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SHORT COMMUNICATION

Antimicrobial Activity of Penicillin G and N-acetylcystein on Planktonic and Sessile Cells of *Streptococcus suis*

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Abstract

The aim of this study was to investigate the capacity of *Streptococcus suis* strains to form biofilms and to evaluate the antimicrobial activity of Penicillin G and N-acetylcystein (NAC) on both *S. suis* sessile and planktonic forms. Only non-typeable isolates of *S. suis* were correlated with a greater biofilm formation capacity. The MCI of Penicillin G and NAC required for inhibiting biofilm growth were higher than the required concentration for inhibiting planktonic growth. The combinations of NAC and Penicillin G showed a strong synergistic activity that inhibited biofilm formation and disrupted the pre-formed biofilm of *S. suis*.

Key words: *Streptococcus suis*, biofilm, N-acetylcystein, Penicillin G

In most parts of the world, *Streptococcus suis* is the predominant agent of streptococcal infections in the swine industry and it is associated with a variety of diseases including meningitis, arthritis and pneumonia (Gottschalk *et al*., 2007; Lun *et al*., 2007). Thirty--five serotypes of *S. suis* have been characterized, the most virulent type to swine and humans, being serotype 2 (Gottschalk *et al*., 2007; Lun *et al*., 2007; Wang *et al*., 2011).

Zoonotic disease due to *S. suis* does occur sporadically in Western countries, but is encountered more commonly in countries such as China and Hong Kong. It affects predominantly individuals with occupational exposure to pigs (Huang *et al*., 2005). The antibiotics are widely used to control *S. suis* infections (in humans and in animals), but the emergence of antibiotic-resistant strains represents a problem for both pig production and public health (Hui *et al*., 2005).

Bacterial biofilm formation is a mechanism that allows them to become persistent colonizers and enhance their resistance to antibiotics (Guo *et al*., 2012). Many drugs and compounds have been described as biofilm inhibitors (Aslam *et al*., 2007), specifically Ampicillin and Penicillin G have been examined on *S. suis* biofilm (Grenier *et al*., 2009); biocides such as N-acetylcysteine (NAC) decrease biofilm formation for a variety of bacteria (Marchese *et al*., 2003; Schwandt *et al*., 2004), but their effect on *S. suis* biofilm remains unknown. Therefore, we investigated the biofilm-forming potential of *S. suis* strains collected from farms in Cuba; the effect of two antimicrobial agents, Penicillin G and N-acetylcysteine, on biofilm formation was also studied.

S. suis strains tested for biofilm formation in this study belonged to different serotypes: two serotype 1 (Ss181, ss1S), one serotype 1/2 (Ss1/2M), two serotype 2 (Ss213, Ss211), two serotype 3 (Ss36, Ss364), one serotype 8 (Ss8O), one serotype 9 (SsS9A), one serotype 16 (Ss16X) and four non-typeable isolates (SsNTF, SsNTV, SsS9Q, SsNTY). The isolates were recovered as predominant bacteria from lungs (pigs with pneumonia) in different farms in Cuba. Appropriate amounts of materials from infected lungs were aseptically transferred to the surface of a Columbia agar plate (Oxoid), supplemented with 5% bovine blood. All the isolates were biochemically typed using the API 20 STREP test kit (Bio Mérieux, France). All the strains are part of the collection of Bacteriology Laboratory at National Centre for Animal and Plant Health (CENSA). Strain confirmation was carried out by PCR assays proposed

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by (Marois *et al*., 2007). Serotyping of *S. suis* was performed by a coagglutination test as previously described (Higgins and Gottschalk, 1990).

The biofilm formation was screened using the conditions previously described (Grenier *et al*., 2009). Briefly, *S. suis* strains were cultured in Todd Hevit Broth (THB) until the late-log phase (1×10^8 CFU $\cdot$ ml^{-1}). Then, aliquots of 0.05 ml were added into wells containing the minimal medium (MM). The adherent cells in the wells were quantified measuring the optical density (OD) at $\lambda = 492$ nm using a microtiter plate reader (SUMA, PR-621, Cuba).

