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Recognition of *Corynebacterium pseudodiphtheriticum* by Toll-like receptors and up-regulation of antimicrobial peptides in human corneal epithelial cells

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Bacterial keratitis is a major cause of corneal ulcers in developing and industrialized nations. In this study, we examined the host innate immune responses to *Corynebacterium pseudodiphtheriticum*, often overlooked as commensal, in human corneal epithelial cells. The expressions of innate immune mediators were determined by quantitative PCR from corneal ulcers of patients and immortalized human corneal epithelial cells (HCEC). We have found an elevated expression of Toll like receptors (TLRs) along with IL-6 and IL-1β from both ulcers and epithelial cells infected with *C. pseudodiphtheriticum*. Activation of NF-κB and MAPK signaling pathways were also observed in HCEC in response to *C. pseudodiphtheriticum*. In addition, we found a significant increase in the expression of antimicrobial peptides S100A8, S100A9 and human β-defensin 1 from both corneal ulcers and HCEC.

Introduction

Microbial keratitis is the most prevalent cause of corneal infection worldwide and is a painful disease with severe corneal opacity and visual impairment. Among microbial keratitis, bacterial infection is a major cause of corneal ulcers in India[1,2] and other developing countries.[3] Gram-positive bacteria are the most common cause of bacterial keratitis among Indian populations and are primarily associated with trauma related to agricultural work.[1,2] Although traditionally *S. aureus* is the most commonly isolated Gram-positive organisms, increasing cases of *Corynebacterium* infections are also being reported. The number of cases of *Corynebacterium* keratitis diagnosed at our institute, L.V. Prasad Eye Institute (LVPEI), India has increased from less than 1% to more than 5% over last 5 years and *Corynebacterium pseudodiphtheriticum* consists of more than 28% of all *Corynebacterium* infection (unpublished data). A study on the microbial keratitis among the elderly also reported an increase in *Corynebacterium* infection from 0.7% to 11% over last 3 decades.[4] Others have also reported several cases of *Corynebacterium* keratitis in last few years and the numbers of cases are on the rise.[5-7]

C. pseudodiphtheriticum is Gram-positive aerobic or facultative anaerobic bacilli found ubiquitously in food, water and soil. Although generally a harmless commensal, it is an opportunistic pathogen and can cause infection in people with predisposing medical conditions like HIV.[8,9] However it can cause keratitis in fully immunocompetent individuals. *C. pseudodiphtheriticum* has been known to cause community based pneumonia[10] and nonrespiratory infections including septic arthritis,[11] endocarditis[9] and dermal infections.[12] The review of the literature indicates that *C. pseudodiphtheriticum* is largely overlooked, especially in developing countries, and should be regarded as an emerging pathogen. However, little is known about pathogenicity and our knowledge regarding host immune responses for *C. pseudodiphtheriticum*, particularly in corneal infections are very limited.

In this study, to understand the basic pathogenic mechanism underlying *C. pseudodiphtheriticum* infections, we looked into host responses in corneal tissues infected with *C. pseudodiphtheriticum* and immune responses of immortalized human corneal epithelial cells exposed to *C. pseudodiphtheriticum*. Our results show that there is significant elicitation of immune responses in the cornea to this bacterium which is characterized by upregulation of Toll like receptors (TLRs), expression of pro-inflammatory cytokines, and antimicrobial peptides and similar responses were also observed in immortalized human corneal epithelial cells. Thus our data will add to our understanding of the pathogenesis of *Corynebacterium* keratitis.

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Materials and Methods

Statement of Ethics

The protocol for obtaining scrapings of corneal ulcer was reviewed and approved by Institutional Review Board of Hyderabad Eye Research Foundation, LVPEI, India and informed consent forms were obtained from patients. For controls, cadaver corneas unsuitable for transplant were obtained from Ramayamma International Eye Bank (LVPEI, India). Tissue procurement was approved by the Institutional Review Board, LVPEI.

