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CHARACTERIZATION OF M*YCOPLASMA PENETRANS* AND *MYCOPLASMA FERMENTANS* IMMUNODOMINANT PROTEINS

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ABSTRACT

Mycoplasmas are a heterogeneous group of the smallest organisms capable of self replication and are known to cause many detrimental diseases in both animals and humans. These wall-less prokaryotes are enveloped by a lipoprotein membrane and their small genomes are sufficient to synthesize molecules required for growth and self-replication. Among sixteen species isolated from humans, *Mycoplasma pneumoniae*, an agent of primary atypical pneumonia, and the urogenital tract species *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum* have been confirmed to be pathogenic. *Mycoplasma penetrans* and *Mycoplasma fermentans*, which are species associated with HIV, have been investigated mainly in research laboratories. In this study we have characterized lipid-associated membrane proteins (LAMP) of *Mycoplasma penetrans* and *Mycoplasma fermentans*, in view of the importance of mycoplasmas in human diseases and the peculiar antigenic variation observed in these species. To characterize proteins with possible diagnostic value, we used ELISA and Western blot in sera of pregnant women whose cervical samples were positive for these species of mycoplasmas when tested by PCR. ELISA showed IgG anti-LAMP-*M. fermentans* antibodies to be present in 57.5% of cases and IgM antibodies to be present in 74.5% of cases. The three samples that were PCR positive for *M. penetrans* showed IgG anti-LAMP-*M. penetrans* antibodies, and one sample was positive for IgM. No IgA antibodies against either species were detected in any of the samples. LAMP analysis by Western blot revealed the 35, 38, 42, 61 and 103 kDa proteins of *M. penetrans* and the 29, 38, 41, 61, 78 and 95 kDa proteins of *M. fermentans*. Among these, will be considered p35 to *M. penetrans* and 29 kDa protein to *M. fermentans*, the main immunoreactive proteins and therefore useful markers for further laboratory diagnosis.

Key words: *Mycoplasma penetrans, Mycoplasma fermentans,* LAMP-proteins, ELISA, Western blot

INTRODUCTION

In spite of the outstanding progress made in our understanding of the nature of mycoplasmas, their taxonomic position and their relationship with other organisms, they still represent an enigma for microbiologists because some mechanisms of pathogenicity are still unknown. Mycoplasma cell is only enveloped by a lipoprotein membrane and the genome, despite its diminutive size, is sufficient for self-replication.

Adherence and intense antigenic variation allow mycoplasmas to escape efficiently from host immune responses, thus allowing chronic infectious diseases to become established (11,16,18). More than 170 mycoplasma species live as parasites on human and animal cellular surfaces by sticking to and colonizing the epithelial coating of the respiratory and urogenital tracts, but rarely invade adjacent tissues (11). The pathogenicity of mycoplasmas is therefore related, among other things, to the absence of the cell wall. The inner contact is possible via adhesion proteins and the high-frequency antigenic variation

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represented by the lipoprotein membrane (21). *Mycoplasma fermentans* and *Mycoplasma penetrans* species isolated from urine and blood of HIV infected patients have been associated with the AIDS progression as possible co-factors in the development of this disease. *Mycoplasma fermentans* was isolated for the first time from the urogenital tract and, later, from other body parts and fluids such as: bone marrow of leukemic patients; the trachea, urine and blood of HIV carriers; saliva of healthy subjects; and synovial liquid of patients with rheumatoid arthritis (9,10,16,22). The significant detection rate of these microorganisms among healthy subjects and among patients remains a paradox. The lipoprotein of *M. fermentans* is abundant in the surface of the membrane and shows strong antigenicity and immunogenicity (24,28). The antigenic variation in these species is clearly demonstrated by the presence of p150, p 95, p78, p61, p48, p43, p41, p38 and p29 proteins. Protein p29 is the main lipid-associated membrane protein and expresses a high frequency of phase variation (15,25).

