30 weeks of age and then persisted at this level. Once again the regression analysis of this data indicates good correlation with R^2 values of 0.85 to 0.96.

Table 4.3. The average *Mycoplasma gallisepticum* RSPA test results taken over a 4 year period and representing 5 million broiler breeders from 112 farms.

Age in wks	Region 1			Region 2			Region 3			TOTAL		
	Sample size (n)	% RSPA Pos	Stdev	Sample size (n)	% RSPA Pos	Stdev	Sample size (n)	% RSPA Pos	Stdev	Sample size (n)	% RSPA Pos	Stdev
5	8600	0	0	8200	0	0	3400	0	0	20200	0	0
10	8600	0	1	8200	0	1	3400	0	0	20200	0	1
15	2600	34	40	6400	56	22	800	49	11	9800	50	29
17	6400	37	26	600	68	6	1600	64	16	8600	44	26
20	3000	43	29	6400	69	23	1600	57	16	11000	60	26
22	6000	36	21	0	-	-	0	-	-	6000	36	21
25	9300	45	23	11100	84	16	4800	72	16	25200	67	26
30	3900	71	24	11400	95	8	4500	93	9	25200	86	19
35	7800	85	24	8400	97	5	3900	100	1	20100	93	16
40	3600	80	25	3300	97	4	1200	100	0	8100	90	18
45	2400	97	6	2100	99	1	1200	100	0	5700	98	4
50	600	100	0	2100	100	0	0	-	-	2700	100	0
55	300	95	0	1200	100	1	0	-	-	1500	99	2
60	300	100	0	900	100	0	0	-	-	1200	100	0

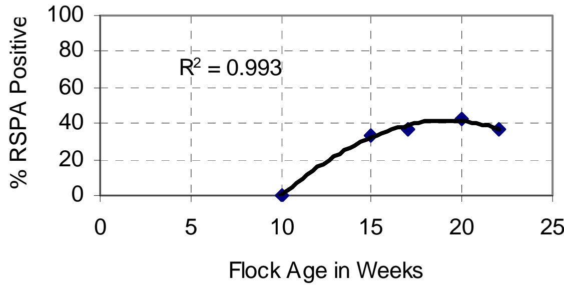

Figure 4.1. The average RSPA status during rear of 43 flocks in region 1 after vaccination with ts-11 at 10 weeks of age.

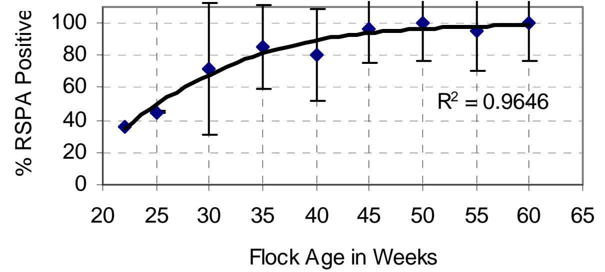

Figure 4.2. The average RSPA status during lay of 43 flocks in region 1 after vaccination with ts-11 at 10 weeks of age.

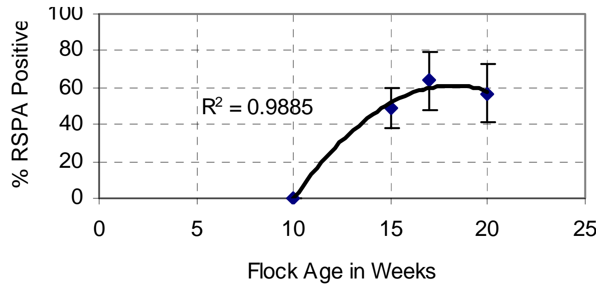

Figure 4.3. The average RSPA status during rear of 40 flocks in region 2 after vaccination with ts-11 at 10 weeks of age.

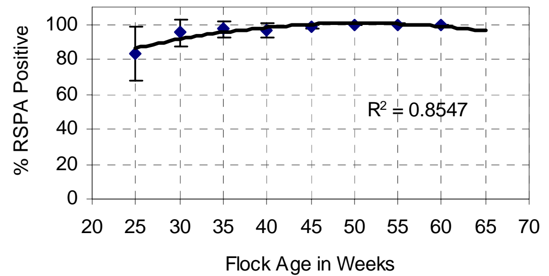

Figure 4.4. The average RSPA status during lay of 40 flocks in region 2 after vaccination with ts-11 at 10 weeks of age.