Identification of bacterial strains

Corneal ulcer materials collected aseptically were placed on glass slides for Gram stain and inoculated in different specific media for bacterial cultures. The inoculated plates were incubated overnight and bacteria were identified by Gram stain and were confirmed by sequencing. For bacterial infection experiments, bacteria were grown overnight in thioglycollate media at 37°C, washed in 1X phosphate buffered saline (PBS) and resuspended in cell culture media (10^8 bacteria/ml).

Cell culture

Human corneal epithelial cell line 10.014 pRSV-T[13] was maintained in keratinocyte serum free media containing bovine pituitary extract and recombinant human epidermal growth factors (Invitrogen, Carlsbad, USA) at 37°C and 5% CO_2. HCEC were grown overnight in 6 well plates (5×10^5 cells/well) and incubated with bacteria for 4 hours. The cells were then washed with 1X PBS and processed further.

RNA isolation, cDNA synthesis and quantitative PCR analysis

Quantitative real-time PCR was used to determine mRNA expression of different genes from corneal scrapings and human corneal epithelial cells. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA), quantity and A_{260}/A_{280} value of RNA were determined by spectrophotometer. The quality of RNA was checked by agarose gel electrophoresis and was reverse transcribed using Reverse Transcriptase Kit (Eurogentec, Belgium) according to the manufacturer's protocol. Quantitative PCR was performed on ABI PRISM 7000HT Sequence Detection System (Applied Biosystems, Grand Island, NY) using the SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY). The primer sequences are shown in **Table 1**. The optimum concentrations of primers used were standardized and was checked to eliminate primer-dimer formation. Relative quantities of mRNA expression of respective genes were normalized using the $2^{-\Delta\Delta ct}$ method with GAPDH as the housekeeping gene. The final PCR products were analyzed by agarose gel electrophoresis and single band of desired size was obtained in each case.

Western blot analysis

HCEC were incubated with *C. pseudodiphtheriticum* at 10:1 ratio of bacteria to cells for indicated time points. The cells were washed and lysed and western blot analysis was done as described before[14] using antibodies against p-IκBα, p-p38, p-ERK, p-JNK and β-actin (Cell Signaling Technology, Beverly, MA).

Table 1. Oligonucleotide Sequences

Gene	Sequence (5′→3′)
TLR1	FWD:TTCAAACGTGAAGCTACAGGG
	REV:CCGAACACATCGCTGACAACT
TLR2	FWD:GCCAAAGTCTTGATTGATTGG
	REV:TTGAAGTTCTCCAGCTCCTG
TLR3	FWD:CAAACACAAGCATTCGGAATC
	REV:AAGGAATCGTTACCAACCACAT
TLR4	FWD:TGAGCAGTCGTGCTGGTATC
	REV:CAGGGCTTTTCTGAGTCGTC
TLR5	FWD:TTCAACTTCCCAAATGAAGGA
	REV:TTGCATCCAGATGCTTTTCA
TLR6	FWD:TGAATGCAAAAACCCTTCACC
	REV:CCAAGTCGTTTCTATGTGGTT
IL-6	FWD:CTCACCTCTTCAGAACGAATTG
	REV:CCATCTTTGGAAGGTTCAGGT
IL-8	FWD:GCAGTTTTGCCAAGGAGTGCT
	REV:GCATCTGGCAACCCTACAAC
IL-1b	FWD:CCACAGACCTTCCAGGAGAA
	REV:GTGCAGTTCAGTGATCGTACAG
BD-1	FWD:GCCTCCAAAGGAGCCAGCCT
	REV:CTTCTGGTCACTCCCAGCTCA
BD-2	FWD:CAGCCATCAGCCATGAGG
	REV:TGGCTTTTTGCAGCATTTT
BD-3	FWD:TCTCAGCGTGGGGTGAAGC
	REV:CGGCCGCCTCTGACTCTG
S100A8	FWD:CCGAGTGTCCTCAGTATATA
	REV:GCCCATCTTTATCACCAGAAT
S100A9	FWD:TGGCTCCTCGGCTTTGG
	REV:CGACATTTTGCAAGTCATCGTC
LL-37	FWD:TCGGATGCTAACCTCTACCG
	REV:ACAGGCTTTGGCGTGTCT
NLRP3	FWD:CGTGAGTCCCATTAAGATGG
	REV:CCCGACAGTGGATATAGAACA
ASC	FWD:GACGGGGCCAATACCACAC
	REV:CTGTAACAAAAGTCGTGCTT
GAPDH	FWD:GATCCCTCCAAAATCAAGTG
	REV:GGCAGAGATGATGACCCTTTT