M. penetrans, although described in 1992, is the most recent species of *Mollicutes* isolated in humans. Properties of adherence, hemadsorption, cytoadsorption and invasion of mammalian epithelial cells have been described. The last property is responsible for its name (6,14,23). Analysis of *M. penetrans* isolated from the urine of HIV homosexual subjects (13,14) has shown that it may act as a co-factor of the virus, accelerating the evolution of this retroviral disease (1,2,8). *M. penetrans* adhesion to the host cell is related to the 35, 38, 61 kDa lipid-associated membrane proteins (LAMP) and to the 103 kDa proteins. Studies with sera of HIV positive patients showed p35 and p38 to be immunodominant proteins (3,6,27). However, p35 and p38 was shown to be the specific lipoproteins for *M. penetrans* in Western blot with a high frequency of phase variation (7,19,20). These lipoproteins and their adhesins act as potent immunogens, determining the nature, intensity and multiple specificity of the host immune response.

The studies of these microorganisms in other clinical conditions and in different populations are very important to know how widespread these infections are and if there is any association with other diseases. Standardization of ELISA using LAMP antigen and characterization of immunodominants proteins would be of great importance for this purpose. The aim of this work was to characterize some antigens in the *Mycoplasma penetrans* and *Mycoplasma fermentans* membranes for further laboratory diagnosis, in view of the importance of mycoplasmas in human diseases and the antigenic variation in the species.

MATERIALS AND METHODS

Mycoplasma strains and culture methods

M. penetrans GTU-54-6A1 was cultured in SP-4 medium at 37ºC under aerobic conditions. *M. fermentans* ATCC 19989 was cultured in SP-4 medium at 37ºC under anaerobic conditions. LAMP antigen preparation was as follows: *Mycoplasma* lipid-associated membrane proteins (LAMP) were obtained in accordance with the method described by Wang *et al.* (27). Briefly, 500 mL of broth cultures were centrifuged at 12,000 x g for 60 min at 4ºC. Mycoplasma pellets were washed three times in phosphate-buffered saline (PBS) pH 7.4 containing timerosal and were re-suspended in 45 mL of Tris-HCl 50 mM, NaCl 150 mM, EDTA 1 mM, pH 8.0, followed by addition of 5 mL of 10% Triton X-114 solution. This mixture was submitted to ultrasonic disruption at three cycles/min. After 90 minutes the solution was centrifuged at 20,000 x g for 30 min at 4ºC. The supernatant was submitted to partition phase by incubation for 5min at 30ºC followed by centrifugation at 2,500 x g for 5 min at room temperature. Detergent phase containing LAMP was collected and submitted to another partition phase as previously described. This detergent phase was named LAMP, and stored at -20ºC after the addition of phenylmethylsulphonylfluoride (PMSF). Characterization of LAMP was carried out by analysis of protein profile using sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) in 7-15% gradient gels by the Laemmli (12) method and by visualization of the proteins pattern using Coomassie Brilliant Blue staining (26).

Patient samples

Serum samples were obtained from 50 pregnant women receiving routine prenatal care at the Gynecology and Obstetrics Department of the Hospital do Servidor Público do Estado de São Paulo, whose cervical samples were PCR positive for *M. penetrans* or *M. fermentans*. Forty seven cervical samples obtained from these 50 patients were PCR-positive for *M. fermentans* and three for *M. penetrans.* Serum samples were stored at -20ºC until used.

Control group

The thirty serum samples in the control group were obtained from healthy male blood donors at the blood bank of the Hospital do Servidor Público do Estado de São Paulo. The results obtained were used for calculating cut-off value.

