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THE EFFECT OF MYCOPLASMA *GALLISEPTICUM TS11* VACCINATION ON EGG PRODUCTION

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Abstract

Chronic respiratory disease caused by *Mycoplasma gallisepticum* (MG) is known to cause economic losses among poultry industries. One of the economic losses is due to egg production drops. Prevention using MG vaccine is reported to have good protection against MG infection and egg production drops. A vaccination trial to determine the effects of TS-11 vaccine, a mutant strain of MG, against MG infection and egg production drops was carried out on 13 week-old of pullets. Three groups of eight pullets were vaccinated with TS-11, MG80083M and MG80083L, respectively. The other 6 groups of pullets were not vaccinated. Each chicken was kept in a single wire cage. Fifteen weeks after vaccination the chickens were challenged. The first three groups were challenged with a virulent Ap3AS strain of MG, and groups 4, 5, 6, 7 and 8 (non vaccinated groups) were challenged with Ap3AS, MG80083M, MG80083L, TS-11 and broth medium via intra-abdominal air sacs respectively, whereas the last group of chickens (group 9) was not challenged kept as controls. The egg production was recorded 7 weeks before and after challenge starting from 22 weeks of age. The results showed that the egg production of the vaccinated hens were not affected by the challenge and was not statistically different from the non-vaccinated non-challenged control chickens (group 9). The egg production of the non-vaccinated hens challenged with Ap3AS (group 4), MG80083M (group 5), MG80083L (group 6) was declined dramatically and was significantly lower ($P<0.01$) than that of the non-vaccinated hens challenged with TS-11 (group 7). The egg production of hens in group 7 was not affected by TS-11 challenge and was not statistically different from the non vaccinated hens injected with broth medium (group 8) or the control chickens (group 9).

Introduction

Chronic respiratory disease (CRD) is a costliest disease confronting poultry industries in the world. This disease has been reported worldwide spread (FAO-WHO-OIE, 1992). The economic losses is mainly due to downgrading of carcasses, loss of body weight gain, poor feed conversion, increased cost of medication and mortality in broilers, whereas in layers decrease egg production, high cost of treatment and mortality. In the case of CRD alone the mortality rate is low but with secondary infection the mortality rate is high (Yoder, Jr., 1991). Economic loss due

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to CRD infection of chicken in the USA and Australia was reported to be million dollars (Biggs, 1982; Bagust, 1989), and in Indonesia it was reported about 30.5 million dollars loss (Soeripto, 2001).

Prevention of the disease can be done by vaccination. Vaccination using MG bacteria has been reported by Hilderbrand (1985) but the result is still variation. Vaccination using MG mutant strain has been reported in the USA (Lam *et al*, 1983, 1984). The mutant strain of TS37 was immunogenic but was still pathogenic whereas TS102 was not pathogenic and gave 70% protection only. The latest strain was found to be pathogenic in turkeys.

Mutant MG strain of TS-11 was selected in the laboratory and reported to be non pathogenic (Soeripto and Whithear, 1996a) and gave immune response against MG challenge (Soeripto and Whithear, 1996b) in young chickens.

The aim of this study is to determine the efficacy of mutant MG strain of TS-11 against MG infection and MG-associated egg production drops in layer chickens

Materials and Methods

Mycoplasma media

Mycoplasma broth (MB) and agar (MA) were used for purification and production of all MG cultures. The medium was made base on the formulation of Frey *et al* (1968). The broth medium containing Mycoplasma broth base (Gibco), cystein HCl, phenol red, thallous acetate and double glass distilled water. The pH of the medium was adjusted to 7.6 prior to autoclaving. When cooled to room temperature a sterile enrichment consisting of 10% swine serum, 1% fresh yeast autolysate, 0.002% DNA, 0.1% glucose and 0.02% ampicillin was added aseptically into the medium. For the MA, 0.01% actidione was added and 1% of Special Noble agar (Difco) was used to solidify the agar. Phenol red was omitted in the MA.

MG strains

Strains Ap3AS and 80083 of MG were obtained from Dr. K. Whithear, Australia. MG strain of Ap3AS was originally isolated from the air sacs of a broiler chicken from a flock which showed severe respiratory disease. This isolate was clone purified 5 times and designated as Ap3AS strain. MG strain of 80083 was isolated from the trachea of a broiler breeder hen. This strain was clone purified 5 and 50 times and designated as MG80083L and MG80083M, respectively. Strain MGTS-11 was a mutant, originally from MG80083L.