p65 Translocation

p65 translocation assay was carried out in HCEC after incubation with *C. pseudodiphtheriticum* for indicated time points as described earlier.[15]

Statistical analysis

Statistical analysis was performed using an unpaired *t* test (Prism; GraphPad Software). *P* values less than 0.05 were considered significant.

Results

Characteristics of the study population

Corneal ulcers were collected from patients presented at LVPEI, India. LVPEI, a tertiary eye care facility, is routinely involved in diagnosis and treatment of corneal ulcers. The samples were taken from keratitis patients but without any systemic illness. Corneal ulcer scrapings were cultured and subsequent

examinations of these isolates confirmed the identity of *C. pseudodiphtheriticum*. The scrapings were collected from 7 patients identified with *C. pseudodiphtheriticum* in a period of 10 months and were within 6 to 61 years old. **Figure 1A** is the representative corneas of a patient with culture proven *C. pseudodiphtheriticum* keratitis and a representative Gram stained corneal ulcer showing Gram-positive rods of *C. pseudodiphtheriticum*.

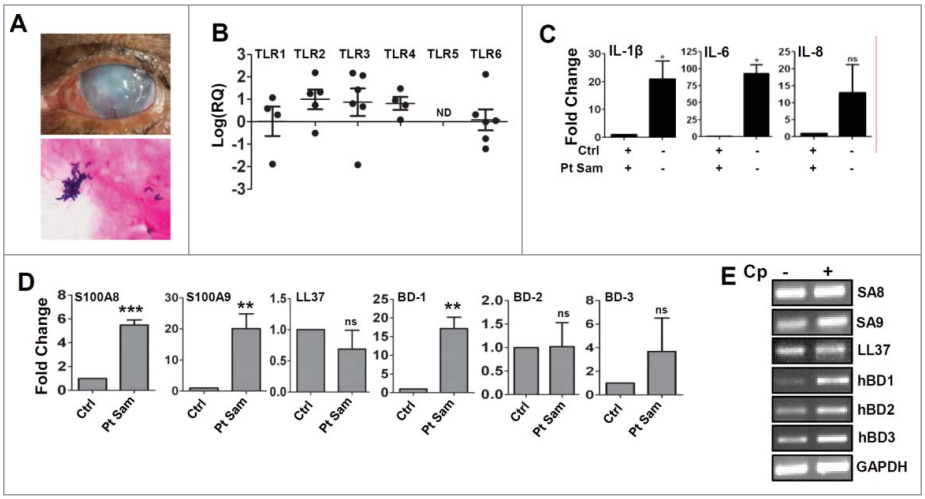

Figure 1. Gene expression of Toll like Receptors, cytokines and antimicrobial peptides in corneal ulcers from patients with *C. pseudodiphtheriticum* keratitis. (**A**) Representative corneal ulcer of patient with *C. pseudodiphtheriticum* keratitis and Gram staining of corneal ulcer material showing Gram-positive *C. pseudodiphtheriticum*. RNA was isolated from corneal ulcers, reverse transcribed and quantitative PCR was done. Data points represent individual patients infected with *C. pseudodiphtheriticum*, and the values are presented either as log of relative expression or fold change in relation to uninfected donor corneas. The expressions of Toll like Receptors (**B**), proinflammatory cytokines (**C**) and antimicrobial peptides are (**D**) are shown. The representative gel images of the amplified products of antimicrobial peptides are shown (**E**). (**) designates $P < 0.005$.

