Studies on the phospholipid composition of pathogenic cell membranes of *Mycoplasma hyopneumoniae*

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The membrane phospholipid and fatty acid compositions of *Mycoplasma hyopneumoniae*, a pathogen of porcine enzootic pneumoniae isolated in China, was studied by thin-layer chromatography and gas chromatography. The results showed that membrane phospholipids consisted predominantly of diphosphatidylglycerol. The percentage of C16 – C18 fatty acids comprised 79% of the total fatty acids, of which oleic acid as well as palmitic acid are the major fatty acids. Some differences were shown in fatty acid composition as compared with membranes of other species of *Mycoplasma*.

(Mycoplasma hyopneumoniae) *Membrane phospholipid* *Gas chromatography* *Diphosphatidylglycerol* *Phosphatidylglycerol*

1. INTRODUCTION

Mycoplasmas are minute prokaryotic micro-organisms with a rather simple structure. As it lacks a cell wall and intracellular membrane system, pure membranes can easily be obtained, thus making them very useful as a model for studies of the plasma membranes. Mycoplasmas are also known as the causative agents of diseases in domestic animal, poultry as well as in human and plants. *Mycoplasma hyopneumoniae* is the pathogen of a worldwide enzootic pneumoniae of pigs. For the overall control of this major chronic problem, the study of structure and function of *M. hyopneumoniae* membrane would be of great importance. But little is known about the pathogenicity and membrane properties especially on the molecular level. The major difficulty lies in the cultivation of *M. hyopneumoniae* to obtain a sufficient amount for the investigation. Investigations on physical, biochemical and morphological aspects of the membranes of *Acholeplasma laidlawii* [1,2], *M. gallisepticum* [3,4], *M. hominis* [5] and *M. pneumoniae* [6] were reported. Studies on the pathogenicity and immunogenicity suggested that the glycolipids were as hapten in *M. pneumoniae* [7]. The cytopathogenic effect of purified *M. hyopneumoniae* membranes on rabbit kidney primary tissue cultures provides evidence of the virulence of the membranes [8]. But so far studies on the membrane lipids of *M. hyopneumoniae* have not been reported. This report describes lipid compositions and fatty acid constituents of pathogenic *M. hyopneumoniae* membranes studied by thin-layer chromatography and gas chromatography.

2. MATERIALS AND METHODS

2.1. *Materials*

Fatty acid methyl ester and phospholipid standards were purchased from Sigma.

M. hyopneumoniae strain 168, isolated in China, was obtained from the Institute of Animal Husbandry and Veterinary Medicine, Jiangsu Academy of Agricultural Sciences. Growth medium was as described by Goodwin et al. [9]. The inoculum containing 10^8 color-changing units (CCU) of *M. hyopneumoniae* per ml was diluted 1:9 with the growth medium and incubated at 37°C for 3–4 days until the pH of the medium dropped to 7.0.

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2.2. *Preparation of cell membranes*

Cells harvested by centrifugation at 14 000 × *g* for 15 min, were lysed by osmotic shock. The supernatant obtained by centrifugation at 14 000 × *g* was recentrifuged at 48 000 × *g* for 30 min. The membrane pellet was washed twice with 1:20 *β* buffer and suspended for use.

2.3. *Phospholipid analysis*

Lipids were extracted by the method of Folch et al. [10] with minor modifications. The membrane suspension was extracted with a mixture of chloroform and methanol (2:1, v/v). A biphasic system was obtained by mixing the combined supernatants with redistilled water and chloroform. The phospholipids contained in the lower phase were applied to a silica gel H thin-layer plate. Chloroform-methanol-acetic acid-water (25:7:3:0.8, v/v) was used as the solvent system. For two-dimensional thin-layer chromatography, the solvents were chloroform, methanol, 7 M ammonium hydroxide and water (180:180:11:11, v/v) in the first dimension and chloroform, methanol, acetic acid and water (25:7:3:70.8, v/v) in the second dimension.

