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IN VITRO BIOFILM FORMING POTENTIAL OF *STREPTOCOCCUS SUIS* ISOLATED FROM HUMAN AND SWINE IN CHINA

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ABSTRACT

Streptococcus suis is a swine pathogen and also a zoonotic agent. The formation of biofilms allows *S. suis* to become persistent colonizers and resist clearance by the host immune system and antibiotics. In this study, biofilm forming potentials of various *S. suis* strains were characterized by confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and tissue culture plates stained with crystal violet. In addition, the effects of five antimicrobial agents on biofilm formation were assayed in this study. *S. suis* produced biofilms on smooth and rough surface. The nutritional contents including glucose and NaCl in the growth medium modulated biofilm formation. There was a significant difference in their biofilm-forming ability among all 46 *S. suis* strains. The biofilm-forming potential of *S. suis* serotype 9 was stronger than type 2 and all other types. However, biofilm formation was inhibited by five commonly used antimicrobial agents, penicillin, erythromycin, azithromycin, ciprofloxacin, and ofloxacin at subinhibitory concentrations, among which inhibition of ciprofloxacin and ofloxacin was stronger than that of other three antimicrobial agents.Our study provides a detailed analysis of biofilm formation potential in *S. suis*, which is a step towards understanding its role in pathogenesis, and eventually lead to a better understanding of how to eradicate *S. suis* growing as biofilms with antibiotic therapy.

Key words: biofilm; *Streptococcus suis;* scanning electron microscopy; confocal laser scanning microscopy; microplate assay; antimicrobial agents

INTRODUCTION

Streptococcus suis is an important swine pathogen, causing a wide range of diseases in pigs, including meningitis, septicaemia, pneumonia, endocarditis, and arthritis (24). It is also a zoonotic organisms and its public health importance was highlighted by a recent large-scale outbreak of human *S. suis* infections in China in 2005, which resulted in 38 deaths (29). Human can also be infected with *S. suis* by direct contact with pigs or its byproducts and the infection leads to development of streptococcal toxic shock syndrome (3, 4) as well as meningitis and endocarditis (2, 13). Thirty five serotypes of *S. suis* (types1 to 34 and type 1/2) have been described, but type 2 is considered to be the most pathogenic for both human and swine (27).

Biofilms are matrix-enclosed bacterial population adherent

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to each other and/or to surfaces or interfaces. The biofilm mode of growth is widespread and is of medical and economic importance in diverse ecological niches (5). The formation of biofilm allows microorganisms to become persistent colonizers and resist clearance by the host immune system. Among pathogenic bacteria including *S. suis*, the formed biofilms are able to attach to the surfaces of various indwelling devices such as vascular catheters, prosthetic joints and artificial heart valves, as well as to host tissues and demonstrate superior resistance to antibiotics, which makes antibiotic therapies less effective or leads to treatment failure (9, 10). The ability of these bacteria to produce biofilms on the surfaces of biomaterials used for surgery is one of the main causes of difficult-to-cure infections. Recently, Grenier *et al*. (10) have investigated the ability of *S. suis* type 2 to form biofilms.

However, biofilm formation by other *S. suis* serotypes has not been extensively examined.

In this study, we extensively investigated the biofilm-forming potential of *S. suis* on smooth glass coverslip and rough organic membrane by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Additionally, the polystyrene 96-well microplate stained with crystal violet was used to analyze the factors in the growth medium contributing to the biofilm formation and biofilm-forming ability of 46 *S. suis* isolated from human and swine from different regions of China as well as the effect of antimicrobial agents at subinhibitory concentrations on biofilm formation.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Forty six *S. suis* strains were isolated from human and swine from 1998 to 2008 from different regions of China except for three strains. Serotyping of *S. suis* was carried out by a coagglutination test, using commercial specific sera against serotype 1–28 (Statens Seruminstitut, Copenhagen, Denmark). Twenty eight of the isolates were serotype 2, 16 were serotype 9, and 2 isolates were serotype 1 and 7, respectively (Table 1). *S. suis* isolates were cultured at 37℃ in THB(Todd-Hewitt yeast Broth) containing (w/v) 0.5% beef extract, 2% peptone, 0.3% yeast extract, 0.5% glucose, 0.2% NaCl, 0.04% Na_2HPO_4, 0.25% Na_2CO_3 and 2% calf serum.