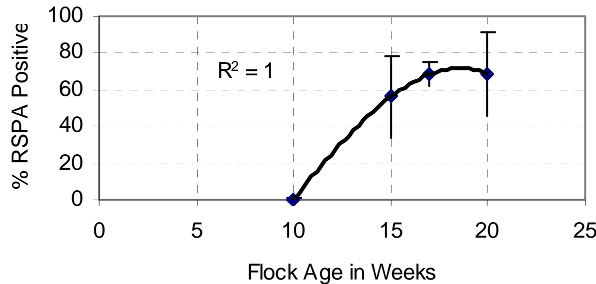

Figure 4.5. The average RSPA status during rear of 21 flocks in region 3 after vaccination with ts-11 at 10 weeks of age.

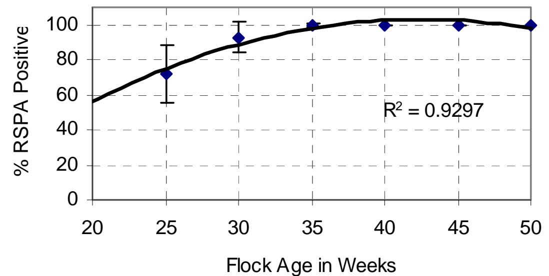

Figure 4.6. The average RSPA status during lay of 21 flocks in region 3 after vaccination with ts-11 at 10 weeks of age.

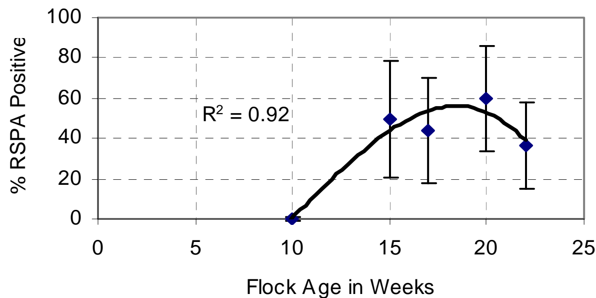

Figure 4.7. The average RSPA status during rear of all 104 flocks after vaccination with ts-11 at 10 weeks of age.

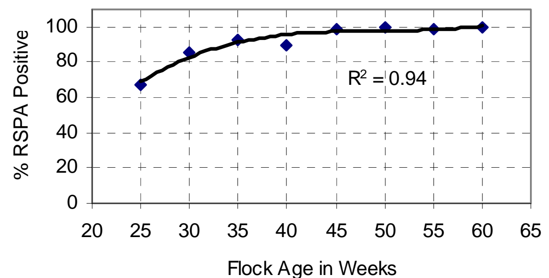

Figure 4,8. The average RSPA status during lay of all 104 flocks in region 1 after vaccination with ts-11 at 10 weeks of age.

3. EVALUATION OF SENTINELS TO MONITOR MG STATUS OF TS-11 VACCINATED BREEDER FLOCKS

The RSPA test results are summarized in Table 4.4 and 4.5 and illustrated graphically in Figures 4.9-4.12.

Although bird groups were compromised at 20 weeks (birds from 4 rearing houses moved into 6 laying houses) the rearing period is represented by the RSPA results to 25 weeks because titre at this time is predominantly rearing dependant. During the rearing period (represented by the RSPA results to 25 weeks) the sentinels showed a gradual increase in the degree of seroconvertion to a peak of 54% at 20 weeks. This peak was followed by a slight, although non-significant, decline to 48% positive by 25 weeks. After transfer to the laying farm there was a rapid increase in the degree of seroconversion such that by 30 weeks in excess of 80% of the sentinels were RSPA positive. The average degree of seroconversion was maintained at above 85% for the following 10 weeks.

The intensity of the agglutination reaction also initially increased and then decreased during the rearing phase. The peak of 1.4 appears to be (best fit polynomial) slightly earlier at 18 weeks of age. After transfer of birds to the laying farm, the strength of agglutination showed a more gradual increase to peak at 2, when the birds were 40 weeks old.

All PCR tests performed on dry choanal swabs at 5 and 15 weeks after vaccination were negative for field strain MG.

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Table 4.4. The percentage positive Rapid Serum Plate Agglutination (RSPA) tests performed on sera from 25 non-vaccinated commingled sentinels in 18 flocks comprising approximately 7 500 broiler breeders each.

	Flock Age in Weeks							
Flock	**10**	**15**	**18**	**20**	**25**	**30**	**35**	**40**
1	0	17	30		72	89	100	
2	0	4	12		56	90	100	
3	0			23	73	95	75	100
4	0			40	28	83	100	100
5	0	9	27		52	90	74	
6	0	0	14		69	82	100	
7	0	21	42		20	89	92	
8	0	22	26		68	96	87	
9	0		8	33	18			
10	0		25	10	14			
11	0		74	75	42			
12	0		100	100	71			
13	0		78	74	40			
14	0		79	74	33			
15	0	40		47	78	100	100	100
16	0	67		69	75	100	100	100
17	0	50		50	25	25	0	0
18	0	25		57	33	100	100	100
Average	0	26	43	54	48	87	86	83
Std. Dev.	0	21	31	26	22	20	29	41