The relative amounts of phospholipids were estimated quantitatively by scanning with a CS-910 double wave thin-layer chromatographic scanner. The fatty acid compositions were analyzed in a GC-7AC instrument with capillary column.

3. RESULTS AND DISCUSSION

Gas chromatographic results (table 1) showed that C16 and C18 fatty acids were 79% of the total fatty acids, in which oleic acid and palmitic acid were 31.13 and 32.66%, respectively, higher than all the others. There were significant differences in fatty acid composition compared with membranes of other Mycoplasma species.

The fatty acid constituents of *M. hyopneumoniae* membrane depend more on the fatty acid species present in the growth medium. Since long chain saturated and unsaturated fatty acids could not be synthesized by many mycoplasmas themselves, these fatty acids in membrane lipids were obtained from culture medium. In a medium supplemented with oleic acid, palmitic acid and stearic acid, significant amounts of these fatty acids in the *A. laidlawii* cell membrane can be detected [11]. These fatty acid contents in *M. pneumoniae* markedly increased 2.5–9.6-times, when cultured in medium which contained saturated, unsaturated and *β*-hydroxy fatty acids [12]. The fatty acid contents with C16 and C18:1 in phosphatidylglycerol (PG) of *M. capricolum* cell membrane were 80% of total

Table 1

Fatty acid composition of total membrane lipid from *M. hyopneumoniae* strain 168 membrane

Lipids	Chain length: no. of double bonds	% of total
Lauric	12:0	8.36
Myristic	14:0	7.21
Palmitic	16:0	31.13
Palmitoleic	16:1	1.61
Stearic	18:0	6.67
Oleic	$18:1_c$	29.16
	$18:1_t$	3.50
Linoleic	18:2	7.08

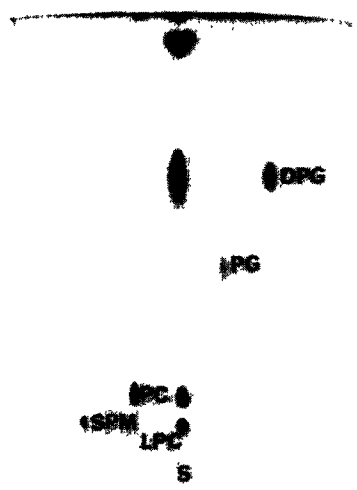

Fig.1. Thin-layer chromatogram of phospholipid composition of *M. hyopneumoniae* strain 168, on laboratory-made silica gel H plate. Eluent: chloroform, methanol, acetic acid, water (25:7:3:0.8, v/v). 1. LPC; 2. SPM; 3. PC; 5. PG; 6. DPG; 4 and 7 unidentified; S. sample.

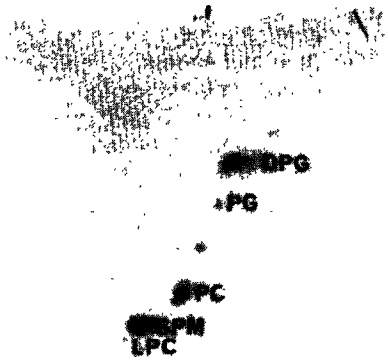

Fig.2. Two-dimensional chromatogram of phospholipid composition of *M. hyopneumoniae* strain 168, on laboratory-made silica gel H plate. Eluents: first direction - chloroform, methanol, ammonia, water (180:108: 11:11, v/v); second direction - chloroform, methanol, acetic acid, water (25:7:3:0.8, v/v).

acids [4] and in *M. hyopneumoniae* strain 168 cell membrane about 3/4 of the total.

As to the phospholipid composition, 7 spots could be separated on the one-dimensional thin-layer chromatogram which were defined as PG, diphosphatidylglycerol (DPG), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyelin (SPM) as compared with the standards (fig. 1). Two-dimensional thin-layer chromatography further demonstrated the above results (fig.2). For quantitative estimation, the relative amount of phospholipids was calculated from the corresponding peak area corrected by lipid standard (fig.3, table 2): DPG, 40.5%; PG, 3.2%; PC, 21.8%; SPM, 29.9% and LPC, 0.9%.

