PROSIDING

TEMU ILMIAH NASIONAL BIDANG VETERINER

Bogor, 12-13 Maret 1996

Penyunting:

Sjamsul Bahri
Sutijono Partoutomo
Darminto
Fachrian Pasaribu
Yulvian Sani

Redaksi Pelaksana:

Iman Salihin
Gerhat

KERJASAMA ANTARA:
BALAI PENELITIAN VETERINER
BADAN PENELITIAN DAN PENGEMBANGAN PERTANIAN
DENGAN
PERHIMPUNAN DOKTER HEWAN INDONESIA

THE VIRULENCE OF 4 TS-MUTANTS AND 80083L OF *MYCOPLASMA GALLISEPTICUM* STRAINS IN 2 WEEK-OLD CHICKENS

SOERIPTO* and K.G. WITHEAR**

** Balai Penelitian Veteriner*
Jalan R.E. Martadinata 30, Kotak Pos 52, Bogor 16114, Indonesia
*** Veterinary School, Melbourne University, Australia*

ABSTRACT

The aim of this study is to determine the virulence of four ts-mutants of *Mycoplasma gallisepticum* strains in 2 week-old chickens. Groups of five 2 week-old SPF CSIRO Hybrid White Leghorn chickens were inoculated intraabdominally either with 0.2 ml of MG cultures of 80083 ts-3, 80083 ts-11, 80083 ts-15, 80083 ts-21 or 80083L strains. The sixth group was inoculated with mycoplasma broth and used as controls. All the chickens were killed 2 weeks after inoculation and examined for air sac lesions and antibody responses. The results showed that chickens inoculated with ts-mutants or 80083L had air sac lesions except with ts-11 mutant. The control and inoculated chickens with 80083 ts-3 or 80083 ts-11 had no antibody responses, whereas chickens inoculated with 80083 ts-15, 80083 ts-21 or 80083L had antibody responses. It is concluded that ts-11 mutant is not virulence in 2 week-old chickens.

Key Words: ***Mycoplasma gallisepticum*, virulence, ts-mutant**

VIRULENSI 4 TS-MUTANT DAN 80083L GALUR *MYCOPLASMA GALLISEPTICUM* PADA ANAK AYAM UMUR 2 MINGGU.

ABSTRAK

Penelitian ini bertujuan untuk mengetahui keganasan empat ts-mutant galur *Mycoplasma gallisepticum* (MG) pada ayam umur 2 minggu. Sebanyak 30 ekor ayam SPF CSIRO Hybrid White Leghorn umur 2 minggu dibagi atas 6 kelompok a 5 ekor. Kelompok 1, 2, 3, 4, dan 5 masing-masing diinokulasi dengan 0,2 ml kultur 80083 ts-3, 80083 ts-11, 80083 ts-15, 80083 ts-21 dan 80083L melalui intraabdominal. Kelompok 6 diinokulasi dengan mycoplasma broth yang dipergunakan untuk kontrol percobaan. Dua minggu setelah inokulasi semua ayam dibunuh dan diperiksa perubahan patologik pada kantung udara dan respon antibodi terhadap MG. Hasilnya memperlihatkan bahwa peradangan pada kantung udara rongga perut terlihat pada ayam yang diinokulasi dengan ts-mutant galur MG dan induknya 80083L kecuali dengan ts-11 mutant. Ayam kontrol dan ayam yang diinokulasi dengan 80083 ts-3 dan 80083 ts-11 tidak memperlihatkan respon antibodi, sedang ayam yang diinokulasi dengan 80083 ts-15, 80083 ts-21 dan 80083L masing-masing memperlihatkan respon antibodi. Kesimpulan dari percobaan ini menunjukkan bahwa ts-11 mutant galur MG tidak ganas pada ayam umur 2 minggu.

Kata kunci: ***Mycoplasma gallisepticum*, virulensi, ts-mutant**

INTRODUCTION

Mycoplasma gallisepticum (MG) is one of the pathogenic species of avian mycoplasmas (JORDAN, 1979; YODER, 1991). It causes predominantly respiratory tract infection and occasionally arthritis and reproductive tract infection (JORDAN, 1979).