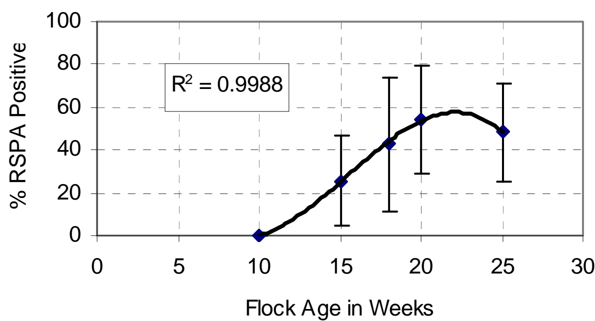

Figure 4.9. The percentage of unvaccinated commingled sentinels showing a positive RSPA test result during rearing, after flock vaccination at 10 weeks of age.

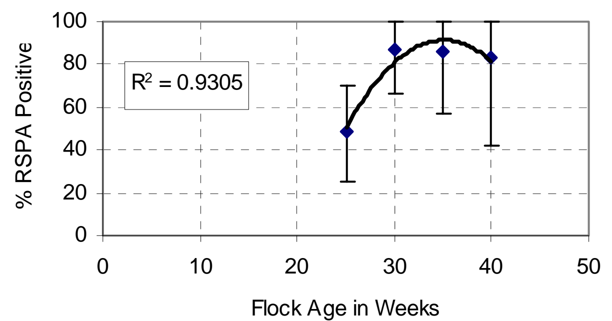

Figure 4.10. The percentage of unvaccinated commingled sentinels showing a positive RSPA test result during lay, after flock vaccination at 10 weeks of age.

Table 4.5. The mean positive result of Rapid Serum Plate Agglutination (RSPA) tests performed on sera from 25 non-vaccinated commingled sentinels in 18 flocks comprising approximately 7500 broiler breeders each.

Flock	Flock Age in Weeks							
	10	**15**	**18**	**20**	**25**	**30**	**35**	**40**
1	0	1.3	1.3		1.6	1.7	1.4	
2	0	1	1		1.1	1.6	2.5	
3	0			1	1.6	1.6	1.5	3
4	0			1.1	1.6	1.4	2.3	3
5	0	1	1		1.4	1.5	1.6	
6	0	0	1.5		1.1	1.6	2	
7	0	1.4	1.2		1.4	1.3	1.7	
8	0	1.4	1.6		1.5	1.4	1.8	
9	0		1	1	1			
10	0		1	1	1			
11	0		1.6	1.4	1.3			
12	0		2	2.1	1.6			
13	0		1.6	1.5	1.4			
14	0		2.1	1.8	1.2			
15	0	1.2		1	1.3	1.3	1	1
16	0	1.6		1.1	1.5	1.2	2.3	2
17	0	2		1	3	2	0	0
18	0	1		1	1	1	1.5	3
Average	**0**	**1**	**1.4**	**1.3**	**1**	**1**	**2**	**2**
Std. Dev.	0	0.52	0.4	0.4	0.45	0.26	0.67	1.3

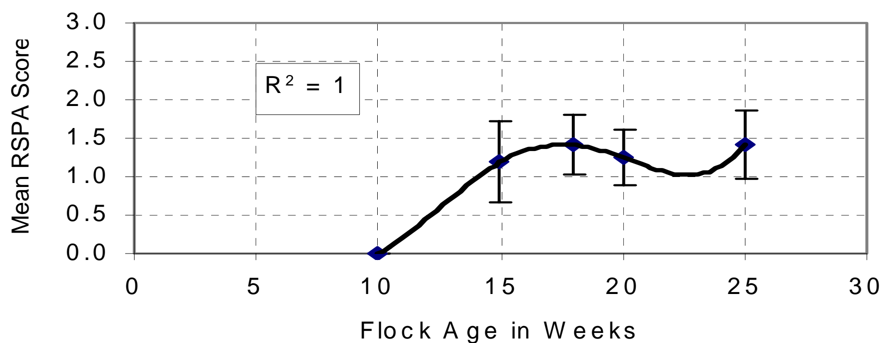

Figure 4.11. The mean positive RSPA test result of non-vaccinated commingled sentinels during rearing after flock vaccination at 10 weeks of age.

