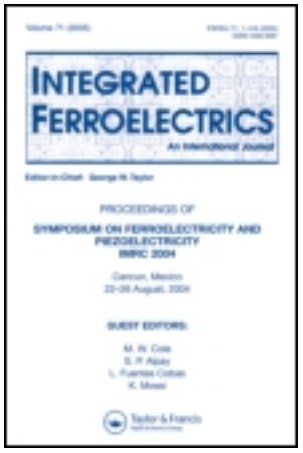

Integrated Ferroelectrics: An International Journal
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ginf20

Rapid and Label-Free Detection of Porcine Reproductive and Respiratory Syndrome Virus on Nanoscale by Biosensor Based on Imaging Ellipsometry

Yanyan Chen [b] , Cheng-Hong Huang [a] , Changjun Hou [a] , Danqun Huo [a] & Gang Jin [c]

[a] Key Laboratory of Biorheological Science and Technology, Ministry of Education, Bioengineering College , Chongqing University , Chongqing , 400030 , China

[b] Suzhou Institute of Nano-Tech and Nano-Bionics, CAS , Suzhou , 215123 , China

[c] Institute of Mechanics, CAS , Beijing , 100190 , China

Published online: 12 Jul 2013.

To cite this article: Yanyan Chen , Cheng-Hong Huang , Changjun Hou , Danqun Huo & Gang Jin (2013) Rapid and Label-Free Detection of Porcine Reproductive and Respiratory Syndrome Virus on Nanoscale by Biosensor Based on Imaging Ellipsometry, Integrated Ferroelectrics: An International Journal, 145:1, 122-129, DOI: 10.1080/10584587.2013.788963

To link to this article: http://dx.doi.org/10.1080/10584587.2013.788963

Integrated Ferroelectrics, 145:122–129, 2013

ISSN: 1058-4587 print / 1607-8489 online
DOI: 10.1080/10584587.2013.788963

Taylor & Francis
Taylor & Francis Group

Rapid and Label-Free Detection of Porcine Reproductive and Respiratory Syndrome Virus on Nanoscale by Biosensor Based on Imaging Ellipsometry

CHENG-HONG HUANG,[1] CHANGJUN HOU,[1]
DANQUN HUO,[1,*] YANYAN CHEN,[2] AND GANG JIN[3,*]

[1]Key Laboratory of Biorheological Science and Technology, Ministry of Education, Bioengineering College, Chongqing University, Chongqing 400030, China
[2]Suzhou Institute of Nano-Tech and Nano-Bionics, CAS, Suzhou 215123, China
[3]Institute of Mechanics, CAS, Beijing 100190, China

This study describes a simple and rapid method for virus detection on nanoscale measurement utilizing biosensor based on imaging ellipsometry. Porcine reproductive and respiratory syndrome virus (PRRSV) was served as an experimental model for demonstration. Staphylococcal protein A was firstly attached onto carboxyl-modified silicon substrate and purified anti-PRRSV IgG obtained from hyperimmune sera combined with Protein A for oriented immobilization to form capture layer. PRRSV in samples can then be specifically grabbed onto the capture layer. The signal (grayscale value) after virus combination is intuitively quantified by imaging ellipsometry. The minimum detection limit was as low as 2.4 × 10^3 median cell infectious dose ($CCID_{50}$ mL^{-1}) infectious virus. Application the proposed method to 20 clinic samples demonstrated that the result was significant correlated ($R = 0.85$) with virus isolation. The detection time was only 20 min expended.

Keywords Biosensor; Imaging Ellipsometry; PRRSV

1. Introduction

Acute lower respiratory tract infection is a major health problem in the world. Viruses that can cause such symptom are usually involved and highly pathogen avian influenza (H5N1) [1], severe acute respiratory syndrome (SARS) [2] and porcine reproductive and respiratory syndrome virus (PRRSV) [3] are mostly concerned. The former two mainly bring serious lesions to human beings together with some animals [4] and the latter mainly infringe pigs. In some cases, specific virus species can transmit between human beings and animals.

