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Research Article

Research: *In vitro* Rifampicin Combination Chemotherapy Confers Rapidly Rifampicin Resistance for Biofilm Formed *Staphylococcus aureus*

Takashi Uno[1,2*], Takumi Sato[1], Mariko Yagi[1], Ryota Ito[1], Masato Kawamura[1], Shigeru Fujimura[1]

[1]Division of Clinical Infectious Diseases and Chemotherapy, Graduate School of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, Sendai, Japan; [2]Department of Pharmacy, Tohoku Medical and Pharmaceutical University Hospital, Sendai, Japan

ABSTRACT

Biofilm-forming *Staphylococcus aureus* makes it difficult to treatment for prosthetic joint infection. To improve the outcome of treatment, rifampicin is used combined with several antimicrobial agents. However, the evidence is not clear that the sterilization effect of antimicrobial agents with the concentration in bone tissue when the usual dose against biofilm-formed staphylococci. Using 10 isolates of *S. aureus*, we made a biofilm formation model on the washer surface which assumed a medical device in this study. The sterilization effect by combined treatment rifampicin and other antimicrobial agents (cefazolin, vancomycin, or clarithromycin) was considered against these models. All biofilm-formed *S. aureus* was not sterilized by 120 hours of exposure with a single antimicrobial agent. Besides, four strains were not sterilized by the exposure of a combination of rifampicin and cefazolin, and these strains acquired rifampicin resistance 8 hours later. Similarly, in rifampicin and vancomycin or and clarithromycin, 2 strains and 3 strains were not sterilized, respectively. Therefore, by all combinations including rifampicin, the biofilm-formed *S. aureus* were not completely sterilized. It was shown that the acquired rifampicin-resistant in 50% out of clinical isolates occurred 8 hours after the exposure of combined antimicrobial agents. Furthermore, with 4 of these 9 strains that were not sterilized, biofilm production was rather promoted. One of the reasons that these strains were not sterilized, probably is reduced the bactericidal effect of other antimicrobial agents due to increased biofilm formation by rifampicin-resistant acquisition. When rifampicin is selected for the treatment of prosthetic joint infection, the acquisition of rifampicin-resistance should be confirmed 24 hours after administration.

Keywords: Biofilm; Combination therapy; Rifampicin; Rifampicin resistance; *S. aureus*

INTRODUCTION

Staphylococcus aureus, known to be the major pathogen involved in device-related infections including Prosthetic Joint Infection (PJI), can sometimes form biofilm. PJI is generally treated by a combination of surgical procedure such as debridement or prosthesis removal and antimicrobial agent administration. The Infectious Diseases Society of America (IDSA) [1] recommends treatment with Rifampicin (RFP) plus nafcillin, Cefazolin (CEZ), or ceftriaxone, in case the joint prosthesis is to be preserved. The administration period should be 3 months for infected knee prosthesis, and 6 months for an infected hip prosthesis. A similar antibiotic protocol for 3 months is recommended when the joint prosthesis is re-implanted. In this way, RFP is used to treat PJI on the assumption of biofilm-formed *S. aureus* is involved in the infection. Regarding the outcome of this antibiotic protocol, a report from Spain showed 76% of patients with PJI due to *S. aureus* were treated effectively with RFP combination therapy [2]. However, in Australia, a research involving patients with PJI due to methicillin-resistant *S. aureus* infection, found that treatment of PJI failed in 25% of the patients who received RFP combination therapy [3]. Additionally, Morata, et al. [4] reported that there were no differences in the rates of successful treatment in patients with PJI between the group receiving Linezolid (LZD) and the group receiving LZD plus RFP. Other than that, multicenter, randomized, double-blind, placebo-controlled trial of patients with *S. aureus* bacteremia, conducted at 29 institutions, in the UK in 2019, found no significant differences between active antibiotic therapy plus RFP group and active antibiotic therapy group, among therapeutic effect, rate of recurrence and number of deaths; however, the incidences of adverse events and drug interactions were rather higher in the RFP-combination group [5]. Thus, the efficacy and safety of RFP combination, which is expected to have an anti-biofilm effect, are not well explained.