The antibacterial activity of Penicillin G and NAC on planktonic cells were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute, document M100-S18 (CLSI, 2008). Briefly, serial two-fold dilutions of Penicillin G (500–0.5 μg $\cdot$ ml^{-1}) and NAC (16–0.016 mg $\cdot$ ml^{-1}) were respectively prepared in THB. Then, aliquots corresponding to a cell concentration of approximately 5×10^5 CFU $\cdot$ ml^{-1} were inoculated into wells containing each antimicrobial. After incubation at 37°C for 24 h, the absorbance at 620 nm was determined.

The effect on biofilm formation was evaluated as described previously, but the dilutions of both antimicrobials (Penicillin G and NAC) were prepared in MM. Each well was inoculated with a final concentration of approximately 5×10^8 CFU $\cdot$ ml^{-1}. The antibacterial activity of Penicillin G in combination with NAC (P + NAC) was investigated as previously described (Mackay *et al*., 2000). Two concentrations of NAC (4 and 8 mg $\cdot$ ml^{-1}) and 10 fold dilutions (500–0.5 μg $\cdot$ ml^{-1}) of Penicillin G were tested. The effect of antimicrobials on preformed biofilms was also evaluated after incubation at 37°C for 4, 8 and 24 h in MM. Preformed biofilms were then exposed to 200 μl of test agent-containing MM broth.

The activity of Penicillin G and NAC was evaluated on planktonic growth of the following strains Ss213, Ss36, SsS9A, while the effect on both planktonic and sessile growth was evaluated in the strains *i.e.* SsNTF, SsS9Q and SsNTV.

The results of the antimicrobial activity were expressed as percentage of the growth inhibition compared with the untreated wells. All experiments were repeated independently three times. All microtiter plate assays were performed in duplicate wells. One-way ANOVA was used to compare groups followed by Bonferroni's multiple comparison post-test by using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). The significance level was $p < 0.05$.

Only one isolate was classified as strongly adherent (SsNTF), one isolate as moderately adherent (SsNTQ), while SsNTV and SsNTY were weakly, the rest were non-adherent. According to our results, only few biofilm-producing isolates could be detected, in agreement with the observations of other researchers who found non-typeable isolates producer of biofilms (Bonifait *et al*., 2008; 2010). The ability to form biofilms is not required for virulence, but instead contributes towards long-term colonization, transmission and difficulties to eradicate these infections (Huaijie *et al*., 2013).

Penicillin G is still the drug of choice for the treatment of *Streptococcus* spp. infections because it remains susceptible to the antibiotic despite its intensive use (Grenier *et al*., 2009). In this work, the minimum effective concentration of Penicillin G for reducing the 50% of planktonic growth of *S. suis* was found to be 2 μg $\cdot$ ml^{-1}; however, the needed concentration for reducing their sessile counterparts was 32 μg $\cdot$ ml^{-1}. On the other hand, MIC for reducing the 90% of planktonic cells were over 128 μg $\cdot$ ml^{-1} and 500 μg $\cdot$ ml^{-1} for sessile cells (Fig. 1A). Significant limitations to biofilm penetration have been reported for beta-lactams antibiotics, which act at the surface of the bacteria due to the impermeability of their stable architecture. Bacteria within a biofilm multiply very slowly and therefore are much less susceptible to growth-dependent antimicrobial killing (Mackay *et al*., 2000; Marchese *et al*., 2003).

Thus, the need for more effective biofilm dissolution treatments becomes imperative. One the ways is to enhance the antimicrobial activity by combining antibiotics with other compounds such as NAC. MIC of NAC for reducing the 90% of planktonic cells were over 2 mg $\cdot$ ml^{-1} (Fig. 1B). Interestingly, the MCI of Penicillin G in the presence of 8 mg $\cdot$ ml^{-1} of NAC was reduced from 500 μg $\cdot$ ml^{-1} to 128 μg $\cdot$ ml^{-1} for sessile bacteria inhibition and to 1 μg $\cdot$ ml^{-1} for the inhibition of planktonic cells (Fig. 1C). Therefore, the combinations of Penicillin G and NAC exerted a synergistic inhibition of both planktonic and sessile forms.

