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PHENOTYPIC AND ANTIGENIC VARIATION OF *MYCOPLASMA GALLISEPTICUM* VACCINE STRAINS

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ABSTRACT

Phenotypic and antigenic variations among *Mycoplasma gallisepticum* vaccine F and Ts-11 strains were investigated by SDS-PAGE and serological methods (hemagglutination-inhibition and immunoblot assays). The SDS-PAGE system followed by densitometer analysis showed weak phenotypic variability between the strains, being the major difference close to the 75 kDa level where a prominent peptide band was detected only in the F vaccine strain. Polyclonal antibodies to the *M. gallisepticum* antigens produced in chickens were used in serological tests for this antigenic variability study. There were strong cross-reactions between the strains and homologous/ heterologous antibodies. The most evident characteristic was the specific response of the vaccine-type F polyclonal antiserum to the 75 kDa peptide band of the homologous strain.

Key words: *Mycoplasma gallisepticum*, phenotypic variation, antigenic variation.

INTRODUCTION

Mycoplasmosis continue to be the major problem in poultry, being *Mycoplasma gallisepticum* (MG) infections responsible for important economic losses (24). To reduce this problem, layer producers commonly have used MG live vaccines in attempt to immunize and protect chicken flocks (2). The MG-F strain is the most used as vaccine to prevent the colonization by the most virulent MG strains. The continuous vaccination may displace field strains from multiple-age poultry production sites (1). However, MG-F is pathogenic for turkeys (13), and has been associated with MG outbreaks in meat and breeder turkeys under field conditions (12).

Attenuated MG Ts-11 strain has been produced commercially as live vaccine. The strain was selected after exposure passage of an immunogenic Australian field isolate (strain 80083) of MG to N-methyl-nitro-N-nitrosoguanidine (22). This strain was found to be poorly transmitted from vaccinated to unvaccinated birds and showed little or no virulence for chickens and turkeys (21). This suggests that it could be safer than the MG-F strain for vaccinated flocks. The ability to replace wild or vaccine MG strain has originated controversy among researchers (11,23).

For use of new MG vaccines knowledge about the variability of these vaccine strains is essential. Phenotypic, antigenic and genotypic heterogeneity among MG strains have been studied by sodium dodecyl sulfate-polyacrylamide gel analysis (6), hemagglutination-inhibition test (8), DNA-DNA hybridization (7), polymerase chain reaction/PCR (15,16), restriction endonuclease DNA analysis (10), flow cytometry (1), southern blot analysis, using a ribosomal RNA gene probe (25), and immunoenzymatic assays (17). These techniques have made it feasible to determine if MG-F strain long-term used as a vaccine in multiple-age commercial layers has resulted in displacement of the original field strain with the F vaccine strain. Random amplified polymorphic DNA analysis was used to discriminate between F and TS-11 strain (3), but one of the disadvantages of current available vaccines is that there is no convenient serological technique to accurately distinguish between vaccinated or naturally infected flocks (23). A MG marker to distinguish vaccine from wild type-strains would be useful as diagnostic tool. MG-F strain has a unique molecular band, a 75 kDa protein, which can be used as a marker to differentiate it from the other MG strains by immunoblot (18).

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The objective of this study was to search for Ts-11 molecular marker that could be used to distinguish it from MG-F and wild strains.

MATERIALS AND METHODS

Microorganisms

MG strains received for study were characterized by PCR (15,16). MG-S6 strain was recovered from a chicken experimentally infected with S6 virulent strain. Originally isolated and maintained by Dr. R. Yamamoto (University of California, USA), this strain was given to Dr. Elmiro R. do Nascimento. (UFF, Niteroi, RJ), and used as positive control. MG-F is a low virulent strain used as vaccine in Brazil marketed by Coopers® as MG-F vaccine. MG Ts-11, attenuated strain used as vaccine in Brazil, marketable by Rhodia-Merieux® as Mycovax Ts-11 vaccine.

Cellular antigen preparation

MG-S6 strain was stored (v/v) in glycerol, MG-F a lyophilized vaccine, was maintained at 4ºC and the MG Ts-11 vaccine was maintained at -20ºC. All strains were cultivated in Hayflick modified medium (5) with 10 % equine serum. The cultures were incubated at 37ºC and the cells were harvested by centrifugation at the begining of the acidification of the medium. Protein concentration from each cellular suspension was determined as described by Lowry *et al.* (14).

Polyclonal chicken antisera (PCA) preparation

Three to four years old pathogen free chickens (*Gallus gallus*), were used for PCA preparation. The birds were immunized through intramuscular inoculation with 1000 μg of cellular antigen, mixed (v/v) with Freundt Complete Adjuvant. The specific antibodies were titred by hemagglutination inhibition (HI) test. PCA was obtained 30 days after inoculation, and stored at -20°C.

