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Clinical Studies

Variation in the formation of persister cells against meropenem in *Klebsiella pneumoniae* bacteremia and analysis of its clinical features

Jin Seo Lee [a], Ji-Young Choi [b], Eun Seon Chung [b], Kyong Ran Peck [c], Kwan Soo Ko [b,*]

[a] Division of Infectious Diseases, Department of Medicine, Hallym University Kangdong Sacred Heart Hospital, Seoul, South Korea
[b] Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon, South Korea
[c] Division of Infectious Diseases, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea

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ABSTRACT

We investigated variations in the rate of persister cell formation against meropenem in 68 *Klebsiella pneumoniae* isolates from blood. The persister cell formation rates varied markedly but were not significantly different between the patient survival group and death group at 30 days. In addition, they were not associated with the patients' underlying diseases. However, the isolates of CC15 and CC23 showed higher survival rates against 10× MIC of meropenem than CC11. The survival rate of persister cells was less for amikacin and colistin than that for ciprofloxacin. When combinations of meropenem and other antibiotics were administered, persister formation rates decreased compared with those against only meropenem. However, no synergistic effect to remove persister cells was observed. Further investigation is needed to understand persister cell formation in *K. pneumoniae* with respect to the mechanism involved and clinical implications and that diverse strategies should be explored to remove persister cells.

1. Introduction

Persister cells were first described in a study about *Staphylococcus aureus* in 1942; ~1% of cells were not killed by penicillin, so the antibiotic was presumed to be effective on actively multiplying cells only (Hobby et al. 1942). Persisters are genetically identical to antibiotic-susceptible cells with similar minimal inhibitory concentration (MIC) values, but they are tolerant to antibiotics at lethal concentrations (Brauner et al. 2016). Compared to resistant bacterial populations with heritable mutations, persisters represent a small subpopulation caused by nonheritable phenotypic variations (Helaine and Kugelberg 2014).

Persisters have been suggested to contribute to the failure of antibiotic treatment and the evolution of antibiotic resistance (Michiels et al. 2016a). Several factors appear to trigger persister formation. First, phenotypic switching of a few organisms to a dormant state occurs spontaneously and reversibly due to fluctuations in gene expression and protein levels within individual cells of an isogenic population (Kussell et al. 2005). According to this postulation, persisters exist independently of the presence of antibiotics and other environmental signals, and antibiotics reveal the persister subpopulation by killing intolerant cells (Helaine and Kugelberg 2014). However, several other environmental conditions have been recently reported to induce formation of persisters such as starvation, carbon source transitions, indole signaling, quorum sensing, host macrophages, SOS response, and antibiotics (Dorr et al. 2010; Moker et al. 2010; Leung and Levesque 2012; Vega et al. 2012; Amato et al. 2013; Bernier et al. 2013; Johnson and Levin 2013; Maisonneuve et al. 2013; Helaine et al. 2014).

Eradication of persisters using antibiotics is a major challenge because traditional antibiotics are mainly effective against actively growing cells. Periodic dosing, rather than continuous dosing, of antibiotics was indicated to be an effective treatment by mathematical simulation of persistence (De Leenheer and Cogan 2009; Cogan et al. 2013). Prolonged treatment of infections with current antibiotics was also suggested to sterilize stationary cultures of *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* (Keren et al. 2012). However, it remains to be evaluated whether these theoretical methods can remove persisters in clinical settings (Helaine and Kugelberg 2014). Strategies to eliminate persisters are largely classified into i) killing persisters in the dormant state, ii) activating persisters and treating with traditional antibiotics, and iii) preventing the formation of persisters (Wood 2016). Persisters have been shown to be rapidly activated by sugars and glycolysis intermediates, and the resulting growing cells are sensitive to aminoglycoside (Allison et al. 2011). Extracellular *cis*-2-decenoic acid was able to activate persisters, which were more efficiently killed by ciprofloxacin (Marques et al. 2014). Prevention of persister formation by attenuating quorum sensing was also reported (Starkey et al. 2014). In addition, treatment with a combination of antibiotics has been proposed as a

* Corresponding author: Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon 16419, South Korea. Tel.: +82-31-299-6223; fax: +82-31-299-6229.
E-mail address: ksko@skku.edu (K.S. Ko).

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way to remove persisters. For example, daptomycin, cefoperazone, and doxycycline combination treatment showed success in eradicating *Borrelia burgdorferi* and may be helpful for treating persistent Lyme disease not resolved with conventional treatment (Feng et al. 2015). The combination of polymyxin B and meropenem was recently reported to eliminate persister cells from *Acinetobacter baumannii*, especially with a high dose (Gallo et al. 2017).

