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Evaluation of Efficacy of Formalin Treated *Mycoplasma gallisepticum* Vaccine in Broiler Chickens

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To minimize the local problem of *Mycoplasma gallisepticum* (MG) the potential of two indigenous MG isolates (MI-203 and MI-211) was utilized for vaccine development. Formalin treated vaccines were developed and efficacy was evaluated in female broiler chickens (Cobb). Both the vaccines were found effective in providing protection against MG-S6 challenge that caused 11.5 to 23.3% body weight losses as compared to negative control as well as vaccinated chickens. Besides these inactivated vaccines, the effectiveness of MG-F live vaccine was compared with that of inactivated test vaccines. MG-F live vaccine expressed its seemingly low pathogenic character by exhibiting 5.5 to 9.3% reduced body weight gain as compared to control chickens. The study suggests that vaccines prepared from local isolates (MI-203 and MI-211) are effective in providing protection against challenge with MG-S6 by providing better growth rate and total body weight gain, feed uptake and conversion efficiency, eliminating morbidity and keeping chickens infection free.

Key words: Evaluation, Mycoplasma vaccine, Formalin treatment, Broiler chicken, Bacterin.

Introduction

Mycoplasmosis caused by wall-less bacterium, *Mycoplasma gallisepticum* (MG), is an economically important disease that is worldwide in occurrence. The infection usually affects nearly all the birds in a flock but is variable in severity and appearance. *M. gallisepticum* is one of the major pathogens of poultry and causes infectious sinusitis in turkeys and chronic respiratory disease in chickens (Yoder 1991). The clinical and subclinical infections are manifested by the lower body weight gain and poor feed conversion (Lin and Kleven 1982; Yoder 1991). Recently the association of *M. gallisepticum* with conjunctivitis in chickens (Nunoya *et al* 1995) and farm game birds (Cookson and Shivasprasad 1994) has been documented. There are reports of isolations of *M. gallisepticum* from infected house sparrows and free flying sparrows (Shimizu *et al* 1979; Kleven and Fletcher 1983) and found that house sparrows are capable of acting as biological carriers. Similarly conjunctivitis in house finches was reported (Luttrell *et al* 1996,1998). *M. gallisepticum* has been identified as the etiology of conjunctivitis in wild song birds (Ley *et al* 1996, 1997; Fischer *et al* 1997). Clinical disease had not been associated with MG in wild passerine birds, although the organisms (Stalknecht *et al* 1982) and antibodies against MG have been detected in these birds (Shimizu *et al* 1979).

In poultry despite the antimicrobial treatment or the development of antibody response infection persists. Transmission of *M. gallisepticum* is reported to be by direct contact, by airborne droplets or dust, and vertically through eggs (Yoder 1991). This persistent problem of *M. gallisepticum* has encouraged the development of both live and killed adjuvant vaccines (Yoder 1979; Rodriguez and Kleven 1980b; Carpenter *et al* 1981; Whithear 1983; Hildebrand *et al* 1983; Lin and Kleven 1984). Control measures by the use of MG vaccines have proved successful in preventing the development of respiratory symptoms, air sac lesions and in reducing the vertical transmission of the disease by eggs (Rodriguez and Kleven 1980a; Glisson and Kleven 1984; Talkington and Kleven 1985; Ley *et al* 1997). Live vaccines are used in multiple - age commercial layers to minimize the losses associated with the decreased egg production (Levisohn and Kleven 1981; Carpenter *et al* 1981; Mohammad *et al* 1987; Kleven *et al* 1990). The F strain of *M. gallisepticum* used as live vaccine for chickens is known to be less virulent (Levisohn 1984) and poorly transmissible (Kleven 1981). The F strain is virulent at low levels for chickens, particularly for broilers, establishes carrier state and does not protect adequately against the virulent field strains (Levisohn and Kleven 1981; Glisson *et al* 1984; Kleven *et al* 1984). The F strain has been reported to have caused overt disease in turkeys and therefore may not be suitable as a vaccine in areas with active turkey production (Rodriguez and Kleven 1980a; Levisohn and Kleven 1981). The F strain decreased egg production when used in layer as well as increased condemnation of broilers as a result of air sac infection (Levisohn and Kleven 1981). Due to the risk involved in the use of live F strain vaccine, a temperature sensitive mutant vaccine has been prepared and known to

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have lowered the incidence of air sacculitis against MG challenge (Karaca and Lam 1986; Lam *et al* 1986; Karaca and Lam 1987). In long-term programmes in view of the fact that live vaccine strains may overtake the field strains, the use of inactivated vaccine should be emphasized to control the problem.

