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Proteomic Analysis of the Interactions between *Mycoplasma hyopneumoniae* and Porcine Tracheal Ciliated Cells

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Abstract *Mycoplasma hyopneumoniae* colonizes at the porcine respiratory-ciliated epithelial cells and causes the enzootic pneumonia of swine. The adhesion step is crucial in the colonization process. A few adhesion molecules have been characterized, and the concurrent receptors from the porcine ciliated cells have also been suggested to recognize the adhesion molecules. In the present study, the interactions between *M. hyopneumoniae* and porcine tracheal ciliated cells were investigated by employing the Far-Western blotting method. The results indicate that aconitase, lamin A/C, and peroxiredoxin of the porcine tracheal ciliated cell may interact specifically with the mycoplasmal proteins. We speculate that these mycoplasmal proteins could be secreted cleavage products, and their relative small size may enable them to penetrate into ciliated cells interfering with important metabolic pathways and other critical cellular processes.

Keywords *Mycoplasma hyopneumoniae* · Pathogen-host interactions · Porcine tracheal ciliated cells · Proteomics · Virulence factors

Abbreviation

2-DE	Two-dimensional gel electrophoresis
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Introduction

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumoniae. Initially, tracheal ciliated cells are colonized by a binding process through specific molecular recognitions and interactions. Colonization may not only disrupt the normal function of the mucociliary escalator through ciliostasis, loss of cilia, but may also predispose the host to severe infections from secondary pathogens [1]. Since *M. hyopneumoniae* does not penetrate its host cells, it is thought that its membrane surface components and unidentified secreted agents may be critically involved in pathogenesis [1, 2].

For the past years, significant progress has been made to identify possible factors by which mycoplasmas may interact with and damage the host cells [2]. Factors such as arginine depletion, IgA protease, and peroxide secretion have been suggested [3-6]. The adhesion of mycoplasmas to host cell surface may also interfere with the functions of various membrane receptors and transporters [7]. For example, it has been demonstrated that *M. hyopneumoniae* may disrupt the potassium channels of porcine tracheal ciliated cells and lead to ciliostasis [7, 8]. It has also been reported that the attachment of *M. hyopneumoniae* may transduce the signal to the nearby G protein and eventually stimulate the phospholipase C pathway to increase intracellular Ca^{+2} concentration [9]. However, neither the mycoplasmal factor nor the receptor molecule on the porcine tracheal ciliated cell has been identified so far.

The present study aims to identify the possible factors involved in the host–pathogen interactions by a proteomic approach. The total proteins of the porcine tracheal ciliated cells were separated by two-dimensional gel electrophoresis (2-DE), blotted onto nitrocellulose membrane, and reacted with *M. hyopneumoniae*. The tracheal ciliated cell proteins which reacted specifically with the mycoplasmal proteins were, thus, identified by the rabbit antiserum against *M. hyopneumoniae* strain 232.

Materials and Methods

Bacterial strains and antisera M. hyopneumoniae strain 232 (passage 28) was a virulent strain isolated and obtained from Dr. Ross at Iowa State University [10]. The mycoplasmal strains were grown in a modified Friis broth at 37°C, 80 rpm for 2 to 3 days and harvested at late-log phase as the indicator (phenol red) turns to yellow [11]. Mycoplasma cells were harvested by centrifugation at 4°C, 18,000×*g*, for 10 min. The cells were washed extensively (three times) with phosphate-buffered saline (PBS; 137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl, pH 7.53) to remove the remaining medium constituents. Protein concentrations were determined by OD_{595} with an ELISA reader (Dynatech, USA) using the PIERCE Bradford Protein Assay Kit (PIERCE, USA). The hyperimmune serum from rabbit was prepared against the total proteins of *M. hyopneumoniae* strain 232, and the IgG antibodies were further purified from the antiserum by a modified protein-A method [11, 12]. The goat anti-rabbit IgGs conjugated with alkaline phosphatase were obtained from Promega (USA).