Klebsiella pneumoniae is a gram-negative bacteria causing infections including urinary tract infection, pneumonia, liver abscesses, and blood stream infections (Navon-Venezia et al. 2017). Antibiotic treatment failure of *K. pneumoniae* infection is largely reported, and persisters may have a role in persistent infection, including bacterial biofilms (Vuotto et al. 2014; Navon-Venezia et al. 2017). However, there are little data on persister formation in *K. pneumoniae* (Li et al. 2018). Furthermore, there are no reports on persister formation in *K. pneumonia* and its relationship with clinical outcome.

In this study, we aimed to investigate the variability in the rate of persister formation against meropenem in *K. pneumoniae* isolates from blood and its relationship with genotype and clinical outcome. We also investigated the effect of treatment with a combination of antibiotics, including meropenem with amikacin, ciprofloxacin, or colistin.

2. Materials and methods

2.1. Bacterial isolates

A total of 68 clinical isolates were collected from patients with *K. pneumoniae* bacteremia from 7 secondary or tertiary hospitals in South Korea (Samsung Medical Center, Seoul; Kyung Hee University Hospital, Seoul; Chungnam National University Hospital, Daejeon; Keimyung University Hospital, Daegu; Samsung Changwon Hospital, Changwon; Daegu Fatima Hospital, Daegu; Changwon Fatima Hospital, Changwon) between January 2012 and December 2013.

2.2. Clinical data

A retrospective study was conducted in the hospitals participated in this study. We reviewed the electronic medical records of the included patients with acute *K. pneumoniae* bacteremia, all of which were treated with meropenem according to information from the package insert. Some cases were treated with other antibiotics before the administration of meropenem by attending physicians based on clinical judgment. Patients ≪18 years old were excluded from the study. Data collected included demographics, underlying diseases, comorbid conditions, acquisition of infections, antimicrobial regimens, and outcome. Severity of illness was determined by the Pitt bacteremia score (Lesens et al. 2003; Paterson et al. 2004).

Neutropenia was defined as an absolute neutrophil count of ≪500 neutrophils/mm^3. Acute kidney injury (AKI) was defined as an absolute increase in serum creatinine of ≥0.3 mg/dL or a percentage increase in serum creatinine of ≥50% from the baseline level within 48 h (Mehta et al. 2007). The primary source of bacteremia was determined based on medical records. Primary bacteremia (PB) was considered when no infection focus was diagnosed. The all-cause mortality at 30 days was calculated from the day of blood sampling at which bacteremia was first detected. It is determined that all mortality came from *K. pneumoniae* infection because all patients included in this study were acute *K. pneumoniae* bacteremia patients who were under critical condition. The definition of appropriate antibiotics was the initiation of an antimicrobial agent that included at least 1 parenteral antibiotic known to be active against *K. pneumoniae* within 48 h in vitro. As this was an observational study, antimicrobial regimens were not standardized but selected by primary care physicians.

2.3. Antimicrobial susceptibility and genotyping

In vitro antimicrobial susceptibility was determined by MIC by the broth microdilution method following the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI 2017). Susceptibility to meropenem was tested for all 68 *K. pneumoniae* isolates, and amikacin, ciprofloxacin, and colistin susceptibilities were tested for the 6 isolates that were selected for evaluating the effect of antibiotic combinations. Susceptibility was interpreted according to CLSI breakpoints (CLSI 2017). *E. coli* ATCC 25922 was used as a quality control strain. Extended spectrum β-lactamase (ESBL) production was confirmed for isolates deemed positive by screening via a double-disk synergy test using BBL Sensi-Disks from Becton, Dickinson and Company (Sparks, MD, USA).

For genotyping, multilocus sequencing typing (MLST) was performed for all *K. pneumoniae* clinical isolates (Diancourt et al. 2005). Clonal complexes (CCs) were determined by including whole *K. pneumoniae* MLST data using eBURST v3 (http://eburst.mlst.net).

2.4. Persister assays

A persister cell formation assay was performed as previously described (Chung et al. 2017) with some modification. Persister formation of *K. pneumoniae* was examined by exposure of stationary phase bacteria to a high concentration of meropenem (10× MIC). Cells were inoculated in Luria–Bertani (LB) media and grown for 16 h at 37 °C until they reached exponential phase ($OD_{600} = 0.5$), and were then exposed to meropenem (10× MIC). The cells were washed 2 h later, and then reinoculated into LB media. After culturing overnight, colony forming unit (CFU) counts were compared before and after meropenem treatment. Two colonies from each sample were reinoculated into fresh LB broth, and in vitro antimicrobial susceptibility tests were performed to ensure that the MIC values had not changed.

2.5. Killing of persister cells by combination treatments

To determine whether treatment with combinations of antibiotics leads to killing of *K. pneumoniae* persister cells, stationary phase bacteria were treated with a high concentration of meropenem, or with single or combination treatment with amikacin, ciprofloxacin, or colistin. For the combination treatment, we selected 5 isolates based on their genotypes and persister cell formation rates. First, persister formation of *K. pneumoniae* against each antibiotic was examined as described above, and then the combination with meropenem was studied in a similar manner. MIC values before and after persister assays were also checked to confirm antibiotic susceptibility.