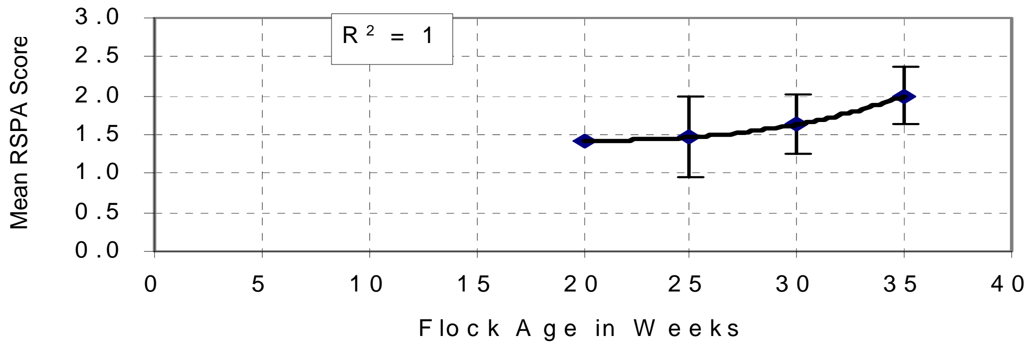

Figure 4.12. The mean positive RSPA test result of non-vaccinated commingled sentinels during lay, after flock vaccination at 10 weeks of age.

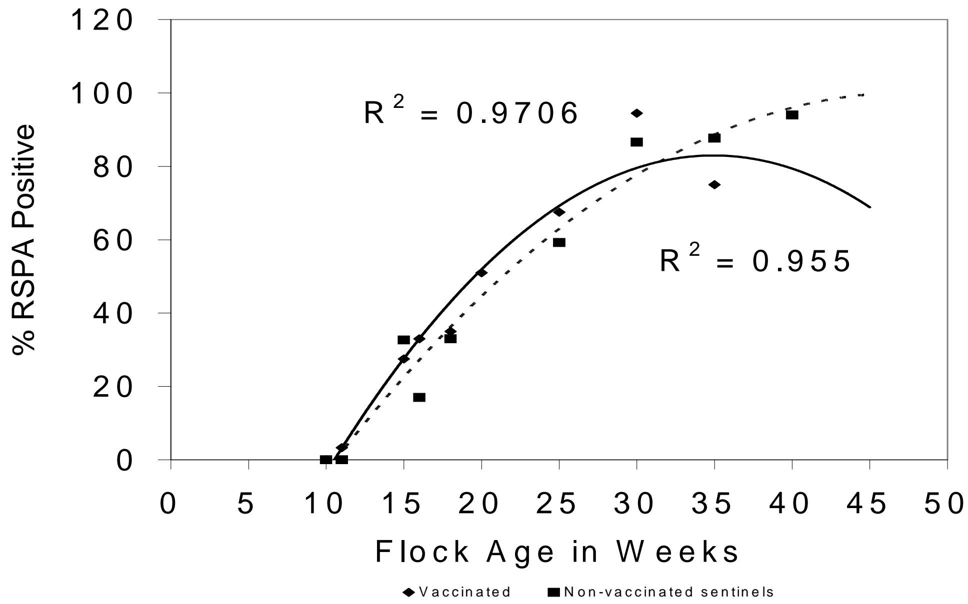

Figure 4.13. Comparison of % positive RSPA tests from vaccinated flock birds and non-vaccinated in-contact sentinels.

CHAPTER 5 - DISCUSSION

1. A FLOOR PEN STUDY TO EVALUATE THE SEROLOGICAL RESPONSE OF BROILER BREEDERS AFTER VACCINATION WITH TS-11 STRAIN *MYCOPLASMA GALLISEPTICUM* VACCINE

The use of the RSPA test for detection and evaluation of *flock Mycoplasma gallisepticum* antibody status has become the subject of intensified research with the increased use of attenuated live MG vaccine (Abd-El-Motelib *et al*., 1993; Ley, *et al*., 1997).

According to the ts-11 vaccine manufacturer, a vaccinated flock RSPA test reactor rate of 30% to 60% indicated effective immunization, more than 60% indicated a field challenge and less than 30% indicated poor vaccination (Cilliers, J. personal communication, 1999). This trial confirms that MG vaccination with ts-11, in the absence of challenge, stimulates a 30-60% positive RSPA reactor rate while previous studies have demonstrated otherwise. (Abd-El-Motelib *et al*., 1993; Ley, *et al*., 1997).

Both Ley *et al*., 1997 and Abd-El-Motelib *et al.,* 1993 recorded in excess of 60% RSPA test positive in ts-11 vaccinated, non-challenged groups, whereas in this trial it remained below 60% positive. These contrasting results may be attributable to several factors.

The vaccine used in this trial was produced by Merial France whereas the vaccine used in previous trials (Abd-El-Motelib *et al*., 1993; Ley, *et al*., 1997) was produced in the USA (Select laboratories, Inc., Gainsville, Ga.) These two countries have different minimum release titer requirements for the ts-11 vaccine. According to the manufacturer, vaccine with a titre of $10^{8.6}$ cfu per dose is known to stimulate a seroconversion rate of 70-80% of the flock while vaccine with a titer of $10^{8.1}$ccu per dose was associated with a 30% seroconversion rate (Cilliers, J. personal communication, 1999).

