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Berberine Effects Biofilm Formation and Expression of LuxS and Virulence Factors in *Streptococcus suis*

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Research Article

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ABSTRACT

Streptococcus suis (*S. suis*) is an important pathogen of pigs, responsible for diverse diseases in swine and human. It is found to form biofilm *in vitro* and *in vivo*. *luxS/AI-2* not only influences the formation of biofilm, but also bacterial virulence factors. Berberine is an isoquinoline-type alkaloid isolated from *Coptidis rhizome* and other herbs against bacteria. In this study, we observed that sub-minimal inhibitory concentrations (sub-MIC) of berberine (62.5 µg/ml) were sufficient to exhibit an antibacterial effect and to inhibit biofilm formation significantly, as shown by the scanning electron microscopy. Real-time PCR showed that berberine decreased the amount of *luxS*-mRNA lower than that of negative control. Quantification of expression levels of known virulence genes by real-time PCR revealed that berberine on the transcription levels of the *ef*, *sly* and *gapdh* genes of biofilm formation were downregulated, while the *gdh*, *cps* and *mrp* genes were upregulated. To summarize the collective data demonstrated that berberine may regulate transcription levels of *luxS/AI-2* and many virulence genes, and inhibit *S. suis* biofilm formation.

INTRODUCTION

S. suis is an important pathogen responsible for important economic losses to the porcine industry worldwide. It causes a variety of life-threatening infections, causing meningitis, septicemia, endocarditis, arthritis and pneumonia in pigs. *S. suis* infections have been reported in over 30 countries, and 35 serotypes (1-34 and 1/2) have been identified on the basis of capsular antigens [1]. *S. suis* has ability to form biofilms, which plays a key role in causing persistent infections [2].

Quorum sensing system plays an important role in the biofilm formation. Quorum sensing is an intercellular communication system by which bacterial cells are capable of indirectly monitoring their own population density through production and exchange of diffusible signal molecules [3]. This regulatory system involves the production of cell signaling molecules via the *luxS*-based autoinducer-2 (AI-2), the extracellular concentration of which increases as the cell density increases [4]. As the extracellular concentration of signaling molecules reaches a threshold, the bacteria can alter their gene expression in unison [5]. The *luxS* gene plays a vital role in biofilm formation, gene expression, motility and drug resistance [6]. In previous study, higher concentrations of AI-2 inhibited *S. suis* biofilm formation and host-cell adherence [7]. The virulence factors such as *cps*, *mrp*, *gapdh*, *sly*, *fbps*, *gdh* and *ef* in the mutant strains, which media the adhesion, invasion, colonization and proliferation capacity of *S. suis* to host cells. Thus, understanding the association between biofilm, virulence and quorum-sensing may lead to the development of novel strategies to fight infections.

There is an urgent need to develop alternative treatments for *S. suis* biofilm infections that are safe, effective and inexpensive. Herbal medicines have been used for centuries to treat various diseases. They also exhibit antibacterial, antiviral [8], anti-inflammatory and anticancer activities [9]. Among all of the strategies that have been exploited to overcome drug resistance, the use of natural substances has shown particular promise, and many natural substances also have antibiofilm properties [10-12]. Previous reports showed that the extracts of *Coptis chinensis* Franch and *Phellodendron amurense* Ruprecht have antimicrobial effect [13]. One of the major compounds of *C. chinensis* and *P. amurense* is berberine. Berberine, one of the most important

members of the protoberberine group of alkaloid, exhibits antimalarial, antisecretory, and anti-inflammatory as well as anticancer activities with relatively low cytotoxicity [14]. It has also been reported that berberine is useful in the treatment of gastroenteritis, diarrhea, and cholera diseases [15]. Previous studies have shown that berberine has antimicrobial activity against several bacterial species, and interferes with the adherence of *Streptococcus pyogenes* to host cells, either by preventing the complexing of lipoteichoic acid with fibronectin or by dissolution of such complexes once they are formed [16].

In most studies of the anti-infective activities of berberine, the focus has been on its antimicrobial effects, and little has been paid to the effects of berberine on the biofilm formation of *S. suis*. Here we focused on investigating the effect of berberine on the *luxS*/AI-2 QS system and virulence factors of biofilm formation of *S. suis*.

MATERIAL AND METHODS

Bacterial Strains and Culture Conditions

S. suis strain ATCC700794 was used in this study. It was grown in Todd-Hewitt broth (THB) containing 5% fetal bovine serum at 37 °C with shaking at 150 rpm to obtain a planktonic culture in mid-exponential growth phase.