2.6. Statistical analysis

Student's *t* test and 1-way ANOVA test were used to compare continuous variables. Spearman's correlation coefficient was used for nonparametric continuous variables. Categorical variables were compared between groups with the χ^2 and Fisher's exact test. A stepwise logistic regression analysis was used to control for potential confounding. Variables with a *P* value ≪0.1 in the univariate analysis were included in the multivariate logistic regression model to control for confounding and identify independent risk factors for mortality. All *P* values were 2-tailed, and a *P* value ≪0.05 was considered to indicate statistical significance for all analyses. Data were analyzed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Persister cell formation against meropenem in K. pneumoniae

All 68 *K. pneumoniae* isolates from blood were meropenem-susceptible, with meropenem MICs ranging from 0.06 mg/L to 1 mg/L.

For all isolates, persister cell formation rates were evaluated against meropenem (Fig. 1). The survival rates against 10× MIC of meropenem were from 2.0×10^{-4} to 2.7×10^{-1}. Six isolates (8.8%) showed a survival rate of $\gg 1.0 \times 10^{-1}$, which was defined arbitrarily to be a high persister formation rate, and the survival rates of 4 isolates (5.9%) were $\ll 1.0 \times 10^{-3}$, which was defined arbitrarily to be a low persister formation rate.

3.2. Genotypes of *K. pneumoniae* isolates and their relationships with persister formation rates

The genotypes of *K. pneumoniae* isolates included in this study were very diverse; a total of 48 distinct sequence types (STs) were identified among the 68 isolates (Table 1). ST11 was identified most frequently (7 isolates, 10.3%), followed by ST48 (5 isolates, 7.4%), ST20 (4 isolates, 5.9%), and ST23 (4 isolates, 5.9%). All but 9 STs were identified in only 1 isolate. Based upon eBURST analysis, 4 CCs were identified (Table 1). CC23 consisting of 10 STs included 16 isolates (23.5%), and both CC11 and CC15 included 8 isolates (11.8% for both). Approximately 50% of the isolates were categorized into singletons.

The persister formation rates were compared between CC11, CC15, and CC23 (Fig. 2). CC11 showed a significantly lower persister formation rate (mean, $2.68 \times 10^{-3} \pm 8.40 \times 10^{-4}$) compared to the other CCs, $2.57 \times 10^{-2} \pm 1.03 \times 10^{-2}$ for CC15 and $4.67 \times 10^{-2} \pm 1.95 \times 10^{-3}$ for CC23 ($P = 0.0218$ and 0.0385, respectively) (Fig. 2A).

3.3. Clinical characteristics of patients with *K. pneumoniae* bacteremia and the relationship with persister formation rates

To examine whether persister formation against meropenem affects patients with *K. pneumoniae* clinically, a retrospective study was performed (Table 2). All patients were initially treated with meropenem. The clinical characteristics and outcomes of patients with *K. pneumoniae* bacteremia were compared with respect to the persister formation rates of bacterial isolates. Among the 68 patients with *K. pneumoniae* bacteremia, 52.9% were male, and ≫50% of patients had hospital-acquired infections (43 cases, 63.2%). One-third of patients had bacteremic episodes in the intensive care unit (ICU) (26 cases, 38.2%). The mortality analysis at 30 days showed that 56 patients (82.4%) survived. The underlying diseases of the 68 patients included in this study were as follows: PB (25, 36.8%), central line associated infection (CRI) (4, 5.9%), urinary tract infection (UTI) (10, 14.7%), hepatobiliary infection (HBI) (10, 14.7%), intra-abdominal infection (IAI) (5, 7.4%), respiratory infection (RI) (10, 17.4%), skin and soft tissue infection (SSTI) (3, 4.4%), and others (1, 1.5%).

We compared the persister formation rates between the survival group and death group of patients at 30 days. The persister formation rates were higher in the survival group than in the death group but were not significantly different ($3.17 \times 10^{-2} \pm 6.71 \times 10^{-3}$ vs. $1.84 \times 10^{-2} \pm 1.07 \times 10^{-2}$, $P = 0.1937$) (Fig. 2B). The persister formation rates in the survival group were also higher than those in the death group at 14 day, which were also not significant ($3.75 \times 10^{-2} \pm 5.18 \times 10^{-2}$ vs $1.78 \times 10^{-2} \pm 2.07 \times 10^{-2}$, $P = 0.215$). The persister formation rates were also not significantly different among the underlying

Table 1
Genotypes of *K. pneumoniae* isolates from blood.