The inactivated vaccines (bacterins) are in use at various commercial poultry farms in Pakistan but proved ineffective in providing protection against MG infection by indigenous field strains probably due to antigenic differences between the indigenous strain and the strain used for bacterin preparation. MG is known to alter its antigenic surface components *in vivo*, which may contribute to its ability to persistently infect by adapting to the host environment and evading the host immune response (Levisohn *et al* 1995). Perhaps the same characteristic of the organism is contributing in ineffectiveness of commercial bacterin.

The present study is undertaken in order to determine the potential of indigenous MG isolates for vaccine development that would effectively minimize morbidity and mortality.

Materials and Methods

M.gallisepticum strains. Local isolates of *M. gallisepticum* MI-203 and MI-201 isolated from active cases of chronic respiratory disease (CRD) were selected for the vaccine preparation.

Medium used. Brain Heart Infusion broth containing 10% egg yolk extract (BHIEY) was used for the cultivation of *M. gallisepticum* (Yagihashi *et al* 1986; Nunoya *et al* 1987).

Cultivation of M. gallisepticum. M. gallisepticum isolates were serially adapted 3-4 times in BHIEY broth (Yagihashi *et al* 1986; Nunoys *et al* 1987), inoculum was prepared and 250 ml of BHIEY was inoculated with 10% inoculum and incubated at 37°C for 48 h. After 48 h CFU/ml was determined by the Miles and Misra technique (Miles and Misra 1938).

Formalin treatment. To inactivate Mycoplasma cells formaldehyde (37%, Merck) in 1:2000 concentration was added into the broth culture, which was incubated for another 24 h at 37°C (Warren *et al* 1968). The cells were harvested by centrifugation at 6000 r.p.m for 2 h. Harvested cells were washed 3 times with phosphate buffered saline (PBS, pH 7.5) and finally suspended in 10 ml PBS. Sterility was confirmed by placing one drop of the vaccine suspension onto mycoplasma agar medium and then incubated at 37°C in a moist chamber for 5 days. For further confirmation 0.1 ml of the vaccine suspension was inoculated into 5 ml of mycoplasma broth and incubated at 37°C for 14 days and subsequently plated onto mycoplasma agar after every 48 h interval. After confirming that no viable cells remained, cell concentration of 10^{10} cells/ml (Hayatsu *et al* 1975) was adjusted taking into consideration CFU/ml count of the live culture before addition of formalin is equivalent to the number of inactivated Mycoplasma cells/ml in the same culture suspension after formalin inactivation.

Alum treatment. The adjuvant vaccine was prepared by adding 5 mg ml^{-1} aluminum hydroxide to the vaccine (Yagihashi *et al* 1986) and stored at 4°C until use.

Evaluation of MG vaccine. Two hundred day-old broiler chicks (Cobb) were brought from a local hatchery and divided randomly into 8 groups, 25 chicks in each group. Each group was housed in separate pen with proper thermostatically controlled brooding arrangement for controlled temperature. All the chickens were fed on the same type of feed. Broiler feed was of two types: (I) starter ration and (II) finisher ration. During first four weeks starter ration was given. In the 5th week mixed ration was given and from 5th week onward chicks were given finisher ration up to the 8th week. Feed and water provided *ad libitum* and temperature was recorded daily. Chicken body weight and feed consumption was recorded weekly. Feed conversion efficiency (FCE) was calculated as follow (Mokady *et al* 1979):

$$\text{Feed conversion efficiency} = \frac{\text{Feed consumed/chicken/week}}{\text{Weight gain/chicken/week}}$$