Collection of porcine tracheal ciliated cells Tracheal ciliated cells were isolated by a modification of the previously described method [13]. The 2- to 3-month-old specific pathogen-free pigs of Lan-Yu mini pig bloodlines were maintained, and the experiments were performed at Tzu-Chi University Animal Center. The protocols in handling animals and in conducting the experiments were permitted by the Regulatory Committees of

Experimental Animals from both National Dong Hwa University and Tzu Chi University. To summarize, the pigs were put into euthanasia with ketamine and isoflurane before exposing their lungs and tracheas. The tracheas were removed, rinsed with PBS, and dissected. The ciliated cells were collected from the tracheas with a cell scraper (Nunclon Corp., USA) and put in PBS buffer, and the cell bodies were removed by centrifugation at 500×*g* for 5 min at 4°C. The supernatant-containing cilia was then harvested by centrifugation at 4°C, 18,000×*g* for 15 min and washed with PBS twice before suspending (approx. 10^5 cilia/mL) in the coating solution (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6). Approximately 5 mg cilia proteins were obtained from a pig weighing 20–25 kg.

2-DE and MALDI-TOF MS analysis The porcine ciliated cells were prepared and washed thoroughly with PBS and suspended in the total protein lysis buffer (8 M urea, 4% CHAPS, 65 mM dethiothritol, 5 mM tributyl phosphine, 0.5% ampholytes, and 0.002% bromophenol blue). After incubation for 15 min, the sample solution was centrifuged (12,000×*g* for 30 min at 4°C), and the supernatant was used for 2-DE analysis. The conductivity of the sample solution was measured (120–200 μS/cm) with the CDM210 conductivity meter (MetaLab, USA). The 2-DE was performed in an IPGphore apparatus (Pharmacia Corp., USA) and a SE600 Ruby gel apparatus (Hoefer Corp., USA) for the SDS-PAGE. To summarize, the protein samples of 350 μL (170–600 μg protein) were loaded onto the IPG strip (17 cm, pI 3–10, BIO-RAD Corp., USA), covered with 1 mL cover oil, and proceeded to IEF focusing at 20°C with the following settings: 50 V, 16 h for active rehydration; 250 V, 40 min; 10,000 V, 8 h until 50,000 V h. After the focusing step, the strips were treated with 3 mL SDS equilibration buffer (6 M urea, 30% (*v*/*v*) glycerol, 2% SDS (*w*/*v*), 0.2% dethiothritol, and 50 mM Tris-HCl, pH 8.8) for 15 min at room temperature prior to SDS-PAGE analysis. The IPG strips were run vertically onto 12% SDS-PAGE. After electrophoresis, the gels were either silver stained or Coomassie blue stained. For the silver staining process, we used Silver Stain Plus Staining Kit, (BIO-RAD, USA). The gels were first incubated 30 min with the fixing solution (40% ethanol, 1.7 M acetic acid), then 30 min in the sensitizing solution (30% ethanol, 0.125% glutaldehyde, 0.2% sodium thiosulfate, 0.83 M sodium acetate), and finally washed three times with dH_2O. To get better resolution in MS analysis, we used the BIO-RAD Coomassie Brilliant Blue R-250 Staining Solutions Kit (BIO-RAD, USA) to stain the gels [14]. The 2-DE gel maps were analyzed with the ImageMaster program (2D Platinum Software version 5.0; Amersham Corp., USA). Protein samples of interests were manually excised from the 2-DE gel and subjected to in-gel digestion step prior to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis.

In-gel digestion Protein spots were excised from the Coomassie blue-stained gels and digested with trypsin. The gel pieces were shrunk by dehydration in acetonitrile, dried, and incubated in 25 mM ammonium bicarbonate containing 10 mM dithiothreitol (DTT) for 30 min at 56°C. After cooling to room temperature, the DTT solution was replaced with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 30 min at room temperature in the dark. The gel pieces were washed two times with (1:1) 50 mM ammonium bicarbonate and acetonitrile, dehydrated by addition of acetonitrile, swollen in 100 mM ammonium bicarbonate, dehydrated in acetonitrile, and vacuum-dried. The gel pieces were swollen in a digestion buffer containing 25 mM ammonium bicarbonate and 20 ng/μL of porcine trypsin (Sigma, USA) at 37°C. After 30 min, the supernatant was first removed, suspended with the digestion buffer without trypsin, and incubated at 37°C overnight. Peptides were extracted and concentrated two times with (1:1) acetonitrile and 0.1% trifluoacetic acid (TFA) in order to get better MALDI spectra.

