

Role of Phosphorylcholine in Streptococcus Pyogenes Adherence to Epithelial Cells

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Abstract

Background: This study aimed to evaluate the influence of Phosphorylcholine (PC) expression on the adherence and invasion of *Streptococcus pyogenes* (*S. pyogenes*) to epithelial cells to clarify the potential effectiveness of a vaccine targeting PC.

Methods: Eight clinical strains of *S. pyogenes* were cultured overnight, and PC expression was evaluated by fluorescence-activated cell sorting. Bacterial adherence and invasion were examined using Detroit 562 cells. An anti-PC-specific monoclonal antibody (TEPC-15) was used to inhibit bacterial PC, and a platelet-activating factor receptor (PAF-R) antagonist (ABT-491) was used to inhibit cellular PAF-R. Additionally, amplification of the *emm* gene was performed using polymerase chain reaction with the standard primers.

Results: The level of PC expressed on the *S. pyogenes* surfaces differed in each strain. Furthermore, PC expression was different even in the same *emm* type. Adherence assay experiments showed that there was a significant negative correlation between TEPC-15 and ABT-491 inhibitory effects and PC expression in *S. pyogenes*. Similarly, intracellular invasion assay experiments showed that there was a significant negative correlation between TEPC-15 and ABT-491 inhibitory effects and PC expression in *S. pyogenes*.

Conclusion: The present study suggests that PC is involved in the cell adhesion and invasion of *S. pyogenes*, regardless of the *emm* type.

Background

Recurrent tonsillitis is one of the most common otolaryngological disorders [1]. The most frequent cause of this disorder is viruses, and the second most frequent cause is bacteria, such as *Streptococcus pyogenes* (*S. pyogenes*), *Haemophilus influenzae*, and *Streptococcus pneumoniae* (Spn) [2]. *S. pyogenes* is a Gram-positive streptococcus with humans as its specific host. It can cause a large variety of infections, ranging from simple benign infections such as sore throats and impetigo to fatal diseases such as streptococcal toxic shock syndrome, necrotizing fasciitis, acute rheumatic fever, and acute glomerulonephritis [3]. The mortality rate of severe *S. pyogenes* infections remains high in both developed and developing countries [4]. Additionally, there are reports of outbreaks of invasive *S. pyogenes* infections in the community and in hospitals [5]. However, there is no effective vaccine against *S. pyogenes*, and development is urgently needed.

There are several candidate protein-based broad-spectrum vaccines, including phosphorylcholine (PC), a structural component of many pathogens including Spn and nontypeable *Haemophilus influenzae* (NTHi) and *emm*, a cell surface protein that is the major virulence and immunological determinant of *S. pyogenes*. Bacterial invasion and colonization are initially induced by bacterial adhesion and fixation onto host mucosal cells, and the binding of PC with platelet-activating factor receptor (PAF-R) expressed on the cell surface is known to play a role in the adherence of *S. pneumoniae* [6] and nontypeable *H.*

influenzae [7] However, more than 170 emm types and 750 emm subtypes of *S. pyogenes* are known [8]. The distribution of emm types reportedly varies among different countries and regions [9].

In the present study, the influence of PC expression and the inhibitory effects of PC and PAF-R on bacterial adherence and invasion in cultured human pharyngeal epithelial cells were examined using several different *S. pyogenes* strains. Based on these results, the effectiveness of a mucosal vaccine targeting PC is discussed.

Results

PC expression and emm genotype on *S. pyogenes*

Fluorescence-activated cell sorting (FACS) analysis of PC expression on *S. pyogenes* strains showed that all the strains examined expressed PC. However, the level of PC expressed on the bacterial cell surfaces, as assessed by mean fluorescence intensity (MFI), differed in each strain (Fig. 1). In this study, emm75 recognized four strains, emm89, emm28, emm12, and emm11 1 (Fig. 1) Furthermore, PC expression tended to be high in emm75 strains, and PC expression was different even in the same emm75 strains (Fig. 1).

Effects of TEPC-15 and ABT-491 on bacterial adherence

A significant positive correlation between PC expression and the number of bacteria adhering to Detroit 562 cells was observed in *S. pyogenes* ($r = 0.76$, $p < 0.05$) (Fig. 2A). Furthermore, when comparing the number of adhered emm75 with other emm types, the number of adhered emm75 was significantly higher ($p < 0.05$) (Fig. 2B). The significant negative correlations were observed between PC expression and the number of adhered bacteria for *S. pyogenes* after pretreatment with TEPC-15 ($r = -0.76$, $p = 0.03$) (Fig. 3A). Moreover, a negative correlation was found with ABT-491 ($r = -0.73$, $p = 0.04$) (Fig. 3B).