Table 1. Origins and the relevant characterstics of *Streptococcus suis* strains

Strains	Serotype	Origin	Geographic
250	2	Diseased human	Sichuang, China, 2005
227	2	Diseased human	Sichuang, China, 2005
SS2-H	2	Diseased pig	Jiangsu, China, 1998
259	2	Diseased human	Sichuang, China, 2005
183	2	Diseased human	Sichuang, China, 2005
X7060821	2	Diseased pig	Xiangtan, China, 2006
SS2-6	2	Diseased pig	Shanghai, China, 1998
YY060816	2	Diseased human	Sichuang, China, 2005
260	2	Diseased human	Sichuang, China, 2005
266	2	Diseased human	Sichuang, China, 2005
05-464	2	Diseased pig	Sichuang, China, 2005
JR05730	2	Diseased pig	Jiangsu, China, 2005
258	2	Diseased human	Sichuang, China, 2005
329	2	Diseased human	Sichuang, China, 2005
ZY05722	2	Diseased pig	Sichuang, China, 2005
CH05806-1	2	Diseased pig	Anhui, China, 2005
SS2-4	2	Diseased pig	Jiangsu, China,
191	2	Diseased human	Sichuang, China, 2005
JDZ05802-1	2	Diseased pig	Jiangxi, China, 2005
BB070119	2	Diseased pig	Bangbu, China, 2007
325	2	Diseased human	Sichuang, China, 2005

GH05-458	2	Diseased pig	Sichuang, China, 2005
719	2	Diseased pig	Sichuan, China, 2005
13	2	Diseased pig	Shanghai, China, 2005
T002	2	Diseased human	Sichuang, China, 2005
251	2	Diseased human	Sichuang, China, 2005
98012	2	Diseased human	Jiangsu, China, 1998
P4254	2	Diseased human	Germany
2083	9	-	Denmark
NJ-2	9	Healthy carrier pig	Suzhou, China, 2006
SH06	9	Diseased pig	Shanghai, China, 2007
NJ-4	9	Diseased pig	Yancheng, China, 2005
CZ0608	9	Healthy carrier pig	Changzhou, China, 2006
55	9	Healthy carrier pig	Jiangxi, China, 2005
SH26	9	Diseased pig	Shanghai, China, 2007
NJ-5	9	Diseased pig	Xuzhou, China, 2005
GZ0565	9	Diseased pig	Guangzhou, China, 2005
SH896	9	Diseased pig	Shanghai, China, 2006
27	9	Healthy carrier pig	Shanghai, China, 2007
NJ-1	9	Diseased pig	Nanjing, China, 2006
NJ-6	9	Diseased pig	Taizhou, China, 2005
L89	9	Diseased pig	Shanghai, China,2006
NJ-3	9	Healthy carrier pig	Yancheng, China, 2006
40	9	Healthy carrier pig	Shanghai, China,2004
SH28	1	-	Canda
SH59	7	Diseased pig	Shanghai, China, 2005

Scanning electron microscopy (SEM)

A mid-exponential growth culture of *S. suis* type 9 NJ-3 and type 2 YY060816 were diluted to an optical density of 0.1 at 600 nm (OD_{600}) and each 200 μL were added to wells of a 6-well microplate (Cosmo) containing an 11 mm×11 mm sterilized rough organic membrane (Mosutech Co., Ltd., Shanghai, China) and a smooth glass coverslip respectively on the bottom. After incubation without shaking for 24 h at 37 ^{o}C, medium and planktonic bacteria on the organic membrane and glass coverslip were removed with sterile PBS. The biofilms and planktonic bacteria were fixed for 6 h with 4% glutaraldehyde and washed three times with 0.1 M PBS in the intervals of 10 min, then fixed to the black transparent in 2% osmium tetroxide. Samples were dehydrated and critical point dried, gold sputtered with ion sputtering instrument (current 15 mA, 2 min) and examined using a scanning electron microscopy (FEI Quanta, Netherland).

Confocal laser scanning microscopy (CLSM)

The biofilm on the glass coverslips was adapted from a procedure as described previously by Takenaka *et al.* (2001). Extracellular polysaccharide in the biofilm formed by *S. suis* NJ-3 was visualized with 50 μg/mL fluorescein isothiocyanate-concanavalin A (FITC-ConA , Sigma , USA) which fluoresces green. The bacterial cells in biofilms were visualized by fluorescent staining with 10 μg/mL propidium iodide (PI, Sigma , USA) solution which fluoresces red. All confocal images were digitized with confocal laser scanning microscope system (CLSM, Leica TCS SP2, Mannheim, Germany) using a 63× or 100× oil immersion objective (Leica, Mannheim, Germany). An argon laser, at 488 nm, was used as the excitation source for the fluorescent probe. A 530/30 Band Pass (BP) filter was utilized for FITC-ConA, and a 605 Long Pass (LP) filter was utilized for PI.

Analysis of factors influenicng *S. suis* biofilm formation with crystal violet (TCP assay)

The biofilm formation assay used in this study was adapted from the method of Grenier *et al.* (10), and is based on the ability of bacteria to form biofilms on solid surfaces

confirmed by SEM and CLSM. Biofilm formation of S. suis NJ-3 was determined in the presence of 0.2%, 0.5%, 1% and 2% glucose. Based on the results of this experiment, biofilm formation of *S. suis* NJ-3 was determined in the presence of 0.2%, 0.5%, 1% and 2% NaCl. The optimized THB medium including 1% glucose and 0.5% NaCl was used to analyze the biofilm-forming ability of 46 *S. suis* clinical isolates. All biofilm assays were run in triplicate and the means ± standard deviations of independent experiments were calculated.