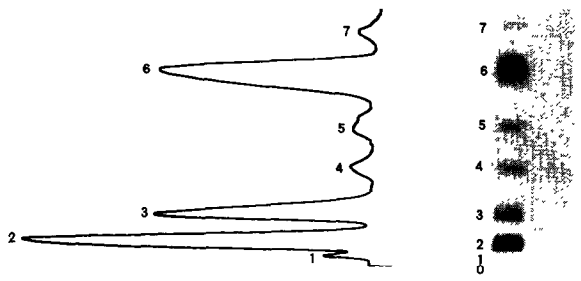

Fig.3. Scanning of thin-layer chromatogram of phospholipid of *M. hyopneumoniae* strain 168; peak sequence corresponds to the spot seen on the chromatogram.

Table 2

Phospholipid composition of *M. hyopneumoniae* strain 168 membrane

Lipid compound	% of total
1 Lysophosphatidylcholine	0.9
2 Sphingomyelin	29.9
3 Phosphatidylcholine	21.8
4 Unidentified	2.4
5 Phosphatidylglycerol	3.2
6 Diphosphatidylglycerol	40.5
7 Unidentified	1.3

In past years the studies on membrane lipids of mycoplasma were mainly in *A. laidlawii, M. gallisepticum* and *M. hominis*. Little was known about the membrane phospholipids of *M. hyopneumoniae*. The results showed that PG and DPG were the major phospholipids in membrane of mycoplasma cells except *A. laidlawii* where glycophospholipids were also found [13]. Only *M. hominis* (ATCC 15056) contained PG, phosphatidic acid (PA) and lysophosphatidylglycerol (LPG) [14]. Our results showed that DPG and PG were also the major phospholipids in *M. hyopneumoniae* strain 168 membrane, DPG about 40% of the total phospholipids and PG 3% only. The amount of PG in phospholipids of *M. gallisepticum* A 5969 was 46%, but no DPG was found. *A. laidlawii* AEF 22 contains 19% PG when equimolar ratios of palmitic acid and oleic acid were added into culture medium [13]. In *M. pneumoniae* membrane PG was about 60%. Rottem and Markowitz [3] reported that the amount of SPM and PC in *M. gallisepticum* A 5969 was increased with increasing of horse serum, however the amount of PG did not change. So they suggested that PG was synthesized by the organism itself, while SPM and PC were incorporated into membrane from culture medium. In our results SPM, PC and LPC in *M. hyopneumoniae* membrane may also have originated from culture medium.

It is well known that protein is antigenic, but only recently was lipid antigenicity recognized [15] and is becoming a new trend in immunology. Good correlation between antigen and immunochemical activity in some diseases and its phospholipid constituents phosphatidylethanolamine (PE), LPC, SPM, PC, PA and DPG has been reported. An-

tigens of high sensitivity and specificity are those antigens which contain DPG and PC. The high sensitivity and specificity of DPG antigen make it a unique lipid antigen in the diagnosis of syphilis. Lipid antigen can also be used in the diagnosis of other infectious diseases such as mycoplasmosis, tuberculosis, etc. Supplement of PC to mycobacteria and gonococcus lipid causes increase or enhanced decrease of immunochemical activities of the antigen. PG which constitutes 87% of the phospholipids in *M. hominis* was reported to be immunologically active [5]. Due to the difficulty in cultivation of *M. hyopneumoniae* and its low yield, the isolation of membrane lipid and study of the lipid composition were not noticed so far. Our report presents the phospholipid composition of *M. hyopneumoniae* membranes in which DPG is the predominant lipid. It is interesting to study the antigenicity of these phospholipids as a whole or individually, which may shed some light on the pathogenicity and immunogenicity of *M. hyopneumoniae*.

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