Effects of TEPC-15 and ABT-491 on bacterial invasion

A significant positive correlation between PC expression and the number of bacteria invading Detroit 562 cells was observed in *S. pyogenes* ($r = 0.94$, $p < 0.05$) (Fig. 4A). Furthermore, when comparing the number of adhered emm75 with other emm types, the number of invaded emm75 was significantly higher ($p < 0.05$) (Fig. 4B). The significant negative correlations were observed between PC expression and the number of invaded bacteria for *S. pyogenes* after pretreatment with TEPC-15 ($r = -0.83$, $p = 0.01$) (Fig. 5A). Moreover, a negative correlation was found with ABT-491 ($r = -0.90$, $p < 0.05$) (Fig. 5B).

Discussion

The present study is the first to report that PC expression was found in *S. pyogenes*, and PC expression was different even for the same emm type. There was a significant positive correlation between PC expression and the number of bacteria adhering to Detroit 562 cells. Significant negative correlations

were observed between PC expression and the number of adhered and invading bacteria after pretreatment with TEPC-15 and ABT-491.

The presence of PC was first discovered in Spn by Tomasz in 1967 [10]. The molecule PC is a small haptenic moiety that is bound to teichoic acid in the cell wall of several Gram-positive bacteria, including *S. pneumoniae* [11]. PC is a structural component of a wide variety of pathogens and possesses immunomodulatory properties, and the amount of PC on the bacterial surface is modulated by phase variation [12]. The present study demonstrates that the level of PC expressed on the cell surface of *S. pyogenes* differs in each strain, as assessed by fluorescence intensity in flow cytometry. Furthermore, PC expression was different, even for the same emm type.

In contrary, typing based on the M protein, a cell surface protein that is the major virulence and immunological determinant of *S. pyogenes*, is the most widely used method [13]. The M protein, which is encoded by the *emm* gene, possesses a hypervariable region of the amino-terminal with 40–50 amino acid residues [14]. Kuhn et al. have reported that the distribution of *emm* genotypes among patients with recurrent pharyngitis was in the order of *emm12* (24.2%), *emm3* (18.2%), *emm1* (15.2%), and *emm4* (12.1%), and *emm12* was the most frequently detected genotype in recurrent streptococcal pharyngitis cases [15]. In this study, *emm75* strains were 50% (4/8), *emm89*, *emm28*, *emm12*, and *emm11* strains were 12.5% (1/8). Moreover, *emm4*, *emm6*, and *emm75* strains showed significantly higher levels of invasion capacity into Detroit 562 cells than strains with other genotypes [16]. Since the bacteria used in this study were collected from patients with recurrent tonsillitis, it is possible that many *emm75* strains that invaded the cells were detected.

High PC expression is considered to increase *S. pneumoniae* and *H. influenzae* virulence [17]. For instance, it has been reported that *S. pneumoniae* with high PC expression causes more invasive infection than that with low PC expression [18]. Andersson et al. have found that the adhesive capacity of *S. pneumoniae* was highest among the strains isolated from patients with acute otitis media (AOM) and suggested that the capacity to attach to the pharyngeal mucosa is a virulence factor in *S. pneumoniae* causing AOM [19]. Furthermore, it has also been reported that isolates recovered from the blood of patients with severe *S. pyogenes* infections adhere more readily to human epithelial cells than isolates from patients with uncomplicated infections [20]. In this study, there was a significant positive correlation between PC expression and the number of bacteria adhering to Detroit 562 cells. Additionally, the adhesiveness of the *emm75* strain was high. These findings suggest that PC expression in *S. pyogenes* is associated with their adhesive activity to mucosal surfaces, which might be one of the reasons that PC-expressing strains exhibit increased virulence.

This study showed that there was a significant negative correlation between TEPC-15 inhibitory effects and PC expression in *S. pyogenes*. The antibody response to PC has been thoroughly described in mice [21], and the cells involved in regulating anti-PC antibody production have been well characterized [22]. Kurono et al. have reported that the adherence of Spn and NTHi was remarkably reduced by treating bacteria with nasopharyngeal secretions with secretory IgA antibody activity against these bacteria [23].

