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Effects of Antibiotics on the Expression of Toxin Genes and their Regulators in *Streptococcus pyogenes*

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ABSTRACT

Background: *Streptococcus pyogenes*, also known as group A streptococcus (GAS) or "the flesh-eating bacteria", is a Gram positive human pathogen that causes many diseases, ranging in severity from milder infections such as pharyngitis to life-threatening necrotizing fasciitis/myonecrosis and streptococcal toxic shock syndrome (1). Although GAS remains susceptible to penicillin, this beta-lactam antibiotic (a cell wall-synthesis inhibitor) is not efficacious in treating severe GAS myonecrosis, whereas treatment with clindamycin (a lincosamide that inhibits protein synthesis) is 80-100% protective. We have previously demonstrated that sub-inhibitory concentrations of the beta-lactam antibiotic, nafcillin, increases and prolongs extracellular protein production in *Staphylococcus aureus*, another Gram positive pathogen (2). The present study seeks to examine the effects of sub-inhibitory concentrations of the antibiotics clindamycin, nafcillin, and penicillin on the expression of toxin genes and toxin gene regulators in *S. pyogenes*. **Methods:** GAS strain 88-003 (M type 3) was isolated from a fatal case of necrotizing fasciitis and Strep TSS. The minimum inhibitory concentrations (MICs) of clindamycin, nafcillin, and penicillin were established through standard microbroth dilution assay. To determine the effects of antibiotics on gene expression, bacteria were cultured in the presence of sub-inhibitory concentrations of antibiotics (i.e., 0.5 x MIC) until the late-log phase of growth (4 h). Expression of toxin genes and global gene regulators were examined by northern analysis and reverse transcriptase (RT)-PCR. **Results:** Clindamycin increased the expression of the genes coding for two exotoxins, namely streptolysin O (SLO,*slo*) and nicotine adenine dinucleotidase (NADase; *nga*). It also increased expression of two global toxin gene regulators, *csrR* and *mga*. By contrast, 0.5 x MIC of nafcillin decreased the expression of these genes. The effects of penicillin at 0.5 x MIC were similar to that of nafcillin, except that penicillin caused a slight increase in *csrR* expression. **Conclusions:** Despite its well known ability to prevent translation of bacterial mRNA into exotoxins, clindamycin at sub-inhibitory doses augments toxin gene transcription in GAS. The effects of beta lactam antibiotics on toxin gene regulation appears to be species-, and perhaps, strain-specific.

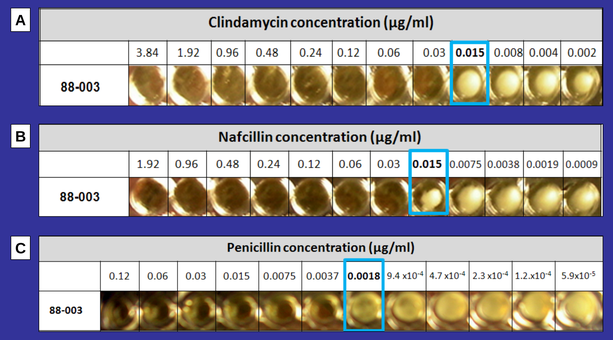
INTRODUCTION

Despite decades of research, severe invasive GAS infections still occur with a frequency of 3.5/100,000 population per year and carry a mortality rate greater than 30%. Survivors often undergo extensive surgical debridement, including amputation, and require prolonged hospitalization and rehabilitation.

Management of severe GAS infections requires immediate and intensive antibiotic treatment. The current consensus for treatment method is administration of high dose clindamycin, together with penicillin (3). We have previously demonstrated *in vitro* that sub-inhibitory concentrations of beta-lactam antibiotics augment the expression and production of toxin genes in *Staphylococcus aureus,* another Gram positive human pathogen, indicating that treatment of infections due to methicillin-resistant *S. aureus* may worsen outcomes (2). Similarly, others have shown that clindamycin increased the levels of toxins SLO, NADase, and streptokinase in a clindamycin-resistant strain of *Streptococcus pyogenes* via its effects on the global regulator, CovR (aka CsrR) (4). However, clindamycin resistance among GAS is rare and the effects of antibiotics on virulence factor gene expression in clindamycin-sensitive strains have not been reported.