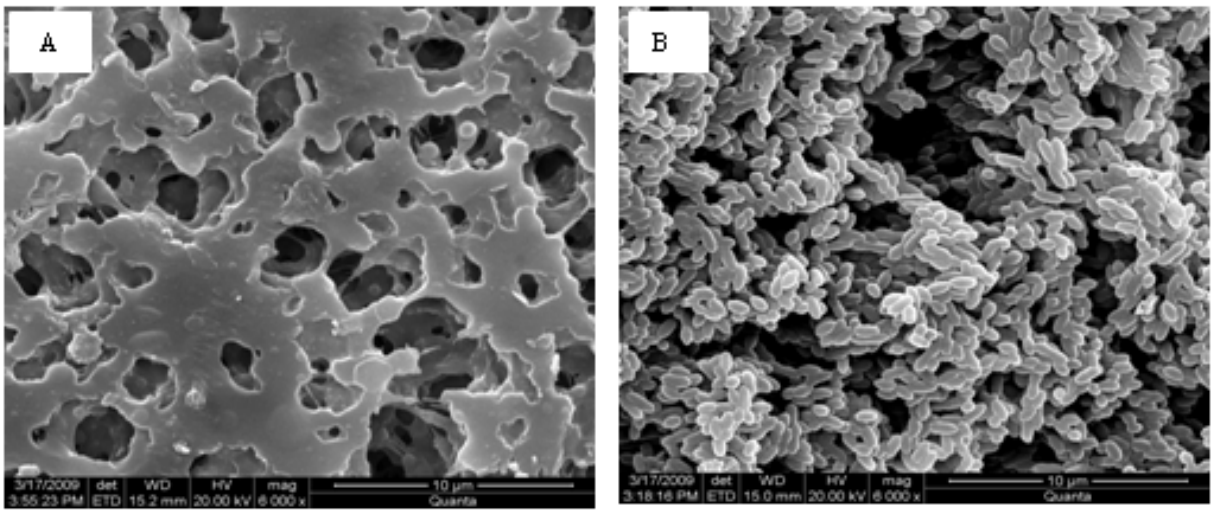
Effect of antimicrobial agents on biofilm formation determined by the TCP assay

Mid-exponential growth phase cultures of 46 *S. suis* isolates were adjusted to an optical density of 0.2 at 600 nm (OD_{600}). One hundred μL of culture and 100 μL of antimicrobial agent solution were added to each well of a 96-well microplate and the final concentrations of each antimicrobial agent were 1 / 2 × MIC, 1 / 4 × MIC, 1 / 8 × MIC and 1 / 16 × MIC, respectively. Wells filled with only sterile growth medium were included as negative controls, and wells including 200 μL culture inoculation without antimicrobial agents were used as positive controls. After incubation without shaking for 24 h at 37 ^{o}C, wells were subsequently rinsed and stained with crystal violet and biofilm formation was quantified as described above.

RESULTS

Direct observation of biofilm formation *in vitro* by SEM

By scanning electron microscopy, we checked the biofilms formed on smooth glass coverslips and rough organic membranes under similar growth conditions by *S. suis* type 9 NJ-3 and type 2 YY060816. Representative images were present, showing the overall appearance of planktonic and biofilm *S. suis* (12000× or 25000×) on different surfaces. Planktonic NJ-3 cells demonstrated an elongated spherical morphology, with no slimy secretion surrounding bacterial cells. However, *S. suis* NJ-3 and YY060816 biofilms cultured for 1 day on glass coverslip and organic membrane corroborated that the massive amounts of mucus-like extracellular material masked the cell surface and aggregated the cells (Fig.1 A-F). In addition, the results showed that it was easier for *S. suis* to form biofilm on rough surface of organic membrane than smooth surface of glass coverslip.



