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Vaccination of Chicken Against Mycoplasma Gallicepticum

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C. ABSTRACT

Field trials were conducted utilizing the F strain of Mycoplasma gallisepticum (MG) as a vaccine in layer chickens. Vaccination with the F strain reduced egg production losses, medication costs, and mortality associated with exposure to field strains of MG.

In laboratory trials the F strain protected against airsacculitis after challenge with virulent MG. It also offered protection against egg production drops and egg transmission. It was less virulent than the highly virulent R strain in vivo as measured by airsacculitis in chickens, egg production losses, and egg transmission, but there was no difference between the strains when presented by aerosol to turkey poults. Its relatively low transmissibility was confirmed. Protection against colonization by superinfecting MG strains was shown

In vivo and in vitro methods were developed to evaluate virulence and vaccine protection induced by numerous MG strains at several in vitro passage levels. These included evaluation of air sac lesions after challenge, ciliostasis and lesions of tracheal ring organ cultures, colonization of tracheal tissue in vivo and in vitro, and pathogenicity in embryos. Virulence observed in some systems was not always apparent in other systems. It appeared that colonization of the trachea was a prerequisite for inducing protection. There was a suggestion that neutralizing antibodies may correlate with protection.

The F strain of MG was shown to be a safe and effective vaccine in naturally infected, multiple-age commercial layers, and it may be a tool for use in eradication programs on such farms.

D. OBJECTIVES OF THE WORK

The present work was primarily concerned with an examination of the efficacy and safety of Mg live strain vaccination against various manifestations of mycoplasmosis in layer hens, and, to a much lesser degree, against respiratory mycoplasmosis in broilers. The main emphasis was on examining the relevant properties of the Mg F strain, but comparison was made with standard virulent strains and other possible vaccine strains, using standard and innovative methods for evaluation of pathogenicity and protection. Within this framework, the specific objectives of the project were as follows.

- 1) The effect of vaccination with Mg F strain on egg production and egg transmission in layer hens and breeders.
- 2) The use of the F strain as a tool in eradication.
- 3) The efficacy of live Mg vaccine (F-strain or other) in the prevention of Mg respiratory disease.
- 4) Methods for evaluation of pathogenicity of Mg and comparison of various Mg strains (virulent, F vaccine, and laboratory derivatives).
- 5) Evaluation of protective immunity against Mg.

E. SUMMARY OF EXPERIMENTAL RESULTS

E-1 Introduction

The F strain of Mycoplasma gallisepticum (Mg) has been widely used in certain areas of the U. S. A. for vaccination of layer hens. Statistical studies and previous field trials indicated that the application of this strain in Mg-free replacement pullets will effectively prevent egg production drops due to Mg infection at the onset of lay. The use of live vaccine was, and remains, a controversial procedure in view of the consensus that the foremost tenet in

mycoplasma control in poultry is eradication of the infective agent. However, it was proposed that judicious use of F strain vaccination may be used as a tool to further eradication in problematic sectors of the industry. Others have suggested that a live vaccine may have merit in Mg control in other sectors of the poultry industry, (such as broilers), especially in countries where Mg disease is a major economic problem and widespread eradication is an unfulfilled hope.

In view of the above it was considered of prime importance to carry out laboratory investigations in order to understand the biological properties of the vaccine strain, and to relate these to the field observations. In particular, it was desired to clarify the status of the F strain with respect to interaction with other Mg strains. The possibility of the use of other attenuated Mg strains as an alternative to the F strain was also investigated.

At the same time it was desired to provide an experimental basis for recommendations with respect to field use of the F strain vaccine, in which area there existed wide variations, mostly empirically based. A series of laboratory trials were carried out to relate vaccination efficacy to mode of preservation and dosage of vaccine. Other studies, including field trials, related to time and route of presentation, rate of transmission and time until seroconversion.

Experimental results are organized into general subject areas, reflecting the general areas of endeavor described above as follows.

- 1) Field trials with F strain
- 2) Egg transmission
- 3) Transmission of Mg from infected to contact chicks
- 4) Attenuated strains for use as live vaccines
- 5) The use of F strain as a tool for eradication
- 6) Pathogenicity
- 7) Protective immunity against Mg.

In some cases reprints of publications relating to one of the subject areas have been included in the relevant section, with the reprint number referring to the list of publications, section (h) of this report.

E-2 Materials and Methods

Experimental animals: Field experiments in Georgia were conducted on commercial egg production operations, using various layer lines, free of Mg and Ms.

Field experiments in Israel were conducted with Mg free replacement pullets, Poultry Breeders Union, line 10 or line 10 (improved). In the first series of experiments (Farm I and Farm IV) fertile eggs were treated to eliminate egg transmitted Mg; in the second series of experiments pullets were obtained from Mg-free breeder flock.

Egg transmission experiments were carried out with leghorn pullets and roosters purchased from commercial sources in Georgia. Two roosters were maintained with each group of 10-15 hens in separate floor pens.

The source of experimental birds for pathogenicity, vaccine and infection studies differed in various experiments, and is described in each report.

Birds for preparation of tracheal rings (TR) for organ culture were 1 d old specific pathogen free (SPF) chicks (SPAFAS, Norwich, Conn). Embronated eggs for Mg inoculation studies were obtained from the following sources; SPF, commercial leghorn layer flocks, or leghorn layers immunized with Mg vaccine.

Mycoplasma strains: M. gallisepticum F strain used in these experiments was obtained from Dr. Gentry (U. of Penn.), and after not more than 10 additional passages was maintained at -70°C as a stock for vaccination and experimentation, both in the U. S. A. and in Israel. The origin of other Mg strains used was as follows: R, (Ritchie, U. of Ga.); S-6. (Adler, U. of California, Davis); A5969 (Tourtellotte, U. of Conn. via Razin, Jerusalem, Israel); 227 (Levisohn, Bet Dagan, Israel).

Strains attenuated by multiple passage in artificial medium (20) have the passage level indicated by subscript, e.g. F₂₅₄ indicates the 254th sequential passage of the F strain.

Cultivation of mycoplasma. Cultures were obtained from trachea of live birds and tracheas and air sacs of necropsied birds with cotton swabs, inoculated into mycoplasma medium (9, 11) and incubated at 37°C. A slightly modified procedure, with isolation from the excised trachea of necropsied birds, was used in some experiments, particularly for reisolation after quantitative intratracheal inoculation (16).