Mutant strain

The production of temperature sensitive (TS) mutant strains following exposure of MG80083L cultures to the mutagen, N-methyl-N-nitro-N-nitrosoguanidine

(NTG) has been reported by Steinberg *et al* (1969) for *M. pneumoniae*, Nonomura and Imada (1982) for *Mycoplasma synoviae* and Lam *et al* (1983) for MG. The MG80083L cultures were exposed to NTG for 30 minutes and incubated at a permissive temperature (33°C) and restrictive temperature (39.5°C). Selections of some mutant strains were carried out in the laboratory. The virulence and immune response of selected mutant MG strain of TS-11 were carried out in young chickens (Soeripto and Whithear, 1996a,b).

Chickens

Commercial MG free Austrolorp and White Leghorn obtained from K&L Thomas Hatchery, Victoria, Australia were used for the experiment. Each chicken was kept in a separate wire battery cages in the same house. When they were 13 weeks old, 3 groups of 8 chickens (groups 1, 2 and 3) were vaccinated with MG80083L, MG80083M and MGTS-11 via eye drops, respectively. The other 6 groups of 8 chickens were left unvaccinated as controls. At 28 weeks of age, hens in groups 1, 2, 3 and 4 were challenged intra-abdominally with 0.2 ml of MG strain of Ap3AS containing 2.4 x 10^8 CCU/ml. Hens in groups 5, 6 and 7 were challenged intra-abdominally with 0.2 ml of MG strains of 80083M, 80083L and MGTS11 containing 1.4 x 10^9, 1.4 x 10^8, 1.1 x 10^8 CCU/ml respectively. Hens of group 8 were challenged intra-abdominally with 0.2 ml of MB, whereas hens of group 9 were left unchallenged used as controls.

Vaccination procedure

The chickens were vaccinated by dropping of 50 µl of MG cultures in the left conjunctiva sac.

Challenge procedure

The hens were challenged with 0.2 ml of the appropriate thawed MG stock cultures into the left abdominal air sacs between the posterior sternum and the os pubis. A 1 ml disposable syringe with a 21 gauge 38 mm needle was used for injection. Control hens were injected in the same manner with 0.2 ml of sterile MB. The method was similar to that described by Adler *et al* (1960).

Calculation of viable MG organisms for vaccination or challenge

Stocks of MG cultures were thawed and 0.5 ml of the cultures was serially diluted in 10-fold steps from 10^{-1} to 10^{-9} in sterile MB supplemented with swine serum. An amount of 25 µl of each dilution was inoculated into 8 replicate wells of a 96-well flat-bottom micro tray containing 0.2 ml of sterile MB, covered with plastic and then incubated at 37°C for 14 days. The wells showing an acid colour shift in the phenol red indicator were recorded as indicating mycoplasma growth and the most probable number of colour changing units (CCU)/ 25 µl was determined using the tables published by Meynell and Meynell (1975).

Isolation and identification of MG

Swabs from air sacs, turbinates and palatine cleft mucosae and the mucosa of the first 2 cm of the trachea were inoculated onto MA and incubated in a moist atmosphere at 37°C for up to 14 days. The same swab was placed into MB and incubated at 37°C for up to 14 days. Colonies occurring on primary MA plates were identified *in situ* using indirect immunofluorescent (Rosendal and Black, 1972). When the pH indicator in MB turned orange yellow the cultures were frozen at – 20°C and held until required. On some occasions growth was observed in MB but not on primary agar plates. When this occurred the MB cultures were thawed, inoculated onto MA and identified by the Growth inhibition test (WHO, 1976).

Serological procedures

Blood samples were collected 15 weeks after vaccination and 8 weeks after challenged. The sera were tested using Rapid serum agglutination (RSA) test and enzyme linked immunosorbent assay (ELISA). For RSA, an amount of 25 µl of tested serum was added to 25 µl of stained antigen in a WHO tray. Mixed thoroughly by tapping the plate. The plate was then rotated for 2 minutes on a rotary agglutinator. The agglutination reaction was scored from 0 to 4+ on the basis of antigen clump size. The ELISA was performed using the method described by Higgins and Whithear (1986). MG membrane antigen of S6 was used for coating the flat bottom tray and incubated at 4°C overnight. Test sera were placed in the coated plate and diluted in 2-fold steps from 1:10 to 1:1280 using a multi channel dispenser. Following washing, an appropriate dilution of horseradish peroxidase anti chicken IgG conjugate was added to each well except those in column 1. Freshly prepared substrate was then added to each well after a washing step. The absorbance was read at 450 nm on a Titertek Multiscan. Geometric mean titre (GMT) was calculated using the method described by Lutz (1973).