Serological tests

ELISA: Polystyrene plates (MaxiSorp F, NUNC, Denmark) were coated with 100 µL of LAMP containing 1.5 µg of protein per well, diluted in TBC buffer (0.015M Na_2CO_3 and 0.04 M $NaHCO_3$) and incubated for 18 hours at 37ºC. After incubation, the plates were washed four times with solution A (PBS, pH 7.4, plus 0.05% Tween 20) and overcoated with 5% powdered skimmed milk (Molico, Nestlé) in solution A for 30 min. at 37ºC. After one washing step, the sera were diluted 1:100 in solution A plus 1% powdered skimmed milk and incubated for 120 min at 37ºC. The plates were washed four times and peroxidase-conjugated anti-γ, -µ, or -α chains (Sigma Chemical Co., St.

Louis, MO, USA) were added to detect IgG, IgM or IgA antibodies respectively. After 2 h of incubation at 37ºC, the color was developed by addition of 2.0 mg of ortho-phenylene-diamine (Sigma Chemical Company, St. Louis, MO, USA) and 20 µL of hydrogen peroxide in 20 mL of 0.2 M citrate/phosphate buffer. The enzymatic reaction was stopped by addition of 100 µL /well of 4 N sulfuric acid. The absorbance at 492 nm was measured in a plate reader (Spectra I - 5082).

Samples with absorbance equal to or higher than the mean of the control group plus two standard deviations (SDs) for IgG, IgM and IgA were considered positive.

Western blot

Western blot was performed to identify immunoreactive proteins and to determine cross-reactive proteins of *M. penetrans* and *M. fermentans* in serum samples of pregnant women.

Polyvinylidene fluoride (PVDF) 0.22 nm membranes containing mycoplasma LAMP fractions that were separated by 10% SDS-PAGE and electroblotted were cut into strips of about 3 mm. The strips were washed under shaking conditions in PBS pH 7.4 containing 0.05% Tween 20 and blocked with 5% skimmed milk (Molico, Nestlé) in PBS pH 7.4 for 2 h. After the washing step, the sera were diluted at 1:50 in 1% powdered skimmed milk in PBS pH 7.4. After 18 h of incubation at 4ºC and three washes, the strips were incubated with specific conjugates: peroxidase-labeled goat anti-human-IgG, anti-human-IgM and anti-human-IgA antibodies diluted respectively at 1:8,000, 1:4,000 and 1:4,000. After 2 h of incubation at 37ºC and the washing steps, DAB/H_2O_2 (5 mg diaminobenzidine in 30 ml PBS pH 7.4 and 150 mL 30% H_2O_2) was added. After 15 mins the strips were washed ten times with distilled water and analyzed by visual inspection.

RESULTS

Characterization of the LAMP fractions of the species studied by SDS-PAGE revealed a proteins profile for *Mycoplasma penetrans* in which the two major antigenic proteins, p35 and p38 were prominent. We also observed 42, 61 and 103 kDa proteins. The profile of the LAMP fraction of *Mycoplasma fermentans* revealed 29, 38, 41, 61, 78, 95 and 150 kDa proteins. The bands p35 and p29 identified in this study are those referred in the literature as being specific for *M. penetrans* and *M. fermentans* (7,15,27) (Fig. 1).

Samples were considered ELISA positive when optical density was equal to or higher than the established cut-off values: 0.23 (IgG), 0.28 (IgM) and 0.20 (IgA) for *M. penetrans*, and 0.22 (IgG), 0.30 (IgM) and 0.22 (IgA) for *M. fermentans*. These cut-off values were obtained from the results of the control group sera.

IgG and IgM antibodies for *M. penetrans* were detected in 3 and 1 respectively of 3 sera samples of pregnant women, using