MG vaccination schedule. At the age of 5th day chicks in group 1,2,3 and 4 were vaccinated with 0.5 ml of inactivated adjuvant vaccines (MI-203 and MI-211) subcutaneously midway in the nape of the neck (Hildebrand *et al* 1983) while chickens in group 5 and 6 were vaccinated by inoculating intratracheally 0.1 ml of 24 h grown live MG-F (10^8CFU/ml) culture (Lin and Kleven 1984). Groups 7 and 8 were kept unvaccinated and served as positive and negative controls. Booster dose was given one week after the 1st dose. Weight, feed consumption and post vaccination titre was recorded weekly up to the 8th week (end of experiment).

Preparation of MG challenge inoculum. M. gallisepticum S-6 was grown in fresh serum containing mycoplasma broth at 37°C for 48 h. After 48 h 1 ml culture was inoculated into 10 ml of fresh broth and incubated at 37°C for 48 h. The cells were harvested by centrifugation. 9 ml of the supernatant was discarded and 1 ml left over broth was used to resuspend the cells. 0.5 ml of this cell suspension was then inoculated into the yolk sac of 8 days old embryonated eggs. The eggs were incubated at 37°C for another 5-7 days. After appropriate time, eggs were opened aseptically and *M. gallisepticum* was recovered from the inoculated chick embryo. This was designated as P-1 after first passage.

The procedure for second, third, fourth and fifth passage remained the same and the recovered *M. gallisepticum* was designated as P-2, P-3, P-4 and P-5. For the MG challenge five times passaged (P5) MG-S6 was used. The challenge inoculum was prepared by growing it in the mycoplasma broth. After 48 h of incubation at 37°C CFU/ml of this preparation was adjusted up to 10^{10} CFU/ml.

MG challenge schedule. Two weeks after the booster dose chickens of group 2,4,6 and 7 were challenged by injecting 0.5 ml challenge preparation of MG-S6 intratracheally (Yagihashi *et al* 1986), keeping the group 1,3,5 and 8 unchallenged.

Evaluation of MG Vaccine efficacy. Reisolation of MG-S6 from experimental chickens. Chickens from each group were randomly selected and dissected for reisolation of MG-S6 at different intervals post challenge.

Body weight studies. Each chicken in each group was weighed weekly and average weight gain per chicken per week was calculated in order to compare the weight gain in vaccinated and unvaccinated groups.

Statistical analysis of performance (body weight gain) data. Factorial analysis of variance (FANOVA) and Duncan's multiple range tests were performed in a combination of weekly weight gain and group treatments by using SPSS computer software.

Results and Discussion

Serological studies. There were neither detectable MG antibodies (rapid slide test detectable antibodies) present in the sera of one-week-old chickens prior to vaccination nor were they found in the serum of unvaccinated unchallenged chickens. Two weeks after primary vaccination, chickens vaccinated with formalin treated vaccines and live MG-F vaccine developed anti MG humoral antibodies. The antibody titre continued to rise in these groups. This peak antibody titre achieved at 5th week post vaccination. Humoral antibodies helped the chickens from getting infection upon challenge with the live culture of virulent strain of MG. Successful use of inactivated MG vaccines against respiratory signs and lesions, egg production losses and egg transmission have been reported (Hayatsu *et al* 1974, 1975; Hildebrand *et al* 1983: Kleven *et al* 1984; Yoder *et al* 1984). Adjuvant vaccines are known to elicit a higher level of antibody titre (Karaca and Lam 1987).

During the present investigation a quick antibody response was observed with MG-F vaccine possibly due to use of live cells. This is in accordance with other findings (Abdul-El-Motelib and Kleven 1993; Kleven *et al* 1998).

In unvaccinated unchallenged (negative control) group no antibody titre was detected throughout the experiment (in each replicate) in spite of the presence of challenged/infected chickens in the adjacent pens. It suggests that although these chickens (negative control) might have been exposed to MG aerosols produced by the challenged chickens but exposure was not enough as to elicit any detectable antibody response.