A variety of methods have been used to quantify the virulence of MG strains, such as by eye drops (AMIN and JORDAN, 1979; LIN and KLEVEN, 1982), nasal sinus (ADLER *et al*, 1962; ROBERTS, 1964; TIMMS and CULLEN, 1972; NAEEM *et al*, 1980), trachea (FABRICANT and LEVINE, 1962; ROBERTS, 1964; GRIMES and ROSENFELD, 1972; LEVISOHN *et al*, 1986) and aerosol (DUNLOP *et al*, 1961; ANDERSON and BEARD, 1967; RODRIGUES and KLEVEN, 1980; LIN and KLEVEN, 1982; LEVISOHN *et al*, 1986). However, MG disease in the chicken is both subtle and compleks, so measuring and comparing the virulence of MG strains is not straightforward. Pure cultures of the organisms given by natural routes of exposure rarely produce clinical or pathological signs of disease (DUNLOP *et al*, 1961; ANDERSON and BEARD, 1967).

The aim of this study is to determine the virulence of four ts-mutants of *M. gallisepticum* strains which were exposed to a mutagenic chemical N-methyl-N-nitro-N-nitroso guanidine (NTG) in 2 week-old chickens.

MATERIALS AND METHODS

MG strains

Strains 80083 and Ap3AS of MG were received from Dr.K. WHITHEAR, School of

Veterinary Science, University of Melbourne. Strain 80083 was erythromycin sensitive and was originally isolated from the trachea of a broiler breeder hen. In the field this strain appeared to be of low virulence and spread slowly to other chickens in the flock. This strain was clone purified 5 times and then designated as strain 80083L. Strain Ap3AS was resistant to erythromycin and was originally isolated from the air sacs of a broiler chicken from a flock which showed severe respiratory disease. This strain was also clone purified 5 times. A single stock culture of each strain was stored in multiple small aliquots at -70°C until required.

Mycoplasma media

Mycoplasma broth (MB) and mycoplasma agar (MA) were made based on the formulation of FREY *et al* (1968). MB contained 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 sterile 10% (v/v) swine serum, 1% (v/v) fresh yeast autolysate, 0.002% (v/v) DNA, 0.1% (w/v) glucose and 0.02% ampicillin was added aseptically. MA contained Mycoplasma broth base (Gibco) and solidified with 1% Spesial Noble Agar (Difco). Cystein HCl and thallous acetate in double glass distilled water were added to the basal medium and the mixture was autoclaved. The pH of the medium was adjusted to 7.6 before autoclaving. After sterilization, the mixture was cooled in a waterbath to 50°C and then supplemented aseptically with a sterile enrichment that contained 10% (v/v) swine serum, 1% (v/v) fresh yeast autolysate, 0.002% (v/v) DNA, 0.01% (w/v) actidione and 200 µg/ml ampicillin. The medium was then dispensed into sterile petri dishes.

Propagation of MG cultures

All MG stock cultures were grown at 37°C in MB enriched with swine serum until the phenol red indicator in the medium changed from red to orange yellow (pH 6.8 to 7.0). Inoculated MA plates were incubated in a humidified atmosphere in a candle jar at 37°C for up to 14 days.

Determination of the number of viable mycoplasma organisms used for inoculation

Stock MG-cultures were thawed and 0.5 ml of the culture was serially diluted in 10-fold steps from 10^{-1} to 10^{-9} in sterile MB supplemented with swine serum. Twenty five ul of each dilution was inoculated into 8 replicate wells of a 96-well flat bottom micro-tray (LINbro) 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).

Inoculation procedures

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

SEROLOGICAL PROCEDURES

Rapid serum agglutination (RSA)

The RSA test was done as follows: 25 ul of serum was added to 25 µl of stained MG antigen (Intervet) in a WHO tray and mixed thoroughly by tapping the plate. The plate was then rotated for 2 minutes on a rotery agglutinator. The agglutination reaction was scored from 0 to 4+ on the basis of antigen clump size and degree of clearing of the back ground fluid.