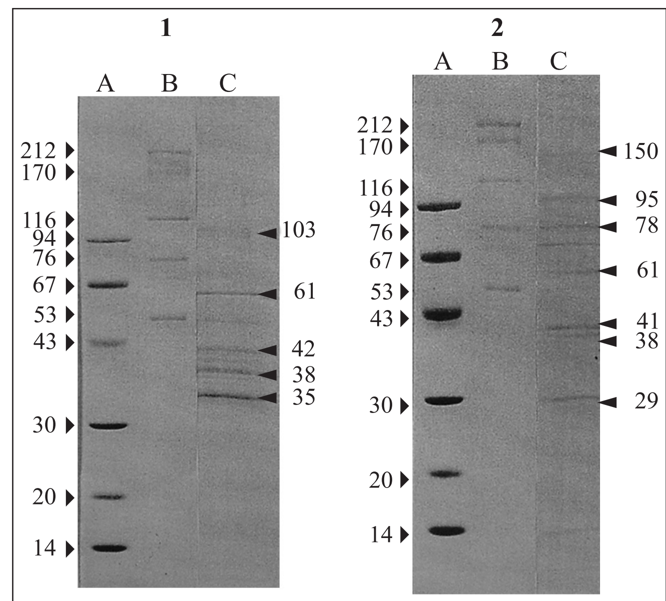

Figure 1. Electrophoretic protein profile in SDS-PAGE 7 to 15% of LAMP preparations of *Mycoplasma penetrans* (1) and *Mycoplasma fermentans* (2) with Coomassie Brilliant Blue (C) stain. 1A and 2A indicate low molecular weight-calibrator; 1B and 2B indicate high molecular weight-calibrator. The arrows indicate the 35, 38, 42, 61 and 103 kDa proteins for *Mycoplasma penetrans* (1) and the 29, 38, 41, 61, 78, 95 and 150 kDa proteins for *Mycoplasma fermentans* (2).

ELISA; for *M. fermentans* the respective figures were 57.5% (27/47) and 74.5% (35/47). IgA antibodies were not found in either case.

Western blot for *M. penetrans* showed IgG antibody reactivity in 3 of samples for 35, 38 and 61 kDa proteins and in 2 of samples for 42 and 103 kDa proteins, others proteins were observed but they were not characteristic for the specie. IgM antibody reactivity showed positive results for 38 and 61 kDa proteins in all 3 samples and 35 and 42 kDa proteins in 2 samples. Specific *M. penetrans* IgA antibodies were not found.

Western blot for *M. fermentans* showed IgG antibody reactivity characteristic of specie against 29, 38, 41, 61, 78 and 95 kDa proteins, with a frequency of 32.0% (15/47), 25.5% (12/47), 53.2% (25/47), 19.1% (9/47), 51.1% (24/47) and 17.0% (8/47) respectively. For the same proteins IgM reactivity was detected in 36.2% (17/47), 40.5% (19/47), 42.6% (20/47), 19.1% (9/47), 27.7% (13/47) and 2.1% (1/47) of samples respectively. Although IgA antibodies were not detected by ELISA, using Western blot we identified a weak reactivity to some proteins no characteristic of the specie (Fig. 2).

The results obtained by ELISA and Western blot assays are shown in Table 1.

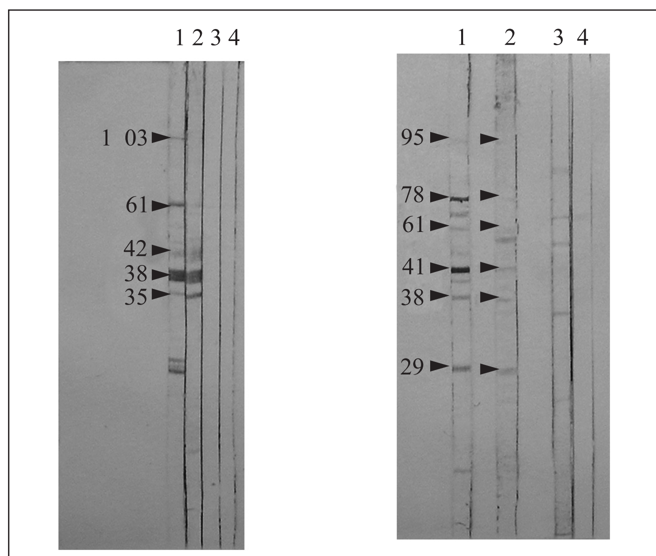

Figure 2. Western blot: reactivity profile of sera of pregnant women for *M. penetrans* (**A**) and *M. fermentans* (**B**) proteins. Strips 1, 2, 3 and 4 show the IgG, IgM and IgA bands and negative serum control respectively. The 35, 38, 42, 61 and 103 kDa proteins of *M. penetrans* and the 29, 38, 41, 61, 78 and 95 kDa proteins of *M. fermentans* were detected.