Furthermore, intranasal immunization of mice with PC induced PC-specific IgA in mucosal secretions such as saliva, which reacted with most Spn and NTHi strains and enhanced the clearance of these bacteria from the nasal cavity [24]. These findings indicate the inhibitory effect of PC-specific IgA on the adherence of these bacteria.

Furthermore, intracellular invasion assay experiments showed that there was a significant negative correlation between TEPC-15 inhibitory effects and PC expression. In this study, there was a significant positive correlation between PC expression and the number of bacteria invading Detroit 562 cells. A number of bacterial species are able to enter host cells by internalization via phagosomes or endosomes [25], including *S. pyogenes* [26]. In general, immediate bacterial degradation with lysosomes follows phagocytosis or endocytosis. However, some bacteria have evolved strategies to avoid the host defense system by escaping from the endocytic compartment into the cytoplasm or modifying the lysosomal acidic environment [27]. *Streptococcus pyogenes* enters human epithelial cells via engulfment by early endosomes, after which endosomes containing bacteria disappear within a few hours, indicating escape into the cytoplasm [27]. Considering these facts, it has been considered that the vaccine targeting PC is also effective against bacteria that invade cells. Additionally, more than half of the emm75 strains temporarily escaped killing by penicillin alone by internalization into epithelial cells, even when the antibiotic concentration used was greater than the 10-fold minimum inhibitory concentration for planktonic *S. pyogenes* [16]. In this study, emm75 strains had high PC expression and also had a large number of intracellular invasions. Vaccines targeting PC may also be effective against emm75 strains, which avoid the effects of antibacterial agents.

To confirm the previously reported association between PC and PAF-R in bacterial adherence, the presence of PAF-R on Detroit 562 cell surfaces and the effects of blocking PAF-R by pretreatment with ABT-491 on the adherence of *S. pyogenes* were examined. PAF-R expression in Detroit 562 cells was confirmed by FACS, as previously reported [28]. Cundell et al. have investigated the attachment of bacterial PC to PAF-R and found that binding between PC and PAF-R enhanced the adherence of Spn and that only virulent Spn engaged with PAF-R [6]. Moreover, the enhanced expression of PAF-R induced by viral antigens is associated with susceptibility of host epithelial cells to Spn infection and contributes to the incidence of recurrent and persistent bacterial upper airway infections [12]. In this study, significant negative correlations were observed between PC expression and the number of adhered and invaded bacteria after pretreatment with ABT-491. These results indicate that PAF-R and its binding to PC play an important role in the adherence of virulent strains of PC-high bacteria.

Our study has some limitations. First, the number of bacterial strains used was small. However, we believe that the reliability was also obtained by examining the emm type. Second, in this study, epithelial cells were 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 similar to those that would have been obtained with the use of normal cells.

Conclusion

In conclusion, the present study demonstrates that the adherence and intracellular invasion of *S. pyogenes* via interaction with PAF-R increased the expression of PC. High PC expression and the adhesive or invasion ability induced by the binding of PC to PAF-R increase bacterial virulence. These findings suggest that a PC-targeting mucosal vaccine is effective against *S. pyogenes*.

Methods

Bacteria and growth conditions

We collected *S. pyogenes* oropharyngeal isolates from patients with recurrent tonsillitis (aged 24–42 years) at Kagoshima University Hospital between March 2019 and December 2020. 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 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 *S. pyogenes* were adjusted to 1.0×10^8 colony-forming units (CFU)/mL at an absorbance of 580 nm.

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 $\mu\text{g}/\text{mL}$; Nacalai Tesque) at 37°C in a 5% CO_2 incubator, as previously described (Iuchi et al., 2020). The cells were harvested using trypsin (final concentration, 0.02%) and ethylenediaminetetraacetic acid (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.

Phosphorylcholine (PC) expression of *Streptococcus pyogenes*

The levels of PC expression on bacterial surfaces were quantified by fluorescence-activated cell sorting (FACS) using a CytoFLEX flow cytometer. Bacteria that had been cultured overnight on blood agar plates, as appropriate, were suspended in PBS and incubated at 4°C for 4 h with TEPC-15 (Sigma-Aldrich, St. Louis, MO, USA), which is a PC-specific monoclonal immunoglobulin (Ig) A antibody (1:100 dilution), or with a purified mouse IgA antibody (1:50 dilution; BD Biosciences) as an isotype control. Next, the bacteria were rinsed in PBS and incubated with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (1:50 dilution; KPL, Gaithersburg, MD, USA) for 30 min at 20°C before analysis.