Haemagglutination inhibition (HI)

The HI test utilised 4 HA units of MG antigen and was performed in 96 well "V" bottom micro-trays. All sera were pre- treated with receptor-destroying enzyme (Wellcome) and adsorbed with chicken red blood cells prior to testing. Antibody titres equal to or greater than 10 were determined as positive. Geometric mean titre (GMT) was calculated using the method described by LUTZ (1973).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed using the method described by HIGGINS and WHITHEAR (1986). MG membrane antigen was coated onto a 96-well flat bottom micro-tray (Linbro) and incubated either at 4°C overnight or at 37°C for 2 hrs. Test sera were placed in the coated plate and diluted in 2-fold steps from 1:10 to 1:1280 using a multichannel dispenser (Titertek, Flow). The initial 1:10 dilution was made in sheep serum (PATTEN *et al*, 1984). Following washing, an appropriate dilution of horseradish peroxidase anti- chicken IgG conjugate was added into 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 (A450) on a Titertek Multiscan (Flow). The MG ELISA titre was the highest dilution of a serum having an A450 of greater than 0.25. Sera having an A450 of less than 0.25 at a 1:10 dilution were considered to be MG negative. Geometric mean titre (GMT) was calculated using the method described by LUTZ (1973).

Isolation and Identification of MG

Swabs from air sacs, nasal sinus 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 epiimmunofluorescence (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 in primary agar plates. When this occured, the MB cultures were thawed, inoculated into MA plates and identified by the growth inhibition (GI) test.

Scoring of air sac membranes

Air sac lesion was scored from 0 to 4+, 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 exudate confined to one side of the air sac membranes; 3+ = air sac membranes somewhat thickened with a large accumulation of caseous exudate extending from one side to the other; 4+ = the air sac membranes were thickened and diffuse accumulation of caseous exudate occured on both side of the air sac membranes.

Method for mutagenesis

The method for exposing MG to N-methyl-N-nitro-N-nitroso guanidine (NTG) closely followed the procedure described by NONOMURA and IMADA (1982). Five ml of a culture of strain 80083L was inoculated into 45 ml of mycoplasma broth (MB), incubated at 37°C overnight and harvested when the phenol red indicator changed from red to orange yellow (pH 6.8 - 7.0). The culture centrifuged at 9,000 G for 30 min. The pellet was washed 3 times in sterile PBS and finally was resuspended in 30 ml of PBS. The suspension of organisms was divided into 10 ml portions in 3 Mc.Cartney bottles and NTG was added to give final concentrations of 25, 50 dan 100 µg/ml. After thorough mixing each suspension was incubated at 37°C for 30 min. The organisms were centrifuged at 9,000 G for 30 min, washed 3 times in PBS and filtered through a 0.45 µm membrane filter (Millipore). The filtrates were then serially diluted in 10-fold steps from 10-1 to 10-5. Twenty five µl of each dilution was inoculated onto mycoplasma agar (MA) and incubated in a humidified atmosphere in a candle jar at 33°C for up to 14 days. Seventy five well isolated colonies from each treatment were selected and inoculated into MB. The cloned cultures were incubated at 33°C until signs of growth were observed after which they were stored at -70°C until required. To assess whether the clone cultures were behaving as ts-mutants the relative plating efficiencies at 33°C and 39.5°C were determined. Each cultures was thawed and diluted from 10^{-1} to 10^{-8}, and then 25 µl of each dilution was inoculated in duplicate onto 2 MA plates. One plate was incubated at 33°C as the permisive temperature while the second was incubated at 39.5°C as the restricted temperature. Ts-mutant strains were defined as clonal populations that showed over 103 decrease in efficiency of colony formation at 39.5°C relative to 33°C. The results of this selection yielded 4 ts-mutants (80083 ts-3, 80083 ts-11, 80083 ts-15 and 80083 ts-21) exposed to 100 µg/ml NTG.