Minimal Inhibitory Concentration (MIC)

MIC was the lowest concentration that inhibited the visible growth. MIC was determined for each compound by using a microdilution assay, as previously described [17]. Briefly, 100 µl 1.0 × 10⁶ cfu/ml *S. suis* (based upon McFarlane standards) was added to each well, microtiter plate was incubated for 24 h at 37 °C.

Quantification of Biofilm Formation

Biofilm formation was performed on the Calgary biofilm device (CBD) according to previously described methods with modifications [18]. Briefly, 200 µl 1.0 × 10⁶ cfu/ml *S. suis* (based upon McFarlane standards) was added to CBD. Incubated at 37 °C for 3 days without shaking.

Effect of Berberine on Biofilm Formation

100 µl THB broth containing berberine were added to the wells of a CBD plate. An overnight culture of *S. suis* was diluted in fresh culture broth to 1.0 × 10⁶ cfu/ml and 100 µl was inoculated into each well. All assays were performed in quintic. After incubation for 3 days at 37 °C, the content of the wells were then discarded and the pegs were washed three times using 200 µl sterile PBS to remove loosely adherent cells. The plate was stained with crystal violet. After fixing of methanol and then staining was measured at 595 nm.

Determination of Colony Forming Units (CFU)

To test the bactericidal activity of berberine against the bacteria in biofilm, 100 µl *S. suis* (1.0 × 10⁶ cells/ml) was added to the CBD and the method as described above. Then the microplate was sealed and sonicated for 10 min. The cells were plated on Petri dishes containing a solid medium in triplicate, and incubated at 37 °C for 2 days before they were counted. The means ± standard deviations were calculated.

Observation on Biofilm Formation by Scanning Electron Microscopy

In addition, Scanning electron microscopy (SEM) images were taken to confirm the prevention of biofilm formation. Biofilm formation on CBD was undertaken as above. Briefly, the Pegs were washed twice with PBS and then initially fixed by 2.5% glutaraldehyde for 20 h at 4°C. The surfaces were washed twice with PBS. The bacteria were then dehydrated by replacing the buffer with increasing concentrations of ethanol (50%, 70%, 90%, 100%) for 10 min each. After critical point drying and coating by gold sputter, samples were examined with a scanning electron microscope.

Real-Time PCR Analysis

Total RNA was isolated from *S. suis* grown as biofilms cells for 3 days. cDNAs were generated by reverse transcription. 16sRNA was used as internal control in real-time PCR (RT-PCR) analysis. RT-PCR was carried out according to the method previously described [19]. RT-PCR was performed in a total volume of 20 µl containing 10 µl of FastStart DNA Master SYBR GreenI, 1.2 µl of cDNA, 7.6 µl of ddH₂O, 0.6 µl of forward primer, and 0.6 µl of reverse primer. The related genes of primers used for the various RT-PCR assays are listed in **Table 1**.

Table 1. Primer Sequence.

Genes	Primer sequence
<i>luxS</i>	Forward: 5'- CGAGTTTGAAGAAATTGCAG -3'
	Reverse: 5'- AGCTGAATGAAGGCTGTGGT -3'
<i>gdh</i>	Forward: 5'- ACCTCTGGCTTTGGTGGT -3'
	Reverse: 5'- TGAAGGATTTACCGTTTGCTG -3'

<i>mrp</i>	Forward: 5'- TGGCACAGTTATCAAGGAACC -3'
	Reverse: 5'- TACCGTCAACACGAACCAAT -3'
<i>gapdh</i>	Forward: 5'- GCTGAAGAAGTAAACGCTGCT -3'
	Reverse: 5'- GTCGCATCAAACAATGAACC -3'
<i>sly</i>	Forward: 5'- AGTCAGTTTGGCACTCGTAGG -3'
	Reverse: 5'- TTGTGGCTCGTAAGTCAAGC -3'
<i>fbps</i>	Forward: 5'- GCTCCTGACCACCTATCTGC -3'
	Reverse: 5'- TCAAAGCCACATCCAATCA -3'
<i>ef</i>	Forward: 5'- GAACCAAGGAACCAATCG -3'
	Reverse: 5'- AACATTCTGACCACTCGCATC -3'
<i>cps</i>	Forward: 5'- TTGAATACGCAGAGCAAGA -3'
	Reverse: 5'- TAACCCTCCCGACAAATCAC -3'
16s RNA	Forward: 5'- TGCTAGTCACCGTAAGGCTAAG -3'
	Reverse: 5'- GGCTGCAAGATTCCTTGAT -3'

Statistical Analysis

Data were expressed as the mean \pm SD. AP value of <0.05 was considered significant. All statistical analyses were performed using a SPSS 20.0 Statistical Software. Data analysis was carried out using Prism Software.