Mass spectrometry and database search MALDI spectra were acquired using an autoflex time-of-flight mass spectrometer (Bruker Daltonic, Germany) equipped with a 337-nm nitrogen laser. Spectral data were obtained in the reflectron mode with an acceleration voltage of 19 kV. Each mass spectrum was derived from a summation of 50 scans. α-Cyano-4-hydroxyl cinnamic acid (CHCA) was used as a MALDI matrix, which was prepared by dissolving CHCA in a solvent mixture of (1:1) acetonitrile and 0.1% TFA at a concentration of 50 mM. The MS analysis of each protein digest was conducted immediately after the in-gel digestion. An aliquot (0.5 μL) of the sample solution was mixed with an equal volume of the matrix solution, applied on the target plate, and dried before MALDI-MS analysis. The spectra were calibrated externally using a peptide mixture that contained angiotensins I and II, substance P, ACTH [1-17], ACTH (18–39), and bombesin.

The peptide ion mass spectra were searched against the NCBInr (http://www.ncbi. nlm. nih.gov) database using the MASCOT (http://www.matrixscience.com) algorithm. Peptide masses obtained from the digestion of keratin and trypsin were excluded from the MS data before the analysis. The maximum number of missed cleavages was set at one, and mass accuracy was set at 150 ppm. Other parameters included enzyme (trypsin), peptide charge (1+), and variable modifications (carbamidomethyl-Cys; oxidation-Met). The top-matched protein, which has a Mowse score above the threshold value with $p<0.05$, was considered as positive identification.

Far-Western experiments—interaction between the porcine ciliated cell proteins and the M. hyopneumoniae 232 proteins The porcine ciliated cell proteins were separated by 2-DE, transferred to NC paper, blocked with skim milk for 2 h, and subsequently reacted with 20 mL solution containing *M. hyopneumoniae* 232 proteins (1 μg/mL in TBST) at 4°C overnight. The nonbound proteins were washed away by PBS (three times, 250 mL each time) at 4°C. The ciliated cell proteins bound with the mycoplasmal proteins were detected by the rabbit IgGs against *M. hyopneumoniae* 232 [11, 12] followed by goat anti-rabbit IgGs conjugated with alkaline phosphatase. The interacting proteins were, thus, identified and the corresponding proteins in the 2-DE gel were picked and analyzed by MALDI-TOF MS.

Results

In the present study, the host (porcine tracheal cell)–pathogen (*M. hyopneumoniae strain 232*) interactions were investigated by the proteomic approach. The 2-DE experiments and MS analysis have been repeated over three times to achieve high degree of reproducibility. As shown in Fig. 1a, the total soluble proteins of porcine ciliated cell were first separated by 2-DE. The silver-stained gels usually resolved over 300 protein spots, while the Coomassie blue stained gels (Fig. 1b) obtained less than 300 spots. Direct recognition of porcine proteins by the IgGs was examined first. As shown in Fig. 2a, the porcine ciliated cell proteins recognized by the IgGs are mainly in region B of pI near 7 and MW near 70 kDa, region C of pI 5–6 and MW around 50 kDa, and region D of pI 5–6 and higher MW. A few spots were also scattered around in region E of pI 6.5–7.5 and MW 33.8–27.0 kDa, a spot in region F near 30 kDa and pI 9.2, and two spots in region H of lower MW near pI 8.