Polymerase chain reaction amplification for the detection of the emm gene

For DNA extraction from the isolates, a commercial extraction and purification kit (Roche, Berlin, Germany) was used according to the manufacturer's instructions. The purity of the extracted DNA was measured with a photobiometer (Eppendorf, Hamburg, Germany) in 260/280 nm UV long waves. To amplify the *emm* gene, the set of primers for *emm1* (50-TATTCGCTTAGAAAATTAA-30) and *emm2* (50-GCAAGTTCTTCAGCTTGTTT-30) were used, which amplified a 914-bp fragment of the target gene. Polymerase chain reaction (PCR) amplification was performed in a final volume of 25 μ L containing 1 \times PCR buffer, 1.5 mM MgCl₂, 200 mM deoxynucleotide, 0.4 mM of each primer, 1.5 U Taq polymerase, and 1 mL of template DNA. All reagents were purchased from QIAGEN (Hilden, Germany). Amplification was performed on a thermocycler nexus gradient (Eppendorf), and the cycling program consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 46°C for 45 s, extension at 72°C for 59 s, and a final extension at 72°C for 7 min. A control positive for *S. pyogenes* ATCC 8668 and a control negative for *S. pyogenes* ATCC 8668 were included in each PCR run. The products were run on a 1.5% agarose gel (w/v) containing 0.5 mg/mL ethidium bromide (QIAGEN, Hilden, Germany). The results were recorded using a gel documentation system (Protein Simple, San Jose, CA, USA). A 100-bp DNA ladder was used as a size marker (Roche). The PCR products were sent for sequence analysis (Bioneer Co., Daejeon, South Korea). The *emm* sequences were blasted against the *emm* database using the BLAST program at the National Center for Biotechnology (www.ncbi.nlm.nih.gov/BLAST/) to determine the *emm* sequence type.

Platelet-activating factor receptor expression on Detroit 562 cells

FACS analysis was performed to determine PAF-R expression on Detroit 562 cell surfaces. The cells were incubated for 1 h at room temperature with mouse anti-human PAF-R monoclonal antibody (1:1000 dilution; Cayman Chemical, Ann Arbor, MI, USA) or mouse IgG2a antibody (1:1000 dilution; DakoCytomation, Glostrup, Denmark) as an isotype control, followed by incubation with FITC-labeled goat anti-mouse antibody (1:20 dilution; DakoCytomation, Glostrup, Denmark) for 30 min at 4°C. The mean fluorescence intensity (MFI) of PAF-R was compared to that of the isotype control.

Adherence assay

Detroit 562 cells were seeded into the wells of a 96-well plate (Thermo Fisher Scientific, Nunc A/S, Roskilde, Denmark). Next, 100 μ L of each bacterial strain (*S. pyogenes*: 1.0 \cdot 10⁵ CFU/mL) was added to the cells and allowed to adhere at 37°C in a 5% CO₂ incubator for 2 h. Next, each well was washed 10 times with 200 μ L of PBS and treated with 100 μ L of saponin at 37°C in a 5% CO₂ incubator for 15 min. Finally, 100 μ L of the solution from each well was plated on sheep blood agar plates, as appropriate, and the number of colonies after 12 h of incubation was evaluated.

To investigate the effects of PC-specific IgA on bacterial adherence, bacterial cells were treated with TEPC-15 (1 μ g/mL) at 37°C in a 5% CO₂ incubator for 1 h. Subsequently, the adherence assay was performed. In addition, PAF-R expressed on Detroit 562 cell surfaces was blocked with 100 μ M of a PAF-R

antagonist (ABT-491; Cayman Chemical) at 37°C in a 5% CO₂ incubator for 1 h, and the influence on bacterial adherence was then examined.