CC	ST	Allele profile [a]	No. of isolates (%)	
11	11	3-3-1-1-1-1-4	7 (10.3)	8 (11.8)
	2193	15-3-1-1-1-1-4	1	
15	15	1-1-1-1-1-1-1	2	8 (11.8)
	14	1-6-1-1-1-1-1	2	
	35	2-1-2-1-10-1-19	1	
	327	2-1-1-1-10-1-19	1	
	412	2-1-2-1-9-1-112	1	
	431	2-1-1-1-1-1-1	1	
23	23	2-1-1-1-9-4-12	4 (5.9)	16 (23.5)
	20	2-3-1-1-4-4-4	4 (5.9)	
	17	2-1-1-1-4-4-4	1	
	218	2-3-1-1-9-4-12	1	
	793	4-1-1-1-9-4-12	1	
	1159	2-1-1-1-16-44-12	1	
	1919	2-1-1-1-12-4-34	1	
	2535	2-3-1-1-9-7-4	1	
	SLV of 10	4-1-1-1-9-1-12	1	
	SLV of 23	2-1-101-1-9-4-12	1	
29	29	2-3-2-2-6-4-4	2	3 (4.4)
	711	2-61-2-2-6-4-4	1	
37	37	2-9-2-1-13-1-16	1	2 (2.9)
	394	2-9-2-1-13-1-82	1	
Singleton	48	2-5-2-2-7-1-10	5 (7.4)	31 (45.6)
	86	9-4-2-1-1-1-27	2	
	111	2-1-5-1-17-4-42	1	
	138	18-22-26-23-31-13-49	1	
	186	2-1-1-37-45-4-9	2	
	249	2-1-44-1-16-4-66	1	
	269	12-1-1-2-5-1-18	1	
	273	3-4-6-1-7-4-4	1	
	299	2-10-1-1-56-24-31	1	
	307	4-1-2-52-1-1-7	1	
	388	16-24-59-27-29-158-105	1	
	414	18-22-26-63-85-20-51	1	
	461	3-1-13-34-63-1-64	1	
	463	2-1-4-1-7-6-19	1	
	628	2-60-11-1-4-8-24	1	
	976	2-3-108-26-3-4-64	1	
	1181	16-24-21-27-81-22-105	1	
	1255	16-24-60-49-47-22-67	1	
	1393	2-1-37-2-3-1-19	1	
	SLV of 1659	2-6-1-1-3-1-9	1	
	SLV of 113	14-1-2-1-21-112-23	1	
	SLV of 2634	28-24-21-27-41-153-341	1	
	SLV of 70	2-6-17-1-20-10-388	1	
	SLV of 87	5-1-5-1-7-4-24	1	

[a] *gapA-infB-mdh-pgi-phoE-rpoB-tonB*.

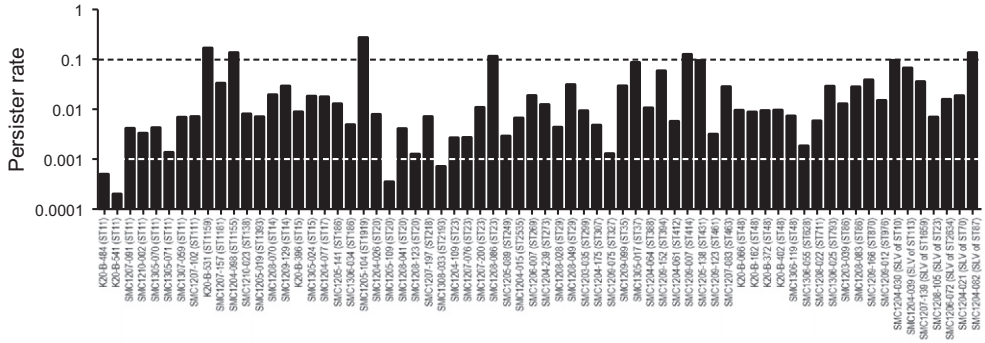

Fig. 1. Persister formation rates of 68 *K. pneumoniae* isolates from blood. The spotted lines indicate survival rates of 0.1 and 0.001, respectively. Six *K. pneumoniae* isolates showed a survival rate of ≫0.1, which was defined arbitrarily to be a high persister formation rate, and 4 isolates showed a survival rate of ≪0.001, which was defined arbitrarily to be a low rate.