Then, the Far-Western experiments were performed in a duplicate blot where the blotted porcine proteins were further interacted with the mycoplasmal proteins. The mycoplasmal proteins interacted nonspecifically with the NC paper were thoroughly washed away, but

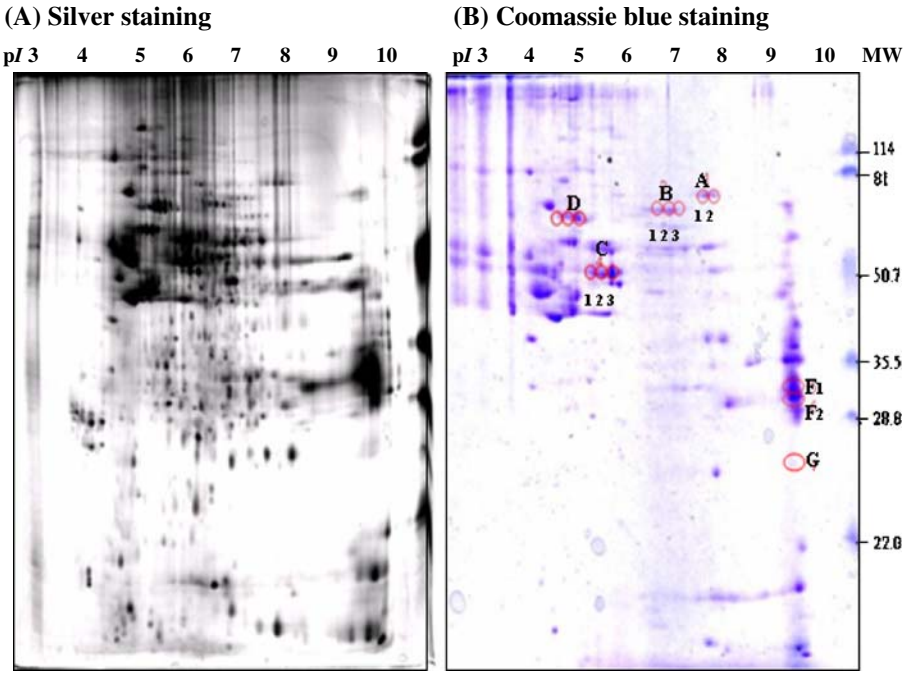

Fig. 1 The 2-DE gel patterns of the total proteins isolated from porcine tracheal ciliated cells. **A** Silver stained gel for reference. **B** Coomassie blue stained gel for protein spots identification

the tightly bound proteins were still there and detected by the IgGs. When comparing Fig. 2a, b, noticeable difference in region A where new spots near pI 7 and approximately 65 kDa were observed. However, spots in regions C and D appear much lighter, while the spots in regions E and H are almost invisible as compared with the corresponding regions in Fig. 2a. The spot at region F becomes denser, and a new low MW spot of approximately 25 kDa, near pI 9–10, appears in region G. All of the proteins that appear more or are newly emerged in Fig. 2b may represent the porcine proteins which interacting specifically with *M. hyopneumoniae* and deserve further identification. The corresponding protein spots were thus excised from the Coomassie blue stained 2-DE gel (Fig. 1b) for MALDI-TOF MS analysis. We used the silver-stained 2-DE gel (Fig. 1a) as a reference to help localize the protein spots, while the confusion and problems caused by the silver staining on MS analysis were avoided by using Coomassie blue staining for the samples in MS analysis. As shown in Fig. 1a, the protein spots A1, A2, and G are the only unique protein signals present after the protein–protein interactions. Proteins in spots B1–B3 and F1 and F2 are more pronounced than the background. On the other hand, the protein spots C1–C3 are about the background due to the direct recognition of porcine proteins by the IgGs. Each of these protein spots were excised for in-gel digestion experiments and further MALDI-TOF MS analysis. Protein spots with Mowse score >100 (according to the probability based Mowse score, protein scores >76 were considered significant, with $p<0.05$) were included in Table 1. Mascot analysis identified the protein spots A1 and A2 as aconitase with reasonably close MW and corresponding pI 7.7 and 7.96. The protein spots B1–B3 were identified as lamin A/C [15], with varied pI values, fairly good sequence coverage, and peptide matching. The protein spot G was identified as peroxiredoxin with good matching.