SDS-Page

The mycoplasmal proteins were submitted to SDS-PAGE electrophoresis with 10% (w/v) of acrylamide/bis 37.5% gels and stained with Coomassie Brilliant Blue R-250 (4). The protein band profile was analysed by densitometry.

Western blot

The electrophoresed proteins were transferred from gels to 0.22 mm nitrocellulose sheets (NS), as cited by Tsang *et al.* (20). The electrotransference was conducted for 1 h at 100 V (19). The NS were stained with Ponceau S to observe the peptide bands of 75 and 64 kDa. The NS were washed with distilled water and stored at -20ºC.

Immunoblot

The NS were incubated with PCA diluted 1/100 in PBS pH 7.2 / 0.3% Tween 20 (PBST) and blocked with 5% skim milk. The NS were washed with PBST four times for 3 min each one. The second peroxidase-labeled antibody (BioManguinhos, FIOCRUZ, Brazil), diluted 1/500 in PBST, was added and the mixture was incubated for 1h. Then, the NS were submitted to additional washing with PBST for 5 min. Freshly prepared developing solution (60 mg de DAB, 100 μL de H_2O_2 30% in 100 mL of Tris 0.15 M pH 7.6) was then added. The reaction was neutralized with distilled water and the NS sheets were air dried. All steps were carried out at room temperature.

Hemagglutination-inhibition (HI) test

HI assays to detect MG antibodies were performed on PCA samples using four-hemaggluination units of antigen obtained from MG-S6 (9). Positive and negative MG stand-sera were used as controls.

RESULTS

Phenotypic analysis

The results for the MG strains are summarized in Fig. 1 and 2. MG strains presented differences on protein banding patterns. However, the patterns were similar, mainly those below 66 kDa level. The peptide band of 64 kDa, characteristic of MG species, was observed in all strains. MG Ts-11 strain presented minor but distinct variation at this peptide band level, particularly by

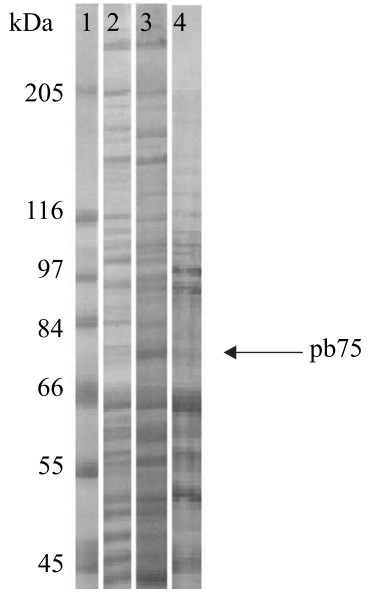

Figure 1. SDS-PAGE of proteins from *Mycoplasma gallisepticum* strains. Lane 1, molecular mass standards (Sigma); Lane 2, MG Ts-11/Mycovax Ts-11 vaccine, Rhodia-Merieux®; Lane 3, MG-F/ MG-F vaccine, Coopers®; Lane 4, MG-S6(208) / virulent strain. The 75 kDa peptide band is marked in the figure as peptide band (pb) 75 and indicate by an arrow.

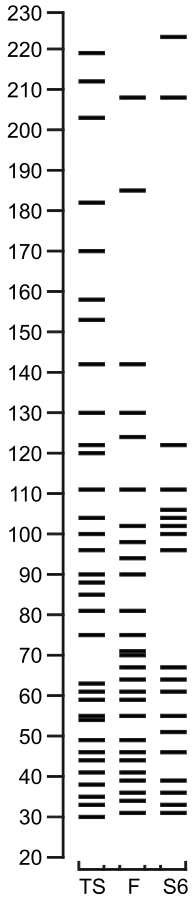

Figure 2. Schematic drawing of protein outline from Ts-11, F and S6 *Mycoplasma gallisepticum* strains obtained after SDS-PAGE densitometer analysis. The arrow shows the 75 kDa position.

densitomer analysis, which showed a distinct band at the 63 kDa level.

Phenotypic variation among vaccine strains were better detected by densitometer scanning above 75 kDa level where the bands in MG Ts-11 strain were very weak or did not match above this level in F strain. On the other hand, a prominent peptide band of 75 kDa in MG-F strain was observed in the gel. The MG Ts-11 strain showed a large band around the 75 kDa level, weakly stained by Coomassie Brilliant Blue. The densitometer analysis indicated where the peptide banding at this level in MG Ts-11 strain had minor band percentage than observed in MG-F strain, 1.68% and 4.08%, respectively (data not shown).

Antigenic analysis

Antigenic differences among vaccine and wild MG strain were studied by HI and Immunoblot assays. Using MG-S6 strain as antigen all PCA showed titres of 80 in HI test. By immunoblot test, PCA reactivity pattern did not vary much and occurrence of high cross-reactivity among the sera was observed (Fig. 3).