The bird group sizes used in this trial were larger than those used previously (Abd-El-Motelib *et al*., 1993; Ley, *et al*., 1997). Following vaccination, ts-11 strain replication in the upper respiratory tract of vaccinates may increase the quantum of pen mate exposure. Since the extent of lateral transmission of ts-11 strain is proportional to challenge dose (Cilliers, J. personal communication, 1999) group size may affect RSPA seroconversion rates.

The RSPA test results of ts-11 vaccinates reported by Ley *et al.,*(1997), Abd-El-Motelib *et al.,* (1993), and Whithear *et al*., (1990) were very similar. Seroconversion in vaccinates was rapid with 88-100% of the birds showing MG antibody on the RSPA test within 3 to 6 weeks. This was followed by a gradual decline to a reactor rate of 20-60% over a 12 to 15 week period after vaccination (Figure 5.1). In contrast, this trial demonstrated a gradual increase in the proportion of RSPA positive pullets in the vaccinated treatments (Table 4.1). The RSPA test detected 16% reaction by 2 weeks post vaccination increasing to 20% by 3 weeks stabilizing at 50- 60% between 8 and 12 weeks post-vaccination (Table 4.1).

In this trial 10% of the commingled pullets were RSPA positive 5 weeks after pen mates were vaccinated. Contrary to the results reported previously (Abd-El-Motelib *et al*., 1993; Ley, *et al*., 1997; Whithear *et al*., 1990) seroconversion progressed to a 30% rate in this group at 10 and 12 weeks post vaccination. Ley *et al.,* (1997) did not identify RSPA positive reactors in non-vaccinated contacts for 12 weeks after vaccination of pen-mates. It is possible that this difference in seroconversion was because in the current trial no effort was made to separate the vaccinated birds from the commingled non-vaccinates during the period immediately after vaccination. Since there were more birds per pen in this trial it is possible that the lateral spread of ts-11 strain vaccine may be enhanced by flock size and density. This requires further study.

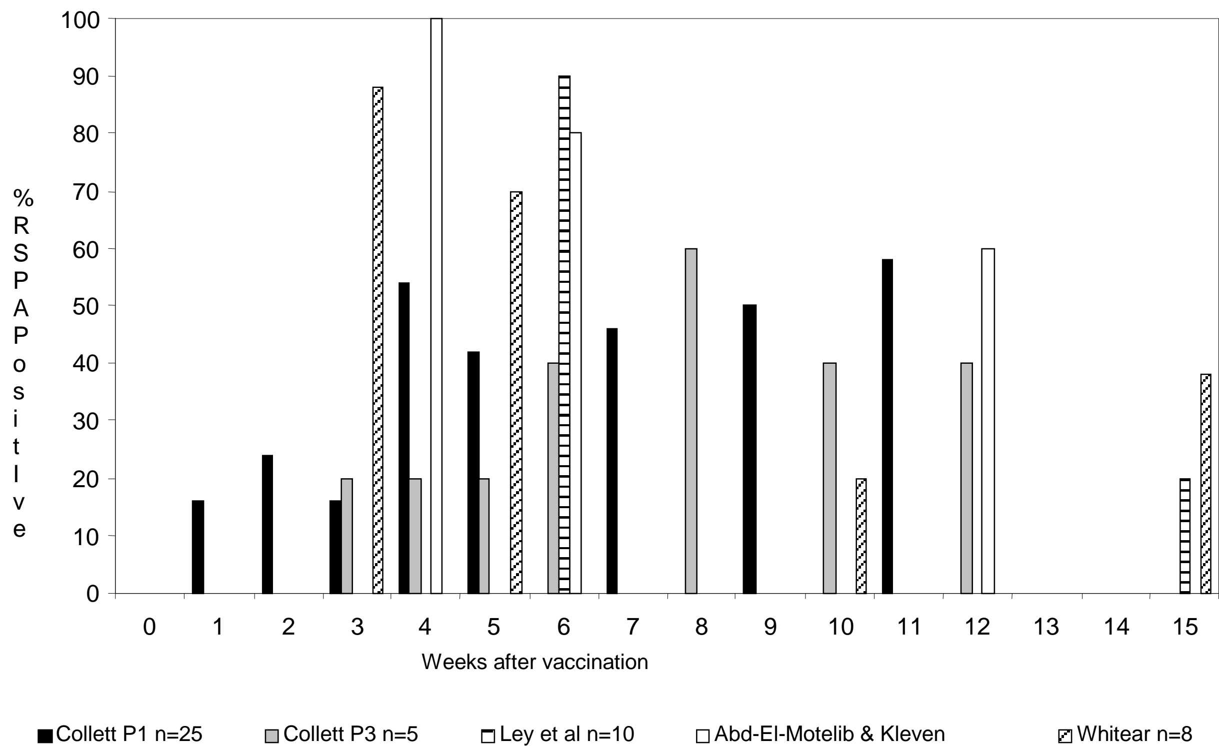

Figure 5.1 A comparison of *Mycoplasma gallisepticum* rapid serum plate agglutination (RSPA) test results after ts-11 vaccination as recorded by various authors.

