J Vet Clin Med 18(2) : 93-97(2001)

Changes of Intracellular Calcium after Administration of Pathogenic and Non-pathogenic Mycoplasmas in Porcine Ciliated Tracheal Cells

Seung-chun Park[1], Walter H. Hsu*, Hyo-in Yun, Kyu-sik Jung, Kwang-ho Jang, Tae-ho Oh, Keun-woo Lee and Seong-joon Yi**

College of Veterinary Medicine, Kungpook National University, Daegu 702-701
**College of Veterinary Medicine, Iowa State University, Ames 50011*
***College of Veterinary Medicine, Chungnam National University, Daejon 305-764*

돼지 기관지 섬모상피에서 병원성 및 비병원성 마이코플라즈마 투여후 세포내 칼슘의 변화

박승춘[1] · Walter H. Hsu* · 윤효인 · 정규식 · 장광호 · 오태호 · 이근우 · 이성준**

경북대학교 수의과대학, *아이오와 수의과대학
**충남대학교 수의과대학

초 록 : 병원성 *Mycoplasma hyopneumoniae* strain 91-3, 비병원성 *M. hyopneumoniae* 그리고 *M. flocculare*를 돼지의 기관지섬모상피에 투여시 세포내 Ca^{2+} 농도 $[Ca^{2+}]_i$의 변화를 본 연구에서 조사하였다. *M. hyopneumoniae strain* 91-3 (300 µg/ml)를 투여시 기관지 섬모상피내의 Ca^{2+}가 투여전과 비교시 투여 후 250±19 nM (net increase) 증가하였다 (10회 반복 47 cells). 이와는 대조적으로 비병원성 *M. hyopneumoniae* (300 µg/ml) (6회반복 18 cells)와 *M. flocculare* (300 µg/ml) (8회 반복 24 cells)는 세포내 Ca^{2+}의 농도를 증가시키지 못하였다. 위의 결과로 병원성 *M. hyopneumoniae* 91-3 균주는 비병원성 mycoplasma와는 다르게 돼지의 섬모상피에서 $[Ca^{2+}]_i$을 유도하였으며 이러한 특성은 mycoplasma 감염증 치료에 중요한 단서를 제공할 뿐만 아니라 새로운 치료법의 개발에 유용한 스크리닝 기술에 응용될 것으로 기대된다.

***Key words* :** Mycoplasma hyopneumoniae, M. flocculare, Intracellular calcium

Introduction

Mycoplasma pneumonia of swine, caused by *Mycoplasma hyopneumoniae*, is a worldwide, economically important swine disease[24,27]. Lack of knowledge about the pathogenic mechanisms and virulence factors involved in *M. hyopneumoniae* infection is a limiting factor in the development of highly effective treatment of mycoplasmal pneumonia of swine. The role of *M. hyopneumoniae* infection in association with other swine respiratory pathogens has gained increased importance. For instance, PRRSV-induced pneumonia is potentiated by M. hyopneumoniae induces pneumonia by damage to ciliated epithelial cells of the trachea, bronchi and bronchioles[3,4,20,24]. However, the mechanisms underlying *M. hyopneumoniae*-induced ciliary damage or loss of cilia are not well-understood. Recently, a tracheal epithelial cell model has been developed to enable us to study the pathogenesis of *M. hyopneumoniae* strain 91-3[17,23,24].

The adherence of *M. hyopneumoniae* strain 91-3 to ciliated epithelium is necessary to induce colonization of the organism on the epithelium, which results in the loss of cilia. The adherence process is mainly mediated by receptor-ligand interactions[6,24-28]. To be consistent with this concept, virulent strains of *M. hyopneumoniae* strain 91-3 adhere to tracheal tissue in vitro, whereas avirulent strains of *M. hyopneumoniae* do not[24]. We noted that an increase in the Ca^{2+} concentrations of the medium resulted in the loss of cilia[5,27]. This indicates that *M. hyopneumoniae* strain 91-3 may increase $[Ca^{2+}]_i$ of respiratory epithelium, which serves as an intracellular signal to induce the cilia loss. The objective of the present study, therefore, was to investigate whether the pathogenic *M. hyopneumoniae* strain 91-3 can induce $[Ca^{2+}]_i$ in epithelial cells.