Also, RFP monotherapy is known to easily induce resistance [6-8].

Correspondence to: Takashi Uno, Division of Clinical Infectious Diseases and Chemotherapy, Graduate School of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, Sendai, Japan, E-mail: t.uno124@gmail.com

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The mutation of rpoB gene, encoding RNA polymerase β-subunit targeted by RFP, reduces affinity of RFP due to structural change of protein results of substitution of amino acid residue [9]. Each amino acid residue of His481, Ala477 or Ile527 was reported as major mutation point that poses RFP-resistance [10-12], and other mutation points were focused in the region from 462 to 530 called cluster I and/or II [10].

In recent years, the increase of the RFP-resistant rate of *S. aureus* has been an issue in South Africa [13] and China [14]. RFP is used with other antimicrobial agents to prevent resistance, but the inhibitory effect on RFP resistance of the combination antibiotic protocol has not been clarified. Although RFP is regarded to have high permeability to bone tissues [15] and it has been shown to readily penetrate into biofilm; however, the anti-biofilm effect of RFP according to its concentration in bone tissue has not been adequately investigated. The aim of this study is to investigate a sterilization effect of in vitro RFP combination chemotherapy against *S. aureus* which formed biofilm on the medical device.

MATERIALS AND METHODS

S. aureus strains and antimicrobial agents

Among 292 strains of *S. aureus* isolated from 16 general hospitals in the Tohoku region, we chose 9 strains, from different institutions, that are susceptible to the all of the following antimicrobial agents, and the standard strain of *S. aureus* ATCC 29213. The used antimicrobial agents were CEZ (sigma-aldrich Co., LLC, Tokyo), Vancomycin (VCM: shionogi Co., Ltd. Osaka), Clarithromycin (CAM: taisho pharmaceutical Co., Ltd. Tokyo), and RFP (WAKO, Osaka).

Measurement of Minimum Inhibitory Concentration (MIC)

MIC of each antimicrobial agent for these 10 strains was measured by the broth microdilution method [16] in accordance with the Clinical and Laboratory Standards Institute (CLSI). According to the breakpoint [17] of CLSI M100-S22, a strain was considered to be resistant if it showed the following values: CEZ, ≥ 32 μg/mL; VCM, ≥ 16 μg/mL; CAM, ≥ 8 μg/mL; or RFP, ≥ 4 μg/mL.

In vitro biofilm formation model

These strains were cultured for 24 hours at 37°C after being inoculated on Mueller-Hinton Agar (MHA: eiken, Tokyo) plates. Single colony was taken into 10 ml of Tryptic Soy Broth (TSB: bectondickinson, Tokyo) including 1% of glucose (TSBG) and the suspension was adjusted to a cell density of 1 × 108 CFU/mL. Sterilized washer (internal diameter 4.0 mm, external diameter 10.0 mm, thickness 0.8 mm) (ohsatoCo., Ltd. Tokyo) were introduced into the cell suspension, after incubation for 48 hours at 37°C in a shaker, biofilm-formed models were established. The established biofilm was confirmed by examination under Scanning Electron Microscope VE-8800 (SEM: keyence, Osaka). Thereafter, the washer showing grown biofilm formation was washed by PBS, fixed in 2.5% glutaraldehyde for 24 hours at room temperature, and then dehydrated by dipping in ethanol (50%, 75%, and 99.5%) for 10 minutes at each step. Sputter coating of the washer with gold [18] was achieved using magnetron sputter (keyence), and observed by SEM at an accelerating voltage of 10 kv.