Table 1. ELISA and Western blot results for sera of pregnant women (cervical samples PCR positive) using *M. penetrans* and *M. fermentans* LAMP fractions.

Mycoplasma species	Antibodies	Sera of pregnant women	
	Isotypes	ELISA p/n (%)	Western blot (kDa)
M. penetrans	IgG	3/3	103, 61, 38, 35
	IgM	1/3	61, 38, 35
	IgA	nd*	nd*
M. fermentans	IgG	27/47(57.5)	95, 78, 61, 48, 41, 29
	IgM	35/47(74.5)	95, 78, 61, 48, 41, 29
	IgA	nd*	nd**

nd*: not detected; nd**: specific bands not detected; p= samples positive by ELISA; n= total number of samples.

A cross-reactivity study was performed using Western blot in sera of pregnant women whose cervical samples were PCR positive for *M. penetrans* and *M. fermentans*. We assayed *M. penetrans* positive sera versus LAMP of *M. fermentans*, and *M. fermentans* positive sera versus LAMP of *M. penetrans*. Two cross-reactive bands were observed: one for the 38 kDa protein and one for the 61 kDa protein.

DISCUSSION

The improvements in diagnosis arising from the use of molecular technology have resulted in the discovery of new species of pathogens which were formerly recognized as commensal organisms, and in the introduction of new concepts of mycoplasma diseases. Although the role of mycoplasmas in the pathogenesis of some human diseases still remains controversial, it has been suggested that these microorganisms contribute significantly as co-factors in disease progression.

The prevalence of mycoplasma infection in our country is not well known, mainly for emergent species such as *M. penetrans* and *M. fermentans*. Some serologic studies were carried out by Cordova *et al.*, (4), who has investigated sera from 106 HIV infected patients by ELISA, presented positivity of 25.5%, 9.1% and 15.1% respectively for IgG, IgM and IgA anti-*M. penetrans*. They found also in 110 sexually transmitted disease (DST) anti-*M. penetrans* by ELISA 17.3%, 9.1% and 17.3% respectively for IgG, IgM and IgA.

Serological tests are a very practical tool to assess the prevalence of mycoplasma species and the distribution of these microorganisms in different populations and in different clinical conditions. However the presence of antigens shared by different species of mycoplasmas interferes in the specificity of serological tests and it may become not useful for diagnostic purposes.

ELISA using LAMP fraction antigen was assayed in sera of pregnant women really infected by *M. penetrans* or by *M. fermentans* to investigate antibody response against these two mycoplasmas. However we observed a low sensitivity using this antigenic fraction. For diagnostic purpose is necessary to improved it.

We found IgG antibody positivity in all three samples obtained from *M. penetrans* infected patients and IgM in two samples. No IgA antibodies were detected.

Due to the small number of samples involved in this study we were not able to reach a definitive conclusion and a study with a larger number of patients would be recommended.

Western blot analysis of LAMP from *M. penetrans* revealed IgG reactivity for p35, p38, p42, p61 and p103. However only p35 is considered specific of the specie (6). IgM antibody reactivity was observed for p38, and p61 in three samples, and p35 and p42 in two samples.

Anti-*M. fermentans* antibodies were identified using ELISA in sera of pregnant women with a frequency of 57.5% for IgG antibodies and 74.5% for IgM antibodies. IgA antibodies were not observed.