Intracellular invasion assay

One hundred microliters of each *S. pyogenes* strain (1.0×10^8 CFU/mL) was added to Detroit 562 cells cultured in a 96-well plate and allowed to adhere at 37°C in a 5% CO₂ incubator for 6 h. Each well was then treated with gentamicin (200 µg/mL) at 37°C in a 5% CO₂ incubator for 1 h. After washing five times with 200 µL of PBS, the cells were treated with 100 µL of saponin at 37°C in a 5% CO₂ incubator for 15 min. Furthermore, 100 µL of the samples from each well was plated on sheep blood agar and cultured overnight, and the number of colonies formed was counted.

Statistical analyses

All statistical data were analyzed using SPSS for Windows software (version 22.0; IBM Corp., Armonk, New York, USA), and the values were presented as mean ± standard deviation. The data were statistically analyzed using the unpaired Student *t*-test and Pearson correlation coefficient (cross-reaction data). Of note, we considered statistically significant differences when the probability values were lower than 5%.

Abbreviations

PC: Phosphorylcholine

S. pyogenes: *Streptococcus pyogenes*

TEPC-15: anti-PC-specific monoclonal antibody

PAF-R: platelet-activating factor receptor

ABT-491: PAF-R antagonist

Spn: *Streptococcus pneumoniae*

NTHi: nontypeable *Haemophilus influenzae*

FACS: Fluorescence-activated cell sorting

MFI: mean fluorescence intensity

PBS: phosphate-buffered saline

CFU: colony-forming units

Declarations

Ethics approval and consent to participate

We obtained written informed consent from all subjects, or from parents or legal guardians of those subjects aged below 18 years. This study was approved by the ethics committee of Kagoshima University (200306).

All methods were performed in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request. The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

H I: methodology, formal analysis, and writing the original draft. J O: software, investigation and validation. T T: resources, project administration, data curation. S K: data curation.

All authors read and approved the final manuscript.

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Figures

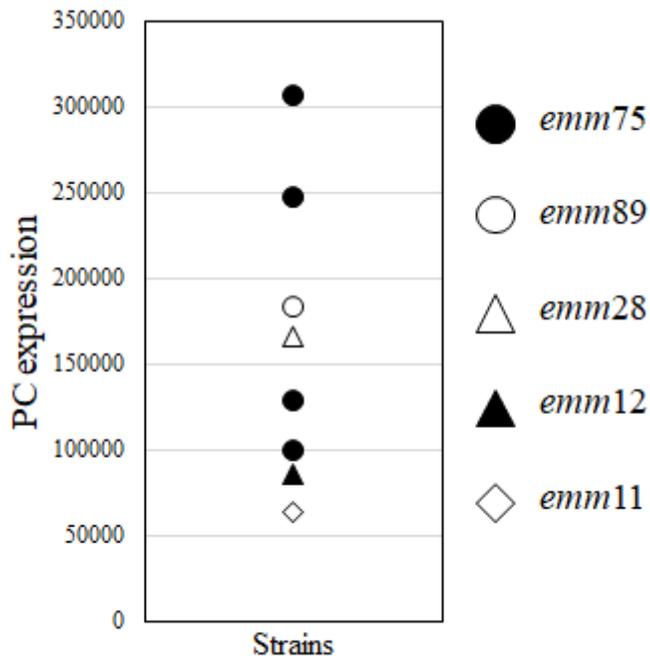


Figure 1

Phosphorylcholine (PC) expression and emm type on *Streptococcus pyogenes* PC expression was different among the bacterial strains. In addition, PC expression was different, even with the same emm type.