Bactericidal effect of each antimicrobial agent with and without Rfp on biofilm-formed *S. aureus*

A total of 7 biofilm-formed models were established per strain, added with each antimicrobial agent every 24 hours in TSBG, and cultured for a total of 120 hours at 37°C. Each washer was taken out one by one at hour 0 and every 24 hours thereafter, washed 3 times, rubbed 50 times with 1mL of PBS [19]. The number of viable bacteria in 100 μL of biofilm-formed *S. aureus* suspension was counted. When the viable count was lower detection limit of 102 CFU/mL, the washer was placed in antibiotic-free MHA and incubated at 37°C for 48 hours. It was determined to be completely sterilized when it did not bacterial growth. The concentration of each antimicrobial agent, referring to the concentration in bone tissue when the usual dose of each agent is administered, was CEZ, 20 μg/mL [20]; VCM, 7 μg/mL [21]; CAM, 1 μg/mL [18]; or RFP, 1 μg/mL [22]. Additionally, the bactericidal effect of RFP plus CEZ, VCM, or CAM was determined. The MICs of each antimicrobial agent for surviving strains after RFP combined with each antimicrobial agent were also measured.

rpoB gene sequence analysis

The *rpoB* gene mutation was analyzed in the strains with confirmed resistanceafterexposuretoRFPcombinedwithanotherantimicrobial agent. The rpoB-Fw (5'-ACCGTCGTTTACGTTCTGTA-3') and the *rpoB*-Rv (5'-TCAGTGATAGCATGTGTATC-3') were used as primers [23] for DNA sequencing. Amplicons were purified using FastGeneTM Gel/PCR extraction Kit (NIPPON Genetics Co., Ltd. Tokyo). The DNA sequence was determined by the dye terminator cycle sequencing method using genomelabGeXP (beckman coulter Inc., CA).

Growth Rate of *S. aureus*

To examine any differences in growth rate of each parental strain between the surviving group after exposure to RFP combined with each antimicrobial agent and the non-surviving group, these 10 strains were divided into to 2 groups after exposure to each combined RFP plus CEZ, VCM, or CAM: the surviving group (MS-2, -14, -18, -19, -20) and the non-surviving group (MS-4, -5, -10, -17, ATCC 29213). Each parental strain was suspended in 100 μL in Mueller-Hinton Broth (MHB: eiken, Tokyo), diluted to a cell density of 104 CFU/mL, and then incubated in 96-well plate for 24 hours at 37°C. The number of viable bacteria was counted by collecting each bacterial culture immediately after the start of the incubation, and after 2, 4, 6, 8, 12, 15, 18, and 24 hours.

Measurement of biofilm amount

The measurement of biofilm amount was determined using a modification of a previously reported method [24,25]. RFP-resistant strain and the parental strain suspended to individually 3×10^6 CFU/mL in TSBG and incubated for 48 hours at 37°C in 96-well plate. The plate in which biofilm-formed *S. aureus* was washed 3 times with PBS after removing the culture media. Then, 100 μL/well of 0.1% (w/v) Crystal Violet solution (CV: WAKO, Osaka), were added and they were stained for 5 minutes. The plate was washed with Milli-Q after removing CV solution. Finally, the bound CV in biofilm was released by adding 30% acetic acid solution (WAKO). The amount of biofilm was determined according to the absorbance at 595 nm using microplate reader model 680 (Bio-Rad, CA).

Statistical analysis

Statistical analysis of the results was performed using Student's t-test. A difference was considered statistically significant at a P value of <0.05.

RESULTS

MIC of each antimicrobial agent

MIC ranges of CEZ, VCM, CAM, or RFP for 9 clinical isolates

of *S. aureus* were 0.5-1 μg/mL, 0.5 μg/mL-1 μg/mL, 0.25 μg/mL-1 μg/mL and 0.0078 μg/mL-0.0156 μg/mL, respectively (Table 1). Similarly, MICs of each antimicrobial agent for ATCC 29213 strain were CEZ, 0.5 μg/mL; VCM, 1 μg/mL; CAM, 0.25 μg/mL; and RFP, 0.0078 μg/mL.