Biofilm formation by the *S. suis* strains was shown to occur earlier than 4 hours. The effects of the tested antibacterial agents on the development of established biofilms is shown in Fig. 2. Among the concentrations of Penicillin G tested, 250 μg $\cdot$ ml^{-1} and 500 μg $\cdot$ ml^{-1} showed the highest reduction activity against preformed biofilm, permitting only the following percentages of biofilm formation $47.16 \pm 5.54\%$; $P < 0.05$ and $48.14 \pm 8.64\%$; $P < 0.05$, respectively (Fig. 1A). N-acetylcysteine (16 mg $\cdot$ ml^{-1}) reduced biofilm development approximately to $60.16 \pm 2.33\%$ ($P < 0.05$) compared to the control group during the first 4 hours (Fig. 1B). However, the combination of Penicillin G and NAC showed a higher disruptive effect on the preformed biofilms and reduced the development of biofilm approximately to $80.84 \pm 18.73\%$ ($P < 0.05$) with respect to the control after 4 hours (Fig. 1C). None of the treatments inhibited biofilm development at more than 50% with respect to the untreated group after 8 or 24 hours.

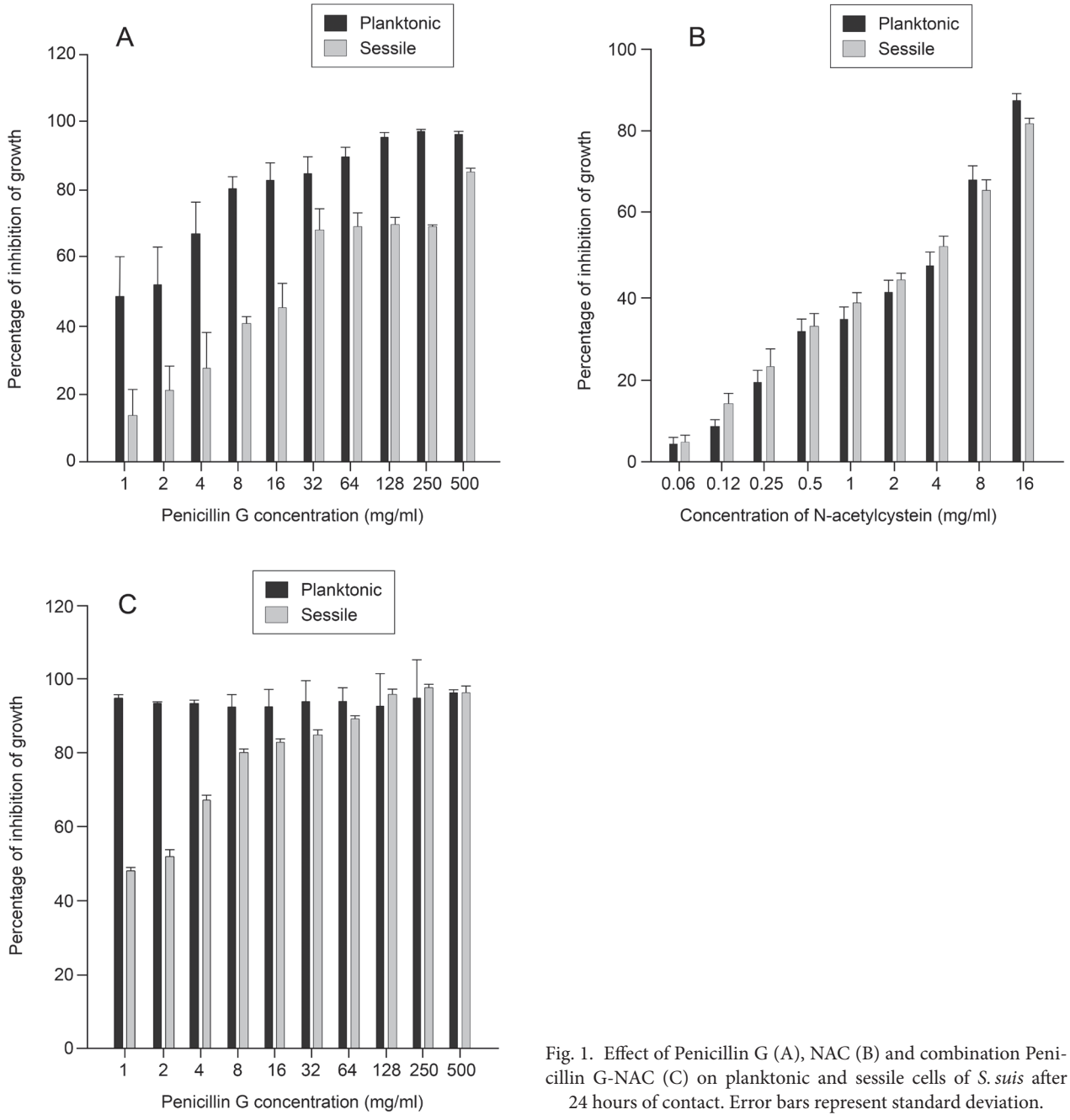

Fig. 1. Effect of Penicillin G (A), NAC (B) and combination Penicillin G-NAC (C) on planktonic and sessile cells of *S. suis* after 24 hours of contact. Error bars represent standard deviation.