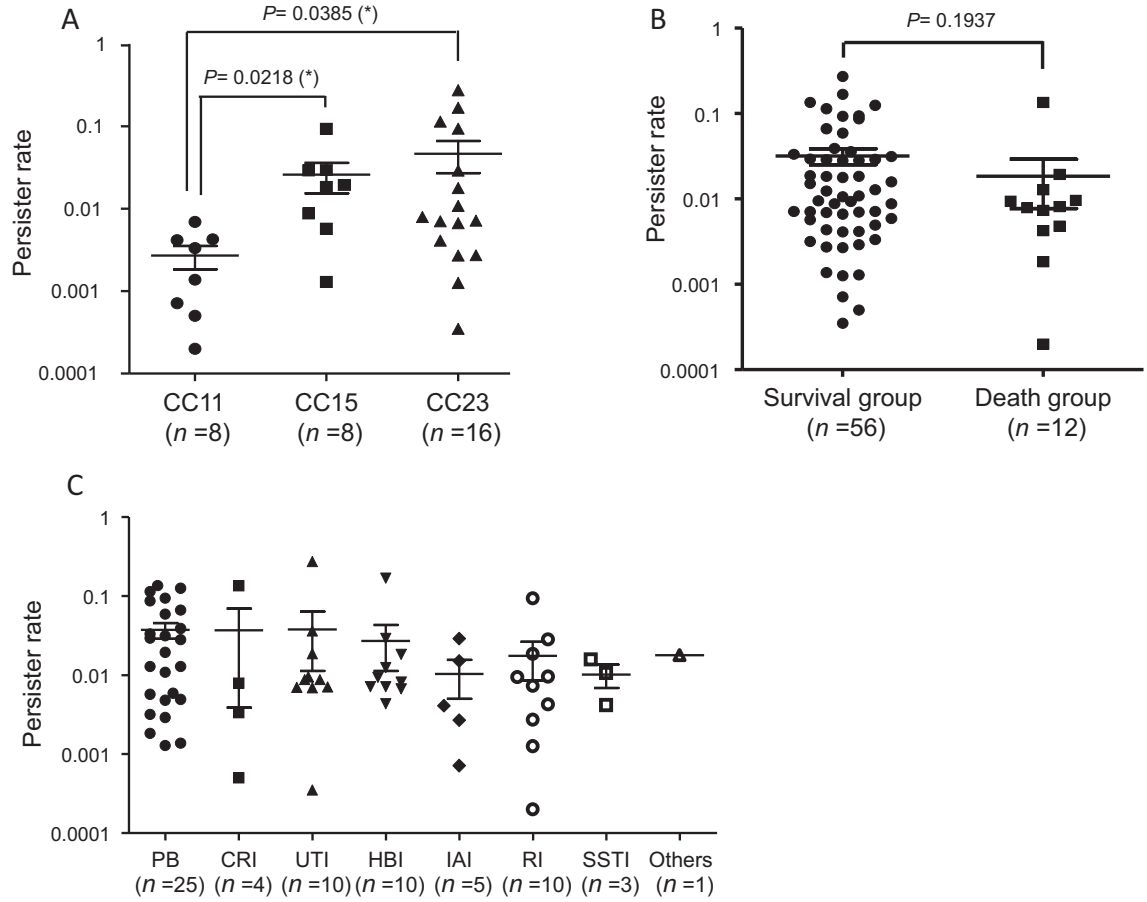

Fig. 2. The relationships between persister formation rates and genotype and clinical features. (A) Persister formation rates in 3 genotypes: CC11, CC15, and CC23. The persister formation rates in CC15 and CC23 were significantly higher than those in CC11. (B) Comparison of persister formation rates between the survival and death groups at 30 days. The persister formation rates between the 2 groups were not significantly different. (C) The persister formation rates in diverse underlying diseases. PB = primary bacteremia; CRI = central line related infection; UTI = urinary tract infection; HBI = hepatobiliary infection; IAI = intra-abdominal infection; RI = respiratory infection; SSTI = skin and soft tissue infection. The persister formation rates did not differ according to underlying diseases.

diseases (Fig. 2C). In addition, neutropenia ($3.43 \times 10^{-2} \pm 5.36 \times 10^{-2}$ vs. $3.56 \times 10^{-2} \pm 4.76 \times 10^{-2}$, $P = 0.906$) or use of immunosuppressants ($4.59 \times 10^{-2} \pm 7.60 \times 10^{-2}$ vs $3.41 \times 10^{-2} \pm 4.69 \times 10^{-2}$, $P = 0.680$) did not affect persister formation rate. Time for defervescence, hospital day, and ICU day were evaluated for association with the persister formation rate, but all these additional clinical outcomes did not show any significant association with persister formation rate (Table 3).

Additionally, we compared the clinical outcome between CC23 and non-CC23 isolates. As a result, there was no significant difference in clinical outcome between CC23 and other CCs except hospitalization days: 6.3% vs. 21.2% for 30-day mortality (*P*, 0.268), 3.06 ± 2.08 vs. 2.81 ± 2.56 days for time to defervescence (*P*, 0.60), 7.00 ± 21.50 vs. 8.46 ± 19.69 days for ICU stay (*P*, 0.81), and 83.26 ± 154.99 vs. 49.84 ± 55.82 days for hospitalization day (*P*, 0.008).

3.4. Effect of antibacterial combination treatment

Persister formation rates against amikacin, ciprofloxacin, and colistin were evaluated separately for 5 selected *K. pneumoniae* isolates. They were relatively lower than that of meropenem (Fig. 3). While the mean persister formation rate of meropenem was $1.20 \times 10^{-1} \pm 1.10 \times 10^{-1}$, those of amikacin, ciprofloxacin, and colistin were $2.34 \times 10^{-5} \pm 4.84 \times 10^{-5}$, $1.82 \times 10^{-3} \pm 2.09 \times 10^{-3}$, and $3.44 \times 10^{-6} \pm 1.35 \times 10^{-6}$, respectively. In particular, persister cells were rarely formed against amikacin in 3 isolates (K20-B-531, SMC1205-104, and SMC1204-098) with rates of $\ll 1.0 \times 10^{-6}$. Persister formation rates against colistin were also very low.