Isolation of mycoplasma from embryonated eggs was performed from the yolk membrane, as described by Glisson and Kleven (10). Viable counts were performed by end point dilution in liquid medium (Georgia) or by plating 5 µl aliquots of appropriate dilutions on solid medium, and counting 3 replicate spots containing 25-250 mycoplasma colonies.

Selective isolation of the tyl⁺Mg strain 227 was performed by plating on mycoplasma agar medium containing 0.5 µg/ml tylosin; at this level there was 100% recovery of 227 and complete suppression of tylosin sensitive Mg strains.

Quantitative isolation was performed by swabbing the entire inner surface of the excised trachea, suspending the material in 1 ml PBS and, determining the number of cfu in the suspension by standard methods for viable count on agar medium.

Cultures for inoculation to tracheal ring (TR) organ culture, intratracheal inoculation in vivo, or embryonated eggs were grown in mycoplasma medium with glucose, but in the absence of thallium acetate. Only active logarithmic phase cultures after a single overnight passage were used (titer 5×10^8 - 1×10^9 cfu/ml).

Isolation of mycoplasma from tracheal ring culture was performed by sampling the supernatant culture medium in which the infected TR were maintained.

Identification of mycoplasma isolates was performed by direct immunofluorescence employing the method of Baas and Jasper (2) in Georgia, and a modified method with staining of excised agar blocks (Israel). Conjugated antisera were prepared independently; results using the different methods and reagents were compared and found equivalent.

In vivo infection: Aerosol administration of Mg culture for challenge and evaluation of air sac lesions was performed as described in previous studies (12). Unless specified otherwise, lesion score and isolation attempts were performed on birds necropsied 2 wks post-infection.

Challenge of layers in peak production (28 weeks of age) was done by a combination of aerosol presentation of Mg strain R and injection of about 1.5×10^7 cfu/bird into the left infraorbital sinus.

Intra tracheal administration of mycoplasma was performed as described previously (17). In order to determine the tracheal infectious dose (ID_{50}), groups of 2 wk old Leghorn chicks were inoculated with serial one hundred-fold dilutions of mycoplasma culture, using Frey's medium - thallium acetate as the diluent. Birds were sacrificed for isolation at 0 or 4 hrs, 3 d, 7 d or 14 d PI; each group composed of 5 birds or 20 birds (at 3 d). Infectious dose was calculated according to the modified method of Spearman-Kärber (24).

Vaccination with Mg: Vaccination of replacement pullets with Mg was carried out by eye drop instillation unless otherwise designated. An overnight culture, grown in Frey's medium without glucose was used in Georgia; the titer was approximately 10^7 cfu/ml in most experiments. In Israel vaccine was grown in mycoplasma medium containing glucose, and the titer was 5×10^8 - 3×10^9 cfu/ml. Vaccination was usually performed at time of transfer of birds to laying cages, which was usually about 18 wks in the U. S. A. Exceptions were trials in N. W.

Georgia, where birds were vaccinated before housing, and trials in Mississippi where birds were vaccinated at 8 wks of age when transferred from brooder units to growing units.

Vaccination of young chickens was performed by eyedrop instillation or by aerosol vaccination, as reported in detail in Reprint 4 or Reprint 15.

Intramuscular vaccination of layer hens with Mg oil-emulsion bacterin (alone or in combination with live F strain vaccine) was performed according to the schedule detailed in Reprint 12 (10).

Serological methods: Evaluation of serological reactions in chickens was by standard methods of rapid slide agglutination (RSA) and hemagglutination inhibition (HI) (1). In Israel the strain used for antigen production was Mg A5969; in Georgia Mg S-6 was used unless noted otherwise.

Growth inhibition activity was determined by the agar disc method of Clyde (6) or by Lin's modification employing agar wells (19).

Hyperimmune chicken serum was prepared by aerosol infection of chickens with Mg R strain followed by one or more intramuscular injections with oil-emulsion Mg bacterin (Mg-Bac, Salisbury Laboratories, Inc., Charles City, Iowa). Pooled sera were filtered sterilized before injection in vivo, in ovo or into TR organ culture.

Tracheal ring organ culture: Tracheal rings were prepared from the excised trachea of 1 d chicks using a Mickle tissue chopper, adjusted to give a 0.5 mm ring. The TR were maintained in the medium of Cherry & Taylor-Robinson (5), adjusted to pH 7.5. Incubation was in a tissue culture drum roller at 37°C. Ciliary activity was scored by daily microscopic examination, expressed as percentage of the TR circumference in which the beating of cilia could be detected.

Light and electron microscopy: Methods for light, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) in the portion of the work carried out in the U. S. A., have been described in detail (7).

Methods for scanning electron microscopy performed in Israel have been included in Reprint 7 (16) which relates to the relevant portion of the project.

Mycoplasma interaction profile: A method for numerical evaluation of interaction between mycoplasma and the TR in organ culture has been devised. Three area of interaction are evaluated:

- 1) Host reaction, as exhibited in surface changes in the respiratory epithelium visualized by 440x SEM overview of the interior surface of the TR.
- 2) Localization of mycoplasma cells, as visualized by 4000x SEM.
- 3) Effect of TR activity, as expressed in microscopic quantitation of ciliary activity.

Each type of interaction was given a numerical score of 0-3, in which 0 expressed the normal condition. Thus a maximum score of 9 indicates the highest degree of pathogenic interaction.

Statistical methods: Egg production data in the Israeli field experiments were analyzed using the t test for paired observations on monthly egg production, comparing vaccinated and non-vaccinated groups for an 8 month lay period.

Analysis of variance was used to analyze egg production data in the field trials in Mississippi, with comparison to data from the non-vaccinated flock immediately preceeding the trial.

Egg transmission data after challenge with the R strain were analyzed by comparison of binomial proportions using Z distribution of standard normal probabilities.

Effect on mortality was determined using a Chi-square analysis.

Air-sac lesion scores were analyzed with Duncan's multiple range test.

E-3 Results

a. Field Trials with F Strain

In Israel field trials were carried out in five layer flocks (about 7000 birds) Reprint 6 describes in detail two simultaneous trials in a single-aged and multi-age small holdings. Later trials were carried out according to the same format.