Body weight gain of chickens. There was no difference in the body weight gain of chickens of group 1,2,3,7 and 8 prior to challenge. Two and three weeks following the challenge a significant decline in body weight gain was observed (Table 1) in unvaccinated but challenged group 7 (positive control). This is an indirect indication that the infection was established.

Chickens of group 1,2,3 and 4 (vaccinated with formalin treated MG vaccine) showed normal growth and body weight gain pattern even after challenge. This was the name as that of unvaccinated unchallenged (negative control) group 8 (Table 1). These results indicate that the inactivated vaccines have the ability to protect the chickens from getting infection even after intratracheal challenge. This might correspond to the production of anti MG antibodies that strengthened the immunological status of the chickens and did not allow the virulent MG cells to cause infection in immunologically competent host when challenged with live virulent MG culture. These results are in agreement with the findings of early workers (Hayatsu *et al* 1974, 1975; Hildebrand *et al* 1983; Kleven *et al* 1984, Yoder *et al* 1984).

Chickens of group 5 and 6 (vaccinated with live MG-F) showed lesser body weight gain as compared to group 1,2,3 and 4 (vaccinated with inactivated MG vaccine) and group 8 (negative control chickens) one week after the vaccination. While working with formalin treated vaccine, 5.5-7.5% weight loss was observed in MG-F vaccinated chickens as compared to the chickens in the negative control group (Table 2). Upon challenge with MG-S6, group 6 did not express any further reduction in body weight gain as compared to group 5. This may be due to the fact that MG-F strain colonize trachea when administered intratracheally and block all receptors providing protection against the colonization of MG-S6 strain that is more virulent as compared to MG-F. This has already been reported by other workers (Cummings and Kleven 1986; Abdul-El-Motelib and Kleven 1993). The extent of loss in body weight gain is lower in magnitude than that of positive control group 7 (MG unvaccinated but MG-S6 challenged chickens) highlighting the less pathogenic character of MG-F strain. As shown in Table 2 infection with MG-S6 caused 11.5-23.3% weight loss as compared to negative control

Table 1

Cumulative weight gain (per week) pattern of female broiler chickens observed while evaluating efficacy of formalin treated (using local isolates) MG vaccine

Groups	Weight gain at various age levels (g)							
	1	2	3	4	5	6	7	8
Group-1	$41.29^{a}\pm3.30$*	$127.29^{a}\pm15.19$	$244.00^{b}\pm28.18$	$299.71^{b}\pm38.12$	$412.71^{b}\pm16.82$	$402.71^{b}\pm24.51$	$315.14^{b}\pm48.80$	$203.86^{b}\pm23.25$
Group-2	$41.14^{a}\pm3.43$	$127.43^{a}\pm15.17$	$243.57^{b}\pm28.08$	$299.43^{b}\pm38.56$	$412.00^{b}\pm16.60$	$402.57^{b}\pm24.86$	$315.57^{b}\pm48.56$	$203.86^{b}\pm23.43$
Group-3	$42.00^{a}\pm3.34$	$126.71^{a}\pm15.36$	$243.57^{b}\pm28.35$	$299.43^{b}\pm38.38$	$410.57^{b}\pm16.69$	$401.14^{b}\pm24.66$	$313.14^{b}\pm48.79$	$203.14^{b}\pm23.48$
Group-4	$41.57^{a}\pm3.37$	$127.43^{a}\pm15.37$	$243.43^{b}\pm28.21$	$299.43^{b}\pm37.98$	$411.29^{b}\pm16.38$	$401.57^{b}\pm24.82$	$312.86^{b}\pm49.01$	$203.14^{b}\pm23.11$
Group-5	$41.14^{a}\pm3.41$	$125.71^{a}\pm15.16$	$227.43^{a}\pm28.40$	$282.86^{a}\pm37.73$	$388.43^{ab}\pm15.17$	$377.71^{ab}\pm24.57$	$286.43^{ab}\pm48.71$	$176.14^{ab}\pm24.67$
Group-6	$41.14^{a}\pm3.53$	$126.86^{a}\pm15.08$	$226.86^{a}\pm28.19$	$282.29^{a}\pm37.24$	$386.57^{ab}\pm15.46$	$377.00^{ab}\pm24.42$	$285.00^{ab}\pm48.51$	$174.71^{ab}\pm24.60$
Group-7	$41.14^{a}\pm3.46$	$128.00^{a}\pm14.99$	$243.29^{b}\pm28.10$	$299.14^{b}\pm38.06$	$307.86^{a}\pm10.19$	$322.14^{a}\pm26.67$	$230.43^{a}\pm42.73$	$112.43^{a}\pm18.95$
Group-8	$40.86^{a}\pm3.43$	$127.00^{a}\pm15.28$	$243.14^{b}\pm28.04$	$299.43^{b}\pm37.66$	$409.57^{b}\pm16.34$	$400.00^{b}\pm24.72$	$312.29^{b}\pm49.43$	$202.29^{b}\pm23.43$