The indirect-ELISA is often carried out by immobilization of a structural protein/whole virus particle onto the solid plate and addition of a monoclonal antibody directed to the structure protein so that structure protein's concentration can be deduced after a secondary

Received August 1, 2012; in final form February 1, 2013.
*Corresponding authors. E-mail: huodq@cqu.edu.cn, gajin@imech.ac.cn

antibody originated from heterologous animals added, on which an indicator (alkaline phosphatase or horseradish peroxidase is usually used.) is previously labeled [5]. Due to an indirect method to verify virus infection by measuring its induced titers this may be confused when the pigs are so young that acquired enough maternal antibodies. RT-PCR/nested-PCR had been used to detect PRRSV but not suitable for variation strain which has not the ability to induce devastating infection, and these methods are complicated in manipulation, particularly requiring experienced operators and professional laboratory. Furthermore, high false positive rates [6] or false negative rates [7] are also occasionally reported. It was reported that PRRSV isolation mainly depends on cell cultivation, which Marco-145 cell is generally adopted [8]. This is more inconvenient with disadvantages of time-consuming, labor-intensive and difficult for acquisition of cell from specific pathogen-free pigs.

Compared with these techniques, biosensor based on imaging ellipsometry (BIE) is a comprehensive method for virus identification with distinguished features of simple and rapid [9–10]. It is a powerful tool for optical characterization of surfaces and thin films on nanoscale with a sub-nanometer resolution of vertical. In this study, PRRSV virus was used as a experimental model to illustrate the direct detection procedure. Herein, strategy by oriented immobilization of antibody for PRRSV detection is demonstrated. Anti-PRRSV immunoglobulin (IgG) is firstly oriented immobilized via protein A which is initially attached on carboxyl-modified silicon substrate to form capture layer. Then, PRRSV in clinic samples or purified sample will be specifically captured and its titer could be accordingly obtained from grayscale change measured by imaging ellipsometry.

2. Experiments

2.1 Reagents

Silicon wafers were purchased from General Research Institute for Nonferrous Metals (China). Purified anti-PRRSV immunoglobulin (IgG) was obtained from Yangzhou Unibio Co.Ltd (China). Clinic serum or tissues were obtained from suspected pigs in Jiangsu of China in 2007. The isolate PRRSV-N strain was used as reference, which has an infectivity titer of 10^8 median cell infectious doses (CCID50/ml) infectious virus. Heterologous species including porcine circovirus type 2 (PCV2), swine influenza virus (SIV), Escherica Coli O157:H7 and actinobacillus pleuropneumoniae (APP) are stored by our laboratory. Bovine serum (FBS) was purchased from Beijing Dingguo Co. (China). 3-aminopropyltriethoxysilane(APTES, 99%),1-(3-Dimethylaminopropyl)-3-ethylcarbobiimide hydrochloride (EDC), N-hydroxy-droxysuccinimde (NHS) and succinimide anhydride were obtained from ACROS. Water was obtained from a millipore Milli-Q ion exchange apparatus. Phosphate-buffered saline (PBS, 8 mM $Na_2PO_4{\cdot}2H_2O$, 2.68 mM KCl, 1.14 Mm KH_2PO_4, 137 mM NaCl; pH 7.4) was prepared in deionized water. PBST (0.05% tween-20) was used as washing solution.

2.2 BIE Detection Platform

BIE consists of an array system and imaging ellipsometry (IE) [9, 11]. The array system is used for surface patterning and array fabrication. IE is used for reading the protein array. The polydimethylsiloxane (PDMS) template in array system contains a 6×4 cell array. When silicon wafer is attached to the template surface, 24 individual chambers are formed independently as each chamber had two access holes where solution could pass in

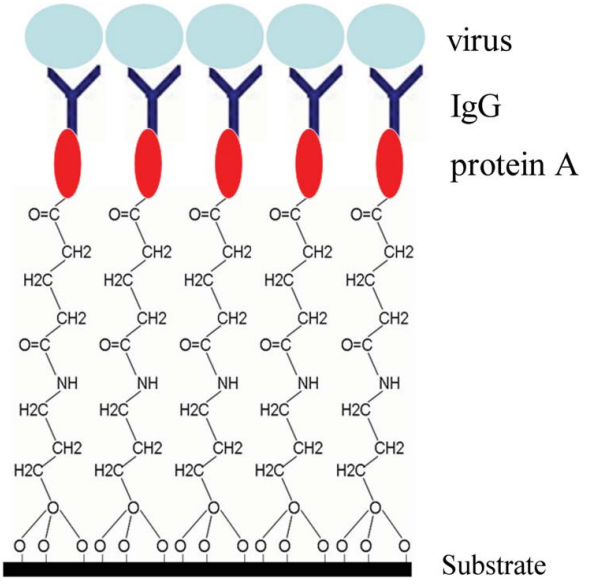

Figure 1. Oriented immobilization of IgG by protein A for virus detection. (Figure available in color online)