Administration of the vaccine resulted in 100% seroconversion and reisolation from vaccinated birds (Reprint 6, Tables 2 and 4). Vaccination did not cause any alteration in the mortality rates or general health status of any of the flocks.

Egg production data (monthly/hen) are reported for two flocks in Tables 1 and 3 Reprint 6. A graphic reproduction of egg production for all 5 farms is shown in Fig 1, in which Farm I and Farm IV are those reported in Reprint 6. Egg production in Mg-free hens was the same or better than in vaccinated birds (Fig 1, A). Egg production in birds infected by older layers in multi-aged units was lower than in vaccinated birds (Fig 1, B). Statistical analysis indicated that the differences in Trial II and Trial III were non-significant; differences in Trials I, IV, and V were significant.

Transmission of the vaccine strain to non-vaccinated birds on single-aged farms differed between the trials, occurring at 17 wks in Farm I, 7 wks (Farm II) and 9 wk (Farm III) post-vaccination. On multi-aged farms infection of the non-vaccinated birds occurred before the onset of lay, at 4 wks PI on Farm IV and 2 wks on Farm V.

At the University of Georgia vaccination programs were carried out on large commercial egg production operations in South Carolina, N.W. Georgia, and Mississippi. Vaccination and monitoring of flocks was in some cases carried out in cooperation with local, state and federal laboratories.

Ongoing experiments were conducted at two sites in South Carolina. Each operation received two successive cycles of vaccination, after which vaccination was stopped. The vaccination program appeared to have stopped all Mg-associated egg production problems previously experienced. Mg free replacement flocks remained free on one farm until monitoring was stopped. On the other operation, a slow-spreading Mg strain, probably the vaccine strain, apparently replaced the field strain. The spread of the strain was slowed even more by tylosin water medication in several houses, and no egg production problems were experienced.

Vaccination was carried out in a multi-aged high rise layer operation in N. W. Georgia where Mg infection of layers was causing major egg production depression and expenses for tylosin medication at time of break. After two cycles of vaccination, medication costs (\$.17 chicken) were almost completely eliminated. Due to technical problems it was not possible to compare egg production data with previous flocks, but the impression was that there was improvement. Plans to replace live Mg vaccination by Mg bacterin as a transitional step in eradication were not implicated because the operation went out of business.

Flock records from a multiple-age commercial egg production unit in Mississippi (approximately 140 000 layers) were analysed to determine the effect of F strain Mg vaccination on 37 weeks of egg production and mortality. Egg production was significantly higher in vaccinated hens, averaging 69.96% on a hen housed production (HHP) basis as compared to 65.38% HHP for non-vaccinated birds. Total mortality for the 37 week period was significantly lower in vaccinated versus non-vaccinated birds (3).

Experiments were carried out on means of preservation of mycoplasma for vaccination (18) and on the correlation between dosage level and immune response (21). There was no clear correlation between the titer of the vaccine and protection against air sac lesions or the level of humoral antibodies in young Leghorn chickens. Field observatio

support the conclusion that the degree of serological response is not directly related to the dose of the vaccine (in the range of 10^6 - 10^9 cfu/ml). However, the time at which 100% seroconversion of vaccinates is detected appeared to be longer in farms in which the vaccine titer was low.

b. Egg Transmission

A comparison between MgF and Mg R strains with respect to levels of egg transmission (Reprint 3) indicated levels of 1.59% and 3.19% respectively after administration to laying hens in peak production by the aerosol challenge route. In a later study (Reprint 12) in which the virulent R strain was administered by a modified challenge method, approximately 18% of the eggs were infected during a comparable 8 wk period, and the overall level of 12% was found during 25 wks of lay. A 70-80% drop in egg production resulted from the administration of virulent Mg (Reprint 3, Fig 1; Reprint 12, Fig. 5) which persisted for 4-6 wks after which egg production increased but never returned to peak levels. Administration of F strain produced a numerical but statistically non-significant decline in egg production (Reprint 3, Fig 1). No egg transmission was detected after administration of Mg F strain by eye drop instillation (Reprint 3, Table 1).

The effects of vaccination with Mg F strain or Mg bacterin are reported in detail in Reprint 12. All methods of vaccination were protective to some degree against egg transmission after challenge with Mg R (Reprint 12, Table 2). However, the pattern of egg transmission in the various experimental groups differed (Reprint 12, Figs 1-4).

A comparison of embryo mortality and recovery of Mg from embryonated eggs inoculated with Mg showed a marked difference between eggs from vaccinated and non-vaccinated layers (19). Table 1 shows the results of titration of Mg R in eggs with and without maternal antibody.

Table 1 : Effect of maternal antibody on embryo mortality and isolation in eggs inoculated with M. gallisepticum strain R_{LOW}

Inoculation level	Embryo mortality		Isolation from live eggs	
	Non-vacc.	Vacc.	Non-vacc.	Vacc.
3.8×10^6 CFU	5/5 ^A	1/5	-	4/4
3.8×10^5	5/5	0/5	-	5/5
3.8×10^4	5/5	1/5	-	3/4
3.8×10^3	4/5	0/5	1/1 ^B	4/5
3.8×10^2	2/5	0/5	1/1	1/5
3.8×10^1	0/5	0/5	5/5	1/5
3.8	0/5	0/5	5/5	1/5
0.38	0/5	0/5	5/5	1/5
Medium	1/5	0/4	0/5	0/4

A) Number embryo deaths/number inoculated.

B) Number positive isolates/number.

C. Transmission of Mg from infected to contact chicks

A series of experiments were carried out to determine the rate of spread of Mg F and derivative strains from infected chicks to Mg negative chicks raised in direct contact.

Table 2 summarizes the results of 3 experiments in which the time of first isolation from in contact birds is shown. The rate of transmission differed in repetitions of the experiment, but there did not appear to be any difference in time of transmission from chick to chick, or chick to poult. Likewise, chicks infected by the aerosol or eye drop route transmitted at the same rate in spite of the differences in the pathogenic effect of Mg F by the different routes of administration. Thus in Exp II 10% (6/60) of the aerosol infected chicks died 7-14 d PI with typical signs of Mg disease at post mortem, whereas only 1/30 eye drop infected chicks died during this period. There was no mortality in the turkey poult infected by contact exposure. Serological reactions in the RSA test were detected in 100% of the birds tested in the spray and eye drop infected groups 2 wk PI (2 wks of age) and in the in contact groups about 2 wks after first detection by isolation.

