Garenoxacin is effective against bacteria invading cells such as Streptococcus pyogenes and Haemophilus influenzae

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Research Article

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Abstract

Background: Recurrent tonsillitis is one of the most common otolaryngological disorders caused by bacteria invading cells such as Streptococcus pyogenes (S. pyogenes) and Haemophilus influenzae. The aim of this study was to investigate the effect of antibacterial drugs against bacteria that have invaded cells.

Results: The intracellular invasion of Detroit 562 cells by five strains of nontypeable *Haemophilus influenzae* (NTHi) and four strains of *Streptococcus pyogenes* was investigated. The antibacterial drugs used were garenoxacin (GRNX), clarithromycin (CAM), amoxicillin (AMPC), cefditoren pivoxil (CDTR-PI), and levofloxacin (LVFX). Both NTHi and *S. pyogenes* fully invaded Detroit 562 cells in 6 h. Both NTHi and *S. pyogenes* had reduced susceptibility to CAM. GRNX, CAM, and LVFX were effective against bacteria invading the cells, but AMPC and CDTR-PI were not effective. GRNX was the most effective.

Conclusion: GRNX was the most effective agent against bacteria invading cells.

Background

Recurrent tonsillitis is one of the most common otolaryngological disorders [1]. The most frequent cause is viruses, and the second most frequent cause is bacteria, such as *Streptococcus pyogenes (S. pyogenes)*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* [2]. Recurrent tonsillitis is caused by nontypeable *H. influenzae* (NTHi) and *S. pyogenes* that enter the cells and escape from the action of antibacterial drugs [1]. Tonsillitis that is not cured by initial treatment requires a change of antibacterial agents or the selection of agents against bacteria that have invaded the cells.

H. influenzae is a leading cause of acute and chronic otitis media, chronic sinusitis, and tonsillitis [3]. It is reported that in otitis media and chronic sinusitis, most strains of *H. influenzae* lack capsular polysaccharides and are referred to as nontypeable *H. influenzae* (NTHi), and that *H. influenzae* bacteria frequently persist within dense biofilm communities that are thought to provide resistance to host clearance and bactericidal activity of some antibiotics [4]. *S. pyogenes* is an important human pathogen that can cause severe, life-threatening, invasive infections such as soft-tissue infection, sepsis, and streptococcal toxic shock syndrome [5]. *S. pyogenes* is generally an extracellular pathogen that can survive and persist within the host by the expression of a broad array of virulence functions directed to circumventing the host immune mechanisms [6]. It is considered that recurrent tonsillitis is caused by NTHi and *S. pyogenes* entering the cells and escaping from the action of antibacterial drugs.

International guidelines recommend penicillin as the first-choice antibiotic treatment for acute sore throat (suspected to be caused by *S. pyogenes*) [7]. However, a recent meta-analysis of clinical studies reported that cephem drugs are more effective than penicillin drugs [8] and are effective as short-term therapy [9]. Therefore, it has become necessary to reconsider the conventional treatment policy based on penicillin. Beta-lactamase-negative ampicillin-resistant *H. influenzae* (BLNAR) is particularly common in

Japan [10]. Therefore, tonsillitis that is not cured by initial treatment requires a change of antibacterial agents or the selection of antibacterial agents against bacteria that have invaded the cells.

Levofloxacin (LVFX), a broad-spectrum fluoroquinolone with potent activity against gram-positive bacteria, is currently recommended to treat respiratory tract infections and pneumonia due to *S. pneumoniae*, one of the most important causative pathogens in community-associated pneumonia (CAP). Similarly, garenoxacin (GRNX) is an oral des fluoro (6)-quinolone with potent antimicrobial activity against common respiratory pathogens [11]. LVFX and GRNX show similar antimicrobial activities against gram-negative bacteria. However, GRNX has higher antimicrobial activity than LVFX against gram-positive bacteria, including staphylococci, streptococci, and pneumococci [12]. Additionally, GRNX has higher broad-spectrum antimicrobial activity against anaerobes than LVFX [13]. These data suggest that GRNX may be an attractive agent for the treatment of CAP.