Western-blot analysis of *M. fermentans* showed 29, 38, 41, 61, 78 and 95 kDa proteins. The 29 kDa protein, which has high-frequency phase variation, was detected in the following frequencies: 31.9% and 36.2% for IgG and IgM antibodies respectively. Although this protein showed low frequency it

is the most specific (25). A higher frequency observed was for 41 kDa protein, although this is not referred to be specific of specie.

Specific reactivity for species

Analysis of Western blot profile of LAMP fraction allowed to identify some specie specific lipoproteins which showed strong immunoreactivity and some lipoproteins with cross-reactivity.

Proteins of 35 and 38 kDa, the two main lipoproteins used by *M. penetrans* to penetrate into the host cell (3,7), are the most abundant in mycoplasma cellular surface had have high-frequency phase variation (17). Our results showed a strong immunoreactivity these two proteins and the specie specificity of 35 kDa lipoprotein as previously found by Ferris *et al.*, (5).

Furthermore, Wang *et al.*, (27) described two 61 and 103 kDa proteins in sera of HIV patients, which were not identified in sera of HIV-negative patients. In our study, however, these two proteins were identified in sera of pregnant women, indicating a possible presence in general population.

Like other species of mycoplasma, *Mycoplasma fermentans* presents proteins that express phase variation. Our results revealed the presence of 29, 38, 41, 61, 78, and 95 kDa proteins, corroborating the results described by Theiss *et al.* (24).

Cross-reactivity profile: anti- *M. fermentans* antibodies revealed cross-reactivity in sera of pregnant women for p38 and p61 of *M. penetrans*. This enabled us to consider p29 as specific to *M. fermentans* and p35 as specific to *M. penetrans*.

Our work consisted of a study into the characterization of the species *Mycoplasma penetrans* and *Mycoplasma fermentans.* The study revealed the presence of proteins already mentioned in the international literature, but also revealed the presence of other strongly reactive proteins.

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RESUMO

Caracterização de proteinas imunodominantes de *Mycoplasma penetrans* e *Mycoplasma fermentans*

Micoplasmas são procariotos diminutos, desprovidos de parede celular e envoltos por uma membrana lipoproteica cujo pequeno genoma sintetiza a maioria das moléculas necessárias para crescimento e replicação. Dentre as dezesseis espécies isoladas do homem, *Mycoplasma pneumoniae*, agente causador da pneumonia atípica primária, e as espécies do trato urogenital como *Mycoplasma hominis*, *Ureaplasma urealyticum* e *Ureaplasma parvum* têm definido seu papel patogênico. *M. penetrans* e *M. fermentans*, espécies associadas ao HIV, têm sido investigadas principalmente em laboratórios de pesquisa. Considerando a importância dos micoplasmas nas doenças humanas e a peculiar variação antigênica observada em tais espécies, foram caracterizadas, neste estudo, as lipoproteínas associadas a membranas (LAMP) de *Mycoplasma penetrans* e *Mycoplasma fermentans*. Para definir peptídeos com possível valor diagnóstico, empregamos as técnicas de ELISA e de Western blot usando soros de gestantes cujas amostras cervicais foram positivas por PCR. Por meio do ELISA foram observados anticorpos IgG anti-LAMP-*M. fermentans* em 57,5% e IgM em 74,5% das amostras. As três amostras PCR positivas para *M. penetrans* apresentaram anticorpos IgG anti-LAMP-*M. penetrans* e uma amostra positiva para IgM. IgA não foi detectada em nenhuma das espécies. A análise da LAMP, por Western blot, revelou como principais proteinas imunoreativas: 35, 38, 42, 61 and 103 kDa para *M. penetrans* e 29, 38, 41, 61, 78 and 95 kDa de *M. fermentans*. Dentre estas podemos considerar p35 específica para *M. penetrans* e p29, *M. fermentans*.Tais proteinas são promissoras como marcadores em diagnóstico.

Palavras-chave: LAMP-peptídeos, *Mycoplasma penetrans*, *Mycoplasma fermentans*, ELISA, Western blot

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