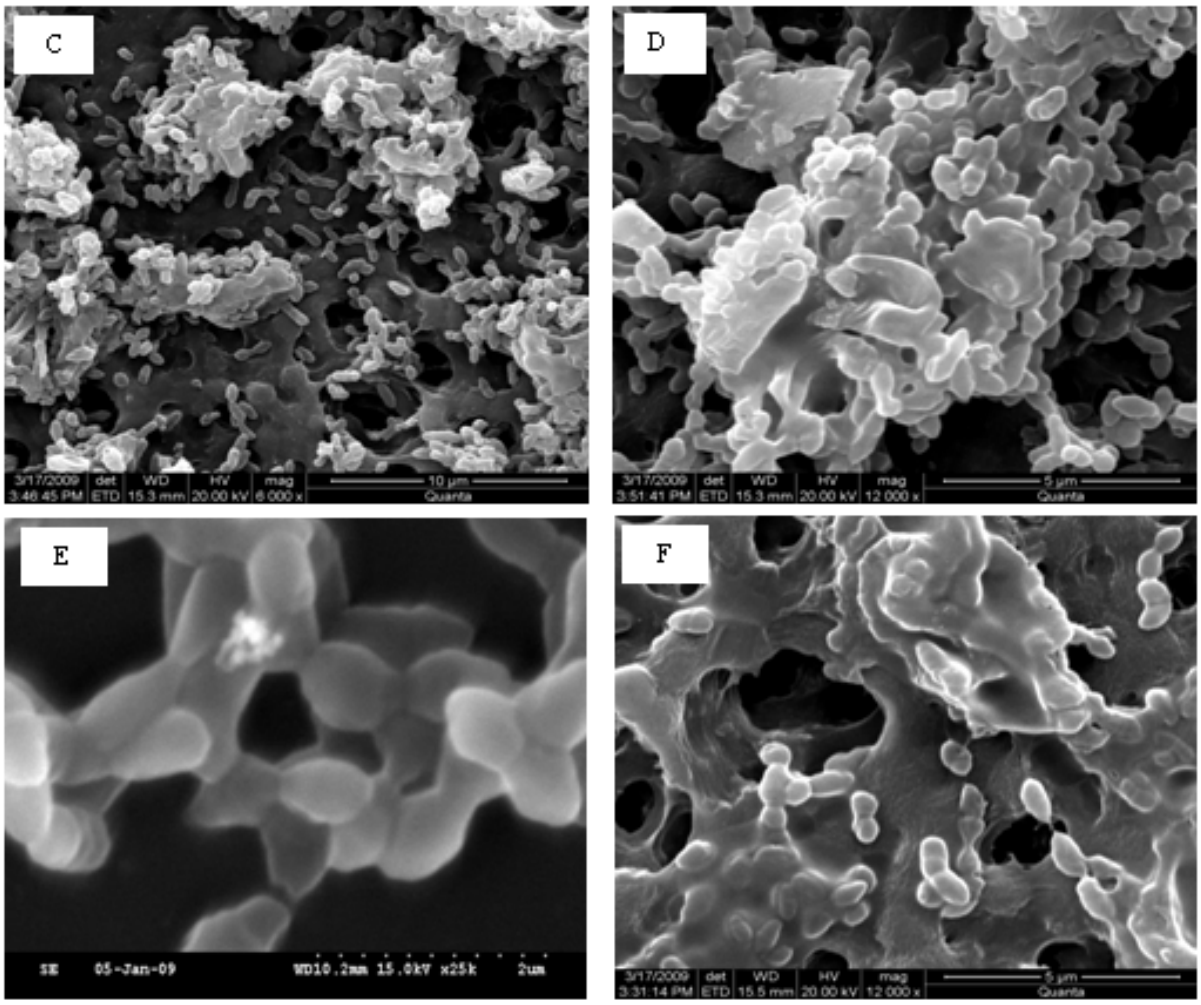


Figure 1. SEM observation of the biofilms formed by *S suis* NJ-3 and YY060816 on rough organic membrane and smooth glass coverslip. Bacteria were grown in six well tissue culture plates with organic membranes and glass coverslips at 37 ^{o}C for 24 h. The massive amounts of mucus-like extracellular materials were observed. A: Rough organic membrane; B: NJ-3 planktonic cells; C: Biofilm of NJ-3 on rough organic membrane (6000×); D: Biofilm of NJ-3 on rough organic membrane (12000×); E: Biofilm of NJ-3 on smooth glass coverslip (25000×); F: Biofilm of *S. suis* YY060816 on rough organic membrane.

CLSM observation of biofilm *in vitro*

In order to further confirm the potential of biofilm formation in *S. suis* NJ-3, the extracellular polysaccharides surrounding bacterial cells were detected by CLSM. The sterile coverslips were not dyed either green or red fluorescence, indicating the coverslips were clean and served as an ideal carrier for visualizing biofilms. Planktonic *S. suis* NJ-3 cells cultured for 12 h stained with FITC-ConA showed no green fluorescent materials, indicating that the planktonic NJ-3 cells did not secrete any polysaccharides. However, biofilm produced by *S. suis* NJ-3 demonstrated green fluorescent materials dispersed among red-fluorescent cells after stained with FITC-ConA (Fig. 2 A-E).