Clarithromycin (CAM) exerts its antibacterial activity through its inhibitory effect on protein synthesis and is therefore effective against atypical pathogens such as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* that do not have cell walls [14]. It also has antibacterial activity against intracellular parasites such as *Legionella* and nontuberculous mycobacteria, reflecting its excellent transferability from tissues to cells [15].

The present study investigated the in vitro antibacterial activity of antibacterial agents against clinical strains of NTHi and *S. pyogenes* isolated in Japan.

Materials And Methods

Antibacterial agents

The following antibacterial agents were used in the study: analytical grade powders of GRNX (FUJIFILM Toyama Chemical Co., Ltd., Tokyo, Japan), CAM (Meiji Seika Pharma, Tokyo, Japan), amoxicillin (AMPC) (Wako Pure Chemical Industries), cefditoren pivoxil (CDTR-PI) (Meiji Seika Pharma, Tokyo, Japan), and LVFX (Sigma-Aldrich, Tokyo, Japan).

Bacteria and growth conditions

We evaluated five clinical strains of NTHi isolated from the nasopharynges of patients with otitis media with effusion and four clinical strains of *S. pyogenes* isolated from tonsillar crypts of patients with recurrent tonsillitis. All bacteria were stored in skimmed milk with glycerol at − 80°C until use. An aliquot of each bacterial stock was thawed and cultured overnight at 37°C in a 5% CO_2 incubator on chocolate II agar (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan) or sheep blood agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) plates, as appropriate. After washing in 0.5% bovine serum albumin–phosphate-buffered saline (PBS), the bacteria were used for intracellular invasion assays. The concentrations of NTHi and *S. pyogenes* were adjusted to 1.0×10^8 colony-forming units (CFU)/mL at an absorbance of 580 nm. This study was approved by the Institutional Review Board of Kagoshima University

Determination of minimum inhibitory concentration (MIC)

The susceptibility of bacteria to antibiotics was studied by the broth microdilution method, performed according to Clinical Laboratory Standards Institute guidelines [15]. The test medium was prepared using cation-adjusted Mueller Hinton broth (Eikenkagaku, Tokyo, Japan) with lysed horse blood (Nippon Biotest Laboratory, Tokyo, Japan). The quinolones evaluated were GRNX, CAM, AMPC, CDTR-PI, and LVFX. In this study, 1 and 2 MIC were used.

Cell culture

Detroit 562 cells (CCL-138; ATCC, Manassas, VA, USA), a human pharyngeal carcinoma epithelial cell line, were grown to confluence in minimal essential medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 1 mM sodium pyruvate (Nacalai Tesque), 10% fetal bovine serum (Invitrogen, San Diego, CA, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL; Nacalai Tesque) at 37ºC in a 5% CO_2 incubator as previous described [16]. The cells were harvested using trypsin (final concentration, 0.02%) and ethylenediaminetetraacetic acid (EDTA; final concentration, 0.02%; Nacalai Tesque) and seeded at a density of 2×10^4 viable cells per well in a 96-well BD Falcon tissue culture plate with a low-evaporation lid (BD Biosciences, Franklin Lakes, NJ, USA). The plates were used when > 90% confluence was observed following overnight incubation.

Intracellular invasion assay

One hundred microliters each of the NTHi and *S. pyogenes* strains (1.0×10^8 CFU/mL) were added to Detroit 562 cells cultured in a 96-well plate and allowed to adhere at 37ºC in a 5% CO_2 incubator for 6 h. Each well was then treated with gentamicin (200 µg/mL) at 37ºC in a 5% CO_2 incubator for 1 h. After washing five times with 200 µL of PBS, the cells were treated with 100 µL of each antibacterial agent at 37ºC in a 5% CO_2 incubator for 6 h. After washing five times with 200 µL of PBS, the cells were treated with 100 µL of saponin at 37ºC in a 5% CO_2 incubator for 15 min. Further, 100 µL of the samples from each well was plated on chocolate II agar plates or sheep blood agar and cultured overnight, and the number of colonies formed was counted as previous decribed [17].