Gross pathology of air sac membranes

Air sac lesion scoring was adapted from the methods described by Adler *et al* (1960) and Kleven *et al* (1972). Score 0: normal; 1+: slight cloudiness of the air sac membranes with the presence of frothy materials; 2+: the air sac membranes slightly thickened with small accumulations of caseous exudates confine to one side of the air sac membranes; 3+: air sac membranes somewhat thickened with a large accumulation of caseous exudates extending from one side to the other; 4+: the air sac membranes were thickened and diffuse accumulation of caseous exudates occurred on both sides of the air sac membranes.

Statistical analysis

One-way classification analysis of variance was used to test the egg production per week for 7 weeks before and after challenge. Duncan's new multiple range test was used to compare the differences between treatment means.

Results

Egg production of hens of vaccinated and non-vaccinated groups over 7 weeks period before and after intra abdominal challenge with MG strain of Ap3AS is presented in Table 1. The egg production of hens of each vaccinated group was not statistically different from that of hens of the non-vaccinated groups prior to challenge. After challenge with MG strain of Ap3AS, the production of hens in groups 1, 2 and 3 were significantly higher ($P<0.01$) than that of non-vaccinated hens (group 4). The production of the vaccinated groups seemed to be unaffected by the challenge and were not statistically different from the production of the non-vaccinated non-challenged control hens (group 9).

Egg production of non-vaccinated hens over 7 weeks period before and after intra abdominal challenge with MG strain of Ap3AS, 80083M, 80083L or TS-11 is presented in Table 2. The egg productions of the non-vaccinated hens challenged with MG strain of Ap3AS (group 4), 80083M (group 5) or 80083L (group 6) were declined dramatically and were significantly lower ($P<0.01$) than that of the non-vaccinated hens challenged with TS-11 (group 7). The egg production of hens of group 7 was not affected by TS-11 challenge and was not statistically different from the hens injected with sterile broth (group 8) or the control hens (group 9).

Serological response over the 15 weeks after vaccination is presented in Table 3, and serological response for the 8 weeks after challenge is shown in Table 4. No MG antibodies were detected in any non-vaccinated hens over the 15 weeks observation period prior to challenge. Chickens vaccinated with TS-11, 80083M or 80083L had low mean MG ELISA antibody unit (AbU) levels over the 15 weeks period after vaccination. About 3, 1 and 3 hens vaccinated with TS-11, 80083M and 80083L respectively gave agglutination reactions, and only 1, 0 and 1 respectively had MG spesific IgG >3 AbU at 2 weeks after vaccination. MG antibody levels in the chickens vaccinated with TS-11 increased slightly up to 4 weeks and then declined. No MG antibodies could be detected in hens of group 1 at 9 weeks post vaccination. MG antibody levels in the chickens vaccinated with TS-11 increased again up to 15 weeks post vaccination. MG ELISA antibody levels in the chickens vaccinated with 80083M and 80083L showed gradual increase up to 15 weeks post vaccination. MG ELISA antibody levels in the vaccinated chickens continued to increase after challenge while those of non-vaccinated groups showed similar pattern, and continued to increase up to 8 weeks post challenge.

The results of air sacs examination and MG isolation from the vaccinated and non-vaccinated hens after challenge are shown in Table 5. No air sac lesions were found 8 weeks after challenge in hens vaccinated either with TS-11 or 80083L. MG was isolated from upper respiratory tract (URT) of all hens but no isolates were obtained from the air sacs. Chickens vaccinated with 80083M and challenged with Ap3AS had a mean air sac lesion score of 1.1. Four out of 8 chickens had air sac lesions ranging from 1+ to 4+ in severity. MG was isolated from the air sacs of 2 hens that had air sac lesions, while from the URT MG was isolated from each chicken.

Non-vaccinated hens showed air sac lesions 8 weeks after challenge with Ap3AS, 80083M or 80083L. Five out of 8 hens from each group had air sac lesions from 2+ to 4+ and MG was isolated from the air sacs of 4 hens from each group. MG was isolated from the URT of 6 chickens challenged either with 80083M or Ap3AS, and from 4 hens challenged with 80083L.

Neither air sac lesions were observed nor MG was isolated from the air sacs of the non-vaccinated hens challenged with TS-11. MG was isolated from the URT of 5 hens.

Discussion and Conclusion

The aim of this study was to develop a save and effective mutant MG vaccine which can reduce MG infection and MG-associated egg production drops in layers. MGTS-11 was evaluated and selected as a possible vaccine. In the previous experiments (Soeripto and Whithear, 1996a,b) were demonstrated that TS-11 did not induce air sac lesions and gave good protection against MG challenge in young chicks..