Innate immune responses during *C. pseudodiphtheriticum* corneal infections

Toll like receptors are expressed and activated in corneal ulcers caused by bacteria, or filamentous fungi leading to cytokine production. In order to study the innate immune responses during *C. pseudodiphtheriticum* corneal infection, RNA was isolated from corneal ulcer scrapings collected from *Corynebacterium* keratitis patients. mRNA expression levels of TLRs, pro-inflammatory cytokines and antimicrobial peptides were determined by quantitative PCR. **Figure 1B** shows significant elevated expression of TLR1, TLR2, TLR3 and TLR4 compared with uninfected control corneas. Cadaveric corneas unsuitable for transplant but free from any infection were used as controls. There is almost 30 fold increase in TLR2 expression in the infected corneal scrapings compared to the uninfected corneas. However, TLR5 was not detected in any of the samples and there was a significant decrease in the expression of TLR6. 100 fold increase in expression of IL-6 and more than 20 fold increases in IL-1β expression were observed, while no significant changes in IL-8 expression was determined (**Fig. 1C**). No expressions of IL-17, IL-22 or IFN-γ were either detected (data not shown). Since epithelial cells are known to express antimicrobial peptides, the expressions of human β-defensins and S100A proteins were determined. There was almost 6 fold increase in S100A8 and more than 20 fold increase in S100A9 expressions. Of the human β-defensins, increase of only β-defensin 1 (nearly 20 fold) was observed while no significant changes were seen for β-defensin 2 or β-defensin 3 compared to control (**Fig. 1D**). There were also no significant changes in the expression of cathelicidin or LL-37. The representative gel images of the antimicrobial peptides are shown (**Fig. 1E**).

C. pseudodiphtheriticum activates of Toll like Receptors and mediate signaling via NF-κB and MAPK pathways in human corneal epithelial cells

As there is increase in expression of TLRs in corneal scrapings during *Corynebacterium* keratitis, we wanted to check the expression of these innate immune mediators in response to *C. pseudodiphtheriticum* in corneal epithelial cells *in vitro*. Immortalized human corneal epithelial cells were exposed to *C. pseudodiphtheriticum* for 4 h and expression of TLRs and pro-inflammatory cytokines were monitored by quantitative PCR. As seen in **Figure 2A**, there is 3-4 fold increase in expression of TLR1, TLR2, and TLR4, compared to uninfected control cells. Surprisingly, TLR3 that was elevated in corneal ulcers of infected patients was found to be down-regulated in immortalized corneal epithelial cell lines. Although there were 3 fold increases in TLR5 and TLR6 expression, the changes were not significant. To determine the pathways by which *C. pseudodiphtheriticum* mediates signaling, HCEC were exposed to *C. pseudodiphtheriticum* for defined time periods and p65 translocation and phosphorylation of IkBα, p38, ERK and JNK were examined. As shown in **Figure 2B**, phosphorylation of IkBα starts by 30 minutes but significantly increases at 60 minutes of exposure to *C. pseudodiphtheriticum* in HCEC. This is also evident from translocation of p65 subunit from the cytoplasm to the nucleus within 30 minutes of exposure to *C. pseudodiphtheriticum*, and is persistent after 60 minutes (**Fig. 2C**). *C. pseudodiphtheriticum* also causes phosphorylation of p38, ERK and JNK in HCEC within 30 minutes which was maximal after 60 minutes of infection (**Fig. 2D**). This clearly shows that *C. pseudodiphtheriticum* mediates cell signaling by both NF-κB and MAPK pathways in human corneal epithelial cells. As cytokines and chemokines transcriptions are induced by NF-κB and are important in TLR-induced corneal infection and inflammation, we examined expression of pro-inflammatory cytokines induced in HCEC by *C. pseudodiphtheriticum*. It is seen that *C. pseudodiphtheriticum* causes significant increase in expression of IL-6 (20 folds) and IL-1β (~3 folds) whereas