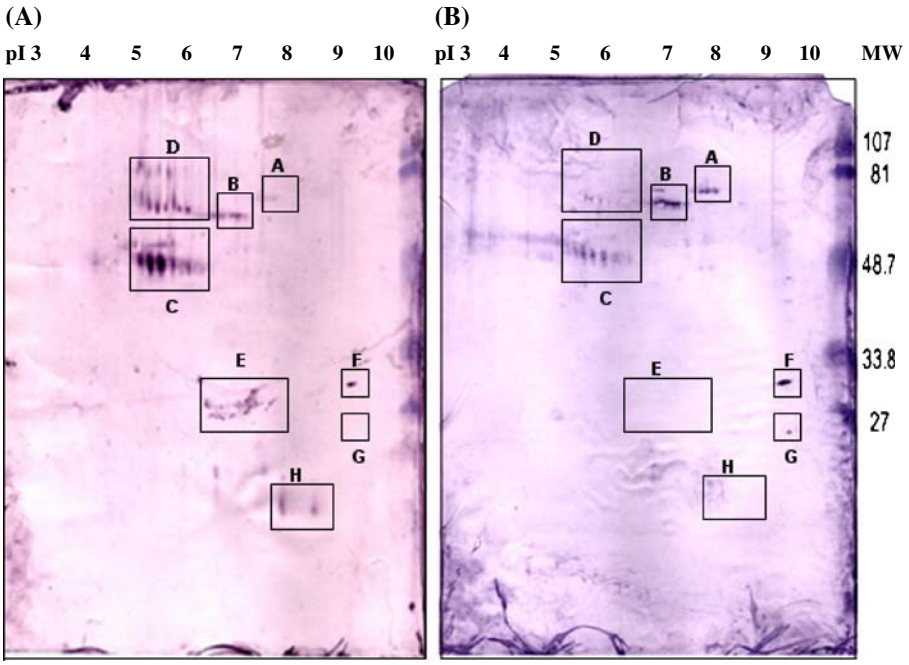

Fig. 2 Far-Western analysis of the interaction between the total proteins of porcine tracheal ciliated cell and *M. hyopneumoniae* strain 232**. A** The total proteins of the porcine ciliated cell were first separated by 2-DE, transferred to NC paper, and detected by using rabbit antiserum against *M. hyopneumoniae* 232. **B** In the duplicate experiment, the porcine ciliated cell proteins were separated by 2-DE, transferred to NC paper, and then reacted with *M. hyopneumoniae* 232 proteins. The ciliated cell proteins bound with *M. hyopneumoniae* 232 proteins were then detected by using the rabbit IgGs against *M. hyopneumoniae* 232

The protein spots C1–C3 were identified as cytokeratin with the same apparent MW and pI 5.6–6.0. The protein spots F1 and F2 expressed at higher levels than the corresponding spot in Fig. 2a were identified as carbonyl reductase.

Discussion

The present study indicates that the porcine ciliated cell proteins including aconitase, lamin A/C, and peroxiredoxin may be involved in the host–pathogen interactions. However, none of the three proteins are located on the membrane surface of the porcine tracheal ciliated cells as we expected intuitively for the sites of interacting proteins. The discrepancy may be partially due to that current 2-DE lysis buffer was for the total cellular proteins so that the crucial proteins on the membrane surface were lost under the experimental condition. Among the three interacting proteins, aconitases, formally called aconitate hydratase, present in both mitochondria and cytosol of cells. It catalyzes the reversible transformation of citrate to isocitrate. Aconitase activity is frequently viewed as a marker of oxidative stress in biological systems because the $[4Fe\text{-}4S]^{2+}$ cluster of the enzyme is readily inactivated by O_2^-, H_2O_2, and $ONOO^-$ [16].

Lamin A/C has been known to have functions in nucleoskeletal structure and cell division. In studying the cell-cycle-dependent dynamics of nuclear pore formation, the inner nuclear membrane proteins lamin A/C were observed richly in the large pore-free

Table 1 Identification of the protein spots from porcine tracheal ciliated cells which may interact with the proteins of *M. hyopneumoniae* strain 232.