Materials and Methods

Mycoplasmas

A pathogenic *M. hyopneumoniae* strain 91-3, originally cloned from strain 232, shows high adherence to cilia in a microtiter adherence assay[27]. A non-pathogenic *M. hyopneumoniae* J, originated from ATCC strain 25934, which does not adhere to cilia[28]. *M. flocculare* strain Ms42, originated

[1]Corresponding author.
E-mail : parksch@bh.knu.ac.kr

from ATCC strain 27399, is known as a non-pathogen in swine[7]. These mycoplasmas were cultured in a waterbath shaker with 50 rpm at 37°C for 48-96 h. Mycoplasmas were grown by logarithmic phase and harvested by centrifugation at 12,000 g for 30 min to remove Friis media. Following centrifugation, the mycoplasma pellets were collected and suspended with 50 ml phosphate buffer saline (PBS). The suspended pellets were centrifuged at 15,000 g for 15 min. This process was repeated once. The pellets were dispersed through a 27-gauge needle in PBS. The concentration of intact mycoplasma proteins were determined using bicinchoninic acid (BCA assay) (Pierce, Rockford, IL). The final cell protein of mycoplasmas was diluted to as 3 mg/ml in PBS (equivalent to 10^{10}-10^{10} CCU). Color changing units (CCU) represented the highest dilution culture changing the color of a tube of Friis mycoplasmal medium from red to yellow.

Ciliated tracheal cells

To prepare ciliated tracheal epithelial cells, the tracheas were removed from 3-month-old mycoplasma free pigs anesthetized with sodium pentobarbital using aseptic techniques[22,24]. The ciliated cells were isolated by enzyme digestion using 0.15% pronase and 0.01% DNase in Ca^{2+}- and Mg^{2+}-free MEM media and incubated at 4°C for 24 h. Fetal bovine serum (10%) was added to stop enzyme reaction. To collect epithelial cells, the tubes were centrifuged at 125 g for 5 min. The cell pellets were resuspended in a mixture of Dulbeccos MEM (high glucose) (DMEM) and Hams F-12 (1:1) containing 5% FBS, 0.12 U/ml of insulin, and 100 U/ml of penicillin-strptomycin. Cell suspensions were transferred to 90 mm tissue culture dishes and incubated in 5% CO_2 for at least 1 h to remove fibroblasts. A portion of the cell suspension was diluted in 0.04% trypan blue for counting and assessing viability. The tracheal epithelial cells were stored at 125°C until use.

$[Ca^{2+}]_i$ measurement in tracheal cells

To measure $[Ca^{2+}]_i$ in single epithelial cells, the tracheal cells were loaded with 4 M fura-2 acetoxymethyl ester (fura-2AM) in Krebs-Ringer bicarbonate (KRB) buffer solution containing (in mM): 136 NaCl, 4.8 KCl, 1.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 HEPES, 5.5 glucose and 0.1% BSA, pH 7.4 and incubated for 30 min at 37°C. The loaded cells were centrifuged (700 g, 2 min), then resuspended with KRB at a concentration of 500-1,000 cells/ml. The tracheal cells loaded with fura-2AM were plated onto the poly-lysine-coated coverslip in a custom-made petri dish. The dish containing fura-2 loaded cells was mounted on the stage of an inverted fluorescence microscope (Carl Zeiss, NY). Fluorescence images were obtained (excitation wavelengths of 334 and 380 nm; emission wavelength of 510±20 nm), background subtracted, and divided on a pixel-by-pixel basis to generate spatially resolved maps of $[Ca^{2+}]_i$. The emitted signals were digitalized, recorded and processed using the Attofluor digital fluorescence imaging system (Atto instruments, Rockville, MD)[9]. After reading fluorescence for 150 sec, mycoplasmas were loaded. $[Ca^{2+}]_i$ was calculated as previously described[12]. To calibrate the fluorescence signals, the ratio of fluorescence at 334 nm to fluorescence at 380 nm was compared with the ratios obtained at maximal extracellular Ca^{2+} (10 mM $CaCl_2$) and Ca^{2+} (10 mM Ethyleneglycoltetraacetic acid).