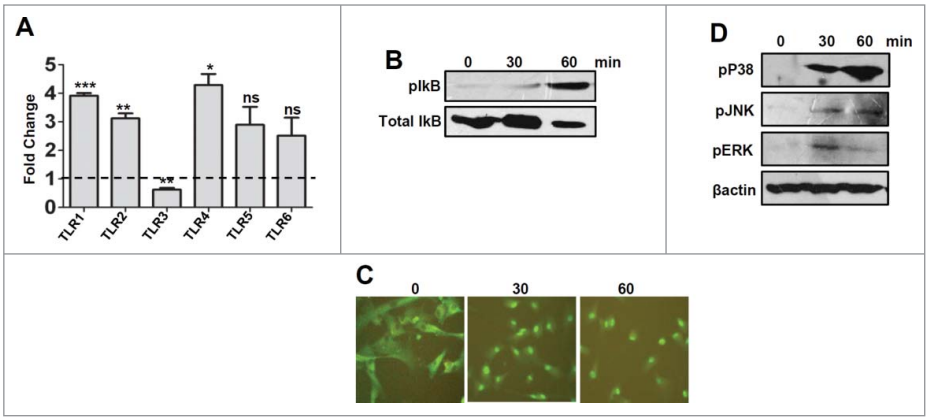

Figure 2. Expression and activation of Toll like Receptors by *C. pseudodiphtheriticum* in human corneal epithelial cells. Human corneal epithelial cells were infected with *C. pseudodiphtheriticum* for 4 hours, washed and RNA was isolated, reverse transcribed and quantitative PCR was done to determine the expression of Toll like Receptors in response to *C. pseudodiphtheriticum* (**A**); Western blot analysis of phosphorylation of IkBα in HCEC in response to *C. pseudodiphtheriticum* (**B**); p65 translocation from cytosol to nucleus in HCEC at 30 and 60 minutes postinfection with *C. pseudodiphtheriticum* was detected by immunocytochemistry (**C**). Activation of MAPK pathways in HCEC after incubation with *C. pseudodiphtheriticum*. At indicated times, cells were harvested and processed for Western blot analysis using antibodies to phospho-p38, phospho-ERK and phospho-JNK and β-actin (**D**).

no change in IL-8 expression was observed, similar to the data obtained from patient's samples (**Fig. 3A**). Since there is an increase in IL-1β expression by *C. pseudodiphtheriticum* infection in human corneal epithelial cells, we also examined the expression of Nod like receptor protein 3 (NLRP3) together with the adaptor molecule, apoptosis associated speck like protein with caspase activation domain (ASC).These together are responsible for the recruitment of caspase 1 that processes IL-1β into mature and biologically active form. We found an increase in expression of both NLRP3 (4 fold) and ASC (~3.5 fold) in HCEC by *C. pseudodiphtheriticum* (**Fig. 3B**).

C. pseudodiphtheriticum induces differential expression of antimicrobial peptides in human corneal epithelial cells

Epithelial cells are known to express repertoire of antimicrobial peptides in response to microbial insults. In order to determine the expressions of antimicrobial peptides in response to *C. pseudodiphtheriticum* infection of human corneal epithelial cells, cells were exposed to *C. pseudodiphtheriticum* for 4 h, RNA isolated and expressions were determined by quantitative PCR.