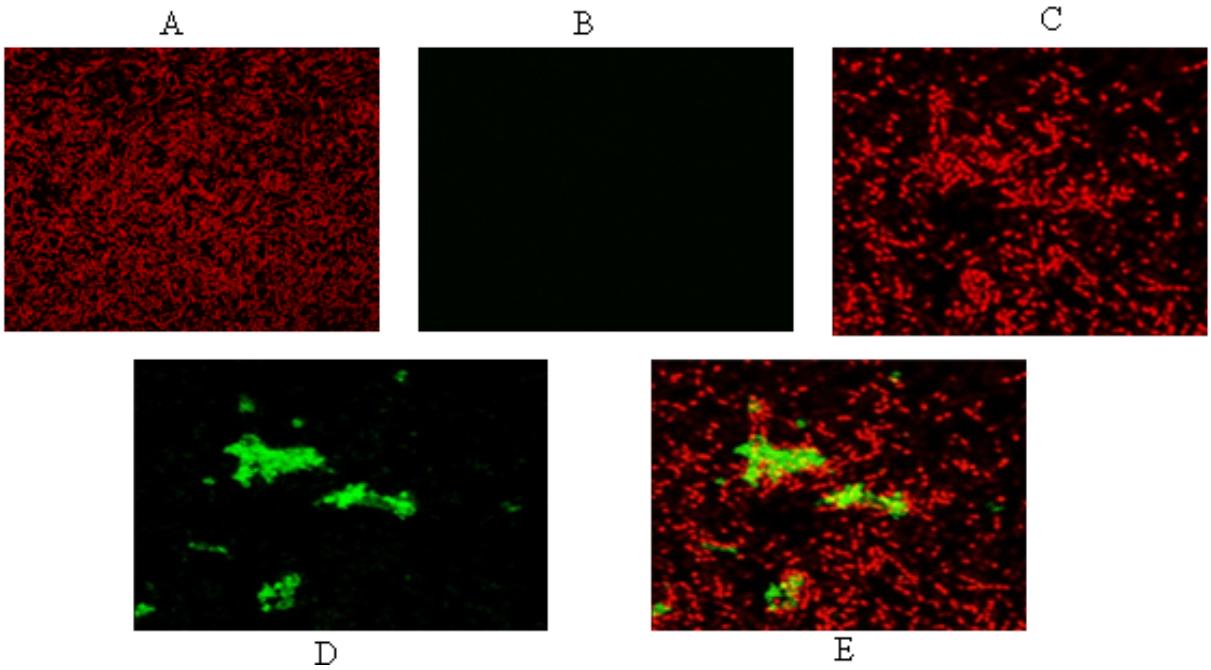


Figure 2. CLSI images of *S. suis* NJ-3 planktonic cells and biofilms on glass coverslips. The extracellular polysaccharide in the biofilm was visualized with 50 μg/ml fluorescein isothiocyanate –concanavalin A (FITC-ConA) which fluoresces green. The bacterial cells in biofilms were visualized by staining with 10 μg/mL propidium iodide (PI) which fluoresces red. A: planktonic cells stained with PI (red); B: Polysaccharide staining of planktonic cells with FITC-ConA; C: NJ-3 cells from biofilm cultures stained with PI (red); D: Polysaccharide of biofilm of NJ-3 stained with FITC-ConA (green); E: Merged image of C and D.

Factors influencing *S. suis* biofilm formation

S. suis biofilm formation was also assayed by crystal violet staining under different conditions including different NaCl and glucose concentrations as well as different strains. The results demonstrated that the nutritional state of the medium had significant effect on biofilm formation. The biofilm formation can be enhanced with the increase of glucose concentration (as showed in Fig.3). Compared to THB including 0.2% glucose, biofilm formation activity significantly increased, about 6.2-fold in THB including 0.5% glucose, 10.2-fold in THB including 1% glucose, and 12-fold in THB including 2% glucose. Based on the results, THB including 1% glucose was used to study the effect of NaCl concentration in medium on biofilm formation. When the final concentration of NaCl in THB was 0.5%, biofilm formation in *S. suis* was the strongest. However, biofilm formation decreased with the NaCl concentration in the medium further increased (Fig. 3). Therefore, the optimized THB including 1% glucose and 0.5% NaCl was used to analyze biofilm-forming ability of different *S. suis* strains. The result showed that different strains had varied biofilm-forming capacities (Fig.4). Especially, the serotypes of *S. suis* had a major influence on biofilm formation. There were 3 strains (10.7%) with OD_{550} exceeding 0.1 among 28 *S. suis* strains of serotype 2, and the rest (89.3%) with OD_{550} less than 0.1. However, among 16 *S. suis* strains of serotype 9, 13 (81.3%) had an OD_{550} value greater than 0.1, and the OD_{550} of the remaining three (18.7%) were less than 0.1. OD_{550} values of the type 1 and type 7 strains were less than 0.1. The study also revealed that two *S. suis* serotype 2 isolates (98012 and P4254) from human, had OD_{550} values 0.311 and 0.418, respectively, among the highest in all 46 strains (Fig.4, Table 3）