Table 3 shows the results of a comparison between Mg F vaccine strain and two high passage derivative strains with respect to recovery and transmission. There was reisolation of Mg from all the chicks inoculated with F_{Low} or F_{254} , but no recovery of Mg F_{300} from infected birds. In this experiment F_{Low} was first detected in the in contact group 5 wk PI whereas F_{254} was not detected even at 6 wk PI. Serological response in the RSA test was detected in the F_{Low} infected chicks 4 wks PI (5/5); the F_{254} infected chicks showed 1/5 at 4 wk and also at 5 wk PI. The in contact groups and the F_{300} infected group did not show positive reactors up to 6 wk PI. Mg-associated mortality was noted in 6/40 chicks infected with F_{Low} , 2/30 F_{Low} in contact chicks; and none in other groups.

Table 2: Time until transmission of Mg F_{LOW} to contact birds

Exposure		Isolation ^A		Serological Response ^A
Aerosol ^B	Exp I	Chicken	3 wk	5 wk
		Turkey	4 wk	6 wk
	Exp II	Chicken	4 wk	5 wk
		Turkey	4 wk	7 wk
	Exp III	Chicken	5 wk	7 wk
Eye drop ^C	Exp II	Chicken	4 wk	6 wk

- A) Time after inoculation in which at least one positive isolation or one positive response in RSA for Mg was found.
- B) Aerosol exposure of 1 d old chicks to Mg F (titers of cultures used, 5×10^8 - 5×10^9 cfu/ml),
- C) Eye drop instillation of culture of Mg F as used for aerosol in Exp II, titer 8×10^8 cfu/ml.

Table 3 : Isolation of Mg from chicks infected at 1 d age by aerosol administration of Mg F, F₂₅₄, or F₃₀₀, or from in contact chicks

Weeks after infection	Strain					
	A F _{LOW}		A F ₂₅₄		A F ₃₀₀	
	Infected	Contact	Infected	Contact	Infected	Contact
2	5/5 ^B	NT	5/5	NT	0/5	NT
3	5/5	0/5	4/5	0/5	0/5	0/5
4	5/5	0/5	4/5	0/5	0/5	0/5
5	8/8 ^C	4/5	5/7 ^C	0/5	0/8 ^C	0/5
6	4/5	4/5	4/5	0/5	0/5	0/5

A) Titer of Mg cultures used for inoculation: F_{LOW} = 7×10^9 cfu; F₂₅₄ = 1.1×10^9 cfu/ml; F₃₀₀ = 1.6×10^9 cfu/ml.

B) Number of positive isolates/ Number of chickens tested. Isolation attempt by tracheal swab from live bird.

C) Isolation attempts include 3 necropsied birds from each group.

d. Attenuated Mg strains for use as live vaccine

Attempts were made to develop an attenuated Mg vaccine by successive passages in artificial medium, starting with various Mg type strains and the F vaccine strain. Representative passage levels (indicated by the subscript) were evaluated with respect to pathogenicity, infectivity, antigenicity, stimulation of protective immunity and stability against back-revision.

Table 4 summarizes the biological properties of the strains tested . Additional information regarding the in vivo pathogenicity of attenuated strains is found in Reprint 15, Table 3. There was no correlation between passage level of the attenuated strains and in vivo pathogenicity or in ovo pathogenicity (16).

The evaluation of selected attenuated strains with respect to vaccination efficacy are summarized in Reprint 15. The results of in vivo pathogenicity of attenuated strains after back-passage in chickens is shown in Table 7 (Reprint 15).

Table 4: Pathogenicity and infectivity of attenuated Mg strains

Strain	Isolation ^A from trachea	ID ₅₀ ^B	A S ^C lesion score	Serology ^D	M
R _{LOW}	100	200	2.3	100	
R ₁₉₈	70	1.7x10 ⁶	0	40	
F _{LOW}	100	350	1.3	100	
F ₂₅₄	90	2200	0.1	100	
F ₃₀₀	20	2800	0	0	
S-6 _{LOW}	100	NT	1.3	100	
S-6 ₂₆₀	100	NT	0.5	100	
A ₅₉₆₅	0	-F	0	0	
Control	0	0	0	0	

A) Per cent positive isolates; ten birds tested.

B) Recovery of Mg from the trachea, 3 d PI; 20 birds/group.

C) Average of ten birds, on a scale of 0-3 proportional to severity of lesions.

D) Rapid slide agglutination test for Mg, 2 wks PI by aerosol route; ten birds/grc

E) Evaluation of tracheal ring organ culture, 5 TR per group; maximum score of 9 indicates highest degree of pathogenicity.

F) No mycoplasma isolated.

e. The use of the F strain as a tool for eradication.

Preliminary laboratory studies were carried out to investigate the degree of retention or elimination of Mg in the trachea of birds which had been previously exposed to Mg either by vaccination or infection. The experiments necessitated the quantitation of mycoplasma in the trachea, and the ability to distinguish between the two strains of Mg (vaccine and superinfecting), as described in Materials and Methods. Preliminary experiments and internal controls were carried out to verify the ability of the selective method to distinguish between Mg R or R and Mg 227, tylosin resistant.

Table 5 summarizes the results of super infection of chicks vaccinated with Mg F at 1 d age by eye drop instillation. Experimental group 1 indicates the level of mycoplasma in the trachea as the result of the vaccination procedure. Groups 3 and 5 indicate the level of mycoplasma present 4 hr or 10 d after infection of non-vaccinated chicks. In these groups all the mycoplasma isolated were resistant to tylosin, indicating their origin in the superinfecting culture. Groups 2 and 4 show the presence of a mixed infection with Mg strains F and 227 in which the proportion of 227 increases with time after infection (Group 6).

Table 6 indicates the effect of prior infection on the degree to which Mg is retained by the chicken trachea. Superinfection was attempted 5 wks after 1 day old chicks were infected intraairsac with either Mg R or Mg F. The presence of tyl sensitive Mg in the trachea, resulting from infection, is shown in Group I. The ability to recover and distinguishing the superinfecting strain is shown in Group II, where 10^5 tyl⁺ Mg are recovered immediately after inoculation. By 24 hrs after superinfection there are only sporadic isolations of the superinfecting strain from most chickens.