Experimental procedure

Groups of five 2-week old SPF CSIRO Hybrid White Leghorn were kept in wire cages. Each group was inoculated either with strains 80083 ts-3, 80083 ts-11, 80083 ts-15, 80083 ts-21 or 80083L. Chickens in the sixth group were injected with MB and used as controls. The chickens were bled prior to inoculation and immediately before euthanasia at 2 weeks post inoculation and examined for air sac lesions and antibody responses. Swabs from nasal sinuses, the first 2 cm of tracheal mucosae and the air sac membranes were also carried out, inoculated into MB and MA.

RESULTS

Table 1 shows the result of selection of ts-mutant strains. Clonal populations derived from cultures exposed to 25 µg/ml or 50 µg/ml NTG gave similar viable counts at both 33°C and 39.5°C. However, 4 out of 75 clones derived from cultures exposed to 100 µg/ml NTG showed >102 decrease in titre when viable counts were done at 39.5°C as compared with 33°C. These 4

ts mutants cultures were designated as 80083 ts-3, 80083 ts-11, 80083 ts-15 and 80083 ts-21.

Table 2 shows the result of the virulence of ts-mutants. No antibodies nor air sac lesions were found in the control chickens. Chickens inoculated with 80083 ts-11 did not show any air sac lesions or MG antibodies and no MG organisms could be isolated from the air sac membranes. However, MG organisms were re-isolated from the upper respiratory tract (URT) of 2 chickens inoculated with 80083 ts-11.

Chickens injected with 80083 ts-11 had no detectable MG antibodies. However, 2 out of 5 birds had moderate to severe (3+) air sac lesions. MG organisms were re-isolated from air sacs of one chicken and from the URT of 4 chickens.

Chickens injected with 80083 ts-15 did not have MG agglutinins. However, 4 out of 5 birds showed MG antibody titres >10 by HI test and ELISA. Three out of 5 birds had moderate to severe (2+ to 4+) air sac lesions. MG organisms were re-isolated from the air sacs and URT of 2 birds that had air sac lesions and from the URT of another bird that did not show air sac lesions.

All of the chickens inoculated with 80083 ts-21 showed severe air sac lesions (3+) and MG organisms were re-isolated from the air sac membranes and URT of all birds. Four out of 5 chickens showed MG antibody titres >10 by HI test and ELISA, and 2 birds showed weak agglutination reactions.

Four out of 5 chickens inoculated with 8083L had moderately severe (3+) to severe (4+) air sac lesions and MG organisms were re-isolated from the air sac membranes and URT of each chicken showing lesions. These chickens had MG antibody titres >10. One chicken showed resistance to inoculation with strain 80083L and no MG antibodies or air sac lesions were present at 2 weeks after inoculation.

DISCUSSION AND CONCLUSION

From 225 clones selected from the NTG treated cultures of strain 80083L, only 4 behaved as ts-mutants. These were designated as 80083 ts-3, 80083 ts-11, 80083 ts-15 and 80083 ts- 21. All of ts-mutant strains were selected from trhe culture treated with 100 µg/ml NTG. Treatment with 50 µg/ml or 25 µg/ml NTG failed to induce detectable ts-mutants.

Chickens injected with 80083 ts-11 showed no air sac lesions or MG antibodies 2 weeks after intraabdominal injection, indicating that 80083 ts-11 is avirulent. MG was not isolated from the air sacs although it was isolated from the nasal sinuses and trachea of 2 chickens. Presumably 8083 ts-11 was unable to survive in the air sacs, although migration organisms from the air sacs to URT did occur. This finding similar to the findings reported by LAM *et al* (1983), while NONOMURA and IMADA (1982) reported that mutant strain of *Mycoplasma synoviae* (MSts44) was not isolated from the air sacs when it was inoculated by intraabdominal injection.

Chickens inoculated with mutant strain 80083 ts-3 also failed to develop MG antibodies by 2 weeks after inoculation, although 2 out of 5 chickens had moderately severe (3+) air sac lesions and MG was recovered from the air sacs and URT. The reason for this is not known.