Spot number	Matching protein name accession number	Mowse score	Theoretical MW/p*I*	Apparent MW/p*I*	Sequence coverage (%)	Peptide matched
A	Aconitase [*Bos taurus*] GI:5821963 Aconitase [*Sus scrofa*] NCBI NP_955119	108	82452/ 7.69	74000/7.7 (A1); 8.0 (A2)	33	20/35
B	Lamin A/C [*Homo sapiens*] GI:55957497	156	65096/6.4	68000/6.8 (B1);7.0 (B2); 7.2 (B3)	51	32/61
C	Cytokeratin 8 (*Bos taurus*) GI:61861942	117	42369/ 5.13	47000/5.6 (C1); 5.8 (C2); 6.0 (C3)	57	25/67
F	Carbonyl reductase [*Sus scrofa*] GI:47523198	138	25969/ 8.60	29000/8.8 (F1); 9.0 (F2)	60	22/65
G	Peroxiredoxin 1 [*Mus musculus*] GI:56103807	120	22162/ 8.26	23000/9.2	43	10/16

Included in this table are protein spots with convincing Mowse scores, the apparent MW/pI, sequence coverage, and peptide matched

islands of the nuclear membrane. Lamin A/C were suggested to play an essential structural and regulatory roles in the nuclear pore distribution [17]. In addition, lamin A/C and emerin were reported to have functions involving in the maintenance of nuclear envelope structure and simultaneously modulating the expression patterns of certain mechanics-sensitive and stress-induced genes [18].

Peroxiredoxin has the role in redox regulation of the cell and in eliminating peroxides generated during metabolism. Taken together, we can speculate that any interference affecting the function of these three enzymes may attenuate the antioxidant capability of the cells [19].

The molecular mechanism of *M. hyopneumoniae* in causing porcine enzootic pneumonia is still poorly understood. Neither does the pathogen invade the cells nor does it attach to the non-ciliated cells of the porcine respiratory tracts [20, 21]. In principle, the adherence process could be mediated by receptor–ligand interactions. So far, the porcine glycolipids La, Lb, and Lc have been reported as possible receptors for the adhesion of *M. hyopneumoniae* [13]. Recent studies also suggested that heparin may function as the binding factor of the mycoplasmal protein P159 [22]. Besides cell adhesion, it has been demonstrated that the pathogenic strain 232 of *M. hyopneumoniae* can increase the $[Ca^{2+}]_i$ in porcine ciliated tracheal cells, but the nonpathogenic J strain failed to do so, suggesting the critical role of the $[Ca^{2+}]_i$ induction in pathogenesis [9]. However, the (anti-*M. hyopneumoniae*) polyclonal antibodies which can inhibit the adherence of *M. hyopneumoniae* failed to block the induced increase of $[Ca^{2+}]_i$ in the tracheal epithelia. This result implies that the adherence alone does not mediate the increase of $[Ca^{2+}]_i$ in tracheal epithelia, and other soluble factors were proposed for the rise of $[Ca^{2+}]_i$ [23]. Based on the Far-Western data, we speculate that the intimate cell–cell contact may trigger the delivery of some soluble factors. The factors could be cleaved membrane components, secreted proteins, or peptides from *M. hyopneumoniae*. These components may interact directly or indirectly with the host counterparts after the cell–cell contact. It is worth noting that *M. hyopneumoniae* possess a sophisticated membrane protein secretion system, consisting of SecA, SecD, SecY, PrsA,

DnaK, Tig, and LepA [24]. Specific cleavages of some surface lipoproteins had been suggested to confer efficient secretion of extracellular products of *M. fermentans* [25]. Furthermore, the integrative conjugal element of *M. hyopneumoniae* has also been suggested to be involved in pathogenesis through delivery of effector molecules into the cells [26].

In conclusion, the proteomic studies reveal that the porcine tracheal ciliated cell proteins aconitase, lamin A/C, and peroxiredoxin may react specifically with either the mycoplasmal proteins or the peptides. Very conceivably, behind the cell–cell contact, some secreted or cleavage products and mycoplasmal membrane components may penetrate into the porcine tracheal ciliated cells and interfering with proteins or receptors which are critically involved in metabolic pathways such as stress responses.