and out of the chamber through Teflon tubes connection. By such a microfluidic system, ligand solutions are delivered individually to different cells for immobilization under the vain pressure generated by micropump so that the surface is patterned homogeneously and simultaneously in array format [12]. IE is used for the visualization and quantification of the protein layer on the surface of silicon substrate. The detection configuration is illustrated in Fig 1. The detected signal density I is related to the thickness (d) of the protein layer according to $I = kd^2$, where k is a constant. The ellipsometry image was focused on the protein area of the CCD camera. A digital image was grabbed by and stored in a computer with a grayscale format (8 bits, 0–255 grayscale) for further evaluation by image-processing software of the program.

2.3 Silicon Treatment

Silicon wafer was initially treated with piranha solution (30% H_2O_2:70% H_2SO_4 = 1:3, v/v) for 30 min. After washed by deionized water, it was immersed in ethanol solution containing 3-aminopropyltriethoxysilane (5% APTES, ACROS) for 2 h incubation. After washed by ethanol and deionized water several times, the modified silicon wafer was placed in saturated succinic anhydride solution of ethanol for 12 h incubation. Then, the treatment was achieved. When the modified silicon wafer was applied to the array system, silicon wafer was initially pressed on the PDMS template. Small checks in array format with 6 × 4 layout will be formed. Each check can pattern a protein dot on silicon wafer.

2.4 Virus Detection by BIE

The treated silicon surface was firstly activated by solution of 1-(3-Dimethylaminopropyl)-3-ethylcarbobiimide hydrochloride and N-hydroxy-droxysuccinimde (EDC/NHS, 0.2/

0.5 mol/mL) at 5 μl/min for 5 min. After the activation completed, protein A (200 ug/mL, Sigma) was added to the corresponding array dot (20 μL/dot, at 1 μL/min) for 20 min immobilization. Later, anti-PRRSV (100 ug/mL, 30 uL) IgG was dispensed to the array dot to interact with protein A for 30 min. Next, the surface was blocked by 1 mg/mL BSA (30 μl, 1 μL/min). The array dots were rinsed with PBST (50 μL/dot, 10 μL/min). Thus, an array containing multiple protein dots was achieved.

Testing sample collected from pigs was pretreated orderly by pulverization with grinder, dissolution with PBS and centrifugation at 10,000 g for five minutes and subsequently served as trials. Concurrently, PRRSV-N strain and healthy sera/cell lysate was served as positive and negative control, respectively. When testing, total 30 μl sample was delivered to the array dot by microfluidic system to react 30 minutes, then, the silicon substrate was taken from the reaction template and flushed repeatedly by PBST and deionized water. The completed silicon wafer was finally dried by a stream of N2 and send to imaging ellipsometry for grayscale measurement.

2.5 Virus Isolation by Cell Culture

Virus isolation in vitro was performed as routine method as literature previously described [13]. Briefly, pulverized tissue was dissolved in PBS solution to form suspension which immediately centrifuged at 10,000 g for 10 minutes and filtered by a 450 nm filter. Two plates, one for trial and the other for blank control, were each inoculated with 500 μl filtrate and incubated at 37°C for 2 h. Then 10 ml Dulbecco's medium was added for further incubation. The plates were cultured continuously and observed daily with an inverted microscope. When obvious cytopathic effect with typical clumping or cleavage emerged and the blank carried normal shapes, the trial was judged as positive.

3. Results and Discussion

3.1 Analytical Sensitivity

To establish quantitative detection, supernatant without PRRSV as negative control and serial diluted positive samples were concurrently analyzed. A calibration curve was obtained by four-parameter logistic function fitting when the grayscale value was plotted to PRRSV titers (Fig 2.). The mean of grayscale value for negative control was 86.4 ± 1.6. The cut-off value of the BIE was calculated from 8 zero standard measurements. Mean of the blank control grayscale value plus three standard deviations $= 86.4 + 3 \times 1.6 = 91.2$. Sensitivity $2.4 \times 10^3 CCID_{50}mL$ infectious virus was obtained after mathematic transformation.