Biofilm formation can be divided into three stages: early, intermediate and mature. During the first stage, the bacteria are still susceptible to antibiotics and perioperative antibiotic prophylaxis can be critical for successful treatment (Merle *et al.*, 2012). Some studies show that, when an antimicrobial agent is used for exopolysaccharide matrix disruption, the penetration of other antimicrobials into the pre-formed biofilm can be facilitated (Hajdu *et al.*, 2009). The results of this study showed the Penicillin G and NAC disrupted preformed biofilms by up to 50% and 38% from microtiter plates when were used separately at the concentrations 500 µg/ml and 16 mg/ml respectively. However the application of both mixed compounds permitted only the development of biofilm by up to 20% at 4 hours. NAC is a sulfhydryl group-containing antioxidant and a mucolytic agent that is used in therapy of bronchitis (Marchese *et al.*, 2003; Aslam *et al.*, 2007). Previous studies showed that NAC could decrease biofilm formation by a variety of bacteria and that it inhibited bacterial adherence, reduced the production of extracellular polysaccharide matrix, while promoting the disruption of mature biofilms, and reduced sessile cell viability (Aslam *et al.*, 2007).

Antibiotic treatment due *S. suis* infections in pigs is rarely successful, probably because of poor antibiotic penetration of the porcine tonsillar tissues, which act as a source of infection. Development of new therapeutic