The intensity of the agglutination reaction in this-experiment was less pronounced than in previous studies (Abd-El-Motelib *et al*., 1993; Ley, *et al*., 1997; Whithear *et al*., 1990). While most of the reactions were classed as 1+, only 2 out of 550 RSPA assays yielded a 3+ reaction.

Since birds used in the trial of Abd-El-Motelib and Kleven (1993) were 10 days of age at the time of vaccination and the birds in this trial were 8 weeks of age it is possible that the differences are attributable to age. Age was not a determinant in the differences in flock seroconversion in the report of Ley *et al.,* (1997) as the pullets were 10 weeks of age when vaccinated.

Another variable that may account for differences in serological response is genetics. White leghorns were used in the previously reported trials (Abd-El-Motelib *et al*., 1993; Ley, *et al*., 1997; Whithear *et al*., 1990) while broiler breeder pullets were used in this trial.

The ts-11 strain-specific PCR assay results at 2 and 3 weeks post vaccination indicate that colonization of the respiratory tract with ts-11 strain occurred in 100% of the vaccinated pullets in Treatment A but only 80% of the vaccinated pullets in Treatment D. This requires further investigation. Although the difference may merely be a reflection of the low number of subjects in treatment D, and therefore not significant the contrasting rate of colonization and seroconversion in this trial and that reported previously (Abd-El-Motelib *et al*., 1993; Ley, *et al*., 1997; Ley, 2003, Whithear *et al*., 1990) indicates that colonization rate may be influenced by group size.

As previously reported (Abd-El-Motelib *et al*., 1993) the ts-11 strain spread to commingled pullets after vaccination of pen-mates. Although spread of ts-11 from vaccinated birds in pen 1 to birds in the adjacent pen 2 did not occur within 3 weeks of vaccination, by 12 weeks after vaccination ts-11 was detected in one of the 12 birds in the non-vaccinated group in pen 2 (treatment C). The spread of ts-11 strain to birds in adjacent pens under experimental conditions has not been reported previously. Although ts-11 was present in the upper respiratory tract of this bird it remained seronegative indicating that either the colonization rate was too low or there had been insufficient time for an antibody response. It is also possible that contamination of the PCR mixes could have taken place.

This trial differs from a previous report (Whithear *et al*., 1990) in flock size and the use of a single caretaker to service more than one flock. In the field flocks are large (± 7 000 pullets) and a single caretaker services several flocks which may explain why spread can occur (Kleven, 1994).

The ts-11 PCR performed on choanal swabs detected specific DNA in up to 80% of the pullets at least 2 weeks prior to the detection of antibodies by RSPA. By 12 weeks post vaccination the proportion of pullets that were reactors on the RSPA test exceeded the number positive on PCR assay of choanal swabs. These results suggest that the ts-11 infection is transitory (or the copy number declines below detectable limits of the PCR) but MG antibody persists. Tracheal swabs detected infection for a longer period after vaccination than choanal swabs when using the PCR procedure. The higher recovery rate from tracheal swabs, with the ts-11 specific PCR assays suggests that this vaccine strain may survive longer in the upper trachea than in the choanal cleft.

A tracheal swab from a non-vaccinated bird in Treatment C was positive on PCR assay at 12 weeks post-vaccination. It is unlikely that this was a result of contamination during sampling since these birds were sampled prior to the vaccinated subjects. Although this may have been a false positive, this observation suggests the possible spread of ts-11 over short distances. Although the caretaker was careful to move from non-vaccinated to vaccinated groups when servicing the birds this may account for the spread of the ts-11 strain. It is also possible that in larger flocks lateral transmission is facilitated by proximity of birds at high density.

None of the swabs derived from any of the pullets in all treatments were positive for non-ts-11 MG (field strain isolates) using PCR. It was concluded that neither natural exposure nor vertical transmission had occurred in any of the treatments.

Although the PCR test is expensive, its sensitivity and specificity make it ideal for monitoring ts-11 vaccinated flocks. It is also possible to offset the cost issue to a degree by pooling samples (Tyrrell *et al*., 1994). The site selected for sampling by swab influenced the apparent sensitivity of the ts-11 specific PCR assay performed on samples taken 10 weeks after vaccination and needs further investigation.