Statistical analysis

All values are presented as means ± standard deviation. The data were statistically analyzed using the unpaired one-way ANOVA with Tukey's method. We considered differences to be statistically significant when the probability values were <5%.

Results

Bacterial invasion time

NTHi bacteria were found inside the cells 2 h after they were attached to the cells (Fig. 1A). After 4 and 6 h, the number of bacteria invading the cells increased in a time-dependent manner (Fig. 1A). However, there was no difference between the numbers of bacteria invading the cells at 6 h and 8 h (Fig. 1A). Similarly, the number of *S. pyogenes* bacteria invading the cells increased in a time-dependent manner, and there was no difference between the numbers of bacteria invading the cells at 6 h and 8 h (Fig. 1B). Based on these results, the time to enter the cells was set to 6 h.

Minimum inhibitory concentration

In invasion by NTHi, no reduction in susceptibility was observed with GRNX, AMPC, and LVFX. However, AMPC and especially CAM showed decreased sensitivity (MIC ≥ 2 μg/mL). As with NTHi, *S. pyogenes* did not show a decrease in sensitivity to GRNX, AMPC, and LVFX. However, LVFX showed decreased sensitivity (MIC ≧ 0.5 μg/mL) (Table 1).

Effects of antibacterial agents on NTHi

Cells invaded by bacteria, treated with saponin, and then treated with PBS served as a control. Treatment with 1 MIC of GRNX, CAM, or LVFX significantly reduced the number of NTHi bacteria entering the cells (Fig. 2A) ($p < 0.05$). GRNX had a significantly higher bactericidal effect than CAM and LVFX ($p < 0.05$). However, no bactericidal effect was observed from treatment with AMPC or CDTR-PI (Fig. 2A).

Similarly, treatment with 2 MIC of GRNX, CAM, or LVFX also had a significant bactericidal effect ($p < 0.05$), but treatment with AMPC or CDTR-PI had no bactericidal effect (Fig. 2B). GRNX had the highest bactericidal effect (Fig. 2B).

Effects of antibacterial agents on *S. pyogenes*

Cells invaded by bacteria, treated with saponin, and then treated with PBS served as a control. Treatment with 1 MIC of GRNX, CAM, or LVFX significantly reduced the number of *S. pyogenes* bacteria entering the cells (Fig. 3A) ($p < 0.05$). GRNX had a significantly higher bactericidal effect than CAM and LVFX (Fig. 3A) ($p < 0.05$). However, no bactericidal effect was observed from treatment with AMPC or CDTR-PI (Fig. 3A).

Similarly, treatment with 2 MIC of GRNX, CAM, or LVFX also had a significant bactericidal effect ($p < 0.05$), but treatment with AMPC or CDTR-PI had no bactericidal effect (Fig. 3B). GRNX had the highest bactericidal effect (Fig. 3B).

Discussion

This study investigated the effects of GRNX, CAM, AMPC, CDTR-PI, and LVFX on the invasion of Detroit 562 cells by NTHi and *S. pyogenes*. The results showed that NTHi and *S. pyogenes* invaded Detroit 562 cells, AMPC and CDTR-PI did not affect the invasion of Detroit 562 cells by NTHi and *S. pyogenes*, and GRNX, CAM, and LVFX reduced the invasion of Detroit 562 cells by NTHi and *S. pyogenes.*

Fibronectin-binding protein (F1 protein) is mentioned as a mechanism by which *S. pyogenes* invades the cells [18]. In Japan, Ma et al. [19] reported that 77.3% of *S. pyogenes* strains possessed F1 protein. More interestingly, many biofilm-producing strains are F1 protein-negative strains [20]. Intracellular invasion ability and biofilm formation ability are negatively correlated, and it is considered that *S. pyogenes* avoids the attack of antibacterial drugs [18]. In addition, phosphorylcholine is mentioned as a mechanism of intracellular invasion by NTHi, and the higher the expression level of phosphorylcholine, the more it penetrates into cells [17]. The present study showed that it takes a certain period of time for bacteria to adhere to cells and enter the cells, which becomes constant within 6 h. Yamanaka's report that *H. influenzae* invades Detroit 562 cells supports the results of the present study [21].