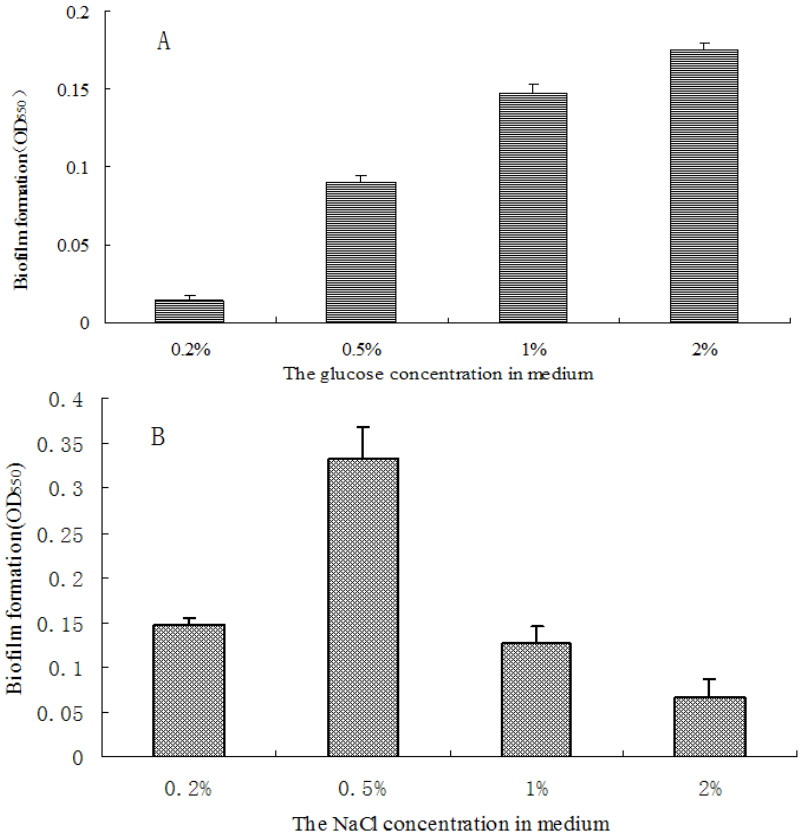


Figure 3. Effect of glucose（A） and NaCl（B） at different concentrations (0.2%, 0.5%, 1% and 2%) on the ability of *S. suis* NJ-3 to form biofilms, as measured by CV staining. Experiments were run in triplicate and each bar represents the mean ± standard deviation from the mean.

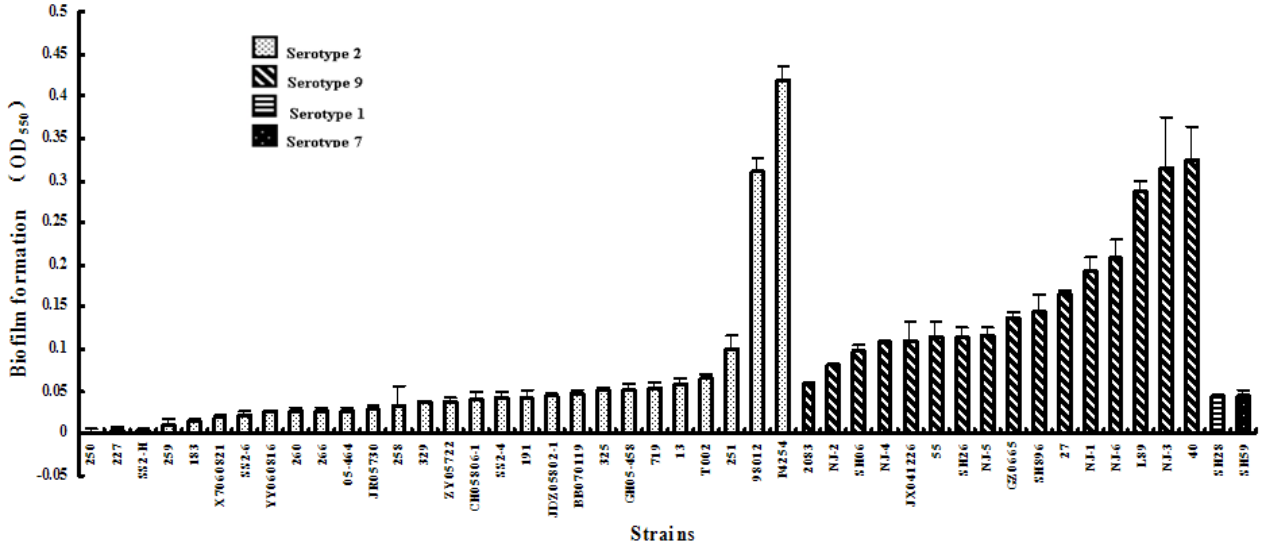


Figure 4. Biofilm-formation by 46 *S. suis* clinical isolates incubated at 37ºC for 24 h in THB including 1% glucose and 0.5% NaCl, as measured by CV staining. Experiments were performed in triplicate and each bar represents the mean ± standard deviation from the mean.