In this trial, it was shown that there was no statistically difference between the production of vaccinated and non-vaccinated hens before challenge. After challenge, there was no statistically difference between the production of groups of hens vaccinated with TS-11, 80083M or 80083L, and the group of non-vaccinated non-challenge hens. However, the egg production of non-vaccinated group of chickens and challenged with Ap3AS (group 4) showed a rapid fall and was significantly lower ($P<0.01$) than that of vaccinated or control chickens. This indicated that TS-11 induced protection against a drop in egg production as high as that induced by the parent strain.

The egg production of non-vaccinated chickens was not affected after intra-abdominal challenge with TS-11 (group 7), whereas the production of the other non-vaccinated chickens challenged with MG strains of Ap3AS (group 4), 88083M (group 5) or 80083L (group 6), declined dramatically and was significantly lower ($P<0.01$) than the production of the chickens challenged with TS-11. This trial

showed further demonstration that TS-11 is a non-virulent and highly immunogenic MG strain which shows great potential as a vaccine.

Serological response of chickens vaccinated with TS-11 starting at 2 weeks after vaccination and then declined at 9 weeks. Why this antibody response declined is undetermined. The antibody response increase again up to 15 weeks after vaccination. Other chickens vaccinated with 80083M or 80083L gave RSA reaction at 4 weeks after vaccination and maintained up to the time of challenge at 15 weeks after vaccination. The antibody levels measured by ELISA showed that hens vaccinated with TS-11 had antibody units substantially lower than those elicited by hens vaccinated with 80083M or 80083L. However, despite this, hens vaccinated with TS-11 showed complete protection against MG challenge and a drop in egg production. This finding similar to those reported by Lam *et al* (1983, 1984) that mutant MG strain of TS37 and TS102 gave good protection against MG challenge but strain TS37 is pathogenic in chickens and strain TS102 pathogenic in turkeys.

Chickens vaccinated with TS-11 and challenged with Ap3AS strain, showed no air sac lesions and no MG organisms were recovered from the air sacs but were re-isolated from the upper respiratory tract. This, provide evidence that TS-11 elicited good protection against MG infection. Non-vaccinated chickens challenged with TS-11 had no air sac lesions. This indicated that mutant strain of TS-11 is not pathogenic strain, similar to that previous experiment (Soeripto and Whithear, 1996a). MG organisms were not able to be isolated from the air sacs but were re-isolated from the upper respiratory tract. This finding is in line with the finding reported by Nonomura and Imada (1982) that mutant strain of *Mycoplasma synoviae* was recovered only from the nostril, nasal sinus and upper part of the trachea.

In a conclusion, this experiment showed that the mutant MG strain of TS-11 had demonstrated good protection against air sac lesions and a drop in egg production. This mutant MG strain of TS-11 is strongly suggested used as a vaccine against chronic respiratory disease in layer chickens.

Table 1. Comparison of egg production between vaccinated and non-vaccinated hens over the 7-week periods before and after IA challenge with MG strain of Ap3AS

Groups	Vaccine strain	Challenge strain	Mean 7 week egg production	
			Pre-challenge 22-28 weeks	Post-challenge 29-35 weeks
1	TS-11	Ap3AS	38.4 [a]	41.3 [a]
2	80083M	Ap3AS	37.4 [a]	38.3 [a]
3	80083L	Ap3AS	37.4 [a]	38.9 [a]
4	NV	Ap3AS	34.6 [a]	25.8 [b]
9	NV	NC	39.9 [a]	39.4 [a]

Dissimilar superscripts between treatment means denote significant difference (P<0.01: a, b)
NV: Non vaccinated
NC: Non challenged

Three groups of eight 13-week-old MG free WL x AO hens were vaccinated by eye drops with 0.05 ml of cultures of TS-11, 80083M or 80083L containing 3.4 x 10^8, 2.0 x 10^8 and 5.5 x 10^8 CCU/ml. Another 6 groups of 8 were not vaccinated. At 28 week of age, all chickens in groups 1, 2, 3 and 4 were challenged intra abdominally with 0.2 ml of Ap3AS containing 2.4 x 10^8 CCU/ml. Hens in group 9 were not vaccinated and non-challenged. Eggs were collected and recorded daily for the 7 weeks before and after challenge.