3.2 Analytical Specificity

Diseased pigs in the field often carried approximate symptoms caused by some heterologous species involved in porcine circovirus type 2 (PCV2), swine influenza virus (SIV), Escherica Coli O157:H7 and actinobacillus pleuropneumoniae (APP) and so on [14–16]. Specificity denoted mathematically as cross-reaction rate with PRRSV was estimated. The virus titer or bacteria concentration for above four species are 50% lethal dose (10^3 LD_{50}) for PCV2, 8log2 Hemagglutimation Inhibition (HI) for SIV, 2.1×10^7 colony formation unit (CFU) for E.Coli and 3.4×10^7 CFU for APP. Evaluation was carried out by calculation of the grayscale response. According to grayscale value changes of PCV2, SIV, *E.coli* and *App*, healthy pig sera (negative control) and PBS (blank control), the grayscale response

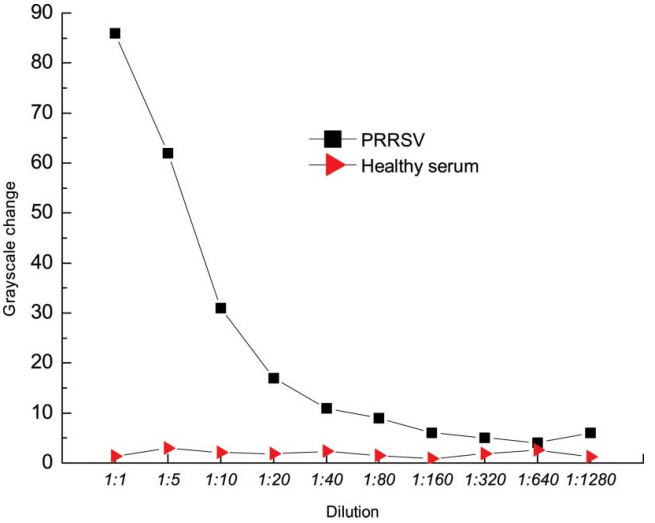

Figure 2. Sensitivity of the BIE detection. Different concentrations of purified PRRSv (from 1:1 dilution to 1:1024 dilutions) were applied to obtain limit of detection. (Figure available in color online)

percentage of these pathogen and PRRSV (2.4×10^8 $CCID_{50}$/mL) infectious virus were calculated (Fig. 3.). The grayscale values of the negative and blank control were both lower than the grayscale value of minimum content of PRRSV. This indicated that BIE has acceptable specificity for PRRSV detection.

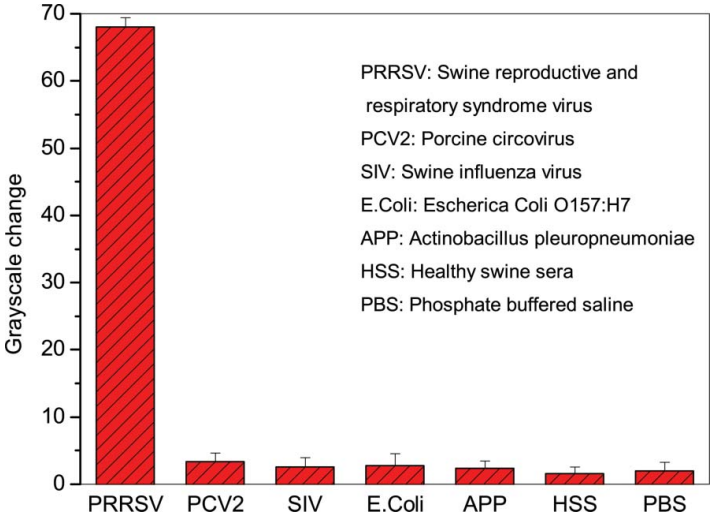

Figure 3. Specificity was assessed by heterologous species including porcine circovirus type 2 (PCV2, 10^3 LD_{50}), swine influenza virus (SIV, 8log2 HI), Escherica Coli O157:H7 (2.1×10^7 CFU) and actinobacillus pleuropneumoniae (APP, 3.4×10^7 CFU). (Figure available in color online)

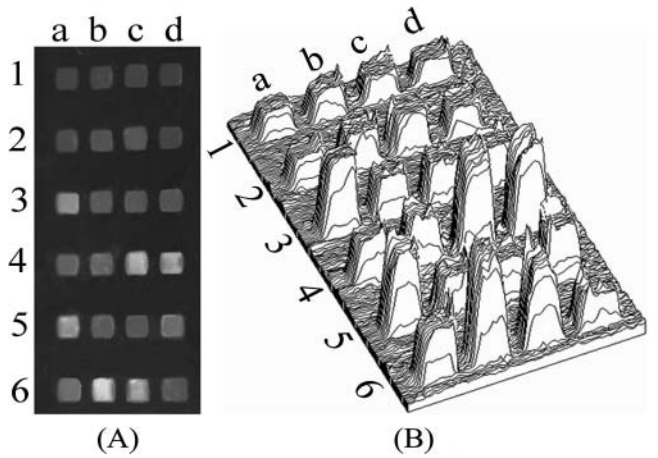

Figure 4. Ellipsometry grayscale images of clinic samples detected by BIE (A) and the corresponding three-dimension map (B). Dots from a1-d1 are negative control and dots from a2-d6 are clinic samples application. Detailed descriptions can be seen in text.