We utilized a clinical isolate of GAS associated with fatal necrotizing fasciitis and StrepTSS (GAS strain 88-003) to investigate the effects of subinhibitory antibiotics on toxin gene transcription by RT-PCR and northern analysis. Our results demonstrated that sub-inhibitory concentrations of clindamycin, and to a lesser extent penicillin, greatly increased transcription of multiple toxin genes and known toxin gene regulators. Gaining insight into the effects of sub-inhibitory concentrations of antibiotics on toxin gene expression could have profound implications on the choice and timing of antibiotics used for treatment of mild to severe GAS infections.

Figure 1. Determination of minimum inhibitory concentrations (MICs) by microbroth dilution. Procedures employed followed NCCLS guidelines. Briefly, 3 colonies of GAS were inoculated in10ml Todd Hewitt Broth (THB). The culture was incubated at 37°C with 5% CO_2 for 18 hours. Cultures were then diluted to an optical density (OD) at 625 nm of 0.08 - 0.1, which is equivalent to ~1x10^8 cfu/ml. The cultures were then diluted 1:1000 to achieve a working concentration of 1x10^5 cfu/ml. Antibiotics used (and their suppliers) were: clindamycin HCl (Pfizer); nafcillin sodium (Bristol-Myers Squibb), and sodium penicillin G (Sigma). Antibiotics were serially diluted 2-fold in THB across the rows of a 96 well plate (100 µl final volume). Then 100 µl of the diluted bacterial culture was added. The plate was incubated at 37°C with 5% CO_2 overnight, and was then examined for bacterial growth. The MIC was defined as the lowest concentration that effectively prevented visible bacterial growth.



A. The MIC for clindamycin against GAS strain 88-003 was 0.03 µg/ml which is within the range reported for 90% of clindamycin-sensitive GAS (5). Experiments testing the effects of antibiotics on gene expression were conducted with 0.015 µg/ml clindamycin (i.e., 0.5 x MIC) and 10^8 cfu/ml of bacteria.

B. The MIC of nafcillin for GAS 88-003 was 0.03 µg/ml. Experiments were conducted with 0.015 µg/ml nafcillin (0.5 x MIC).

C. The MIC for penicillin against GAS 88-003 was 0.0037 µg/ml. Experiments were conducted with 0.0018 µg/ml penicillin (0.5 x MIC). The MIC_{90} of penicillin for GAS is 0.0075 – 0.06 µg/ml (5).

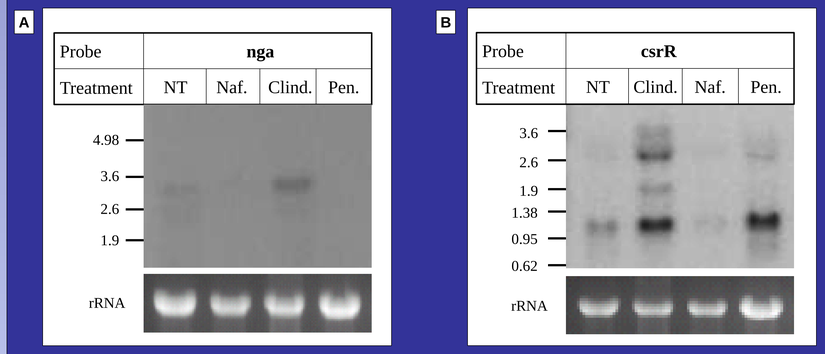


Figure 3. Validation of RT-PCR results by northern analysis. 3 µg of total RNA was hybridized with a ^{32}P-labeled probe that was specific for either A) the gene coding for the exotoxin NADase (*nga*) or B) the global toxin gene response regulator, *csrR*. rRNA was used to show consistency in the amounts of total RNA loaded per lane.