Table 5: Superinfection with Mg 227 (tyl⁺) in chickens vaccinated with Mg F by the eye drop route, 2 wk post-vaccination.

Treatment	Recovery of Mg (cfu/ml)		Proportion 227/total
	Total Mg ^B	Mg 227 ^B	
1. Vaccinated No.S.I.	3.4x10 ⁶	<10 ²	< 3x10 ⁻⁵
2. Vaccinated S.I. + 4 hr.	5.4x10 ⁶	6.6x10 ⁵	6.6/54=0.12
3. Non-vaccinated S.I. + 24 hr.	1.5x10 ⁵	3.6x10 ⁵	$\frac{3.6}{1.5} = 2.4$
4. Vaccinated S.I. + 24 hr.	1.6x10 ⁵	9.5x10 ⁵	$\frac{1.6}{9.5} = 0.17$
5. Non-vaccinated S.I. + 10 d	2.8x10 ⁶	3.2x10 ⁶	$\frac{3.2}{2.8} = 1.1$
6. Vaccinated S.I. + 10 d	4.5x10 ⁶	2.4x10 ⁶	$\frac{2.4}{4.5} = 0.53$

A) Titer of superinfecting culture 1.3×10^9 cfu/ml on selective medium. Superinfection performed by deposition of 0.1 ml ON culture of Mg 227 into the trachea of 2 wk old chickens vaccinated at 1 d age with Mg F by the eye drop route.

B) Average of number of cfu Mg for individual birds in each group. Number of birds in treatment groups: Group 1, 3 birds; Group 2, 4 birds; Group 3, 2 birds; Group 4, 4 birds; Group 5, 2 birds; Group 6, 4 birds.

Table 6: Effect of prior infection (5 wk PI) by MgR or F on retention of superinfected Mg.

	Prior ^A Infection	Super ^B Infection (227)	Time after ^C SI	Total ^D Myco.	227 ^D
Group I	R	-	-	4.4 x 10 ⁶	< 10 ²
				0.8 x 10 ⁶	< 10 ²
	F	-	-	8.6 x 10 ⁷	< 10 ²
				4.7 x 10 ⁷	< 10 ²
				2 x 10 ⁵	< 10 ²
Group II	R	+	Immediate	4.2 x 10 ⁶	9 x 10 ⁵
		+	"	4 x 10 ⁵	3 x 10 ⁵
	F	+	"	2.7 x 10 ⁶	2 x 10 ⁵
		+	"	3.6 x 10 ⁶	1.3 x 10 ⁵
		+	"	2.4 x 10 ⁶	9 x 10 ⁵
Group III	R	+	24 hr.	1 x 10 ⁴	< 10 ²
		+	"	8 x 10 ³	2.4 x 10 ³
	F	+	"	6.6 x 10 ⁵	1 col
		+	"	2 x 10 ⁷	2 col
		+	"	5.8 x 10 ⁵	1 col
		+	"	1.2 x 10 ⁷	< 10 ²
		+	"	5.7 x 10 ⁶	< 10 ²
		+	"	3 x 10 ⁵	2 x 10 ⁵

- A) Infected by aerosol route at 1 d; 5 wks prior to use in experiment. All birds were serologically positive for Mg.
- B) Superinfection with 0.1 ml culture Mg 227, tyl resistant. Titer 2.8x10⁹ cfu/ml grown in the presence or absence of 0.5 µg/ml tylosin
- C) Birds sacrificed within 1 min of infection (immediate) or 23-25 hrs after superinfection
- D) Determination of cfu/trachea by growth on standard mycoplasma medium (total mycoplasma) or in the presence of 0.5 µg/ml tyl.

f. Pathogenicity

A series of studies were carried out in order to clarify the status of Mg F with respect to pathogenicity. Reprint 7 summarizes the results of an in vivo infection experiment comparing MgR and Mg F with respect to clinical status, colonization and changes in the surface morphology of the diseased organ.

The question of the relationship between infectivity (the ability to colonize), pathogenicity, and antigenicity was examined by comparison of a series of Mg strains with respect to these properties. Table 7 shows the results of a series of in vivo infection experiments in which the pathogenicity of the Mg is evaluated by air sac lesion score and the ability to colonize is measured by the ID_{50} of tracheal infection.

Infection of turkeys by the eyedrop route showed minimal lesions, but aerosol exposure of turkey poults with either Mg R or F produced severe air sac lesions (Reprint 1). Likewise, inoculation of 6d embryonated eggs (Table 8) and introduction of Mg into the anterior air sac of 1 d chicks (Table 9) showed little difference in virulence between F and known pathogenic strains.

The use of organ culture of tracheal rings (TR) from 1d SPAFAS chicks as an in vitro model system for the evaluation of pathogenicity was examined. The course of infection in the trachea of chicks infected in in vivo and in TR infected in vitro was followed by light microscopy, scanning electron microscopy, and transmission electron microscopy. Sequential changes induced by infection with pathogenic Mg included cellular edema and release of mucus granules followed by exfoliation of ciliated and non-ciliated epithelial cells. Repair of the damaged tracheal surface was observed at later times (14 - 21 d) after infection in vivo. Observation of TR organ culture indicated the presence of a well-organized epithelial mucosa. The changes in the TR following infection closely paralleled those in vivo (as seen in SEM), indicating the "Natural" progressive pattern of infection in the model system.

The cytopathic effects of Mg on TR organ culture may be summarized as follows:

- 1) Early cytopathic changes are manifested by irregularities in ciliary movement and partial or complete ciliostasis. The time interval until the occurrence of complete ciliostasis is inversely related to inoculum size for a given strain (Table 10).
- 2) Strains differ in their ability to cause ciliostasis. Highly pathogenic strains will cause 100% ciliostasis when as few as 5-10 cfu are added to TR culture. Other strains will cause only negligible ciliostasis at any level of infection, even after long incubation and with extensive multiplication. In strains with intermediate levels of pathogenicity or marked colonization ability both the time of occurrence of ciliostasis and the degree are related to these properties.
- 3) Ciliostasis and cytopathological changes visualized by SEM could be prevented by the addition of an appropriate antibiotic to the culture medium. A strain which showed an increase in the minimal inhibitory concentration to tylosin and which was resistant to tylosin in vivo was also not inhibited by tylosin in TR culture.