RESULTS AND DISCUSSION

Effect of Berberine on Biofilm Formation

As biofilm formation is thought to be the leading to cause of persistent infection caused by *S. suis*. Therefore, we studied the influence of berberine on *S. suis* biofilm formation and biofilm CFU counts. Various concentrations of berberine were added to the biofilm plate to test whether berberine regulates *S. suis* biofilm formation. Wang et al. reported that berberine is active in vitro against *S. epidermidis* and that sub-MICs of berberine may play a role in the prevention of biofilm formation of *S. epidermidis* [20]. As well, we found the MIC of berberine against *S. suis* was determined as 62.5 $\mu\text{g/ml}$. After 3 days of exposure, we observed a decreased in the biofilm CFU counts. The biofilm was markedly decreased by incubation with 31.25 $\mu\text{g/ml}$ berberine for 72 h ($P<0.01$) (**Figure 1**). Decreased biomass was observed for berberine-treated bacteria compared to un-treated control in **Figure 2**. The *S. suis* cfu/ml decreased from 9.16 ± 0.90 cfu/ml in controls to 6.71 ± 0.11 cfu/ml in berberine-exposed isolates ($P<0.05$). The biofilm CFU counts significantly decreased when the cells were incubated with berberine.

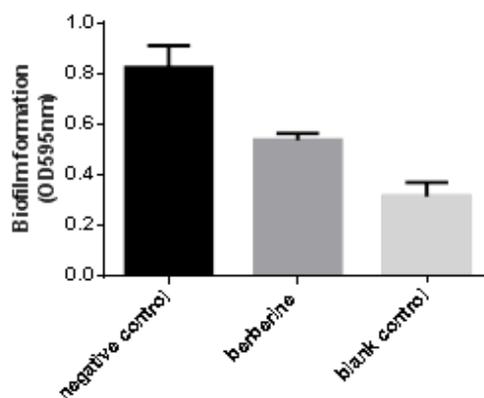


Figure 1. Effect of berberine on the biofilm formation by *S. suis*. Growth of *S. suis* treated with berberine was significantly inhibited compared with that of bacteria without berberine ($P<0.01$). Data are presented as the mean \pm SD of five repeats.

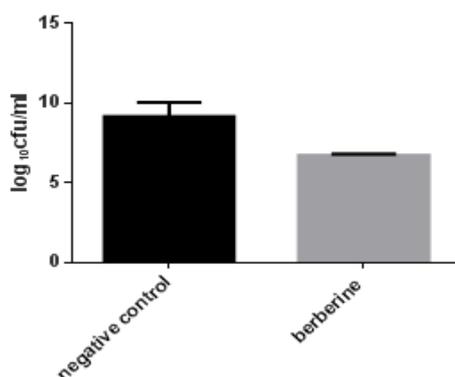


Figure 2. Effects of berberine on cfu for *S. suis*. Growth of *S. suis* treated with berberine was significantly inhibited compared with that of bacteria without berberine ($P<0.05$). Data are presented as the mean \pm SD of five repeats.

The *S. suis* observed by SEM are shown in **Figure 3**. The strain formed a thick, heterogeneous layer with columnar clumps on the CBD when incubated for 72 h without berberine. When strain is treated with berberine at concentration of 31.25 µg/ml, no biofilm was obviously formed and only a few bacterial microcolonies could be seen. The results showed that berberine could inhibit biofilm formation in a dose-dependent manner.

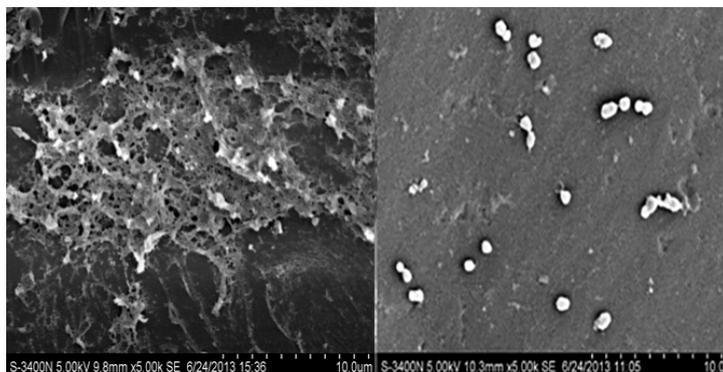


Figure 3. Scanning electron microscopy of biofilm of *S. suis* grown in THB broth A. negative control; with sub-MIC concentration of *B. berberine*.