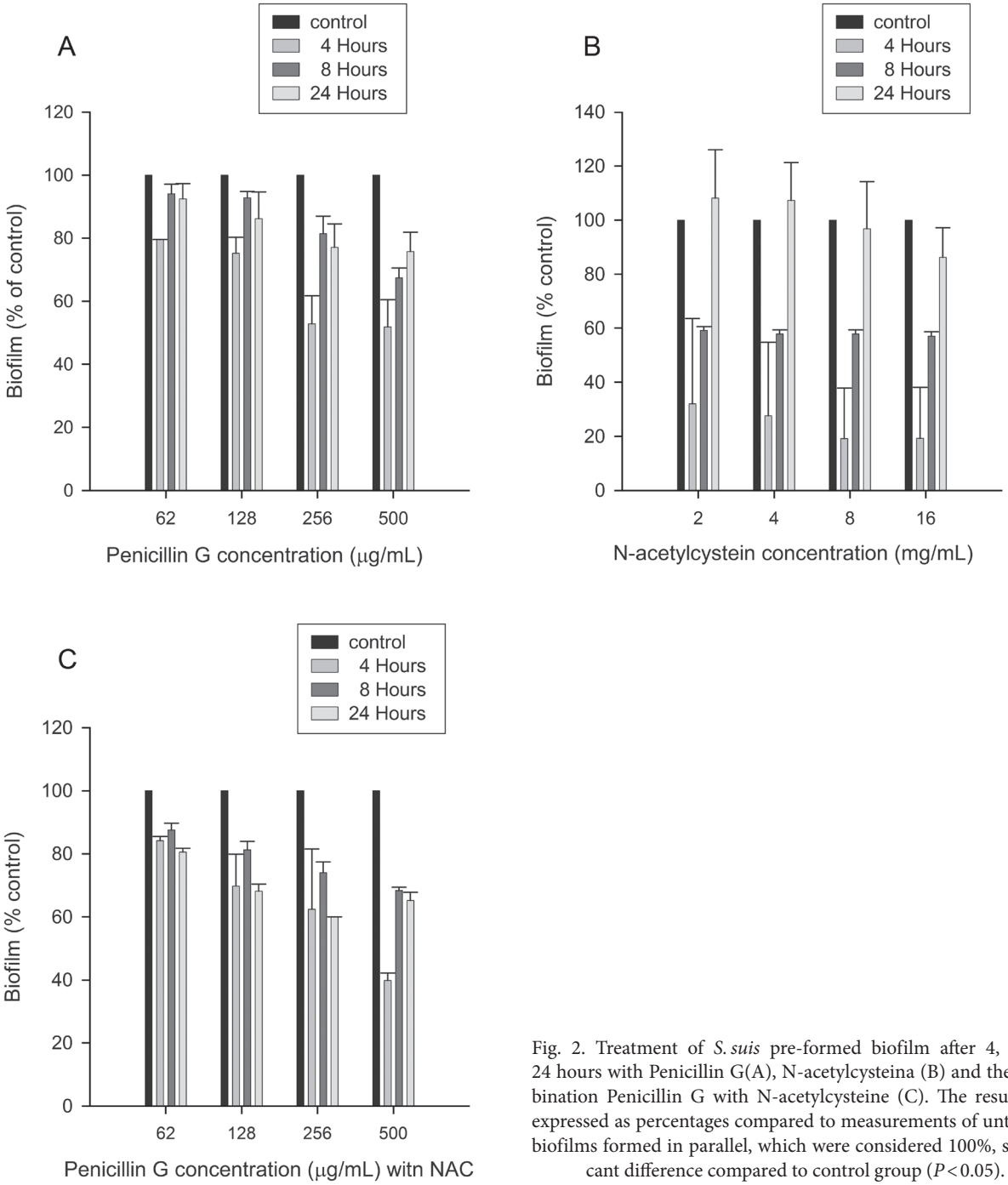

Fig. 2. Treatment of *S. suis* pre-formed biofilm after 4, 8 and 24 hours with Penicillin G(A), N-acetylcysteina (B) and the combination Penicillin G with N-acetylcysteine (C). The results are expressed as percentages compared to measurements of untreated biofilms formed in parallel, which were considered 100%, significant difference compared to control group ($P < 0.05$).

options against the most prevalent strains might be one possible way to prevent colonization of female pigs and to protect those working with pigs.

Traditionally, microbiologists have evaluated the efficacy of an antibiotic by measuring the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). These measurements are made on freely floating, planktonic, laboratory phenotypes. In conclusion, the results of this study agrees with other works in emphasizing the importance of considering the growth in biofilms when evaluating antimicrobials to control *S. suis* – associated infections, and suggests to use combinations of NAC and Penicillin G to increase their antibiotic activity against *S. suis* because of a strong synergic effect.

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