Quantitation of mycoplasma pathogenicity by examination of interaction with TR organ culture was expressed as the Mycoplasma Interaction Profile (MIP), as described in Materials and Methods. The results for a series of Mg strains is shown in Table 11. Correlation with in vivo infection and pathogenicity studies (15) showed marked differences between highly virulent and highly attenuated Mg strains. Mg strains which colonize the chicken trachea profusely with only a moderate degree of virulence may exhibit a relatively high degree of cytopathogenicity for TR in culture.

Table 7: Colonization of the chicken trachea (I D₅₀) vs. air sac lesion score in various Mg strains

Strain	A		B		C	
	Lesion score		RSA		I D ₅₀	
R _{LOW}	2.3		100		250	
R ₁₉₈	0		40		1.7x10 ⁶	
F _{LOW}	1.3		100		350	
F ₂₅₄	0.1		100		2200	
F ₃₀₀	0		0		2800	
A ₅₉₆₉	0		0		(D)	
Non-infected	0		0		-	

A) Average air sac lesions in 10 birds necropsied 14 d post-infection by aerosol challenge route.

B) Per cent positive reactors (10 birds) in the RSA test for Mg.

C) I D₅₀ of tracheal infection, determined 3 d post-infection.

D) No mycoplasma isolated.

Table 8: In ovo pathogenicity of M. gallisepticum strains

Strain	LD ₅₀ ^A	
	I	II
F _{LOW}	80 ^B	47
R _{LOW}	870	190
S-6 _{LOW}	560	-

A) Intra yolk sac inoculation of Mg into 6 d embryonated SPAFAS eggs.
Based on embryo mortality 3-11 d post inoculation. I and II are repetitions of LD₅₀ determination.

B) Colony-forming units/egg.

Table 9: Effect of inoculation intra-air sac in 1 d old chicks with Mg strain R, F or in vivo back-passage of strain F.

Inoculum dilution	R		Strain ----F----		F(back)	
	Mortality ^A	Serology ^B	Mortality	Serology	Mortality	Serology
10 ^{0C}	4/10	6/10	4/10	6/6	6/9	3/3
10 ⁻²	2/10	8/8	4/10	5/5	7/10	3/3
10 ⁻⁴	2/9	7/7	1/10	8/9	0/8	8/8
10 ⁻⁶	2/10	8/8	1/9	6/8	0/8	8/8
10 ⁻⁷	0/9	3/9	0/10	1/10	0/9	3/9
In contact	3/10	0/7	0/9	0/9	0/10	0/10

- A) Total mortality during 3 wk of experiment. Death within 24 hr. of injection was considered non-specific.
- B) RSA test of serum of all surviving chickens at 3 wk PI; number of positive reactors/number of birds tested.
- C) Titer of inoculum in 10⁰ dilution was 1.1 x 10⁸ cfu/chick for strain R; 2.4 x 10⁸ cfu/ml (F); 1.6 x 10⁸ cfu/ml (F, back passage)

Table 10: Relationship between time of ciliostasis and inoculum size in chick tracheal rings infected by various Mg strains

Inoculum size (cfu)	<u>Mg strain</u>			
	R _{LOW}	F _{LOW}	F ₂₅₄	S-6 _{HIGH}
5 x 10 ⁵	2*	1	2	2½
5 x 10 ³	3	2	3	2½
500	3	3	3	5
5	4	3	5	6

* Number of days until 100% ciliostasis in TR cultures infected with Mg strain at indicated level
(average of 5 TR/group).

Table 11: Mycoplasma interaction profile of chick tracheal ring organ culture infected by Mycoplasma gallisepticum strains.

STRAIN	TRACHEAL SURFACE	LOCALIZATION OF MYCOPLASMA	CILIOSTASIS	TOTAL
R _{LOW}	3	3	3	9*
R _{HIGH}	1	1	1	3
F _{LOW}	2	2	3	7
F _(254P)	1.5	2.5	2	6
F _(300P)	0	1	0	1
S-6 _{LOW}	2	2	3	7
S-6 _{HIGH}	1	2	2	5
227	3	2.5	3	8.5
A5969	0	1	0	1
NON-INFECTED	0	0	0	0

* A maximum score of 9 indicates the highest level of pathogenicity according to all three criteria (see Materials and Methods).

g. Protective immunity against Mg

As reported previously (15) chickens vaccinated with live pathogenic Mg are protected against aerosol challenge with the homologous strain. Reprint 4 summarizes experiments designed to test cross-immunity between Mg strains. Strains R and F were found to fall within the same general serological category as the species type strains (such as A5969 and S-6) and differ from the so-called variant strains of Mg (such as 730 and 503). This was reflected in the pattern of cross-immunity in protection against aerosol challenge (Reprint 4; Tables 4,5,6,7).

Studies on transferred humoral immunity to Mg in chickens have been summarized (Reprint 14). Partial protection (statistically significant effect on lesion score) was observed after subcutaneous presentation of high titer anti-Mg chicken serum (Reprint 14, Table 3).

Preincubation of Mg R in the presence of Mg^+ AS before infection of the chick trachea results in an increase of about 10-fold in the ID_{50} (see Table 12). There was no significant decrease in cfu/ml during the 2 hr preincubation with antiserum. However, there was a 10^{-3} decrease in cfu at 24 hrs, in vitro.

A similar experiment was carried out in which antiserum (Mg^- or Mg^+) was deposited in the chicken trachea 1 hr before determination of the ID_{50} . The ID_{50} was increased by the presence of antiserum; the effect of Mg^+ AS was markedly greater than Mg^- AS, but the calculation of the ID_{50} was not technically possible. It may be that the effect was so marked that the dilution levels chosen to detect infection were negative on isolation attempts.

The effects of high titer Mg^+ antiserum on inhibition of the cytopathic effects of Mg in TR organ culture system were tested at various serum concentrations and level of infection with Mg. Fig 2 shows an experiment in which the effect of addition of Mg^+ A to TR cultures prior to (or simultaneously with) Mg infection is compared to the effect

of addition of Mg^+AS . Control non-infected cultures contained the same level of Mg^+ or Mg^+AS . Incubation of the diluted mycoplasma culture with Mg^+AS prior to infection of the TR had little or no effect on the cytopathogenicity. The pooled Mg^+AS was positive in the GI test (agar disc method), but there was no significant mycoplasmicidal activity in broth for at least 12 hrs. incubation in vitro. The TR "protected" by Mg^+AS appeared normal in SEM examination and no mycoplasma organisms or colonies were identified on the epithelial surface.