Administration of Mycoplasmas

To compare the $[Ca^{2+}]_i$ of tracheal cells response to pathogenic *M. hyopneumoniae* strain 91-3, and avirulent *M. hyopneumoniae* and *M. flocculare*, the cells were treated with the same concentration of 300 μg/ml. We did further investigate on the relationship of response-concentration of M. hyopneumoniae strain 91-3 with concentrations of 100 μg/ml and 30 μg/ml. Four to ten ciliated single tracheal cells in each were selected to investigate the $[Ca^{2+}]_i$ changes. Results were analyzed by analysis of variance or by Student *t*-test. The significance was evaluated at $P<0.05$.

Results

Changes after administration of mycoplasmas in ciliated tracheal cells

To investigate the differences of three mycoplasmas in terms of $[Ca^{2+}]_i$ in the ciliated tracheal cells, we firstly selected only ciliated tracheal cells, which were beating, by calcium measuring instrument equipped with microscope (×400). As shown in Fig 1A and 1B, the $[Ca^{2+}]_i$ in the ciliated cell increased (to) 250±19 nM (47 cells in 10 experiments) after administration of pathogenic *M. hyopneumoniae* strain 91-3 (300 μg/ml). The induced $[Ca^{2+}]_i$ was varied from cell (206 nM) to cell (308 nM). However, non-pathogenic M. hyopneumoniae (300 μg/ml) and *M. flocculare* (300 μg/ml) did not significantly increase $[Ca^{2+}]_i$ (8±2 nM, 18 cells in 6 experiments and 2±2 nM, 24 cells in 8 experiments) in the selected tracheal cells (Fig 1C, D, E and F). This indicates that the ciliated tracheal epithelial cells may have specific receptors to the pathogenic *M. hyopneumoniae* starin 91-3, but not to the non-pathogenic *M. hyopneumoniae* and *M. flocculare*.

Response-concentration of the pathogenic *M. hyopneumoniae* 91-3 in ciliated tracheal cells

To investigate the level of $[Ca^{2+}]_i$ after administration of 30, 100 and 300 μg/ml of *M. hyopneumoniae* 91-3, the density of the intact pathogenic *M. hyopneumoniae* strain 91-3 was calculated as mg/ml to increase accuracy of density. The reason is that CCU has deviation of 100-fold in the number of mycoplasma. In addition, this may be a convenient method in the study of exact dosing. After administration of 300,