Antibacterial activity and MIC values for biofilm-formed *S. aureus*

In all strains, a three-dimensional clump observed at the biofilm formation was confirmed on the washer by SEM (Figure 1). The single exposure by CEZ, VCM, CAM and RFP was not able to sterilize 4, 7, 8 and 7 strains, respectively. Whereas, by the combination exposure to RFP, the viable bacterial number of 29 strains except one (MS-20) of the RFP plus CAM exposure became lower than detection limit 96 hours later (Figure 2). However, nine strains (MS-2 exposed with RFP + CEZ (RCE), -2 exposed with RFP+VCM (RV), -2 exposed with RFP+CAM (RCA), -14RCE, -18RCE, -18RV, -18RCA, -19RCE, -20RCA) in these 30 strains of the combination exposure group did not completely sterilized (Table 2), and the MICs of RFP were >128 μg/mL (Table 3). All these RFP resistant strains retained susceptibility to other antimicrobial agents.

Table 1: Minimum inhibitory concentrations of antimicrobial agents for *Staphylococcus aureus* isolates.

Strain	MIC (μg/mL)			
	CEZ	VCM	CAM	RFP
MS-2	0.5	1	1	0.0156
MS-4	0.5	1	0.25	0.0156
MS-5	1	1	0.25	0.0156
MS-10	1	1	0.25	0.0078
MS-14	0.5	1	0.25	0.0156
MS-17	1	1	0.25	0.0078
MS-18	0.5	1	0.25	0.0156
MS-19	1	0.5	0.25	0.0078
MS-20	0.5	1	0.25	0.0078
ATCC 29213	0.5	1	0.25	0.0078

CEZ: Cefazolin; VCM: Vancomycin; CAM: Clarithromycin; RFP: Rifampicin

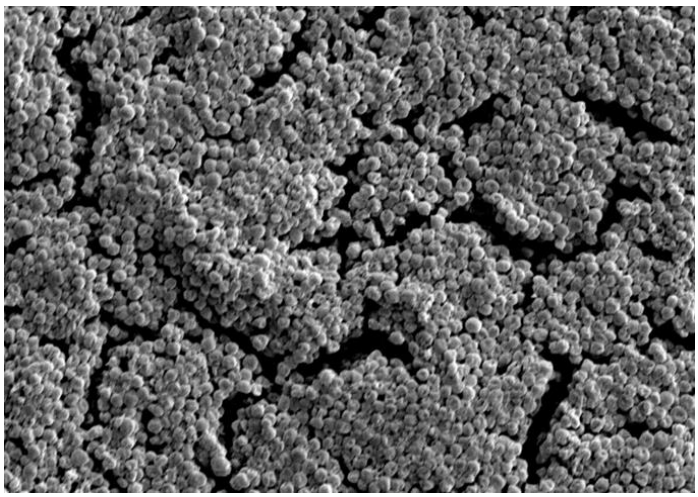

Figure 1: SEM image of biofilm on the washer. Sterilized stainless washer was placed on the medium, then strain (10^8 CFU/mL) was inoculated, and incubation for 48 hours at 37°C in a shaker. Representative images are shown.

Sequence analyses of *rpoB* gene mutation in RFP-resistant strains

Some different *rpoB* mutation points in 9 strains with acquired resistance to RFP were confirmed. In these amino acid mutations, His481>Tyr was confirmed in 4 strains: MS-14RCE, -18RCE, -18RV, and -18RCA. Ile527>His was confirmed in 3 strains: MS-2RCE, -2RV, -2RCA. Ser486>Leu and Ser486>Phe were confirmed in MS-19RCE and -20RCA strains, respectively (Table 3).