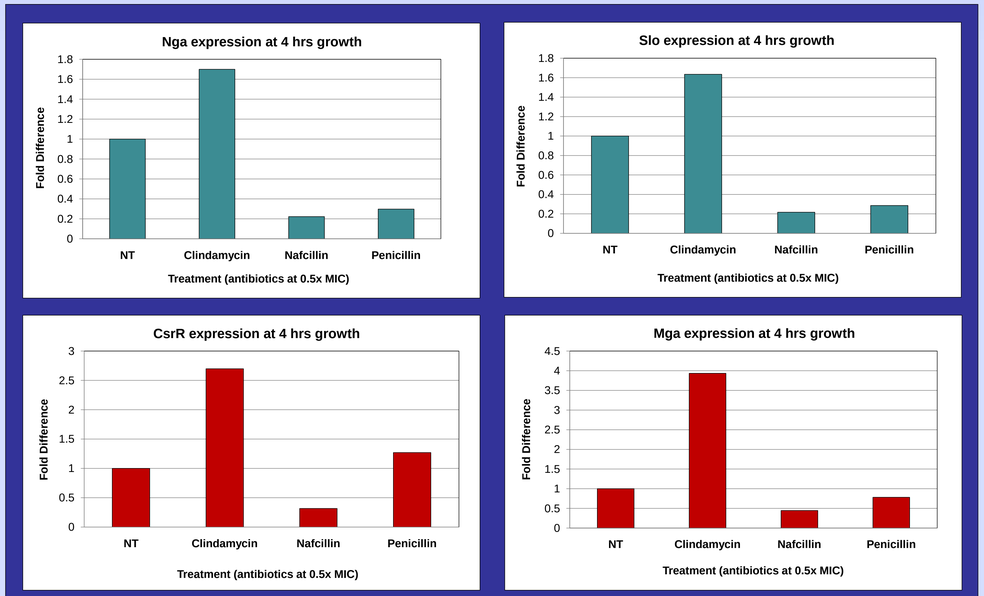


Figure 2. Gene expression analysis by real-time RT-PCR 0.5 µg RNA was isolated from late-log phase cultures (4 h) grown with 0.5 x MIC of specified antibiotics, and used as template for PCR (25µl). cDNA synthesis and PCR was performed using SuperScript™III Platinum® One-Step Quantitative Kit (Invitrogen cat#11732-020) according to the manufacturer's instructions. PCR settings were: 50^{0}C for 30 min, followed by 95^{0}C denaturation for 2 min, and then forty cycles of 15 seconds at 95^{0}C and 30 seconds at 55^{0}C. Fluorescence was detected at each 55^{0}C time point. Four oligonucleotide primer pairs and FAM-labeled probe sets (specific for *nga*, *slo, csrR,* or *mga*) were used to perform target amplification and detection. The transcript abundance is expressed relative to the abundance in the GAS 88-003 with no treatment (NT).

CONCLUSIONS

1. **In contrast to *S. aureus* (reviewed in reference 2), sub-inhibitory concentrations of nafcillin decreased toxin gene expression in GAS 88-003. Thus, the effects of beta lactam antibiotics on toxin gene regulation appears to be species-, and perhaps, strain-specific.**
2. **Sub-inhibitory concentrations of clindamycin significantly upregulated toxin gene transcription, though additional studies such as western blot analysis need to be performed to verify the absence of toxin production by clindamycin-treated GAS strain 88-003.**
3. **The question remains as to whether the observed increase in toxin gene expression is due to direct effects on the toxin genes themselves, or whether it follows upregulation of toxin gene regulators. Future studies to address this could utilize isogenic CsrR- and/or Mga-deficient GAS strains.**

ACKNOWLEDGMENTS

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