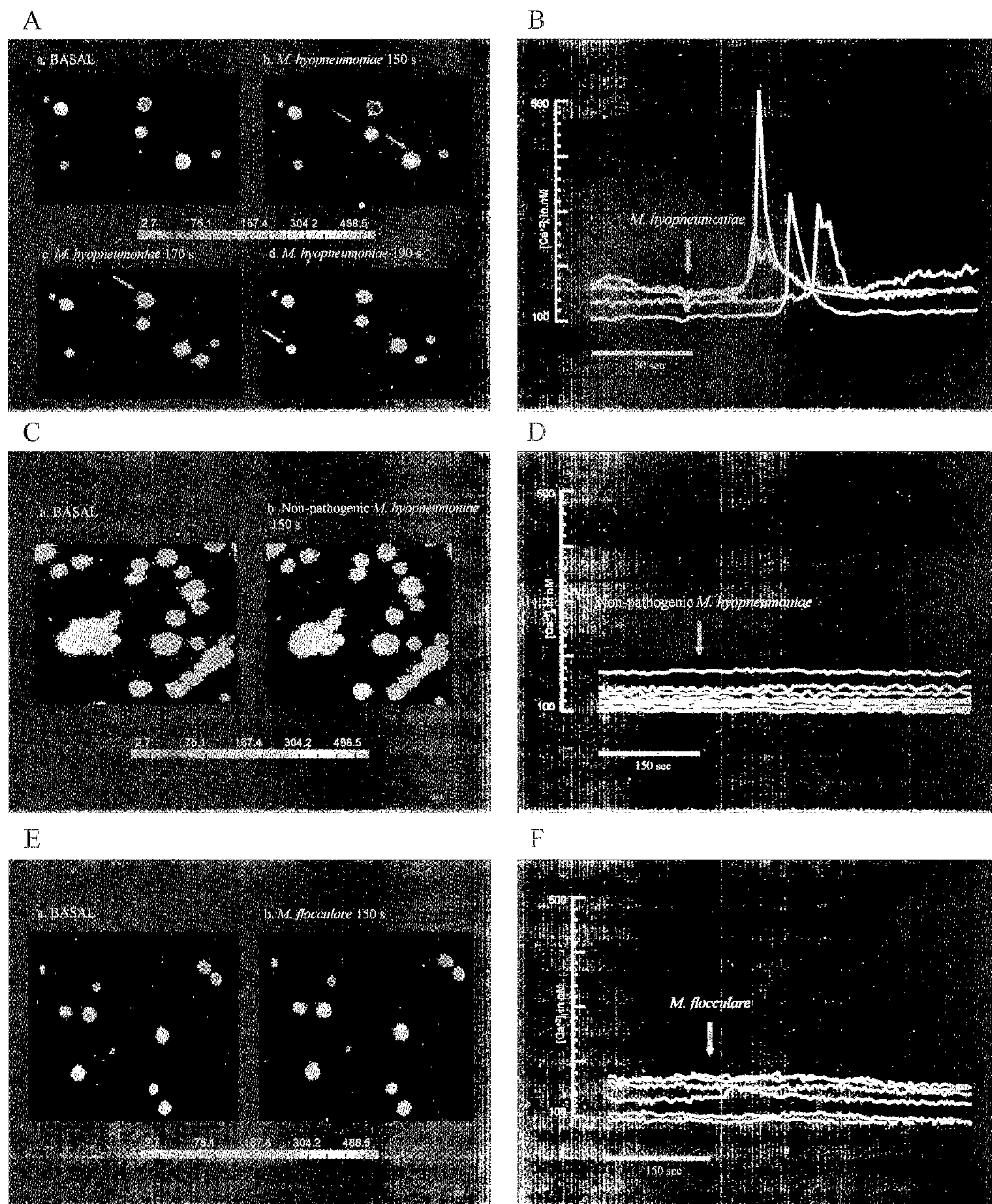

Fig 1. Representive photographs (A) and traces (B) showing the effects of the pathogenic *M. hyopneumoniae* strain 91-3 (300 μg/ml), photographs (C) and traces (D) showing the effects of the non-pathogenic *M. hyopneumoniae* (300 μg/ml), and photographs (E) and traces (F) showing the effects of the non-pathogenic *M. flocculare* (300 μg/ml) in the fura 2AM-loaded porcine cilliated tracheal cells. Mycoplasmal strains were administered at 150 sec.

100 and 30 μg/ml of the organism, $[Ca^{2+}]_i$ in the ciliated cells was significantly increased in a dose dependent manner with ranges of 22±9, 110±9 and 250±19 nM, respectively (Fig 2). This indicates that the interaction of *M. hyopneumoniae* 91-3 and ciliated tracheal cells may be involved in receptor reactions.