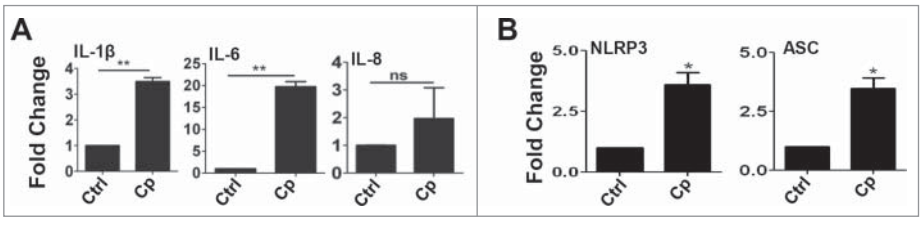

Figure 3. *C. pseudodiphtheriticum* causes expression of cytokines and activation of inflammasome. HCEC were infected with *C. pseudodiphtheriticum* for 4 hours, washed and RNA was isolated, reverse transcribed and expressions of cytokines (**A**) and inflammasomes (**B**) were determined by quantitative PCR. (*) designates $P < 0.05$.

Figure 4A shows 2-8 fold elevated expression of S100A8, S100A9, LL-37 and β-defensin 1 compared with uninfected control cells, however there was no significant difference in the levels of β-defensin 2 and 3. Representative gel images are shown in **Figure 4B**.

Taken together, we conclude that human corneal epithelial cells respond to *C. pseudodiphtheriticum* infection by activation of TLRs and by eliciting expression of pro-inflammatory cytokines and antimicrobial peptides.

Discussion

C. pseudodiphtheriticum, a common commensal of healthy individuals, is an opportunistic pathogen and usually detected in the skin and upper respiratory tract. It can cause respiratory infection in immunocompromised individuals, however keratitis can take place in fully immunocompetent person. In this study, we determined host responses in corneal scrapings from patients with *C. pseudodiphtheriticum* keratitis and detected increased expression of pattern recognition receptors TLR1, TLR2, TLR3 and TLR4, pro-inflammatory cytokines IL-6 and IL-1β and antimicrobial peptides S100A8, S100A9 and β-defensin1 compared with control uninfected cadaveric corneas. We have also demonstrated that human corneal epithelial cells respond to *C. pseudodiphtheriticum* infection *in vitro* that causes up-regulation of TLRs, cytokines and antimicrobial peptides.

We have earlier reported that in murine model of bacterial keratitis, TLRs are activated on resident corneal epithelial cells and macrophages that induce the production of chemotactic and pro-inflammatory cytokines and mediate neutrophil recruitment to corneal stroma.[14] Epithelial cells are the first line of defense against pathogens and responds through identification of pathogen associated molecular patterns (PAMPs). It has already known for corneal epithelial cells and epithelial cells of other origin that cells respond to several bacterial components including lipopolysachharides, lipoproteins and flagellin of Gram positive and Gram-negative bacteria. We have also shown that HCEC responds to LPS in presence of IFNγ by upregulation of MD-2.[15] TLR2 has shown to be activated by *S. aureus* leading to β-defensins production in human corneal epithelial cells.[16] Activation of TLRs and pro-inflammatory cytokines has been recently reported in case of *Pseudomonas* and *Streptococcus* keratitis.[17] Gram-positive bacteria are known to activate and mediate downstream signaling

through heterodimerization of either TLR2/1 or TLR2/6.[18] Since TLR6 is downregulated in ulcers obtained from patients, *C. pseudodiphtheriticum* might initiate cell signaling via TLR2/1. However, the case is little different in immortalized cell lines, where both TLR1 and TLR6 is up-regulated and thus both TLR2/1 and TLR2/6 might be activated.