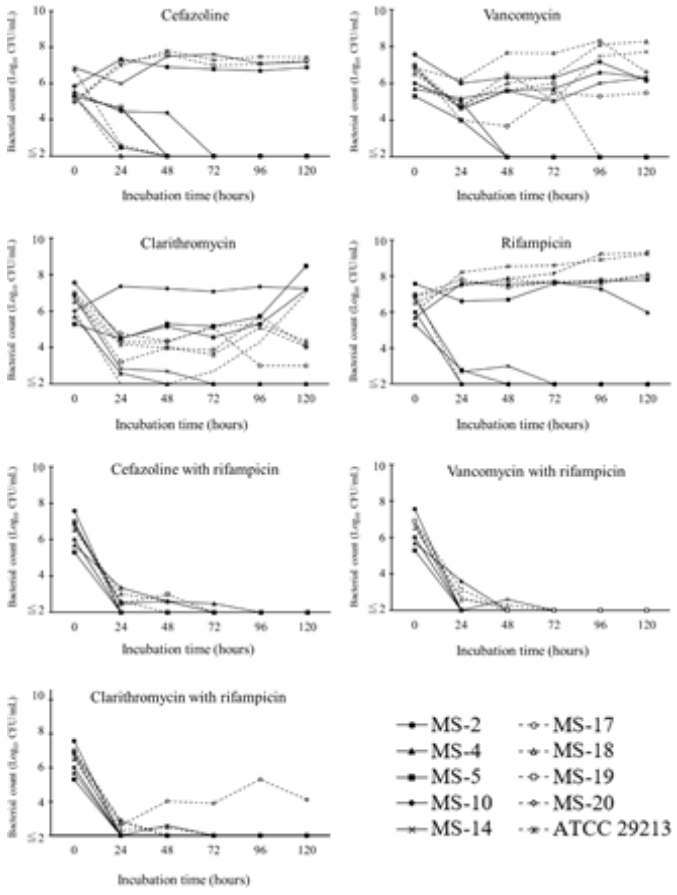

Figure 2: Changes of bacterial count exposure to single antimicrobial agent and combined rifampicin. The vertical axis shows the bacterial counts after exposure of *Staphylococcus aureus* that formed biofilm for a certain period of time to antimicrobial agent. The detection limit is lesser than 10^2 CFU/mL.

Table 2: Sterilize effect of exposure to each antimicrobial agent with and without rifampicin.

Strain	Antibiotic				Antibiotic combined with RFP		
	CEZ	VCM	CAM	RFP	CEZ	VCM	CAM
MS-2	F	F	F	F	F	F	F
MS-4	S	F	S	F	S	S	S
MS-5	S	S	F	S	S	S	S
MS-10	S	S	F	S	S	S	S
MS-14	F	F	S	F	F	S	S
MS-17	F	F	F	S	S	S	S
MS-18	S	F	F	F	F	F	F
MS-19	S	S	F	F	F	S	S
MS-20	S	F	F	F	S	S	F
ATCC 29213	F	F	F	F	S	S	S

CEZ: Cefazolin; VCM: Vancomycin; CAM: Clarithromycin; RFP: Rifampicin; F: Failed; S: Sterilized

Table 3: Changes in minimum inhibitory concentration of strains before and after the combined exposure to rifampicin and other antimicrobial agents, and rpoB mutation after combined exposure to rifampicin.

Strain	RFP with	MIC (mg/mL) CEZ	VCM	CAM	RFP	rpoB mutation
MS-2	CEZ	0.5 →1			0.0156 → >128	Ile527>His
	VCM		1 → 1		0.0156 → >128	Ile527>His
	CAM			1 → 0.125	0.0156 → >128	Ile527>His
MS-14	CEZ	0.5 → 1			0.0156 → >128	His481>Tyr
MS-18	CEZ	0.5 → 1			0.0156 → >128	His481>Tyr
	VCM		1 → 1		0.0156 → >128	His481>Tyr
	CAM			0.25 → 0.125	0.0156 → >128	His481>Tyr
MS-19	CEZ	1 → 1			0.0078 → >128	Ser486>Leu
MS-20	CAM			0.25 → 0.25	0.0156 → >128	Ser486>Phe