Since the late 1990s, respiratory tract infections caused by resistant strains of *S. pneumoniae* and *H. influenzae* have been rapidly increasing worldwide. Penicillin-resistant *S. pneumoniae*, such as penicillin intermediately-resistant *S. pneumoniae* (PISP), penicillin-resistant *S. pneumoniae* (PRSP), and BLNAR, are particularly common in Japan [10]. In this study, no bacteria resistant to β-lactam were found, but decreased sensitivity to LVFX by *S. pyogenes* and to CAM by NTHi and *S. pyogenes* was observed. Quinolone inhibits bacterial growth by disrupting the DNA replication of type II topoisomerase [22]. Type II topoisomerases are currently recognized to include DNA gyrase, which is responsible for the formation and elimination of supercoiled structures in DNA strands, and topoisomerase IV, which cuts and re-ligates tangled DNA during DNA replication [22]. Both of these enzymes are composed of two dimers of subunit types A and B, which together form a tetramer. Amino acid substitutions in either enzyme may lead to the inhibition of such complex formations, and in particular, mutations in the quinolone resistance-determining regions (QRDRs) within subunits A and B are closely related to resistance [23]. Shoji et al. [24] reported that of the 14 *S. pyogenes* strains, 12 (85.7%) had two or more mutations in QRDRs. This is considered one of the reasons that streptococcal susceptibility to LVFX was reduced.

Invasion of cells by bacteria has been cited as a cause of repeated tonsillitis. In this study, neither AMPC nor CDTR-PI was found to have a bactericidal effect on bacteria invading the cells. It is known that β-lactam antibacterial drugs have low intracellular transmissibility, and their antibacterial action is reduced against *H. influenzae* that has entered the cells [24]. Therefore, it is suggested that another antimicrobial treatment is necessary for recurrent tonsillitis.

GRNX shows a favorable pharmacokinetic profile, with good penetration into sputum and otorhinolaryngological tissues, and it is highly effective in the treatment of patients with upper and lower respiratory tract infections [11]. Recent studies have established that the $AUC_{0\text{-}24}$/MIC ratio is an important pharmacodynamic parameter influencing quinolone efficacy. Lister demonstrated that garenoxacin exhibits pharmacodynamics similar to those of clinically available quinolones [25]. In addition, Takagi et al. [26] reported that GRNX concentrations in plasma and tissues of subjects receiving GRNX 400 mg once a day were higher than the MIC_{90} of major causative pathogens. The trough concentration (Cmin) in plasma was 1.92 g/mL, a level that was higher than the mutant prevention concentration, suggesting that GRNX is unlikely to induce the selection of resistant strains during

treatment. The efficacy rates of GRNX in otorhinolaryngological infections were 91.3% for sinusitis, 81.8% for otitis media, 89.5% for pharyngolaryngitis, and 95.0% for tonsillitis [26]. A double-blind study was conducted comparing GRNX 400 mg once a day with LVFX 100 mg three times a day for 10 days in patients with bacterial pneumonia. The efficacy rate was 94.9% (94/99) in the GRNX group and 92.8% (77/83) in the LVFX group at the 7th day after completion of treatment [27]. No significant difference in efficacy rates was found between GRNX and LVFX, with a 95% CI of – 4.9% to 9.2%, indicating that GRNX is not inferior to LVFX. The bacterial eradication rate was 100% (53/53) in the GRNX group and 87.8% (36/41) in the LVFX group. This difference in the eradication rate was statistically significant, with a 95% CI of 2.4% to 23.9% [27]. In the present study, GRNX was also effective against bacteria invading cells. Since LVFX showed decreased sensitivity, GRNX is effective for recurrent tonsillitis.