MG agglutinins were not detected in chickens inoculated with mutant strain 80083 ts-15, but 4 out of 5 chickens had MG antibodies determined by HI and ELISA. Three out of 5 chickens had moderate (2+) to severe (4+) air sac lesions, this indicating that mutant strain 80083 ts-15 retained residual virulence.

All of the chickens inoculated with 80083 ts-21 had moderately severe (3+) air sac lesions and MG was isolated from the air sacs and URT. It would appear that strain 80083 ts-21 was at least as virulent as the parent strain 80083L. The finding that mutant strains 80083 ts-3, 80083 ts-15 and 80083 ts-21 produced air sac lesions was similar to the finding made by LAM *et al* (1983) with their mutant strain TS100.

Chickens inoculated with 80083L showed moderately severe (3+) to severe (4+) air sac lesions in 4 out of 5 birds. One chicken had no MG antibodies nor air sac lesions, and no MG was isolated either from air sacs or URT. The possibly reason for this is could be due to variation in individual resistance of some chickens against intraabdominal injection of strain 80083L.

As a conclusion of this study is that mutant strain 80083 ts-11 of MG is avirulent and the other mutant strains 80083 ts-3, 80083 ts-15 and 80083 ts-21 of MG were remained virulent.

REFERENCES

ADLER, H.E., MCMARTIN, D. and SHIFRIN, M. (1960). Immunization against mycoplasma infections of poultry. *Am. J. Vet. Res.* 21: 482 - 485.

ADLER, H.E.. MC.MARTIN, D. and ORTMAYER,H. (1962). The effect of infectious bronchitis virus on chickens infected with *Mycoplasma gallisepticum*. *Avian Dis.* 6 : 267 - 274.

AMIN, M.M. and JORDAN, F.T.W. (1979). Infection of the chicken with a virulent and avirulent strain of *Mycoplasma gallisepticum*. alone and together with

Newcastle disease virus or *E. coli* or both. *Vet. Microbiol.* 4: 35 - 45.

ANDERSON, D.P. and BEARD, C.W. (1967). Aerosol studies with avian mycoplasma. 2. Infectivity of *Mycoplasma gallisepticum* for chickens and turkeys. *Avian Dis.* 11 : 60 - 64.

DUNLOP, W.R., PARKE, G. and STROUT, R.G. (1961). Summary concerning aerosol infection with mycoplasma alone and combined with viruses. *Avian Dis.* 5 : 455.

FREY, M.C., HANSON, R.P. and ANDERSON, D.P. (1968). A medium for the isolation of avian mycoplasma. *Am. J. Vet. Res.* 29: 2164 - 2171.

FABRICANT, J. and LEVINE, P.P. (1962). Experimental production of complicated chronic respiratory disease infection ("air sac disease"). *Avian Dis.* 6: 13-23.

GRIMES, T.M. and ROSENFELD, L.E. (1972). Experimental respiratory disease and airsacculitis in fowls caused by *Mycoplasma gallisepticum* . *Aust. Vet. J.* 48: 113 - 116.

HIGGINS, P. A. and WHITHEAR, K. G. (1986). Detection and differentiation of *Mycoplasma gallisepticum* and *M. synoviae* antibodies using enzyme-linked immunosorbent assay. *Avian Dis.* 30: 160 - 168.

JORDAN, F.T.W. (1979). Avian Mycoplasmas. In: The Mycoplasmas volume II. Human and Animal Mycoplasmas. J.G. Tully and R.F. Whitcomb. Academic Press, New York, San Francisco, London. pp.1-40.

KLEVEN, S.H., KING, D. and ANDERSON, D.P. (1972). Air-sacculitis in broilers from *Mycoplasma synoviae*: effect on air sac lesions of vaccinating with infectious bronchitis and Newcastle disease virus. *Avian Dis* 16: 915 - 924.

LAM, K. M., LIN, W., YAMAMOTO, R. and FARVER, T.B. (1983). Immunization of chickens with temperature sensitive mutants of *Mycoplasma gallisepticum*. *Avian Dis.* 27: 803 - 812.