3.3 Analytical Reproducibility

Reproducibility was assessed by within-run and between-run analysis. The within-run was performed by 8 measurements in a same silicon substrate and the between-run was performed by 6 measurements in distinct silicon substrate. Coefficient variation (CVs) values of within-run and between-run are 5.8%, 4.6%, 7.6% and 6.5%, 6.9%, 7.8% for 2.4×10^6, 2.4×10^7 and 2.4×10^8 $CCID_{50}$/mL infectious virus, respectively. The CVs value denoted that the BIE detection for PRRSV has excellent reproducibility.

3.4 Methodology Comparison

Samples were taken from suspicious PRRSV pigs and total 20 samples were collected. Each (Lung tissue contains alveolar macrophage, AM) was frozen and thawed, and lysate was divided into two components. One was cultured in vitro and subsequently examined for PRRSV-induced cytopathic lesions. The other was applied to BIE system for virus detection. Fig. 4A shows the ellipsometric image. Four dots in row1 (a-d1) were served as negative control. Twenty dots (a2-d6) were samples application. According to the principle of off-null ellipsometry, on which the IE system is based, the optical intensity of the reflected beam is proportional to the square of thin thickness on a solid surface. It can be see from the image, as concentration increased, the protein dots turned more brightness and thickness distribution (Fig. 4B.). Each clinic sample was detected twice and the mean value was calculated. By virus isolation, 10 positive and 10 negative was acquired. By BIE analysis, 7 positives (dot a3, a5, a6, b6, c4, c6, and d5), 10 negatives (a2, b2, b3, b5, c2, c3, d2, d3, d4 and d6) and 3 suspicious (a4, b4 and c5) were obtained. According calculation, there was 85% accordance between BIE (7 positives and 10 negatives) and virus isolation (10 positives and 10 negatives).

4. Conclusions

PRRSV infection results in clinically normal, but persistently exist in serum, semen, lung tissue and other organs [17]. Positive rate by virus isolation in our results was a little

higher than the reported data. This contributed to the susceptible sample collected in acute stage with typical symptoms was available for virus isolation [18]. To achieve PRRSV detection for disease control, laboratory essentially needs more rapid and less cumbersome methods for pathogen direct identification. Current methods evolved are virus isolation, ELISA and RT-PCR. These methods are unable to detect antigen quickly due to its inherent sophisticated procedure, e.g. sample preparation, virus purification, dying, and so on, let alone requiring expensive equipment and skilled operators. However, BIE constructed a platform for rapid identification of virus (only 30 min was required). This method provided the platform that is capable of mass screening of clinic samples.

The use of polyclonal antibodies may improve the sensitivity and specificity. Although the components of polyclonal antibodies are complex, and the specificity is inferior to that of tests that use monoclonal antibody, some researchers are still inclined to choose polyclonal antibodies for the detection of antigens, because of their higher sensitivity [19]. The result in this study demonstrated that polyclonal antibody can be sensitive to PRRSV detection. The sensitivity of BIE for detection of PRRSV was approximately 2.4×10^3 $CCID_{50}$/mL. In fact, the specificity for polyclonal antibody is also excellent. Further progress should be placed on BIE miniaturization for large-scale popularization.

In summary, PRRSV can be detected by oriented immobilization of IgG via protein A initially attached on the silicon substrate. The proposed method has high sensitivity and excellent specificity. With simple array layout, multiple samples could be analyzed simultaneously with single experiment. Application of 20 clinic samples demonstrated that the result is correlated with virus isolation. It could be a alternative tool for PRRSV detection.

Acknowledgments

The authors are gratefully to Yuhe Li for his gift of purified PRRSV antisera and Junbin Liu for his collection of clinic sample, and they are both worked in Yangzhou Unibio Ltd., Yangzhou, China. Thanks also gifts to Natural Science Foundation (10804083, 20845003) of China and open funds (NJ2009-04) from key laboratory of Sichuan, China.