When antibiotic combinations were administered, the persister formation rates decreased compared with meropenem treatment only (Fig. 4). However, the persister formation rates were not significantly lower compared with treatment of amikacin, ciprofloxacin, or colistin only. Although persister cells were removed significantly when treated with antibiotic combinations in some isolates, including SMC1208-077, no general synergistic effects were observed. Thus, the killing effect of antibiotic combinations might be attributable to the antibiotics other than meropenem.

4. Discussion

Few studies have reported on persister cells in *K. pneumoniae* (Vuotto et al. 2014; Ren et al. 2015; Michiels et al. 2016b). In this study, we investigated persister cell formation against meropenem in *K. pneumoniae* isolates from blood, including a relatively large number of isolates as compared with previous studies. In particular, persister formation against meropenem is important because meropenem is one of the antibiotics of last resort for treatment of gram-negative pathogen infections, including *K. pneumoniae* (Papp-Wallace et al. 2011).

All *K. pneumoniae* isolates included in this study formed persister cells against meropenem, indicating that persister cell formation is likely an inevitable phenomenon in this bacterial species, which was shown in a previous study (Li et al. 2018). In the previous study, the *K. pneumoniae* persister level was growth phase-dependent: the

Table 2
Demographic and clinical characteristics of patients with *K. pneumoniae* bacteremia according to 30-day mortality.

Patient characteristics	Survival ($n = 56$)	Death ($n = 12$)	*P*
Age (yr)	55.66 ± 21.26	58.33 ± 22.31	.697
Male	28 (50.0)	8 (66.7)	.353
Comorbid condition			
Diabetes mellitus	13 (23.2)	2 (16.7)	.723
Cardiovascular disease	21 (37.5)	3 (25.0)	.518
Chronic lung disease	5 (8.9)	3 (25.0)	.141
Renal impairment	3 (5.4)	2 (16.7)	.211
Chronic liver disease	8 (14.3)	2 (16.7)	.99
Solid tumor	23 (41.1)	5 (41.7)	.99
Hematologic malignancy	17 (30.4)	4 (33.3)	.99
Solid organ transplantation	3 (5.4)	0 (0)	.99
Hematopoietic stem cell transplantation	4 (7.1)	2 (16.7)	.285
Pitt bacteremia score	2.09 ± 2.28	4.33 ± 3.05	.005
Persister rate	$3.17 \times 10^{-2} \pm 5.02 \times 10^{-2}$	$1.84 \times 10^{-2} \pm 3.71 \times 10^{-2}$	.387
Neutropenia	22 (39.3)	7 (58.3)	.336
Steroid	1 (1.8)	0 (0)	.99
Immunosuppressant use	7 (12.5)	0 (0)	.338
Recent surgery	7 (12.5)	2 (16.7)	.654
Urinary catheter	22 (39.3)	8 (66.7)	.083
Invasive procedure	11 (19.6)	4 (33.3)	.442
ICU	20 (35.7%)	6 (50.0)	.514
AKI	9 (16.1)	4 (33.3)	.223
Ventilator	11 (19.6)	3 (25.0)	.701
ESBL	25 (44.6)	10 (83.3)	.024
Appropriate antibiotics	45 (80.4)	7 (58.3)	.136
Infection sites			
PB	21 (37.5)	4 (33.3)	.99
CRI	2 (3.6%)	2 (16.7)	.141
UTI	10 (17.9)	0 (0)	.189
HI	9 (16.1)	1 (8.3)	.678
IAI	5 (8.9)	0 (0)	.577
RI	5 (8.9)	5 (41.7)	.012
SSTI	3 (5.4)	0 (0)	.99

Data are presented as median value (interquartile range) for continuous variables, and as *n* (%) of patients for categorical variables. ICU = intensive care unit; AKI = acute kidney injury; ESBL = expended spectrum beta lactamase; PB = primary bacteremia; CRI = central line associated infection; UTI = urinary tract infection; HI = hepatobiliary infection; IAI = intra-abdominal infection; RI = respiratory infection; SSTI = skin and soft tissue infection.

bacterial cells in stationary phase are more tolerant against meropenem than those in exponential phase (Li et al. 2018). As a preliminary experiment, we measured the survival rates repeatedly for a single isolate. As a result, the survival rates showed no significant differences, indicating that the persister formation rate may be consistent feature for each isolate against each antibiotic and that the method used in this study would be reliable. We found that the survival rates against 10× MIC of meropenem, that is, persister formation rates, were variable. While some isolates (8.8%) showed a survival rate of ≫10%, there were isolates (5.9%) that had a survival rate of ≪0.1%. Such variable persister formation rates were reported in *A. baumannii* clinical isolates against colistin (Barth Jr et al. 2013; Chung et al. 2017). The selected isolates also showed variable persister formation rates against other antibiotics, including amikacin, ciprofloxacin, and colistin. The persister formation rates were relatively low against amikacin and colistin. However, the persister formation rate in response to one antibiotic was not related to that of other antibiotics. Thus, it is possible that the mechanism underlying persister formation differs for each antibiotic.