* = Mean $\pm$ SEM a,b,c = Mean followed by the same letters in a column are not significantly different (P = 0.05).

Table 2

Body weights of diferent groups of broiler chikens in different experimental trials (formalin treated vaccine)

Groups	Total body weight in different experiments (g)						
	1	2	3	4	5	6	7
Group-1	2040	2070	2030	2070	2042	2022	2079
Group-2	2013	2066	2025	2074	2043	2017	2081
Group-3	2011	2072	2014	2066	2036	2008	2071
Group-4	2013	2069	2007	2069	2040	2015	2072
Group-5	1864	1941	1871	1944	1926	1864	1931
	(7.3)*	(6.0)	(6.8)	(5.8)	(5.5)	(6.6)	(6.2)
Group-6	1860	1931	1869	1944	1919	1857	1923
	(7.5)	(6.5)	(6.9)	(5.8)	(5.8)	(7.0)	(6.6)
Group-7	1655	1827	1540	1610	1717	1694	1748
	(17.7)	(11.5)	(23.3)	(21.9)	(15.8)	(15.1)	(15.1)
Group-8	2010	2066	2008	2064	2038	1997	2059

* = Figures in parentheses are percent weight loss (in grams) as compared to untreated negative control chickens.

Table 3

Feed conversion efficiency rate* in female broiler chickens exposed to formalin treated MG vaccine

Groups	Feed conversion rate in week							
	1	2	3	4	5	6	7	8
Group-1	2.52	3.48	2.32	3.52	2.72	3.00	3.82	2.40
Group-2	2.48	3.52	2.31	3.50	2.71	3.01	3.80	2.38
Group-3	2.50	3.51	2.29	3.49	2.68	2.98	3.78	2.35
Group-4	2.45	3.47	2.28	3.48	2.68	2.96	3.77	2.34
Group-5	2.52	3.33	1.90	3.00	2.20	2.68	3.30	2.00
Group-6	2.56	3.34	1.91	3.02	2.19	2.66	3.28	2.10
Group-7	2.60	3.49	2.28	3.47	1.60	2.12	2.30	1.52
Group-8	2.52	3.46	2.29	3.48	2.66	2.90	3.75	2.30

Results are based on the average values of seven replicate experiments.

Table 4

Reisolation of MG from vaccinated (formalin treated vaccine) and unvaccinated chickens upon intratracheal challenge by MG-S6 at various intervals post challenge

Groups	Isolation from trachea after PC						Isolation from lung after PC					
	6h	24h	48h	1 week	2 week	3 week	6h	24h	48h	1 week	2 week	3 week
Group-1	-	-	-	-	-	-	-	-	-	-	-	-
Group-2	+	+	-	-	-	-	+	-	-	-	-	-
Group-3	-	-	-	-	-	-		-	-	-	-	-
Group-4	+	+	-	-	-	-	+	-	-	-	-	-
Group-5	+	+	+	+	+	+	+	+	+	+	+	+
Group-6	+	+	+	+	+	+	+	+	+	+	+	+
Group-7	+	+	+	+	+	+	+	+	+	+	+	+
Group-8	-	-	-	-	-	-	-	-	-	-	-	-