The present study showed that CAM was effective against bacteria invading cells. Patel et al. reported that the concentration of CAM in alveolar macrophages of healthy subjects reached a maximum of 1996 μg/mL at 4 h after administration of 500 mg of CAM [28]. Chou et al. [29] reported that cultured human gingival fibroblasts and SCC-25 cells took up CAM via a concentrative active transport system. However, the concentration of CAM used in this study is far beyond the amount used in actual clinical practice and therefore could not be used in actual clinical practice.

Our study has some limitations. First, BLNAS and other resistant strains were not investigated. Since the number of strains of resistant bacteria is increasing, more resistant strains should be included in future studies. The second limitation pertains to the epithelial cells used. Although the use of normal human epithelial cells may be more clinically relevant, we used a pharyngeal cancer-derived cell line. Because these cells were of human origin, we consider that the results of this study were not different from those that would have been obtained with the use of normal cells.

In conclusion, GRNX was the most effective agent against bacteria invading cells. Administration of GRNX should be considered when the efficacy of penicillin and cephem antibiotics and of β-lactam is insufficient in daily medical practice.

Declarations

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Ethics declarations

Ethics approval and consent to participate

We obtained written informed consent all subjects or, if subjects are under 18, from a parent or legal guardian. This study was approved by Kagoshima University ethics committee (200313).

All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

Contributions

Hiroyuki Iuchi: methodology, formal analysis, and writing the original draft. Junichiro Ohori: software, investigation and validation. Satoshi Klyama: resources, visualization, project administration, data curation, conceptualization, supervision. All authors read and approved the final manuscript.

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Table

Table 1. Minimum inhibitory concentrations of antibacterial agents against nontypeable *Haemophilus influenzae* and *Streptococcus pyogenes*

A

strain	MIC (μg/ml)				
	GRNX	CAM	AMPC	CDTR	LVFX
NTHi 1	0.06	4	0.5	0.06	0.06
NTHi 2	0.06	4	0.5	0.06	0.06
NTHi 3	0.06	8	0.5	0.06	0.06
NTHi 4	0.06	2	0.5	0.06	0.06
NTHi 5	0.06	4	0.5	0.06	0.06

B

strain	MIC (μg/ml)				
	GRNX	CAM	AMPC	CDTR	LVFX
S. pyogenes 1	0.06	0.25	0.06	0.06	0.5
S. pyogenes 2	0.06	16	0.06	0.06	0.5
S. pyogenes 3	0.12	0.25	0.06	0.06	2
S. pyogenes 4	0.12	0.25	0.06	0.06	0.5

MIC, minimum inhibitory concentration; NTHi, nontypeable *Haemophilus influenzae*; GRNX, garenoxacin; CAM, clarithromycin; AMPC, amoxicillin; CDTR-PI, cefditoren pivoxil; LVFX, levofloxacin.