References

1. M. Hatta and Y. Kawaoka, The continued pandemic threat posed by avian influenza viruses in Hong Kong. *Trends in Microbiology*. **10**, 340–344 (2002).
2. C. Drosten, S. Günther, W. Preiser, S. v. d. Werf, H.-R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. M. Fouchier, A. Berger, A.-M. Burguière, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J.-C. Manuguerra, S. Müller, V. Rickerts, M. Stürmer, S. Vieth, H.-D. Klenk, A. D. M. E. Osterhaus, H. Schmitz, and H. W. Doerr, Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *The New England journal of Medical.* **348**, 1967–1976 (2003).
3. Y. Li, Emergence of a highly pathogenic porcine reproductive and respiratory syndrome virus in the Mid-Eastern region of China. *The Veterinary Journal*. **174**, 577–584 (2007).
4. Y. Guan, B. J. Zheng, Y. Q. He, X. L. Liu, Z. X. Zhuang, C. L. Cheung, S. W. Luo, P. H. Li, L. J. Zhang, Y. J. Guan, K. M. Butt, K. L. Wong, K. W. Chan, W. Lim, K. F. Shortridge, K. Y. Yuen, J. S. M. Peiris, and L. L. M. Poon, Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science*. **302**, 276–278 (2003).
5. P. G. W. Plagemann, Peptide ELISA for measuring antibodies to N-protein of porcine reproductive and respiratory syndrome virus. *Journal of Virological Methods*. **134**, 99–118 (2006).

6. C. Fetzer, S. Pesch, and V. F. Ohlinger, High risk of false positive results in a widely used diagnostic test for detection of the porcine reproductive and respiratory syndrome virus (PRRSV). *veterinary microbiology*, **115**, 21–31 (2006).
7. M. L. Landry, T. Eid, S. Bannykh, and E. Major, False negative PCR despite high levels of JC virus DNA in spinal fluid: Implications for diagnostic testing. *Journal of Clinical Virology*. **43**, 247–249 (2008).
8. K. D. Rossow, Porcine Reproductive and Respiratory Syndrome. *Veterinary Pathology.* **35** 1–20 (1998).
9. G. Jin, P. Tengvall, I. Lundström, and H. Arwin, A biosensor concept based on imaging ellipsometry for visualization of biomolecular interactions. *Anal Biochem*, **232**, 69–72 (1995).
10. C. Huang, J. Li, Y. Tang, Y. Chen, and G. Jin, Detection of duck hepatitis virus serotype1 by biosensor based on imaging ellipsometry. *Current Applied Physics*. **11**, 353–357 (2011).
11. G. Jin, R. Jansson, and H. Arwina, Imaging ellipsometry revisited: developments for visualization of thin transparent layers on silicon substrates. *Rev. Sci. Instrum.* **67**, 2930–2936 (1996).
12. Z.H. Wang and G. Jin, A Label-Free Multisensing Immunosensor Based on Imaging Ellipsometry. *Anal. Chem.* **75**, 6119–6123 (2003).
13. P. Jiang, P. Y. Chen, Y. Y. Dong, J. L. Cai, B. X. Cai, and Z. H. Jiang, Isolation and genome characterization of porcine reproductive and respiratory syndrome virus in P. R. China. *J Vet Diagn Invest*. **12**, 156–158 (2000).
14. T. Opriessnig, X.-J. Meng, and P. G. Halbur, Porcine circovirus type 2-associated disease: Update on current terminology, clinical anifestations, pathogenesis, diagnosis, and intervention strategies. *J Vet Diagn Invest*. **19**, 591–615 (2007).
15. R. C. Meyer, S. P. Saxena, and H. E. Rhodes, Polyserositis Induced by Escherichia coli in Gnotobiotic Swine. *Infection and Immnuity*. 41–44 (1971).
16. T. A. Marsteller and B. Fenwick, Actinobacillus pleuropneumoniae disease and serology. *Swine Health Prod*, **7**, 161–165 (1999).
17. Dennis, Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. *Veterinary Microbiology*. **86**, 213–228 (2002).
18. W. L. Mengeling, K. M. Lager, and A. C. Vorwald, Diagnosis of porcine reproductive and respiratory syndrome. *J Vet Diagn Invest*. **7**, 3–16 (1995).
19. W. Al-Nakib El-Mekki and R. Bibi, Factors affecting the detection of cytomegalovirus in urine by sandwich enzyme immunoassays. *Journal of Virological Methods*. **15**, 75–83 (1987).