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References

1. Ciprian, A., Pijoan, C., Cruz, T., Camacho, J., Torota, J., Colmenares, G., et al. (1988). *Canadian Journal of Veterinary Research, 52*, 434–438.
2. Razin, S., Yogev, D., & Nato, Y. (1998). *Mol Biol Rev, 62*, 1094–1156.
3. Gong, H., Zolzer, F., von Rechlinghausen, G., Rossler, J., Breit, S., Havers, W., et al. (1999). *Biochemical and Biophysical Research Communications, 261*, 10–14.
4. Cole, B., & Wells, D. (1990). *Infection and Immunity, 58*, 228–236.
5. Kapatais-Zoumbos, K., Chandler, D., & Barile, M. F. (1985). *Infection and Immunity, 47*, 704–709.
6. Almagor, M., Kahane, I., Gilon, C., & Yatziv, S. (1986). *Infection and Immunity, 52*, 240–244.
7. Izutsu, K. T., Fatherazi, S., Belton, C. M., Oda, D., Cartwright, F. D., & Kenny, G. E. (1996). *In vitro cellular and Developmental Biology Animal, 32*, 361–365.
8. Debey, M. C., & Ross, R. F. (1994). *Infection and Immunity, 62*, 5312–5318.
9. Park, S. C., Yibchok-anun, S., Cheng, H., & Young, T. F. (2002). *Infection and Immunity, 70*, 2502–2506.
10. Bereiter, M., Young, T. F., Joo, H. S., & Ross, R. F. (1990). *Veterinary Microbiology, 25*, 177–192.
11. Ro, L. H., Chen, R. J., & Shiuan, D. (1994). *Journal of Biochemical and Biophysical Perspectives Methods, 28*, 155–159.
12. Yang, W. J., Lai, R. F., Peng, K. C., Chiang, H. J., & Shiuan, D. (2003). *Journal of Immunological Methods, 304*, 15–29.
13. Zhang, Q., Young, T. F., & Ross, R. F. (1994). *Infection and Immunity, 62*, 1616–1622.
14. Shevchenko, A., Wilm, M., Vorm, O., & Mann, M. (1996). *Analytical Chemistry, 68*, 850–858.
15. Maeshima, K., Yahata, K., Sasaki, Y., & Nakatomi, R. (2006). *Journal of Cell Science, 119*, 4442–4451.
16. Han, D., Canali, R., Garcia, J., & Aguilera, R. (2005). *Biochemistry, 44*, 11986–11996.
17. Maeshima, K., Yahata, K., Sasaki, Y., Nakatomi, R., Tachibana, T., Hashikawa, T., et al. (2006). *Journal of Cell Science, 119*, 4442–4451.
18. Roux, K. J., & Burke, B. (2007). *Biochimica et Biophysica Acta, 1772*, 118–127.
19. Rabilloud, T., Heller, M., Gasnier, F., & Luche, S. (2002). *Journal of Biological Chemistry, 277*, 19396–19401.
20. Ross, R. F. (1992). In A. D. Leman, B. E. Straw, W. L. Mengeling, S. D. Allaire & D. J. Taylor (Eds.), *Diseases of swine* (pp. 537–551). Ames: Iowa State University Press.
21. Zielinski, G. C., & Ross, R. F. (1993). *Amer J Vet Res, 54*, 1262–1269.
22. Burnett, T. A., Dinkla, K., Rohde, M., Chhatwal, G. S., Uphoff, C., Srivastava, M., et al. (2006). *Molecular Microbiology, 60*, 669–686.
23. Hwang, M. H., Lim, J. H., Yun, H. I., Kim, J. C., Jung, B. Y., Hsu, W. H., et al. (2006). *Veterinary Journal, 172*, 556–560.
24. Binnewies, T. T., Hallin, P. F., Staerfeldt, H., & Ussery, D. W. (2005). *Microbiology , 151*, 1–4.
25. Davis, K. L., & Wise, K. S. (2002). *Infection and Immunity, 70*, 1129–1135.
26. Vasconcelos, A. T., Ferreira, H. B., Bizarro, C. V., et al. (2005). *Journal of Bacteriology, 187*, 5568–5577.