LEVISOHN, S., DYKSTRA, M.J., LIN, M.Y. and KLEVEN, S.H. (1986). Comparison of in vivo and in vitro methods for pathogenicity evaluation of *Mycoplasma gallisepticum*. *Avian Pathol.*. 15: 233 - 246.

LIN, M. Y. and KLEVEN, S.H. (1982). Pathogenicity of two strains of *Mycoplasma gallisepticum* in turkeys. *Avian Dis.* 26: 360 - 364.

LUTZ, W. (1973). Appendix 1. Statistical methods as applied to immunological data. In Handbook of Experimental Immunology. Ed.by Weir, D.M. BLACKwell Scientific Publications. Oxford, London, Edinburg, Melbourne. pp A 1.1 - A 1.27.

MEYNELL, G.G. and MEYNELL E. (1975). Theory and Practice in Experimental Bacteriology. Cambridge University Press, London, New York, Melbourne, p.231 - 236.

NAEEM, M., MUNEER, M. A., VAID, M. Y., HASHMI, H. A., RIZVI, S.A. and CHAUDHRY, M. A.(1980). Studies on the economical losses caused by *Mycoplasma gallisepticum* in poultry. *Pakistan J. Sci.* 32 : 47 - 49.

NONOMURA, I. and IMADA, Y. (1982). Temperature-sensitive mutant of *Mycoplasma synoviae* I. Production and selection of a non-pathogenic but immunogenic clone. *Avian Dis.* 25: 763 - 775.

PATTEN, B.E., HIGGINS, P.A. and WHITHEAR, K.G. (1984). A urease ELISA for the detection of mycoplasma infections in poultry. *Aust. Vet. J.* 61: 151 - 154.

ROBERTS, D.H. (1964). Experimental infection of chickens with *Mycoplasma gallisepticum*. and subsequently re-isolation of the organisms from the body tissues. *Vet. Record.* 76: 798 - 801.

RODRIQUEZ, R. and KLEVEN, S. H. (1980). Pathogenicity of two strains of *Mycoplasma gallisepticum* in broilers. *Avian Dis.* 24 : 800 - 807.

ROSENDAL, S. and BLACK, F.T. (1972). Direct and indirect immuno- fluorescent of unfixed and fixed mycoplasma colonies. Acta Path. Microbiol. *Scand. Sect. B.* 80: 615 - 622.

TIMMS. L.M. and CULLEN, T.L. (1972). Comparative efficiency of 4 *Mycoplasma gallisepticum* strains as antigens in detecting heterologues infection. *Res. Vet. Sci* 13: 523 - 528.

YODER, H.W.Jr. (1991). *Mycoplasma gallisepticum* infection. In: Diseases of Poultry. 9th ed. B.W. Calnek; H.J. Barnes; C.W. BEARD; W.M. Reid and H.W. YODER Jr., eds. Iowa State Univ. Press, Ames, Iowa. pp. 198 - 212.

Table 1. Viable counts of ts-mutants incubated at 33°C and 39.5°C

Mutant strain	33°C CFU/ 25 µl	39.5°C CFU/ 25 µl
80083 ts-3	5 x 105	3 x 102
80083 ts-11	2 x 106	6 x 102
80083 ts-15	6 x 105	8 x 102
80083 ts-21	8 x 105	1 x 103

CFU : Colony forming units

Table 2. Comparison of the virulence of 4 ts-mutants and 80083L of MG strains in 2 week-old chickens

Strain	Serology			Air sac examination			MG Isolation from URT
	RSA GMT	HI GMT	ELISA	Lesions	Mean Score	MG Isolation	
80083 ts-3	0[a]	0.0	0.0	2[a]	1.2	1[a]	7[b]
80083 ts-11	0	0.0	0.0	0	0.0	0	4
80083 ts-15	0	7.2	8.3	3	1.8	2	5
80083 ts-21	2	7.2	9.5	5	3.0	5	10
80083L	3	6.3	8.3	4	2.8	4	8

a Number out of 5 giving response
b Number of MG isolates from 10 sites cultured