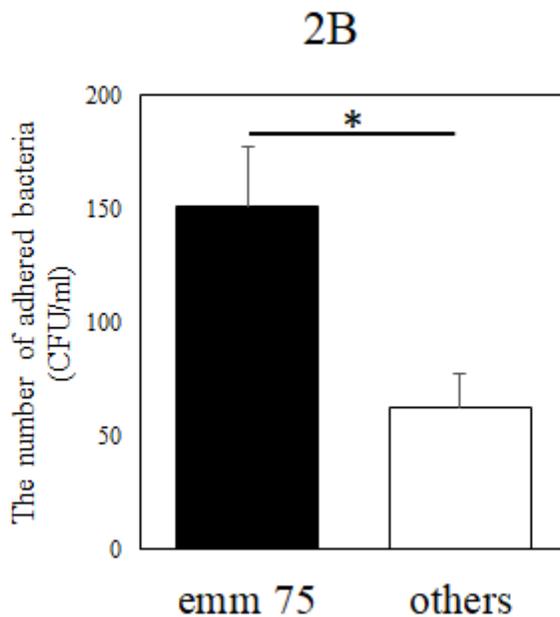
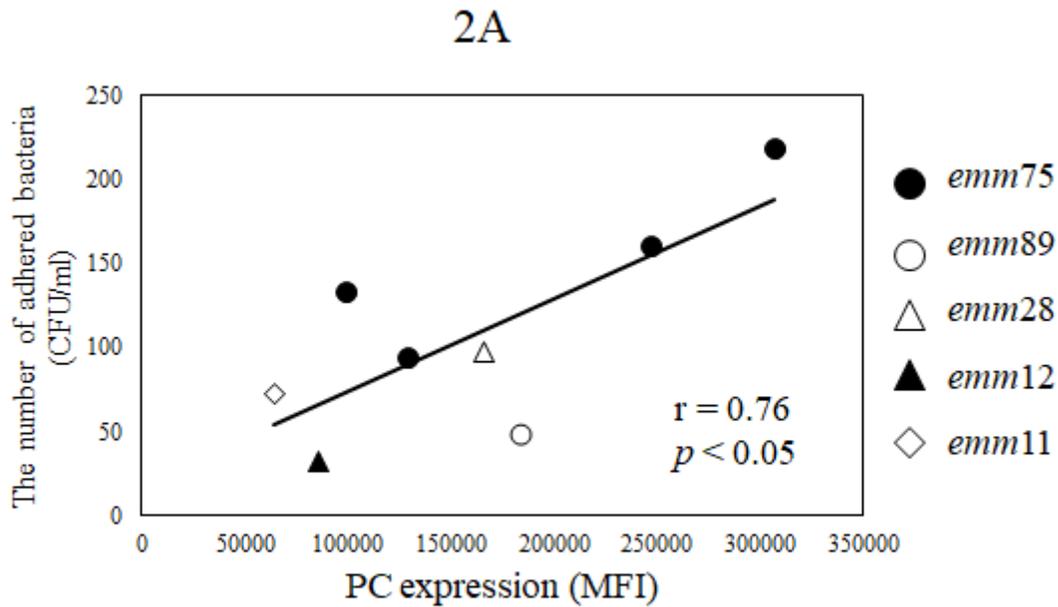
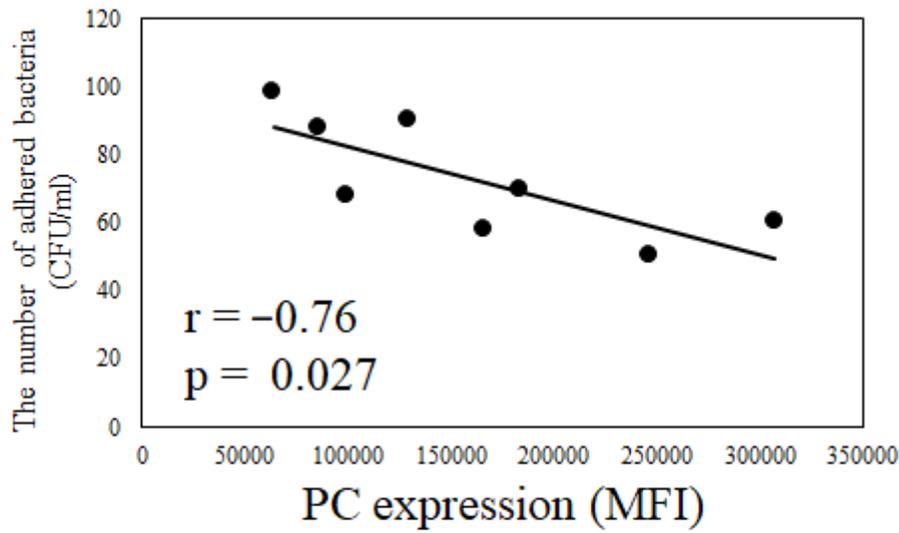


Figure 2

Association between phosphorylcholine (PC) expression and bacterial adhesion PC expression showed a strong positive correlation with the adhesive ability of *Streptococcus pyogenes* ($r = 0.76$, $p < 0.05$). (2A) Furthermore, *emm75* had higher adhesiveness than the other types. (2B) $*p < 0.05$