It has been proposed that the persister cell formation is associated with antibiotic therapy failure (Lewis 2007; Fauvart et al. 2011; Michiels et al. 2016a). However, the clinical outcome, such as the results for 30-day treatment, was not associated with the persister formation rate. In our previous study, no significant relationship between persister formation rates and 7-day treatment results was observed in *A. baumannii* against colistin (Chung et al. 2017). Instead, treatment failure was not observed in most *A. baumannii* clinical isolates showing no persister cell formation. In this study, 3 out of 4 patients infected with *K. pneumoniae* isolates showing a low persister formation rate (≪1.0×10^{-3}) survived, which is not significantly different with the survival rate in patients infected with isolates showing a high persister formation rate (≫1.0×10^{-1}), where 5 out of 6 patients survived. A small number of persisters are likely not of clinical concern when a patient's immune system is normal, but biofilm formation is strongly linked with chronic and recurrent infections (Mulcahy et al. 2010; Michiels et al. 2016b); unfortunately, we could not include cases of biofilm-related infections. In addition to the variability of persister formation rates, we cannot predict clinical outcomes based on persister formation rates of bacterial isolates. However, further study should be carried out with more cases to determine if persister cells play an important role in biofilm-associated infections. In addition, studies are needed to determine the roles of persister cells in both chronic persistent and acute rapidly progressive infection.

Table 3
Relationship between persister formation rate and time for defervescence, hospital day, and ICU day.

	Persister formation rate	
	r	*P*
Time for defervescence	−0.105	0.386
Hospital day	0.034	0.780
ICU day	0.036	0.765

r = Spearman correlation coefficient; *P* = probability.

While persister formation rates had no association with clinical outcomes and underlying diseases, they were higher significantly in some genotypes. The most frequently identified genotypes in this study were CC11 (6 isolates, 17.1%), CC15 (4 isolates, 11.4%), and CC23 (7 isolates, 20.0%). That is, 75.0%, 50.0%, and 43.8% of CC11, CC15, and CC23 isolates produced ESBL, respectively. CC11 and CC15 have been identified most frequently in ESBL-producing *K. pneumoniae* isolates from Korea (Shin et al. 2011), and CC23 is the representative clone causing liver abscess in Asian countries (Chung et al. 2007; Siu et al. 2011).

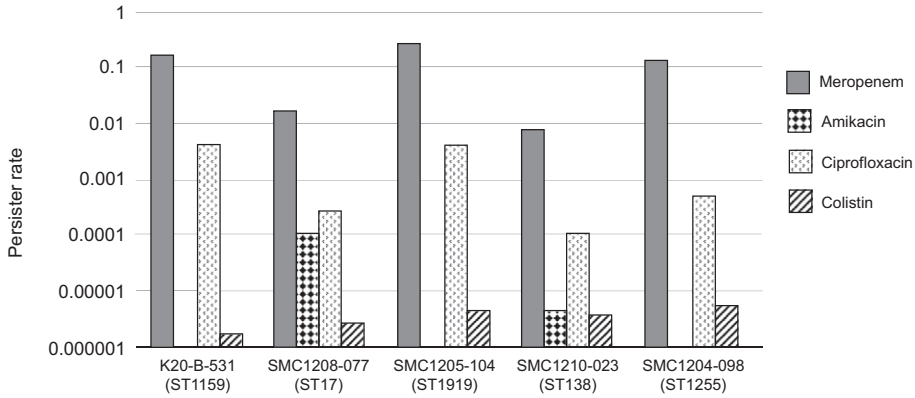

Fig. 3. Comparison of persister formation rates against 4 different antibiotics, meropenem, amikacin, ciprofloxacin, and colistin, in 5 selected *K. pneumoniae* isolates. The persister formation rates against amikacin and colistin were lower than that against meropenem.

CC15 and CC23 showed higher persister formation rates than CC11, which were statistically significant. This implies that some genotypes have an inherent ability to form more persister cells, but there is no experimental or epidemiological evidence to support this hypothesis. However, it is notable that a high persister formation rate was observed in CC23, a highly virulent genotype associated with hypermucoviscosity (Struve et al. 2015), although there was no significant difference in clinical outcome between CC23 and other CCs except hospitalization days in the present study. We confirmed that the hypermucoviscosity was preserved in the persister cells through a string test (Choi and Ko 2015).