The prevalence of RSPA test reactors is an inadequate means of monitoring a ts-11 vaccinated group of broiler breeders for vaccine response or field exposure. Using the

RSPA test response of non-vaccinated commingled sentinels to monitor a group of broiler breeders will not enhance the value of this assay method. Spread of the ts-11 strain to commingled non-vaccinates occurs rapidly and seroconversion patterns parallel those of the vaccinated birds.

It is recommended that a PCR assay performed on tracheal swabs be used to monitor the efficiency of vaccination during rearing and to confirm that point of lay broiler breeder pullets with RSPA agglutination reactions are free of field-strain MG infection.

The potential for spread of the ts-11 strain to adjacently housed non-vaccinated birds needs further investigation.

2. RETROSPECTIVE STUDY ON THE USE OF THE RSPA TEST IN TS-11 VACCINATED FLOCKS

The small-scale trial on SPF birds kept in isolators demonstrated that the vaccine colonized the upper respiratory tract of the vaccinated birds for the entire 8-week trial period. However despite the fact that ts-11 was detected by PCR in 100% of these vaccinates; only 60 % became RSPA positive within the 8 week trial period. This was in excess of the 30% quoted by the vaccine supplier as being the normal level of flock reactor rate following ts-11 vaccination.

Research data, the vaccine manufacturer's recommendation and the in house pilot study suggested that the MG infection status of a ts-11- vaccinated flocks could be determined by assessing the rate and intensity of RSPA test results.

Under field conditions a ts-11 vaccinated flock was classed as field strain MG infected if more than 60% of the birds were RSPA positive and MG free if less than 60% tested positive. It was also assumed that flocks with 30 and 60% of the birds RSPA positive had been well vaccinated and those with less than 30% positive had been poorly vaccinated.

These determinant criteria were applied to serological data obtained from 116 breeder farms over a 4-year period.

Contrary to expectation the downward trend in RSPA reactor rate late in the rearing phase was reversed from 25 weeks (after transfer to the laying farm) and then continued to increase to 100% at 35 to 50 weeks. This second peak was however not followed by a decline and flocks remained at, or very close to 100% positive until depletion.

Based on the preceding assumptions it was initially concluded that all these flocks were exposed to field strain MG during the early laying period. It is however possible that this rapid conversion to 100% RSPA test positive was stimulated by ts-11 strain itself. Vaccine with a titre of $10^{8.6}$ cfu per dose is known to stimulate a flock seroconversion rate of 70-80% while vaccine with a titre of $10^{8.1}$ccu per dose a 30% seroconversion rate (Cilliers, personal communication 1999). Clearly the degree of seroconversion is a function of the challenge dose.

It is possible that the stress experienced by birds during lay could induce sufficient immune suppression to allow the ts-11 population in the upper respiratory tract to increase. This would in turn have the effect of increasing the challenge dose to which these birds and their non-colonized pen-mates were exposed and thus stimulated increased seroconversion. In addition this second stage response could be exaggerated since the immune system had effectively been primed by vaccination at 10 weeks.

It would appear that either exposure to field strain MG or the stress of coming into lay stimulated a second stage seroconversion to 100% RSPA positive. It is not possible with this data to distinguish between seroconversion due to vaccination strain or field strain MG. The presence of field strain MG would need to be confirmed by other means such as culture or PCR.

The incidence of MG in broilers in region 1 was lower than that in regions 2 and 3. Since MG is vertically transmitted it is possible that the flocks in region 1 are MG

negative and the flocks in regions 2 and 3 MG positive. Since the seroconversion profile in region 1 was different from that in regions 2 and 3 it is possible that the pattern of seroconversion is more useful than the degree of seroconversion in distinguishing exposed from non-exposed flocks. This may explain why the rate of seroconversion was faster in regions 2 and 3 as compared to region 1.

While the initial isolator trial (outlined in the introduction) demonstrated that ts-11 vaccine did not spread laterally to commingled sentinels, field data and personal experience and research has shown this to be incorrect. (Ley *et al.*, 1997; Ley *et al.*, 1997b). Vaccine available in South Africa at the time and hence the one used in the initial trial was produced by Merial France, whereas the vaccine used in other trials (Ley *et al.*, 1997b, Abd-El-Motelib *et al.*, 1993) was produced in America (Select Laboratories, Inc., Gainesville, Ga.). According to the manufacturer this change has meant a slight increase in vaccine titre, although the minimum release titer of $10^{7.9}$ ccu per dose remained (Cilliers 1999). Since this titre change could cause a difference in flock seroconversion it is highly likely that it also influenced the degree of lateral transmission. In addition the psychological, physiological and environmental stress imposed on larger groups could enhance the spread of ts-11.