3A



3B

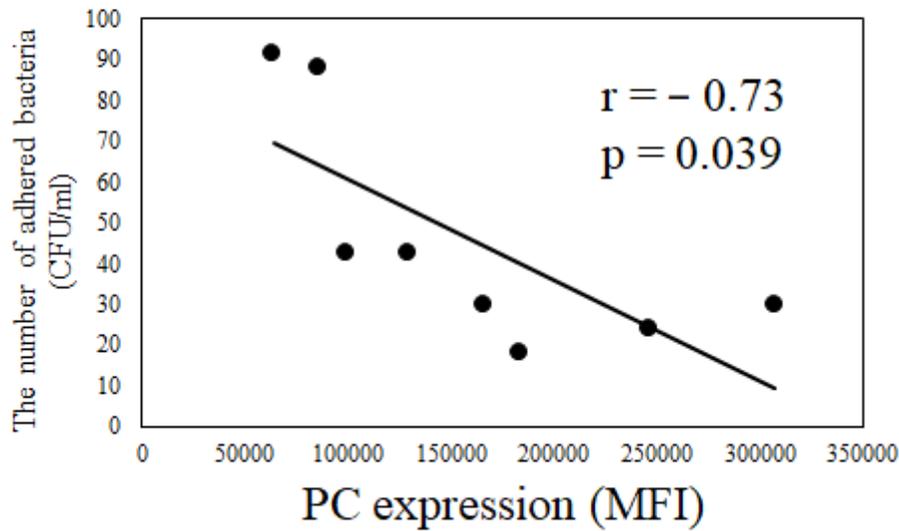
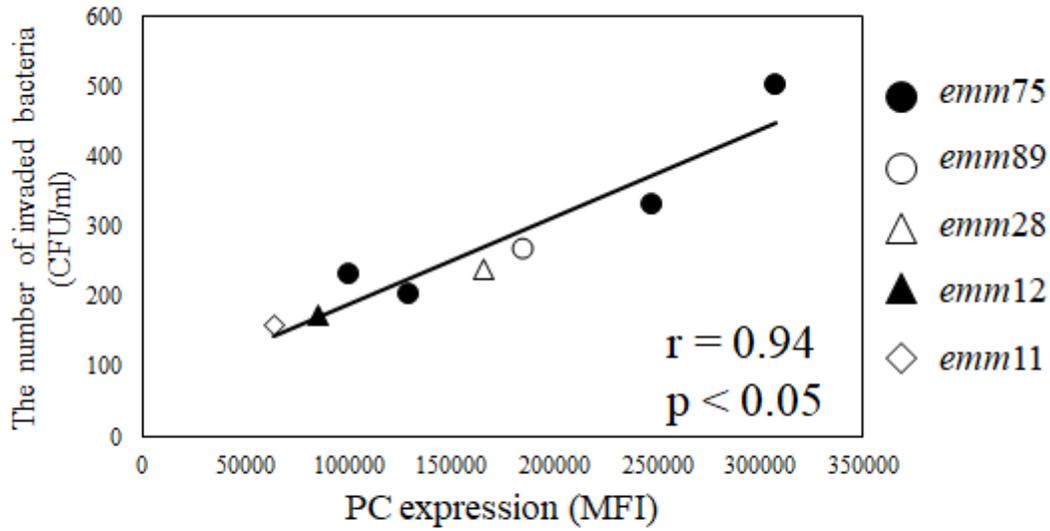


Figure 3

Effects of TEPC-15 and ABT-491 on the adherence of *Streptococcus pyogenes*. Inhibition of adherence by pretreatment with TEPC-15 ($r = -0.76$, $p = 0.027$) (3A) and ABT-491 ($r = -0.73$, $p = 0.039$) (3B) showed a negative correlation with phosphorylcholine expression in *S. pyogenes*.