+ = MG was isolated; - = MG was not isolated; PC = Post challenge; G-1 = Vaccinted with MI-203 formalin treated vaccine; G-2 = Vaccinated with MI-203 formaline treated vaccine and challnged with MG-S6; G-3 = Vaccinated with MI-211 formalin treated vaccine; G-4 = Vaccinated with MI-211 formalin treated vaccine and challenged with MG-S6; G-5 = Vaccianted with live MG-F vaccine; G-6 = Vaccinated with live MG-F vaccine and challenged with MG-S6; G-7 = Unvaccinated challenged group (Positive control); G-8 = Unvaccintaed unchallenged group (Negative control).

chickens. These results show that although MG-F strain is used as a vaccine strain but still it appears to be pathogenic for the young broiler chickens. Inoculation of live cultures of *M. gallisepticum* into nasal sinus and/or trachea has been reported to cause signs of respiratory disease in chickens (Grimes and Rosenfeld 1972, Hildebrand *et al* 1983) and reduced body weight gain in broilers (Naeem *et al* 1980). In a similar laboratory based study Rodriguez and Kleven (1980a) compared and reported the milder pathogenic character of MG-F strain than the R strain in uncomplicated cases.

As seen in the present study that vaccination with F strain blocks the colonization by other pathogenic strains of MG and is known to displace the field strain upon continuous use on multiple age poultry production sites (Levisohn and Kleven 1981; Kleven *et al* 1990). In another study MG-F was found effective against air sacculitis by a Taiwan local strain isolate and MG-R as the co-challenge (Lin 1986). Moreover, our results are similar to another previous study with layer flocks (Branton and Deaton 1985).

From the available literature it appears that MG-F live cells in the form of vaccine have been used especially in broilers for protection but not without risk. MG-F strain has been reported to enhance condemnation rate in broilers due to air sac infection. Although better antibody response is achieved with MG-F vaccination but the possibility of risk of air sacculitis cannot be ignored. During the present study the use of MI-203 and MI-211 as vaccine strains (formalin treated) has not been found to cause condemnation of chickens due to infection.

Feed conversion efficiency (FCE). Average feed consumption per chicken per week as calculated for efficacy evaluation of formalin treated vaccine was same in all the groups irrespective of the loss in body weight gain in positive control group. Feed conversion efficiency (FCE) was calculated at the end of each week (Mokady *et al* 1979) and is illustrated in Table 3. Poor feed conversion efficiency was observed in group 7 (positive control) after 4 weeks of age due to the MG infection. In group 5 and 6 relatively reduced feed conversion was observed one week after intratracheal vaccination with live MG-F strain. The lesser reduction in feed conversion efficiency in group 5 and 6 as compared to group 7 corresponds to the less pathogenic character of MG-F strain.

Reisolation and identification of MG from experimental chickens. Prior to challenge isolations were positive from group 5 and 6 only. These 2 groups were vaccinated using live MG-F strain. MG could not be isolated from the rest of the groups, because either they were vaccinated using inactivated vaccines (group 1,2,3 and 4) or kept unvaccinated (group 7 and 8). It is evident from Table 4 that MG was isolated from the trachea of the vaccinated chickens of group 2 and 4 at 6 and 24 h post challenge. MG was not recovered from these groups at 48 h post challenge indicating that anti MG antibodies produced in response to MG vaccine was strong enough to wash out MG within 48 h. In group 5 (MG-F vaccinated) and 6 (MG-F vaccinated and MG-S6 challenged) MG-F continued to be isolated till the end of experiment. In unvaccinated but challenged group (positive control) MG was continuously isolated from 6 h PC to 3 weeks PC (i.e. end of the experiment). All isolated cultures were identified by immunofluorescent staining and by the rapid slide agglutination method.

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