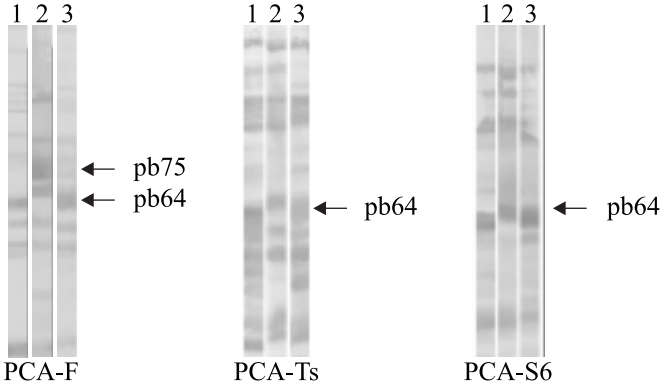

Figure 3. Immunoblots from three *Mycoplasma gallisepticum* strains. Lane 1, MG Ts-11; lane 2, MG-F and lane 3, MG-S6, reacted respectively with chicken sera PCA-Ts, PCA-F and PCA-S6. The 75 and 64 kDa immunoreactive locus are indicated as pb 64 and pb 75, respectively and are marked by an arrow.

The wide and specific response of PCA F serum to the peptide band (75 kDa) of its homologous strain was the unique characteristic in immunoblot test.

DISCUSSION

This study showed that the protein SDS-PAGE gel profiles of vaccine and wild type MG strains were similar. A 64 kDa peptide band was already observed in all strains of *M. gallisepticum* (12,17). In this study, we describe that the major difference among the strains tested was the detection of a 75 kDa band exclusively in MG-F but not in MG S6, or weakly detected on MG Ts-11. These results confirm the previously reported phenotypic diversity between F and S6 MG strains (6,10,17).

Although a darkly stained 75 kDa band was observed in the MG-F strain, Khan *et al.* described that this 75 kDa peptide band was present only in some MG-F seeds (6). This variation among MG-F seeds may constitute a problem for the use of this peptide band as a vaccine molecular marker. The MG-F seed (MG-F vaccine, Coopers), used as vaccine in Brazil, shows this peptide band, which may be useful for monitoring the prevalence of the MG wild type in places where such vaccine is used. The MG Ts-11 vaccine strain (Mycovax Ts-11/ Rhodia-Merieux) also showed a similar protein pattern when compared to the F vaccine strain. However, this strain did not present the 75 kDa peptide band.

When chicken antibody response to the MG strains was evaluated by hemagglutination and immunoblot assays, no antigenic variability was detected when comparing the vaccine and wild type strains. Previous report has showed that the HI assay is not suitable to distinguish variability among strains responsible for infection and serum conversion (17). Similary, using the immunoblot test, we observed that the PCA reactivity pattern showed a high cross-reaction among the sera.

Rabbit policlonal antibodies for MG-F, K810, S6 and A5969 strains were previously produced by Thomas and Sharp (17). These antibodies recognized most peptide bands from heterologous strains. Since heterologous and homologous antibodies to MG-S6 and MG-F recognized both pb63 (MG Ts-11) and pb64 (MG-S6 and MG-F), the peptide band profile may be similar. Therefore, differences detected in protein profile may reflect differences on peptide molecular weight.

Eventhough the Ts-11 strain showed a faint 75 kDa band when stained with Coomassie blue, this band was not detected when gels were blotted with anti-Ts-11 or anti F sera. However the anti-F serum clearly detected a 75 kDa band in the MG-F strain. These data suggest that the MG-F vaccine strain is the only isolate that shows a molecular marker that can differentiate it from the wild MG strain. No specific molecular marker was detected in the Ts-11 strain.

RESUMO

Variabilidade fenotípica e antigênica de cepas variáveis de *Mycoplasma gallisepticum*

Cepas vacinais de *Mycoplasma gallisepticum*, F e TS-11, foram examinadas quanto às suas variações fenotípicas e antigênicas, por SDS-PAGE e através de dois métodos sorológicos (inibição da hemaglutinação e imunoeletroforese). A análise densitométrica das bandas obtidas nos géis de poliacrilamida mostrou pequena variabilidade fenotípica entre as amostras, sendo a banda peptidica de 75 kDa detectada apenas na amostra vacinal F. Anticorpos policlonais produzidos em galinha foram utilizados nos ensaios sorológicos para estudar a variabilidade antigênica das amostras. Houve elevada reatividade cruzada entre as amostras e os anticorpos homólogos e heterólogos. A característica mais evidente foi a resposta específica da banda peptídica de 75 kDa da vacina F ao anticorpo homólogo.

Palavras-chave: *Mycoplasma gallisepticum*, variação fenotípica, variação antigênica.

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