The course of development of cytopathic inhibition activity in chickens infected with pathogenic Mg R was followed at weekly intervals for 7 wks PI. At 5 wks half the birds received a booster with Mg bacterin. Activity was compared to preinfectious serum from the same chickens, and to serum from the non-infected control group 6 wk PI, added at the same level to the TR test system. Figure 3 shows the development with time after infection of a serum factor protecting the TR. The % ciliary activity was scored at 24 hr after infection of the TR, at which time TR infected in the presence of Mg^+AS showed about 9% activity. The cytopathic inhibiting activity increased in the serum with time after infection up to about 5 wks PI, at which time half the group (4 birds) received bacterin booster. In this group the activity appeared to be on the rise, after an initial decrease, when the experiment was terminated.

Table 13 summarizes results with respect to other serological activities in the sera of the individual chickens, before pooling for test in the TR system. All sera were highly positive in the RSA test from the first time of testing, 2 wk PI.

Fig 2: Effect of hyperimmune chicken serum on cytopathic effects of M. gallisepticum in tracheal ring organ culture.

Table 12: ID₅₀ of tracheal infection with Mg R strain, with or without preincubation with chicken serum

Preincubation with	ID ₅₀		
	0 hr ^A	4 hr	3 d
No AS	500	500	400
Mg-AS	NT	1990	400
Mg+AS	1260	5000	2500

A) Time after inoculation of Mg into trachea; 0 or 4 hr (5 chicks/dilution);
3 d (10 chicks/dilution).

E-4. Discussion

The merit of Mg F strain vaccination in prevention of clinical manifestations of mycoplasmosis was repeatedly demonstrated in this work. This can be seen in the University of Georgia field trials, which were carried out on 4 layer premises differing markedly husbandry practices and clinical manifestations of Mg infection. There was a complete elimination of the Mg-associated drop in egg production previously characteristic of the flocks in these companies, and the elimination of the expensive medication regime which had previously been necessary to maintain production.

Likewise in Israel trials carried out on five layer farms, with internal controls of non-vaccinated birds, showed significant protection against egg production drop resulting from infection by field strains of Mg in multi-age production flocks. The clear-cut effects seen in these small scale experiments bear out the observations from much larger experiments conducted in the U. S. A. within the framework of the project, and by others. However, by serological and mycoplasma isolation control throughout the experiment, also on non-vaccinated hens in the same flock and house, it was possible to follow the course of vaccination, immune response and infection.

In addition, it was found in laboratory pen trials that vaccination of layers with F strain protected against egg production drop due to a heavy challenge with virulent Mg; there were also significant lower levels of egg transmission after challenge than in unvaccinated layers.

With respect to Mg respiratory disease in broilers, eye drop vaccination of 1 d or 7 d old chickens with F strain gave statistically significant protection against air sac lesions upon subsequent challenge. However, in these experiments there was no protection against air sac infection; in fact the F strain was reisolated from air sacs after eye drop vaccination, although with no air sac lesions recorded.

The protective capacity of the F strain against respiratory disease in broilers resembled that of fully pathogenic strains (R, S-6) which were also protective when given by the eye drop route, and which appeared relatively innocuous when given by eye-drop (20, 22).

The critical point in evaluation of F strain vaccination lay in determination of the safety of the procedure - the pathogenicity of the vaccine strain in absolute terms or relative to the disease-causing (field) strain. We found a paucity of clear data relating to pathogenicity in the Mg disease system, and to the F strain in particular. Hence we undertook a systematic examination of Mg pathogenicity.

In the first place the degree to which a pathogenic effect is seen is dependent upon the model system used for the test. Natural infection by Mg can undoubtedly occur in many different ways, according to age, route and environmental conditions, and any test system can only approximate the natural situation. We chose three methods by which the pathological effects of Mg on the avian respiratory tract were evaluated; 1) fine aerosol spray presentation, with air sac lesion evaluation 2 wk P 2) quantitative intratracheal inoculation with scanning electron microscope evaluation of tracheal lesion and ID_{50} determination; 3) tracheal ring organ culture. By combining results from all the tests we felt that it is possible to separate the general phenomenon of pathogenicity into the component aspects. By all the criteria in each of the methods the standard Mg strains such as R_{LOW} effected pathogenic changes in the chicken respiratory tract. A highly attenuated strain (such as A596) was completely non-infective and produced no detectable host reaction or pathologic changes.

Other strains studied, including the F vaccine strain, showed intermediate reaction in the various tests. All strains which produced air sac lesions or a

serological response after inoculation were effective colonizers, but not all strains which colonized the respiratory epithelium produced lesions. Thus the F strain is a highly prolific colonizer, but produces only moderate host reaction or clinical effect when introduced directly to the trachea. This is in agreement with the well-based field observations that eye drop administration of this strain to layers has no harmful effects.

On the other hand, the route of administration of Mg appears to have a strong influence on the pathogenic effects; this was found true with respect to the F vaccine strain. Whereas eye drop instillation or tracheal inoculation produces essentially no pathogenic effect, introduction to chickens by aerosol administration indicates significant residual pathogenicity, with marked variability between repetitive trials. By aerosol administration to turkey poults the F strain is fully pathogenic. Likewise in systems in which the normal host defense mechanisms are not present (embryonated eggs, AS inoculation of 1 d chicks, or TR organ culture) the F strain shows a high degree of "pathogenicity", which undoubtedly is connected to its outstanding ability to multiply under these conditions. It may be speculated that under circumstances in which the host defense mechanisms in the upper respiratory tract are drastically impaired the F strain may appear more virulent, which may account for the variability seen between trials with this strain.

Previous studies (8), borne out by the present work, found a general relationship between the virulence of a mycoplasma strain and the ability to protect against subsequent disease. Thus the general state of understanding with respect to Mg (and mycoplasma in general) was that only strains with a fairly high residual degree of virulence could be expected to impart significant infection. In view of the present results in which the protective effect of F strain is evident in systems in which the virulence of the strain is negligible, it appears that the critical element in the interaction is the ability of the strain to colonize. Our results also indicate

that prior infection by Mg F strain can greatly reduce the possibility of super-infection by another Mg strain (Table 6). The degree of protection against super-infection was more evident at 5 wk post vaccination than at 2 wk (Table 6), favoring a possible immunological basis to the phenomenon rather than interference.