Discussion

In a report, *M. hyopneumoniae* 91-3 strain was studied in the differential epithelial cells for the interaction between mycoplasmas (*M. hyopneumoniae* 91-3, *M. hyopneumoniae* and *M. flocculare*) and tracheal epithelial cells[18,24]. They administerd 5×10^8-10^9 of mycoplasmas to the above system. According to their results, the pathogenic *M. hyopneumoniae* 91-3 strain attached to the cilia, inducing tangling,

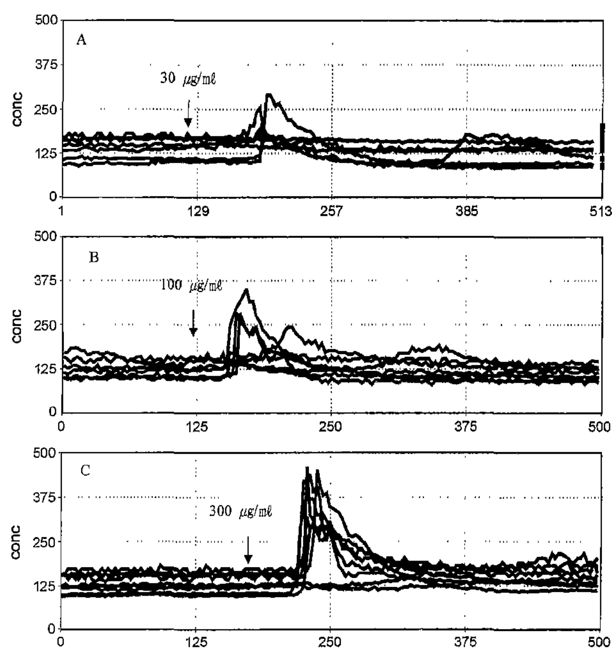

Fig 2. Representive traces showing the effects of $[Ca^{2+}]_i$ response in ciliated porcine tracheal cells to pathogenic *M. hyopneumoniae* strain 91-3. The pathogenic *M. hyopneumoniae* strain 91-3 was administered with dose of (A) 30 (n = 6, total 18 cells), (B) 100 (n = 7, total 16 cells), and (C) 300 µg/ml (n = 10, total 47 cells).

clumping and longitudinal splitting within 90 min after the addition of mycoplasmas to the cells[25]. However, adherence to the cilia by the mycoplasma organism is not sufficient to explain the pathogenesis of mycoplasmal pneumoniae.

In this study, ciliated epithelial cells were selected by microscope (×400) for investigating $[Ca^{2+}]_i$ in the different porcine tracheal cells. The reason is that *M. hyopneumoniae* strain 91-3 causes ciliary damage by interaction between its surface protein (adhesins) and receptors located on the cilia [10,14,23,24,26,28]. After the administration of *M. hyopneumoniae* strain 91-3, the magnitude of the $[Ca^{2+}]_i$ increase in response to M. hyopneumoniae strain 91-3 varied greatly from cell to cell. In addition, we found that the higher the density of *M. hyopneumoniae* strain 91-3 was loaded to ciliated tracheal cells, the greater Ca^{2+} responses was induced, even but no response at all at low density of mycoplasma cilia cells at low density of mycoplasma in the present study. The basis of this different sensitivity was not determined but may arise from variations in the expression, density, or sensitivity of the mycoplasma receptor[6,10,18]. This heterogenicity of Ca^{2+} response in the intact airway epithelial cells was very similar to the heterogenous Ca^{2+} responses to ATP_o reported in glial cells[22], bile duct cells[15], megakaryocytes[19] and chondrocytes[2]. In airway cells of rabbits, Korngreen et al[13] also reported that the heterogenicity of Ca^{2+} response was due in the sensitivity of individual cells to ATP_o.

The pathogenic *M. hyopneumoniae* strain 91-3 increased $[Ca^{2+}]_i$ in porcine ciliated tracheal cells, but non-pathogenic M. hyopneumoniae and *M. flocculare* failed to do so. The $[Ca^{2+}]_i$ response was a rapid event and the $[Ca^{2+}]_i$ increase was dependent on the mycoplasma density. Debey et al[7] reported that $[Ca^{2+}]_i$ increase was induced after administration of *M. hyopneumoniae* strain 91-3 (10^7-10^{10} CCU) in neutorphils isolated from pigs but a non-pathogenic stain did not so. The mechanism to increase $[Ca^{2+}]_i$ in the neutrophils has not yet been elucidated. Also, the role of neutrophils in the pathogenesis of *M. hyopneumoniae*-induced disease is not known.