Figures

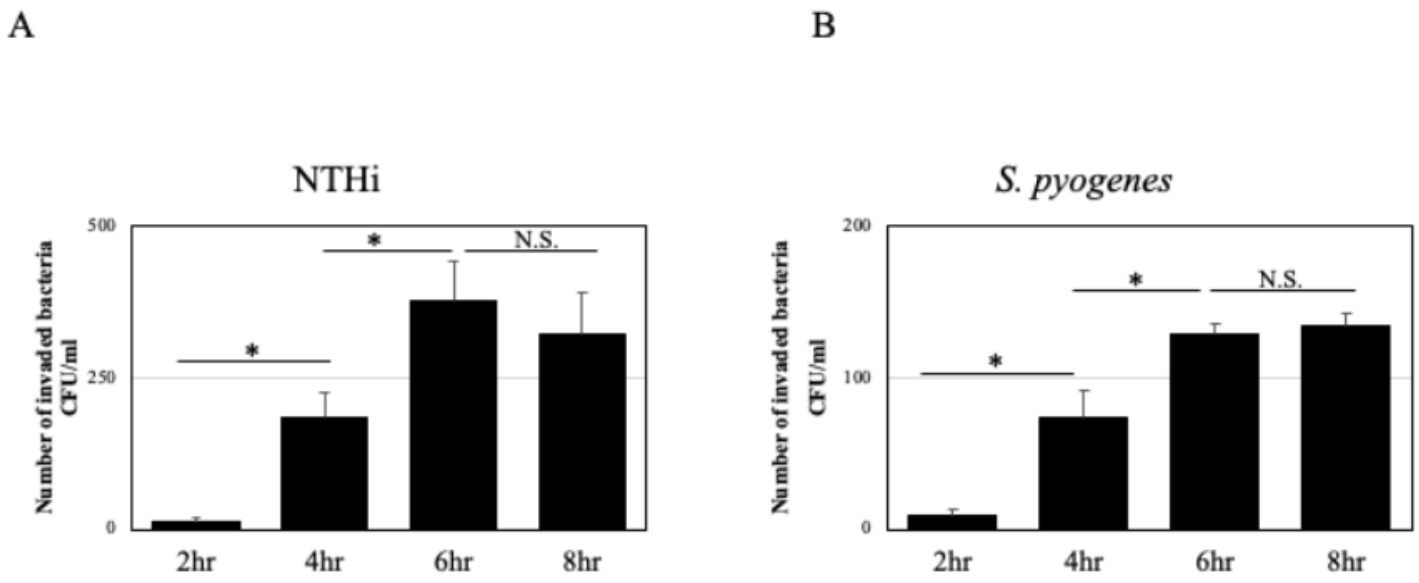

Figure 1

Bacterial invasion time Nontypeable Haemophilus influenzae (NTHi) entered the cells 2 h after adhered to the cells（A）. The maximum invasion was at 6 h（A）. There were no differences between the numbers of bacteria invading the cells after 6 h and 8 h（A）. Similar results were also observed with Streptococcus pyogenes. Streptococcus pyogenes entered the cells 2 h after adhered to the cells（B）. The maximum invasion was at 6 h（B）. There were no differences between the numbers of bacteria invading the cells after 6 h and 8 h（B）. *p < 0.05. N.S., not significant; CFU, colony-forming units.