Table 2. Comparison of egg production in non-vaccinated hens over the 7-week periods before and after IA challenge with MG strains of Ap3AS, 80083M, 80083L or TS11

Groups	Vaccine strain	Challenge strain	Mean 7 week egg production	
			Pre-challenge 22-28 weeks	Post-challenge 29-35 weeks
4	NV	Ap3AS	34.6^{a}	25.8^{bcy}
5	NV	80083M	35.8^{a}	23.4^{cy}
6	NV	80083L	37.9^{a}	23.6^{cy}
7	NV	TS-11	36.4^{a}	42.1^{ax}
8	NV	MB	34.3^{a}	39.4^{abx}
9	NV	NC	39.9^{a}	40.1^{abx}

Differing superscripts between treatment means denote significant difference (P< 0.01: a,b,c; P<0.05: x,y).
NV: Non vaccinated; NC: Non-challenged
MB: Mycoplasma broth

Non-vaccinated 28 week old MG free WL x AO hens in groups 4, 5, 6 and 7 were challenged intra abdominally with 0.2 ml of Ap3AS, 80083M, 80083L or TS-11 containing 2.4 x 10^8, 1.4 x 10^9, 1.4 x 10^8, 1.1 x 10^8 CCU/ml respectively. Hens in group 8 were challenged with 0.2 ml of sterile MB and the 9th group was not challenged. Eggs were collected and recorded daily for the 7 weeks before and after challenge.

Table 3. Serological response over a 15 week period after vaccination of 13 week-old pullets with MG strains TS-11, 80083M or 80083L

MG strains	Serology - Weeks after vaccination									
	2		4		9		13		15	
	RSA	ELISA AbU	RSA	ELISA AbU	RSA	ELISA AbU	RSA	ELISA AbU	RSA	ELISA AbU
TS-11	3	1.1	8	1.6	0	0.0	2	1.4	3	2.7
80083M	1	0.0	5	1.4	8	5.2	8	7.5	8	8.7
80083L	3	1.1	8	3.5	8	9.1	8	11.4	8	11.3

AbU: Antibody units.

Table 4. Serological response of vaccinated and non vaccinated hens over an 8 week period after challenge.

Groups	MG vaccine strain	Challenge strain	Serology - Weeks after challenge							
			2		4		6		8	
			RSA	ELISA AbU	RSA	ELISA AbU	RSA	ELISA AbU	RSA	ELISA AbU
1	TS-11	Ap3AS	5	4.7	7	4.3	7	5.9	6	10.6
2	80083M	Ap3AS	8	15.3	8	22.0	8	19.5	7	12.9
3	80083L	Ap3AS	8	14.2	8	14.1	8	15.1	8	14.0
4	NV	Ap3AS	5	4.0	5	6.0	5	5.4	6	8.9
5	NV	80083M	7	6.8	5	9.2	5	9.2	5	24.1
6	NV	80083L	4	4.0	4	6.3	4	6.3	4	12.8
7	NV	TS-11	1	1.2	6	2.1	6	2.1	6	8.1
8	NV	MB	0	0	0	0	0	0	0	0
9	NV	NC	0	0	0	0	0	0	0	0

NV: Non vaccinated; NC: Non-challenged
MB: Mycoplasma broth; AbU: antibody units

Table 5. Air sacs examination and MG isolation from URT of vaccinated and non-vaccinated chickens after IA challenge

Groups	Vaccine strain	Challenge strain	Air sacs examination			MG isolation from URT
			Lesions	Mean score	MG isolation	
1	TS-11	Ap3AS	0	0.0	0	8
2	80083M	Ap3AS	4	1.1	2	10
3	80083L	Ap3AS	0	0.0	0	13
4	NV	Ap3AS	5	2.0	4	10
5	NV	80083M	5	2.2	4	8
6	NV	80083L	5	1.8	4	88
7	NV	TS-11	0	0.0	0	6
8	NV	MB	0	0.0	0	0
9	NV	NC	0	0.0	0	0

NV: Non vaccinated ; NC: Non challenged
MB: Mycoplasma broth; URT: upper respiratory tract

Three groups of eight 13-week-old MG free WL x AO hens were vaccinated by eye drops with 0.05 ml of cultures of TS-11, 80083M or 80083L containing 3.4 x 10^8, 2.0 x 10^8 and 5.5 x 10^8 CCU/ml. Another 6 groups of 8 were not vaccinated. At 28 week of age, all chickens in groups 1, 2, 3 and 4 were challenged intra abdominally with 0.2 ml of Ap3AS containing 2.4 x 10^8 CCU/ml, whereas hens in groups 5, 6 and 7 were challenged intra abdominally with 0.2 ml of 80083M, 80083L or TS-11 containing 2.4 x 10^8, 1.4 x 10^9, 1.4 x 10^8, 1.1 x 10^8 CCU/ml respectively. Hens in group 8 were challenged with 0.2 ml of sterile MB and the 9th group was not challenged. All hens were bled and killed 8 weeks after challenge.

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