The pathogenic *M. hyopneumoniae* strain 91-3 (10^9 CCU) has known to adhere to cilia, inducing tangling, clumping and longitudinal splitting but the non-pathogenic *M. hyopneumoniae* strain did not show obvious ciliary damages[24]. The reason for the above phenomenon may be explained by the presence of receptors on the ciliated tracheal epithelial cells for the pathogenic *M. hyopneumoniae* strain 91-33[24,25-28]. Therefore $[Ca^{2+}]_i$-changes in the tracheal epithelial cells could be provided as a key in the pathogenesis of maycoplasma infection.

Ismaili et al[12] have investigated on $[Ca^{2+}]_i$ increase by intact verocytotoxin-producing Escherichia coli (5×10^9 CFU) in cultured Hep-2 cells, comparing with enteropathogenic E. *coli* (5×10^9 CFU). They also found that $[Ca^{2+}]_i$ was increased by intact verocytotoxin-producing *E. coli* via IP_3 pathway1. Intact *S. typhimurium* (1.6×10^{10} CFU/ml) induces $[Ca^{2+}]_i$ increase, which mediates Ca^{2+}-mediated activation of the NF-kB pathyway for epithelial IL-8 expression[8,16]. To our knowledge, this is the first report showing $[Ca^{2+}]_i$ increase by pathogenic mycoplasmas in the ciliated tracheal cells. Along with this line, we are recently investigating how to increase $[Ca^{2+}]_i$ in the ciliated cells.

Acknowlegments

The work was supported by the Korea Research Foundation Grant KRF-99-G019.

References

1. Baldwin TJ, Ward W, Aitken A, Knutton S, Williams PH. Elevation of intracellular free calcium levels in HEp-2 cells infected with enteropathogenic *Escherichia coli*. Infect. Immun 1991; 59: 1599-1604.
2. Andrew P, Vittur F. $[Ca^{2+}]_i$ oscillations and intercellular Ca^{2+} waves in ATP-stimulated articular chondrocytes. J Bone Miner Res 1996; 11: 946-954.
3. DeBey MC, Ross, RF. Ciliostasis and loss of cilia induced by *Mycoplasma hyopneumoniae* in porcine tracheal organ cultures. Infect Immun 1994; 62: 5312-5318.
4. DeBey MC, Jacobson CD, Ross RF. Histochemical and morphologic changes of porcine airway epithelial cells in