3. EVALUATION OF SENTINELS TO MONITOR MG STATUS OF TS-11 VACCINATED BREEDER FLOCKS

Vaccination of commercial broiler breeders with ts-11 in a field situation stimulates production of sufficient IgM to elicit a positive RSPA test in a proportion of the birds. Flocks that are negative for MG on PCR could have in excess of 80% of the birds RSPA positive.

The spread of ts-11 to in-contact sentinels is so rapid that the serological response of in-contact sentinels parallels that of the vaccinated flock (Figure 4.13).

CHAPTER 6 - CONCLUSIONS AND RECOMMENDATIONS

The floor pen study to evaluate the serological response of broiler breeders after vaccination with ts-11 strain *Mycoplasma gallisepticum* vaccine demonstrated that:

1. The pattern of RSPA seroconversion of broiler breeders was different to that previously reported. MG vaccination of rearing pullets with ts-11 strain MG at 10 weeks of age stimulated a gradual increase in RSPA reactor rate over a three week period and stabilized at 30-60% positive in the absence of challenge. This seroconversion pattern is in accordance with that suggested by the ts-11 vaccine manufacturer to indicate effective immunization.
2. Spread of the ts-11 strain of MG to commingled non-vaccinates occurred rapidly and seroconversion patterns, although delayed by three weeks paralleled those of the vaccinated birds. Using the RSPA test response of non-vaccinated commingled sentinels to monitor a group of broiler breeders would not enhance the value of this assay method under experimental conditions.
3. The potential for spread of the ts-11 strain MG over short distances (2 meters) to adjacently penned (same house) non-vaccinated birds was suggested as a possibility and needs further investigation.
4. Although the number of samples tested with the ts-11 specific PCR assay was too small to make statistically significant conclusions there were indications that this technique of monitoring flocks definitely warrants further investigation. The ts-11 strain-specific PCR assay was effective at detecting ts-11 strain MG colonization of the upper respiratory tract within two to three weeks of vaccination. Tracheal swabs appeared to be a more sensitive sampling technique (detected colonization for a longer period after vaccination than choanal swabs) when using the PCR assay.

The retrospective study on the use of the RSPA test in ts-11 vaccinated flocks demonstrated that:

1. The RSPA reactor rate in commercial broiler breeder flocks is quite different to that previously reported in both the rearing and laying phases.
2. During the rearing phase (0-20weeks) all the flocks in this survey showed a consistent pattern of seroconversion similar to those recorded in the floor pen trial. The percentage of birds positive on RSPA test increased gradually after vaccination to a peak of 40-70% within 8 to 10 weeks of vaccination. This peak was followed by a slight decline in the remaining weeks before birds were transferred to the laying farm at 20 weeks. As suggested by the vaccine manufacturer it is likely to be possible to distinguish immunized from non-immunized and field strain challenged flocks based on RSPA reactor rate during rearing (0-20 weeks).
3. Once on the laying farm all flocks became 100% RSPA test positive within 15 to 30 weeks making it impossible to distinguish field strain challenged from well-immunized laying flocks using the RSPA test results. The prevalence of RSPA test reactors is an inadequate means of monitoring a ts-11 vaccinated group of broiler breeders for vaccine response or field exposure.

Using sentinels to monitor MG serological response to ts-11 vaccination of breeder flocks demonstrated:

1. Spread of the ts-11 strain of MG to commingled non-vaccinates occurred rapidly and seroconversion patterns mimicked those of the vaccinated birds.
2. Serological monitoring of in-contact sentinels with the RSPA test does not improve the efficiency of the monitoring system under field conditions.

The RSPA test is useful to determine the serological response of a rearing flock to ts-11 vaccination in the absence of MG exposure but it is not a reliable means of monitoring broiler breeders in the laying cycle or for distinguishing vaccine response from field exposure.

It is recommended that:

1. The RSPA assay is used to monitor the efficiency of vaccination during rearing and a seroconversion rate of 30-60% taken to indicate effective vaccination.
2. The PCR assay performed on tracheal swabs is used to:
    a. Confirm that point-of-lay broiler breeder pullets (especially those with RSPA agglutination reactions in excess of 60%) are free of field-strain MG infection.
    b. Monitor broiler breeder flocks for MG challenge during the laying cycle. Flocks should be tested every 2 weeks to detect infection early enough to avoid vertical transmission from infecting broiler flocks. In the case of low risk flocks the testing interval can be extended to 4 weeks especially where the opportunity for egg identification and removal from the setters is possible.
3. Where possible, flocks with a confirmed field strain challenge should be eliminated and all hatching eggs removed from the hatchery and destroyed.

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University of Pretoria etd – Collett, S R (2005)

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