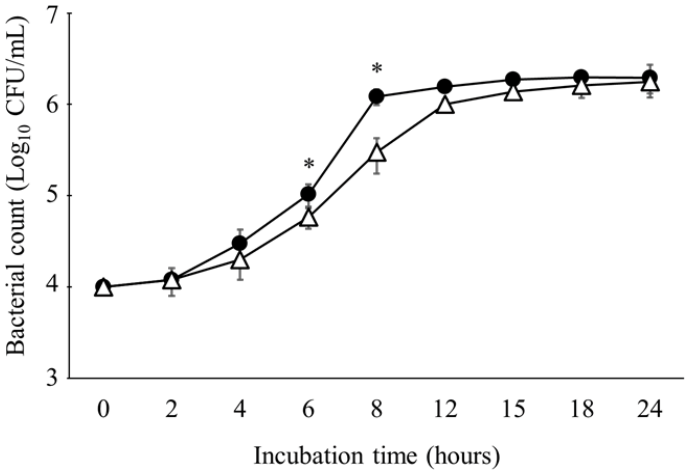

Figure 3: Comparison of growth rate between parental strains of the surviving group and of the non-surviving group. The vertical axis shows the bacterial counts and the abscissa axis shows incubation time. Filled circle, Surviving group (N=5); open triangle, Non-surviving group (N=5). *p<0.05.

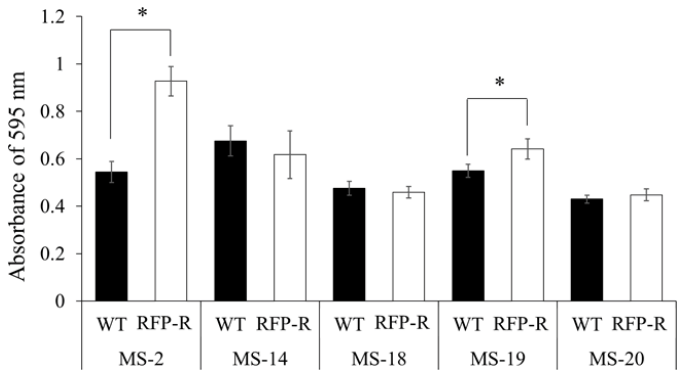

Figure 4: Comparison of biofilm formation amounts between rifampicin-resistant strains and their parental strains. Black bars, parental strains; White bars, rifampicin-resistant strains. WT, wild type; RFP-R, rifampicin resistance.*p<0.05.

Comparison of growth rate of strains

The growth rate of both parental strains of the non-surviving group and the surviving group was compared in MHB. In exposure 6 hours later and 8 hours later, growth bacterial counts of the surviving group were significantly higher than the non-surviving group ($p<0.05$). Namely, parental strains of the surviving group increased to bacterial counts of 10^6 CFU/ml in 7.6 hours, but that of the non-surviving group was 12 hours (Figure 3).

Comparison of biofilm amount

The amount of biofilm in 9 strains (MS-2RCE, -2RV, -2RCA, -14RCE, -18RCE, -18RV, -18RCA, -19RCE, -20RCA) and their parental strains (MS-2, -14, -18, -19, -20) that showed resistance to RFP after combined exposure to RFP plus each antimicrobial agent were compared. Amount of biofilm in each parental strain of MS-2 and MS-19 were absorbance of 0.54 ± 0.04 and 0.55 ± 0.02, respectively. On the other hand, each average of these strains acquired RFP-resistance was 0.93 ± 0.06 and 0.64 ± 0.04, respectively. In other words, the amounts of biofilm in RFP-resistant strains were significantly higher than those in these parental strains ($p<0.05$) (Figure 4).

DISCUSSION

PJI is a devastating complication of total joint replacement surgery and in 60% or more of the cases, the pathogenic bacteria are *S. aureus* and coagulase-negative staphylococci [26,27]. Moreover, staphylococci can lead to biofilm formation on the device, which makes them hard to eliminate due to their resistance to antimicrobial agents. Therefore, antimicrobial chemotherapy that combines antimicrobial agents with RFP is expected to penetration into biofilm [28-32]. In this study, we investigated the bactericidal effect of single exposure to CEZ, VCM, CAM, and RFP on biofilm-formed *S. aureus* and of combined exposure to RFP plus CEZ, VCM, or CAM. Biofilm-formed *S. aureus* strains survived 40%-80% by exposure of single antimicrobial agent. It was confirmed that the strain exposed to RFP acquired RFP-resistance 24 hours later. This early acquisition of RFP resistance supports the findings of previous reports [33,34].