During the course of this study other possible candidates for live vaccine strains were examined with respect to infectivity, pathogenicity, and ability to stimulate protection (20). As indicated in section F, a high passage derivative of the F strain (F₂₅₄) retains the ability to colonize but shows essentially no pathogenicity in any system tested. It is suggested that this strain may be of value as alternative or supplement to the F strain in current usage, and further testing is underway.

One of the characteristic properties of the F strain is the low level of horizontal transmissibility. In the field experiments reported in this work, transmission of F strain to non-vaccinated caged layers occurred at 7, 9 or 17 wks PI, the variation probably being due to differences in environmental conditions. Field strains of Mg were apparently transmitted at 2 or 4 wks after exposure of non-vaccinated pullets to infected older layers.

Transmission of the F strain from aerosol or eye drop infected 1 d old chicks or poults raised together in battery brooders occurred only after about 4 wks. There was no transmission of F from one level of the battery to another for the 8 wks PI duration of the experiment. Transmission of strain F₂₅₄ was not detected for 6 wk PI.

One of the problematic aspects of studying Mg disease, and the effects of vaccination is the inability to identify the protective immune response. While there is

a general consensus (12) that the antibody activities measured by the RSA or HI tests are non-protective, and not correlated with ability to withstand challenge, an alternative serological test has not been found. We have suggested in our proposal on the basis of preliminary experiments (15) that the humoral antibody measured by the GI test may be indicative of protection. The time scale of the appearance of GI activity in the relatively insensitive tests we used, and the appearance of resistance to challenge, appeared suggestively similar. However, in view of our current understanding of Mg as a mucosal membrane surface pathogen, it appeared unlikely that the mechanism of specific protection occurs via circulating antibody. A more likely candidate is a local reaction, either secretory antibody or cellular immunity. If the key to protection is secretory antibody at the level of infection, the effect may be mimicked by high levels of serum antibody, as has been suggested elsewhere (23). This may occur naturally as the result of strong antigen stimulation (live or bacterin vaccination with booster) or passive immunization, or may be reproduced experimentally.

Using the model of TR organ culture infected by pathogenic Mg, we showed that chicken serum possessing a high level of activity against Mg (also in the GI test) blocks Mg cytopathogenic effects. This activity develops with time after infection; the effectivity appears to peak at about 5-6 wks after infection, at which time there is usually appearance of GI activity. However, the appearance of the protective effect and GI activity in the serum may be coincidental rather than essential, for the sera used in our experiment showed essentially no GI activity at the time of peak cytopathogenicity blocking activity.

These antibodies may act by blocking an effective interaction between the infecting Mg and the target organ. In our experiment there was only negligible mycoplasmicidal activity, although in vivo (in the presence of complement) this may be

different. Preliminary experiments in vivo showed an apparent effect of the hyper-immune Mg⁺ serum on the ID₅₀ of tracheal infection. In recent experiments with Mg bacterin immunized chickens, Kleven et al (14) showed an increase in the ID₅₀ of tracheal infection in immunized birds. Small changes in ID₅₀ may be significant under conditions of natural infection of birds, especially in birds possessing the normal respiratory clearance activity. The time scale of appearance of the protective effect agrees with previous results concerning the appearance of protection. This may explain certain apparent contradictions in experimental results, our own and those of others. Thus, for example, in Reprint 15, Table 5 protection against air sac challenge was better in birds eye drop vaccinated 5 wks before challenge, than in birds aerosol vaccinated with the same strain, but only 2½ wks prior to challenge. Other examples abound, supporting the observation that the critical time for development of protection is more than 4 wks PI. This necessitates consideration in evaluation of vaccine effectivity results, especially considering treatments at different ages or comparison between separate trials.

Our results suggest that the biological basis of protection conferred by F strain vaccine lies in its unique mode of interaction with the respiratory epithelium - an interaction which is characterized by profuse colonization but only moderate host damage. The introduction of the live vaccine results in long-lasting protection against disease manifestations in layer hens (egg production drop, egg transmission), and at least partial protection against respiratory mycoplasma disease (air sac lesions). Partial protection against superinfection with homologous mycoplasma was also found in F strain vaccinated birds and there was an increase in the ID₅₀ of tracheal infection by antiserum containing antibodies which neutralize the cytopathic effects of Mg. These observations support suggestions that F strain may reduce or block infection by field strains of Mg, especially at low levels of infection. However, since the F vaccine strain retains certain virulence factors and has significant potential for pathogenicity, the necessity for stringent guidelines and professional supervision is strongly reiterated.

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F. DESCRIPTION OF COOPERATION

There was the fullest degree of cooperation and coordination between the participating laboratories. Dr. Kleven's visit to Israel included visits to farms proposed for vaccine trials. The visit, and consultations with the Israeli veterinary authorities were instrumental in obtaining permission for the experimental use of the F strain in layers in Israel. Due to differences in structure, requirements and restrictions of the poultry industry, different experimental design was possible and necessary in the two countries, and the two series of field experiments were complementary.

Personal reasons necessitated that Ms. Levisohn spend an extended period in the U. S. A. in 1982-83, during which time she continued certain aspects of the BARI project originally planned to be carried out in Israel. There was full cooperation support and productive interchange during this period, which provided a very strong basis for coordination in the final stages of the project.

G. BENEFIT TO AGRICULTURE

The immediate contribution of this project was in the clear demonstration of the positive effect of F strain vaccination of layers in reducing egg production losses, medication costs, and morbidity associated with exposure to field strains of Mg. and possible limitations with respect to residual pathogenicity in other poultry sectors. The feasibility of live strain vaccination for protection of broilers against Mg airsacculitis was also investigated. The long term field studies, and the laboratory investigations of the properties of the vaccine strain have been influential in developing guidelines for the continued use of this vaccine in various parts of the U. S. A. and outside.

A further contribution was made by the development of in vivo and in vitro methods for the systematic evaluation of mycoplasma virulence and vaccinal protection. Within the framework of this project the methods were employed in the study of Mg and mycoplasma disease in chickens. However, it is suggested that there is a general applicability to other mycoplasma diseases of veterinary significance, both in methodology and in experimental conclusions.

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