Effect of Berberine on the *LuxS*/AI-2 of *S. suis* Biofilm

Previous studies have found that the *luxS*/AI-2 QS system and virulence factors affects biofilm development in *S. suis*, and the results of these studies revealed that AI-2 and virulence factors limits biofilm formation [21]. It has been reported that berberine may play a role in the prevention of biofilm formation of *S. epidermidis* [20], but its effect on *S. suis* is not clear. In this study, we focused on investigating the effect of berberine on the *luxS*/AI-2 QS system and virulence factors of biofilm formation of *S. suis*.

Biofilm development and quorum sensing are closely interconnected processes. Quorum sensing is the cell population density-dependent regulation of gene expression by small signaling molecules, called AI [22]. When the AIs accumulate to a threshold concentration, the system is activated and directly or indirectly controls the transcription of target genes. A gene called *luxS* is required for the synthesis of AI-2 [23]. The result of real-time PCR showed that berberine decreased the amount of *luxS*-mRNA to 4.35-fold lower than that of *S. suis* cells grown in THB ($P < 0.05$) (**Figure 4**). In conclusion, berberine may regulate transcription levels of *luxS*/AI-2 and inhibit *S. suis* biofilm formation.

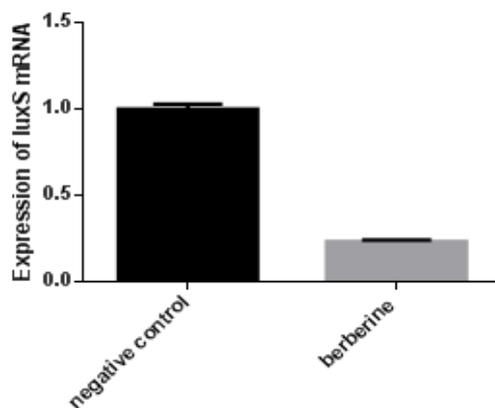


Figure 4. Effects of berberine on *luxS* gene expression of *S. suis* biofilm. Data are presented as the mean \pm SD of five repeats.

Effects of Berberine on Virulence gene Expression of *S. suis* Biofilm

Previous research on the virulence-associated factors of *S. suis* has focused mainly on the capsular polysaccharide (*cps*), muramidase-released protein (*mrp*) (*mrp* gene), extracellular protein factor (*ef*) and suilysin (*sly*) [24]. These molecules have been suggested to be virulence-related factors. Recently, a large number of putative virulence factors associated with *S. suis* 2 have been described [25-27]. These include fibronectin, fibrinogen-binding protein (*fbps*) [28], glyceraldehyde-3-phosphate dehydrogenase (*gadph*) [29], and glutamate dehydrogenase (*gdh*) [30]. Extensive research during the last two decades has identified many factors involved in the virulence of this pathogen including notably the *cps*, which plays a major protective role against host immunity [31]. So far, different genes have been identified as critical for *S. suis* virulence [32]. Hammerschmidt et al., [33] found invasive pneumococci exhibited an enhanced capacity to adhere and invade epithelial cells, appearing to cause a reduction in capsular material, which potentially increases biofilm formation. In this study, berberine increased the *cps* to 1.54-fold than negative control (**Figure 5**). The result showed that *cps* as a surface structure seems to be essential for regulation of biofilm development and virulence. *mrp*, originally associated with virulent strains, has been reported in serotype 2 strains [34].

The N-terminal region of *BaoA1* is homologous to the *mrp* from *S. suis*. Previous study showed that the *BaoA1* deficiency inhibited bacterial biofilm formation [35]. Yang et al. reported the expression level of the *mrp* gene was downregulated between