Jørgensen et al. [35] evaluated the effects of RFP plus daptomycin, LZD, or VCM administered for 14 days in mouse models infected with biofilm-formed *S. aureus* on the surface of implants, and reported that the biofilm on implants could not be eliminated completely. As shown in Table 2, although all of the 30 strains exposed to 3 combinations of RFP (10 strains per combination) showed a decrease of the bacterial count to about 10^2 CFU/mL after 24 hours, 9 strains survived even after being exposed for 120 hours. Those 9 strains retained susceptibility to CEZ, VCM, and CAM, but acquired high resistance to RFP (Table 3). The subsequent investigation revealed that those 9 strains had acquired resistance to RFP already after 8 hours of exposure to RFP combined with another antimicrobial agent (data not shown). The mutation frequency of *rpoB* gene related with RFP resistance is 10^{-7} to 10^{-8} [9,36]. These resistant mutants are selected easily by RFP exposure. Therefore, after exposure, it was suggested that a resistant strain was confirmed rapidly.

Additionally, all those 9 strains were found to have a single mutation on rpoB. His481>Tyr mutation was confirmed in MS-14 and -18 strains and Ser486>Leu mutation in MS-19 strain, which are related to high resistance to RFP [10,11,23]. Ile527>His and Ser486>Phe mutation were newly confirmed.

El Haj et al. [37] reported that the resistance to RFP was acquired by biofilm-formed *S. aureus*, after exposure for 8 hours to RFP plus trimethoprim/sulfamethoxazole. The existence of strains that are resistant to RFP after 8 hours of exposure may be one of the causes whereby a bactericidal effect cannot be achieved by combined administration of RFP. Then, we focused on the differences in the growth rate between the parental strains of the 5 surviving strains and 5 non-surviving strains after the combined exposure to RFP. The bacterial count of the surviving group after 8 hours of incubation was approximately 10^6 CFU/mL, which was significantly higher compared with the that of the non-surviving group (approximately 10^5 CFU/mL) ($p<0.05$). This investigation revealed that there are strains with excellent growth potential among clinical isolates, and those strains acquire resistance to RFP after 8 hours of combined exposure of RFP.

Besides, in 2 of 5 survived strains that were resistant to RFP, the amount of biofilm in the RFP-resistant strains was significantly increased compared with their parental strains ($p<0.05$) (Figure 4). Therefore, prolonged combined administration of RFP may promote biofilm formation. In addition, although both strains of MS-18 and -19 were sterilized only by CEZ, these strains survived by combination exposure of RFP (Table 2). Namely, the strain which acquired RFP-resistance by combination exposure of RFP immediately may reduce bactericidal effect of other antimicrobial agent because amount of biofilm formation increases. Bacteria usually reduce the metabolism activity in the bacterial body, ability for growth and toxigenicity for cost of the drug-resistant acquisition [38,39]. As a survival strategy in such environment, the bacteria may promote the biofilm formation. However, the mechanism is not understood sufficiently.

When various bacteria acquired drug resistance, it is known that amount of biofilm formation increases. Whereas this tendency is uneven in bacterial individual difference [40,41]. The reason is unexplained, and future study is expected.

CONCLUSION

The single agent exposure with each anti *S. aureus* agent hardly showed a bactericidal effect for biofilm-formed *S. aureus* on the washer. Additionally, 50% of the strains acquired resistance 8 hours after combined exposure with RFP. It was found that the biofilm formation was promoted in the strain which acquired RFP-resistance. We believe that RFP-resistant early detection in the RFP combination therapy affects PJI treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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