response to infection with *Mycoplasma hyopneumoniae*. Am J Vet Res 1992; 53: 1705-1710.
5. Debey MC, Roth JA, Ross RF. Enhancement of the increase in intracellular calcium concentration in stimulated neutrophils by *Mycoplasma hyopneumoniae*. Vet Res Commun 1993; 17: 249-257.
6. Franzoso G, Hu PC. Meloni GA, Barile MF. The immunodominant 90-kilodalton protein is localized on the terminal tip structure of *Mycoplasma pneumoniae*. Infect Immun 1993; 61: 1523-1530.
7. Friis NF. Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare*: a survey. *Nord Vet Med* 1975; 27: 337-339.
8. Gewirtz AT, Rao AS, Simon PO, Merlin D, Carnes D, Madara JL, Neish AS. *Salmonella typhimurium* induces epithelial IL-8 expression via Ca^{2+}-mediated activation of the NF-kB pathway. *J Clin Invest* 2000; 105: 79-92.
9. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260: 3440-3450.
10. Hsu TS, Minion FC. Cloning and functional analysis of the p97 swine cilium adhesin gene of *Mycoplasma hyopneumoniae*. J Bacteriol 1997; 179: 1317-1323.
11. Hsuan SL, Kannan MS, Jeyaseelan S, Prakash YS, Sieck GC. Maheswaran SK. *Pasteurella haemolytica* A1-Derived leukotoxin and endotoxin induce intracellular calcium elevation in bovine alveolar macrophages by different signaling pathways. Infect Immun 1998; 66: 2836-2844.
12. Ismaili A, Philpott DJ, Dytoc MT, Sherman PM. Signal transduction responses following adhesion of verocytotoxin-producing *Escherichia coli*. Infect Immun 1995; 63: 3316-3326.
13. Korngreen A, Ma W, Priel Z, Silberberg, SD. Extracellular ATP directly gates a cation-selective channel in rabbit airway ciliated epithelial cells. J Physiol 1998; 508: 703-720.
14. Mebus CA, Underdahl NR. Scanning electron microscopy of trachea and bronchi from gnotobiotic pigs inoculated with *Mycoplasma hyopneumoniae*. Am J Vet Res 1997; 38: 1249-1254.
15. Nathanson MH, Burgstahler AD, Mennone A. Boyer JL. Characterization of cytosolic Ca^{2+} signaling in rat bile duct epithelia. Am J Physiol 1996; 271: G86-96.
16. Pace J, Hayman MJ. Galan JE. Signal tranduction and invasion of epithelial cells by *S. typhimurium*. Cell 1993; 72: 505-514.
17. Rogers MJ, Simmons J, Walker RT, Weisburg WG, Woese CR, Tanner RS, Robinson IM, Stahl DA, Olsen G, Leach RH. Maniloff J. Construction of the mycoplasma evolutionary tree from 5s rRNA sequence data. Proc Natl Acad Sci 1985; 82: 1160-1164.
18. Tajima M, Yagihashi T. Interaction of *Mycoplasma hyopneu-moniae* with the porcine respiratory epithelium as observed by electron microscopy. Infect Immun 1982; 37: 1162-1169.
19. Tertyshnikova S. Fein A. $[Ca^{2+}]_i$ oscillation and $[Ca^{2+}]_i$ waves in rat megakaryocytes. Cell Calcium 1997; 21: 331-344.
20. Thacker E, Halbur P, Ross RF, Thanawongnuwech R. & Thacker BJ. *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. J Clin Microbiol 1999; 37: 620-627.
21. Van den Pol, AN, Finkbeiner SM & Cornell-Bell, A.H. Calcium excitability and oscillations in suprachiasmatic nucleus neurons and glia in vitro. *J Neurosci* 1992; 12, 2648-2664.
22. Wallace P, Kennedy JR, Menndicino J. Transdifferenctiation of outgrowth cells and cultured epithelial cells from swine trachea. In Vitro Cell Dep Biol 1994; 30: 168-180.
23. Woese CR, Stackebrandt E, Ludwig W. What are mycoplasmas: the relationship of tempo and mode in bacterial evolution. J Mol Evol 1984; 21: 305-316.
24. Young TF, Thacker ET, Zimmerman BE, Ross RF. A tissue culture system to study respiratory ciliary epithelial adherence of selected swine mycoplasmas. Vet Microbiol 1999; 71: 269-79.
25. Zhang Q, Young TF, Ross RF. Glycolipid receptors for attachment of *Mycoplasma hyopneumoniae* to porcine respiratory ciliated cells. Infect Immun 1994; 62: 4367-4373.
26. Zhang Q, Young TF, Ross RF. Microtiter plate adherence assay and receptor analogs for *Mycoplasma hyopneumoniae*. Infect Immun 1994; 62: 1616-1622.
27. Zhang Q, Young TF, Ross RF. Identification and characterization of *Mycoplasma hyopneumoniae* adhesin. Infect Immun 1995; 63: 1013-1019.
28. Zielinski GC, Ross RF. Adherence of *Mycoplasma hyopneu-moniae* to porcine ciliated respiratory tract cells. Am J Vet Res. 1993; 54: 1262-1269, 1993.