biofilms and planktonic cells [36]. In this study the expression data demonstrated that berberine increased the *mrp* to 1.71-fold than negative control. Therefore, berberine could regulate the expression of *mrp* and inhibit *S. suis* biofilm formation. *gapdh* possesses a binding activity for albumin and the presence of albumin at the bacterial surface increases the virulence of *S. suis* [37]. *S. suis gapdh* also plays a role in the bacterial adhesion [29]. The expression data demonstrated that berberine decreased the *gapdh* to 3.85 fold. It stands to reason that berberine deal with *gapdh* reduced the bacterial adherence and therefore weakened the virulence of *S. suis*. *sly* belongs to the family of toxins known as thiolactivated toxins or related cholesterol-binding cytolytic toxins that can regulate hemolysin [4]. The expression data demonstrated that berberine decreased the *sly* to 3.13 fold. In this study, berberine may regulate the expression level of the *sly*, which was effect on the *S. suis*. Yang et al. reported three virulence genes were downregulated in the expression level of the *gdh*, *cps2* and *mrp* genes between biofilms and planktonic cells, while *gapdh* and *sly* were upregulated in biofilms [36]. And in this study, three virulence genes (*ef*, *sly*, and *gapdh*) were downregulated and three virulence genes (*gdh*, *cps*, *mrp*) were upregualted addition of 31.25 $\mu\text{g/ml}$ berberine. However, berberine did not dramatically increase the amount of *fbps*-mRNA compared to negative control. A similar result has been found by Yang et al. The results showed that berberine could regulate the expression of virulence genes of *S. suis*.

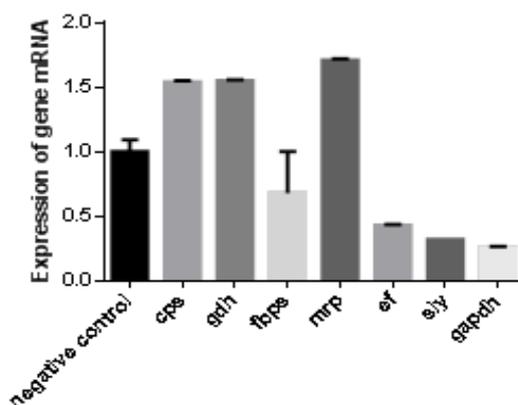


Figure 5. Effects of berberine on virulence gene *cps*, *gdh*, *fbps*, *mrp*, *ef*, *sly* and *gapdh* expression of *S. suis* biofilm. Data are presented as the mean \pm SD of five repeats.

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REFERENCES

1. Wisselink HJ, et al. Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of *Streptococcus suis* strains isolated from diseased pigs in seven European countries. *Vet Microbiol* 2000; 74: 237-248.
2. Hong K. Characterization of group a streptococcal M23 protein and comparison of the M3 and M23 protein's ligand-binding domains. *Current Microbiology* 2007; 55: 427-434.
3. Gonzalez JE and Keshavan ND. Messing with bacterial quorum sensing. *Microbio Mol Biol Reviews* 2006; 70: 859.
4. Gottschalk MG, et al. Characterization of *Streptococcus suis* capsular type 2 haemolysin. *Microbiology (Reading, England)* 1995; 141: 189-195.
5. Hall-Stoodley L, et al. Bacterial biofilms: From the natural environment to infectious diseases. *Nat Rev Microbiol* 2004; 2: 95-108.
6. Han X and Lu C. Biological activity and identification of a peptide inhibitor of *LuxS* from *Streptococcus suis* serotype 2. *Fems Microbiol Letters* 2009; 294: 16-23.
7. Wang Y, et al. Biofilm Formation, Host-Cell Adherence, and Virulence Genes Regulation of *Streptococcus suis* in Response to Autoinducer-2 Signaling. *Current Microbiol* 2014; 68: 575-580.
8. Gao B and Yang G. Immunoregulatory effect and antitumor, antiviral, antivirus activity of ganoderma applanatum polysaccharide. *Inter J Immunopharmacol* 1991; 13: 731.
9. Tigrine C, et al. Cleome arabica leaf extract has anticancer properties in human cancer cells. *Pharmaceutical Biol* 2013; 51: 1508-1514.
10. Figueiredo NL, et al. The inhibitory effect of *Plectranthus barbatus* and *Plectranthus ecklonii* leaves on the viability,