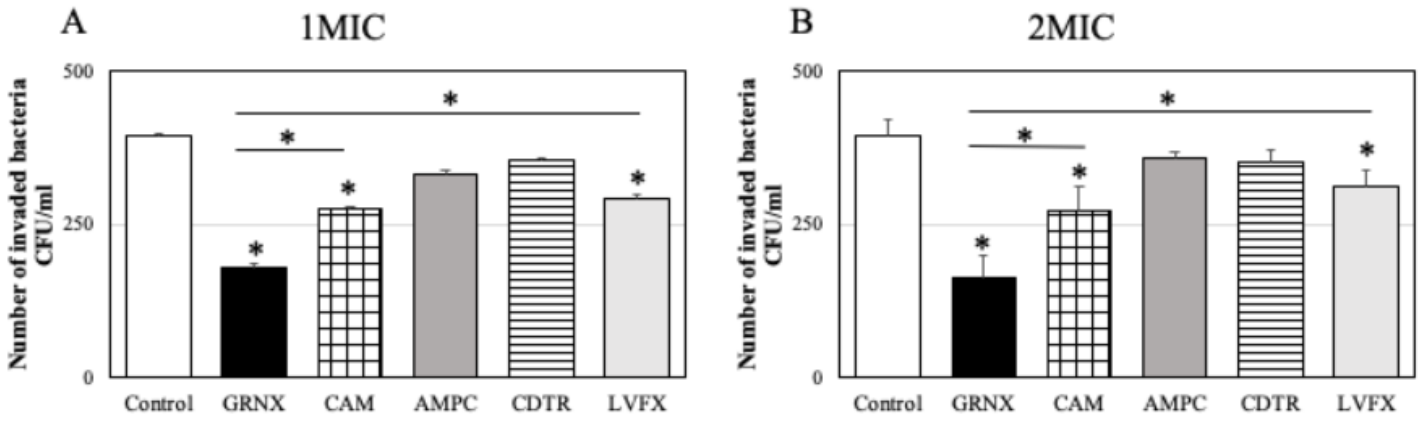

Figure 2

Effects of antibacterial agents on nontypeable Haemophilus influenzae Cells invaded by bacteria, treated with saponin, and then treated with phosphate-buffered saline (PBS) served as a control. A significant bactericidal effect on NTHi was observed when 1 MIC of GRNX, CAM, or LVFX was used (A) ($p < 0.05$). However, no bactericidal effect was observed from treatment with AMPC or CDTR-PI (A). Similarly, treatment with 2 MIC of GRNX, CAM, or LVFX also had a significant bactericidal effect ($p < 0.05$), but when treated with AMPC or CDTR-PI, no bactericidal effect was observed (B). MIC, minimum inhibitory concentration; NTHi, nontypeable Haemophilus influenzae; GRNX, garenoxacin; CAM, clarithromycin; AMPC, amoxicillin; CDTR-PI, cefditoren pivoxil; LVFX, levofloxacin; N.S., not significant; CFU, colony-forming units.

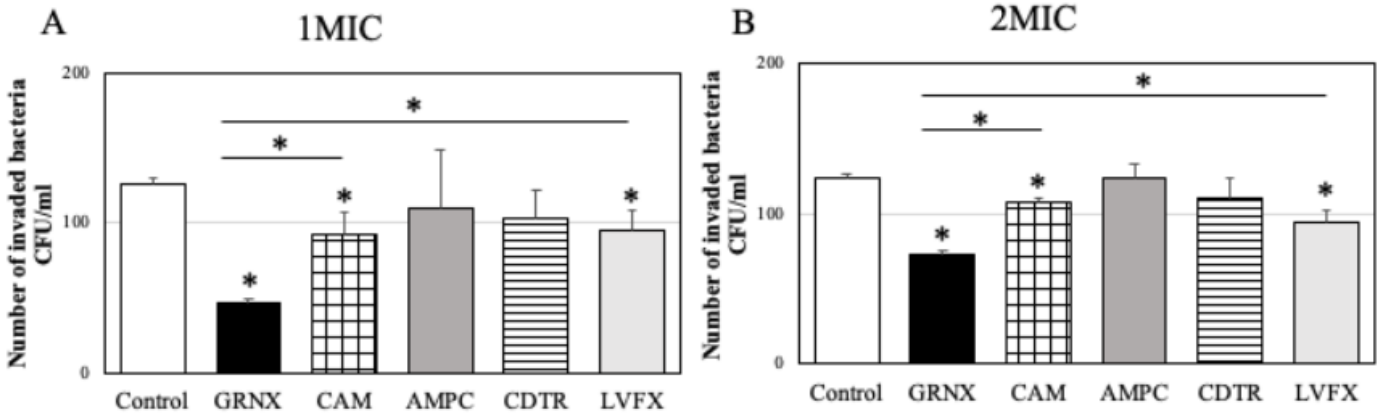

Figure 3

Effects of antibacterial agents on Streptococcus pyogenes Cells invaded by bacteria, treated with saponin, and then treated with phosphate-buffered saline (PBS) served as a control. A significant

bactericidal effect on S. pyogenes was observed when 1 MIC of GRNX, CAM, or LVFX was used (A) ($p < 0.05$). However, no bactericidal effect was observed from treatment with AMPC or CDTR-PI (A). Similarly, treatment with 2 MIC of GRNX, CAM, or LVFX also had a significant bactericidal effect ($p < 0.05$), but when treated with AMPC or CDTR-PI, no bactericidal effect was observed (B). MIC, minimum inhibitory concentration; NTHi, GRNX, garenoxacin; CAM, clarithromycin; AMPC, amoxicillin; CDTR-PI, cefditoren pivoxil; LVFX, levofloxacin; N.S., not significant; CFU, colony-forming units.