Antimicrobial peptides have been identified to play an important role in innate host defense at mucosal barriers and also in ocular infections.[19,20] These often have immunomodulatory properties along with antimicrobial activities. There are several reports of antimicrobial peptides successfully preventing invasion by various bacterial infection. However, the knowledge of antimicrobial peptides elicited during keratitis particularly in case of bacterial keratitis is still very limited. S100 proteins are low molecular weight cationic proteins and involved in a variety of cellular processes. So far, microbicidal activity has been described for S100A7, S100A8, S100A9 and S100A12. Calprotectin, dimer formed by S100A8 and S100A9, are expressed by neutrophils, monocytes and activated epithelial cells and have been shown to exert antimicrobial activity against bacteria such as *E. coli, Klebsiella spp., S. aureus* and *S. epidermis*.[21,22] This suggests that our finding of up regulation of S100A8 and S100A9 by *C. pseudodiphtheriticum* is consistent with the earlier reports. LL-37 has also been reported earlier to be upregulated during both bacterial and fungal infections and expressed by epithelial cells of ocular surface, oral cavity, respiratory and gastrointestinal tracts.[23,24] *C. pseudodiphtheriticum* also causes increased expression of the lone member of human cathelicidin antimicrobial peptide. Human β-defensins (hBDs), a family of epithelial cell derived cationic peptides, has been demonstrated to have activity against Gram-positive and negative bacteria, mycobacterium and even fungi at low concentration.[25] Although human β-defensin 1 is known to be constitutively expressed by epithelial cells, we found that *C. pseudodiphtheriticum* infection induces increased expression of hBD-1.

In conclusion, the data from this study shows that *C. pseudodiphtheriticum* activates Toll like Receptors and induce pro-inflammatory cytokine production by human corneal epithelial cells both during corneal infection and *in vitro*. It also induces the expression of antimicrobial peptides of at least 3 different groups, S100 proteins, cathelicidins and β-defensins, as a host defense mechanism. Further studies on the interactions between corneal epithelial cells and *C. pseudodiphtheriticum* will help us understand the host responses better and what makes these commensal bacteria a virulent one.

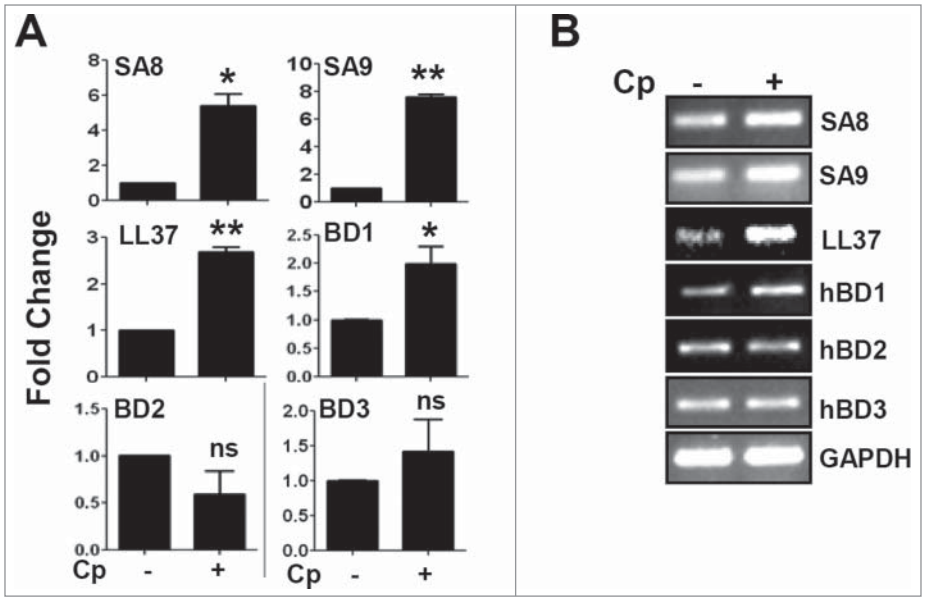

Figure 4. Up-regulation of antimicrobial peptide expression in response to *C. pseudodiphtheriticum* in human corneal epithelial cells. Immortalized human corneal epithelial cells were infected with *C. pseudodiphtheriticum* for 4 hours, washed and RNA was isolated, reverse transcribed and expressions of antimicrobial peptides were determined by quantitative PCR (**A**). The representative gel images of antimicrobial peptides are shown (**B**). (*) designates $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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