- glucosyltransferase activity and biofilm formation of *Streptococcus sobrinus* and *Streptococcus mutans*. Food Chem 2010; 119: 664-668.
11. Naidoo R, et al. Inhibitory activity of *Dodonaea viscosa* var. *angustifolia* extract against *Streptococcus mutans* and its biofilm. J Ethnopharmacol 2012; 144: 171-174.
 12. Kumar L, et al. Zingerone inhibit biofilm formation and improve antibiofilm efficacy of ciprofloxacin against *Pseudomonas aeruginosa* PAO1. Fitoterapia 2013; 90: 73-78.
 13. Higaki S, et al. The correlation of Kampo formulations and their ingredients on anti-bacterial activities against *Propionibacterium acnes*. J dermatol 1995; 22: 4-9.
 14. Chung JG, et al. Effects of berberine on arylamine N-acetyltransferase activity in human bladder tumour cells. Food chem toxicol 1999; 37: 319-326.
 15. Hong SW, et al. Antimicrobial activity of 9-O-acyl- and 9-O-benzoyl-substituted berberrubines. Planta medica 2000; 66: 361-363.
 16. Sun D, et al. Berberine sulfate blocks adherence of *Streptococcus pyogenes* to epithelial cells, fibronectin, and hexadecane. Antimicrob Agents Chemother 1988; 32: 1370-1374.
 17. Brackman G, et al. Use of quorum sensing inhibitors to interfere with biofilm formation and development in *Burkholderia multivorans* and *Burkholderia cenocepacia*. Res Microbiol 2009; 160: 144-151.
 18. Yi Z, et al. Evaluation of the antimicrobial mode of berberine by LC/ESI-MS combined with principal component analysis. J Pharmaceut Biomed Anal 2007; 44: 301-304.
 19. Wang Y, et al. Comparative Proteomic Analysis of *Streptococcus suis* Biofilms and Planktonic Cells That Identified Biofilm Infection-Related Immunogenic Proteins. Plos One 2012; 7.
 20. Wang XQ, et al. Effect of berberine on *Staphylococcus epidermidis* biofilm formation. International J Antimicrob Agents 2009; 34: 60-66.
 21. Wang Y, et al. Functional analysis of *luxS* in *Streptococcus suis* reveals a key role in biofilm formation and virulence. Veter Microbiol 2011; 152: 151-160.
 22. Yun-Peng SUN and Shui-Shan S. Bacterial Signaling Pathways by Small-Molecules. Chemist Life 2007; 27: 396-398.
 23. Surette MG, et al. Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proceedings of the National Academy of Sciences of the USA 1999; 96: 1639-1644.
 24. Gottschalk M and Segura M. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet Microbiol 2000; 76: 259-272.
 25. Smith HE, et al. Selection of virulence-associated determinants of *Streptococcus suis* serotype 2 by in vivo complementation. Infect Immun 2001; 69: 1961-1966.
 26. Jiang H, et al. Identification and distribution of putative virulent genes in strains of *Streptococcus suis* serotype 2. Vet Microbiol 2009; 133: 309-316.
 27. Vanier G, et al. New putative virulence factors of *Streptococcus suis* involved in invasion of porcine brain microvascular endothelial cells. Microb Pathogen 2009; 46: 13-20.
 28. de Greeff A, et al. Contribution of fibronectin-binding protein to pathogenesis of *Streptococcus suis* serotype 2. Infect Immun 2002; 70: 1319-1325.
 29. Brassard J, et al. Cloning and purification of the *Streptococcus suis* serotype 2 glyceraldehyde-3-phosphate dehydrogenase and its involvement as an adhesin. Vet Microbiol 2004; 102: 87-94.
 30. Okwumabua O, et al. Cloning and characterization of the gene encoding the glutamate dehydrogenase of *Streptococcus suis* serotype 2. Clin Diag Lab Immunol 2001; 8: 251-257.
 31. Baums CG and Valentin-Weigand P. Surface-associated and secreted factors of *Streptococcus suis* in epidemiology, pathogenesis and vaccine development. Animal health research reviews/Conference of Research Workers in Animal Diseases 2009; 10: 65-83.
 32. Fittipaldi N, et al. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent *Streptococcus suis*. Future Microbiol 2012; 7: 259-279.
 33. Hammerschmidt S, et al. Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. Infect Immun 2005; 73: 4653-4667.
 34. Smith HE, et al. Virulence markers of *Streptococcus suis* type 1 and 2. Adv Experi Med Bio 1997; 418: 651-655.

35. Liang X, et al. New Cell Surface Protein Involved in Biofilm Formation by *Streptococcus parasanguinis*. *Infect Immun* 2011; 79: 3239-3248.
36. Wang Y, et al. Reduced virulence is an important characteristic of biofilm infection of *Streptococcus suis*. *Fems Microbiol Letters* 2011; 316: 36-43.
37. Quesy S, et al. Description of an albumin binding activity for *Streptococcus suis* serotype 2. *FEMS Microbiol Letters* 1997; 147: 245-250.