4A



4B

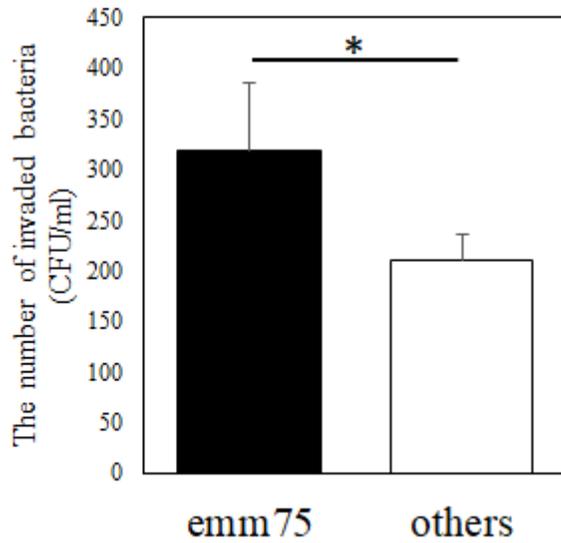
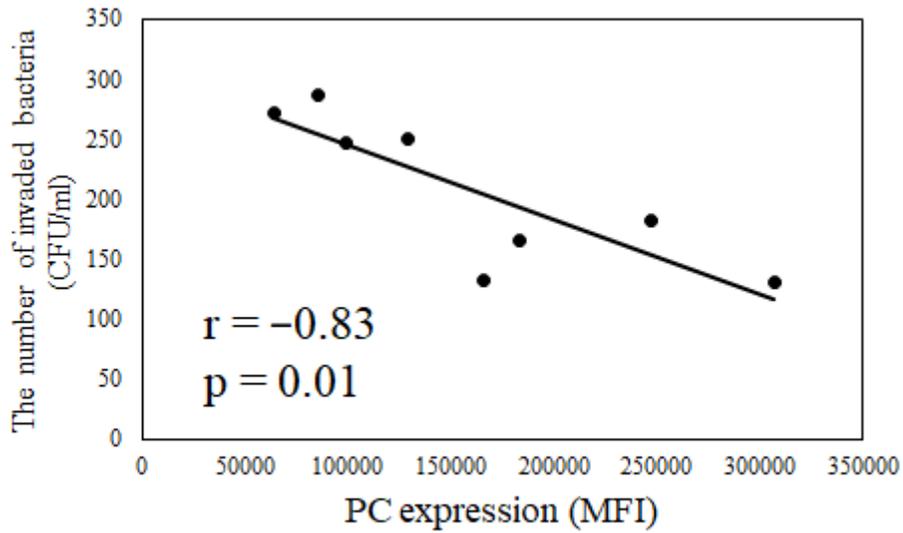


Figure 4

Association between phosphorylcholine (PC) expression and bacterial invasion PC expression showed a strong positive correlation with the invasion ability of *Streptococcus pyogenes* ($r = 0.94$, $p < 0.05$). (4A) Furthermore, *emm75* was more invasive than the other types. (4B) * $p < 0.05$

5A



5B

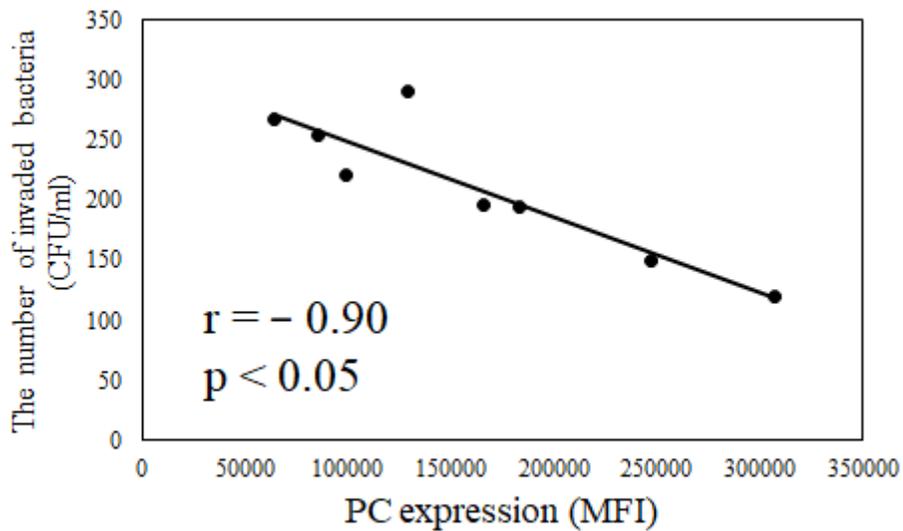


Figure 5

Effects of TEPC-15 and ABT-491 on the invasion of *Streptococcus pyogenes*. Inhibition of invasion by pretreatment with TEPC-15 ($r = -0.83$, $p = 0.01$) (5A) and ABT-491 ($r = -0.90$, $p < 0.05$) (5B) showed a negative correlation with phosphorylcholine expression in *S. pyogenes*.