Table 2. Comparison of biofilm formation activity by *S. suis* of different serotypes

Serotype	Strain number		OD_{550} value	Ratio in 46 strains（%）	Ratio in strains of the same serotype（%）
2	28	25	⩽0.1	54.4	89.3
		3	⩾0.1	6.5	10.7
9	16	3	⩽0.1	6.5	18.7
		13	⩾0.1	28.2	81.3
1	1	1	⩽0.1	2.2	100
7	1	1	⩽0.1	2.2	100

Effect of antimicrobial agents against biofilm formation *in vitro* by the TCP assay

Five antimicrobial agents commonly used for treatment of *S. suis* infection in clinics, include penicillin, erythromycin, azithromycin, ciprofloxacin, and ofloxacin. Their MICs against *S. suis* NJ-3 were 0.063, 1, 0.0039, 1 and 1mg·L^{-1} respectively. They inhibited biofilm formation of *S. suis* NJ-3 at subinhibitory concentration1 / 16 × MIC, 1 / 8 × MIC, 1 / 4 × MIC and1 / 2 × MIC. The inhibitory effect gradually increased by increasing the concentration of antibiotics. The inhibitory effect of biofilm formation by different antimicrobial agents varied substantially. Among the five antimicrobial agents, the inhibition by ciprofloxacin and ofloxacin at 1/8 × MIC, 1/4 × MIC and1/2 × MIC was stronger than other three antimicrobial agents. The inhibition of biofilm formation by azithromycin at 1/8 × MIC and 1/4 × MIC was not stronger than that of erythromycin, while at 1/2 × MIC, the inhibitory activity of azithromycin was stronger than that of erythromycin (Fig. 5).

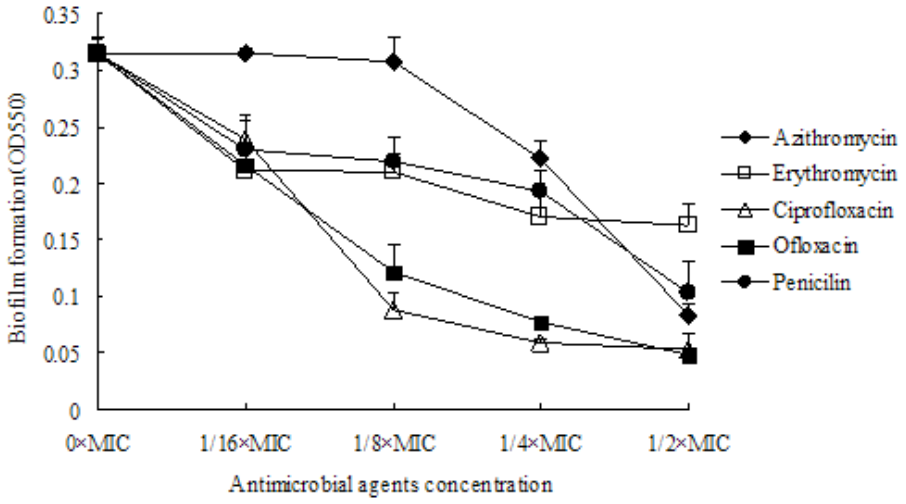


Figure 5. Effect of penicillin, erythromycin, azithromycin, ciprofloxacin, and ofloxacin at different concentrations on *S. suis* NJ-3 biofilm formation, as measured by CV staining.

DISCUSSION

A wide range of bacteria can form biofilms under various conditions. In many cases, biofilms are surface-attached microbial communities that are protected by a self-generated organic polymer matrix, which can act as a diffusion barrier and limit the penetration of antimicrobials to the innermost cells (19). In *S. suis*, biofilm formation is recently reported to

be prevalent especially in field isolates, suggesting possible role of biofilm in *S. suis* pathogenesis (11, 14). Early detection and management of potentially pathogenic *S. suis* biofilm can be one of the essential steps towards prevention and management of device-associated infections. There is also a need to evaluate a simple method for detection of biofilm forming by *S. suis*. Based on these objectives, we investigated *S. suis* adhesion and biofilm formation on rough and smooth surfaces by three different methods.

TCP assay is based on the ability of bacteria to form biofilms on the bottom of tissue culture plates, which is widely used to determine the formation of bacteria biofilms (14, 17, 20). Bacteria are cultured in the wells of tissues culture plates, and the presence or absence of a biofilm is detected by staining the wells with crystal violet. Mathur *et al.* (14) reported the correlation between OD values and biofilm formation. Based on this, comparison of the ability of 46 *S. suis* isolates to form biofilms on plastic surfaces was conducted by this method in this study.

Previous studies have indicated that the nutritional content of the growth medium can regulate biofilm formation (7, 16). In general, bacteria tend to adhere to available surfaces and form mature biofilm in environments that provide sufficient nutrients but will not adhere to surfaces in environments that are nutrients deficient. *S. suis* NJ-3 biofilm formation was tested in a variety of glucose concentration. The results proved that the increased glucose concentration promoted biofilm formation in *S. suis* (Fig. 3), which is in agreement with the results of previous studies done on other bacteria (4, 18). It is also found that 0.5% NaCl in medium is the optimal concentration of *S. suis* biofilm formation (Fig.3). These results support the notion that bacteria form biofilm under favorable nutrient conditions (6). Therefore, the optimized THB including 1% glucose and 0.5% NaCl was used to analyze biofilm-forming ability of different *S. suis* strains. The result showed that the biofilm forming ability was different in all 46 *S. suis* stains at 24 h (Fig.4). Grenier *et al.* (10) reported that 25 *S. suis* strains posses their ability to form biofilms, among which a strain, 95-8242, of *S. suis* serotype 2 isolated from a case of meningitis in pigs formed a significant biofilm. Our results showed difference with that of Grenier *et al.* In this study, the average biofilm-forming ability of *S. suis* serotype 9 stains was stronger than that of type 2 strains. The reason for this discrepancy remains to be further studied. In our study we also found that two *S. suis* type 2 isolates (98012 and P4254) obtained from human infected with *S. suis*, showed the strongest biofilm forming abilities. It is possible that the biofilm-forming ability of *S. suis* may be related with its virulence but possibility remains to be determined.

The TCP method helps the researchers to rapidly analyze adhesion of multiple bacterial strains on growth conditions. But the major disadvantage is that the microplate method is an indirect indication of the level of biofilm produced by a bacterial organism. In this assay, the adsorption of the crystal violet in the destaining solution was used as an indicator of the amount of biofilm. In order to further confirm *S. suis* biofilm formation, the structure of *S. suis* biofilm was identified by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). Biofilm examination relied heavily on scanning electron microscopy. Because of its high magnification, biofilm microstructure is observed clearly. In addition, the results from SEM also indicated surface roughness may promote bacterial adhesion to metallic implants due to the increased surface area or better attachment sites for bacteria. More mucus-like substance was discovered on the rough organic membrane than on the smooth glass coverslip (Fig.1). However, SEM technique utilizes graded alcohol to gradually dehydrate the specimen prior to examination. This dehydration process results in significant sample distortion and artifacts. Therefore, CLSI was used to detect microorganisms and exopolysaccharides of biofilms in situ by an appropriate staining method. Here, FITC-ConA and PI were used to mark exopolysaccharides and bacteria, respectively. Culturing *S. suis* on the glass coverslips results in the adhesion of the masses of

bacteria (red) and the production of exopolysaccharides (green) (Fig. 2). As a matter of fact, polysaccharides are belived to be a major prerequisite for the production of biofilm (2). These findings further confirm that *S. suis* NJ-3 can form biofilm *in vitro*. Although these methods have their advantages, the TCP method has a high specificity, sensitivity, and positive predictive value. Thus, it is suitable for screening biofilm formtion in large numbers of samples.

Most antibiotics of clinical relevance are derivatives of naturally occurring microbial products that probably function in microbial competition within environment niches. β-lactam antibiotics, macrolide antibiotics, and fluoroquinolone antimicrobial agents are commonly used drugs in the treatment of *S. suis* infection. Upon treatment with these antimicrobial agents, a fraction of *S. suis* is inevitably exposed to subinhibitory level of the agents. Therefore, we studied the effect of these commonly used antimicrobial agents in the subinhibitory concentrations on *S. suis* biofilm formation. Five antimicrobial agents, including penicillin, erythromycin, azithromycin, ciprofloxacin, and ofloxacin, can inhibit biofilm formation of *S. suis* NJ-3 at subinhibitory concentration, and this inhibition is a dose-dependent manner, i.e., the higher the concentration of antimicrobial agents, the stronger the inhibition of biofilm formation. In addition, the inhibition of biofilm formation varied among the antimicrobial agents. Numerous reports have described biofilm formation in the presence of subinhibitory concentrations of antimicrobial agents (3, 9, 21, 22). Yassien *et al.* (28) reported that subinhibitory concentrations (1/2, 1/4, and 1/8 of the MIC) of fluoroquinolones (ciprofloxacin, norfloxacin, pefloxacin, and ofloxacin) reduced the adherence of *P. aeruginosa* to 30 to 33, 44 to 47, and 61 to 67% of that of controls, respectively. Shibl (23) found that subinhibitory concentrations of erythromycin decreased adherence of *S. aureus* to tissue culture plates. Our results are in agreement with these previous findings (3, 21, 22). Although our results showed a better activity of ciprofloxacin and ofloxacin against *S. suis* biofilm formation, further studies should be conducted to confirm and clarify the relationship between the relative adherence-inhibiting properties of ciprofloxacin and ofloxacin and their mechanisms. We also need to detect if the biofilm has formed, whether the common used antibiotics could killed the bacteria aggregated in biofilm or not. It will provide information for clinical use of antimicrobial agents against biofilm formed bacteria.

The study could be extrapolated on more isolates with respect to biofilm property along with analysis of the biofilm encoding genes that may lead to better understanding of the determinants involved in the adherence of the organisms growing as biofilms. Further studies are also required to define more precisely the extent to which the organism forms biofilms during colonization.

CONCLUSIONS

In summary, this study provides a detailed analysis in understanding biofilm formation potential that could be one of the important virulent and antimicrobial resistant mechanisms associated with *S. suis* induced pathogenesis in human and animals. This study may eventually lead to a better understanding of how to eradicate *S. suis* growing as biofilms with antibiotic therapy.

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