Antibiotic combination treatment has been proposed as a strategy to remove persister cells (Lechner et al. 2012). It was reported that a combination of polymyxin B and meropenem eradicated *A. baumannii* persister cells (Gallo et al. 2017). In this study, we evaluated the effect of antibiotic combinations on the eradication of *K. pneumoniae* persisters, using different classes of antibiotics from meropenem. All 5 *K. pneumoniae* isolates showed lower persister formation rates in combination treatment for meropenem with other antibiotics compared with treatment with meropenem only. However, the killing effect induced by combination therapy is likely due to the other antibiotics combined with meropenem. Although a synergic effect by combination treatment was observed in 1 isolate, SMC1208-077, the persister formation rates for combination treatments were similar to those for single treatment with the other antibiotics. Thus, it can be postulated that

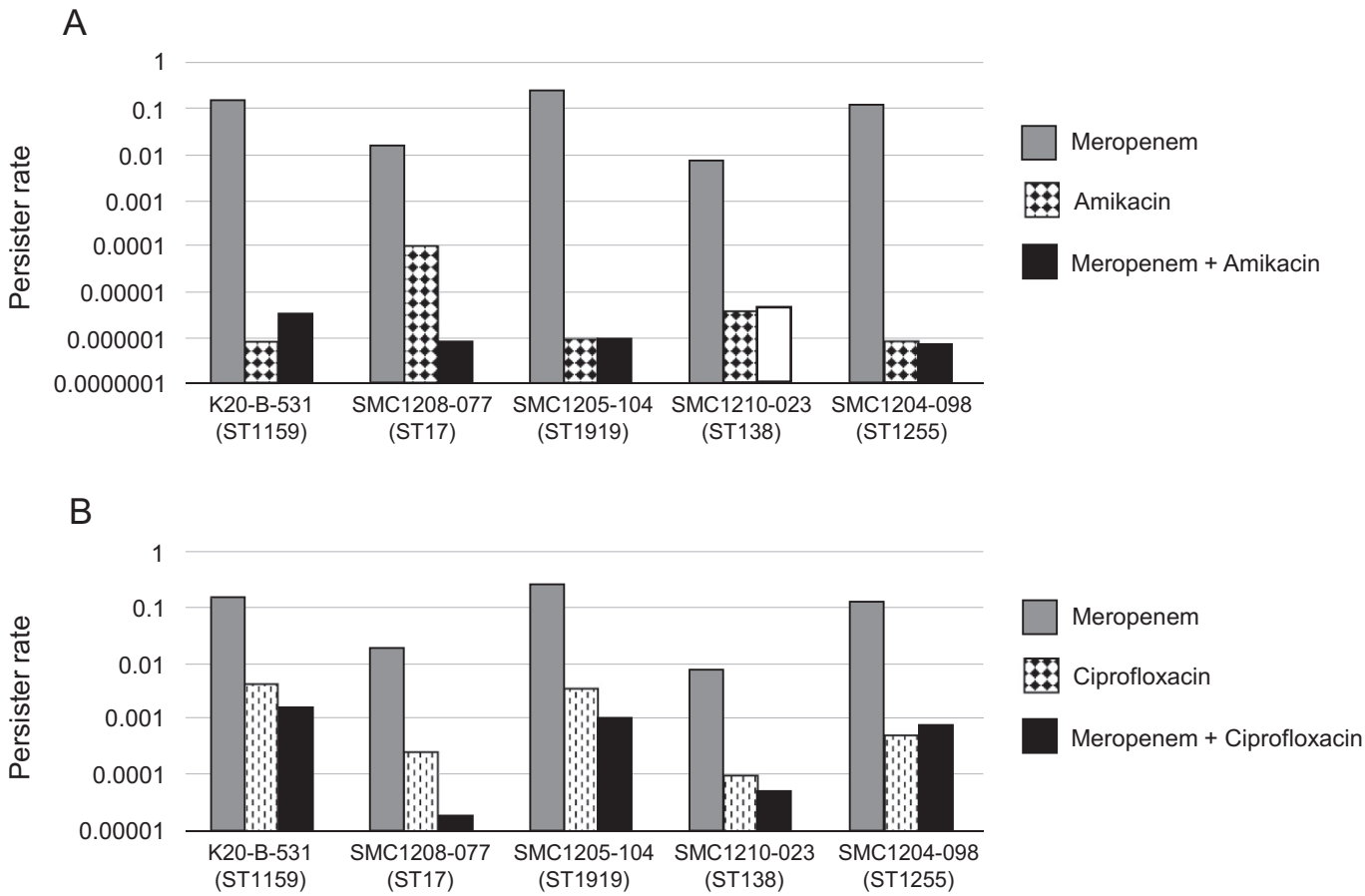

Fig. 4. Effect of combination antibiotic treatment to remove persister cells in 5 *K. pneumoniae* isolates: meropenem and amikacin (A), meropenem and ciprofloxacin (B), and meropenem, meropenem, and colistin (C).

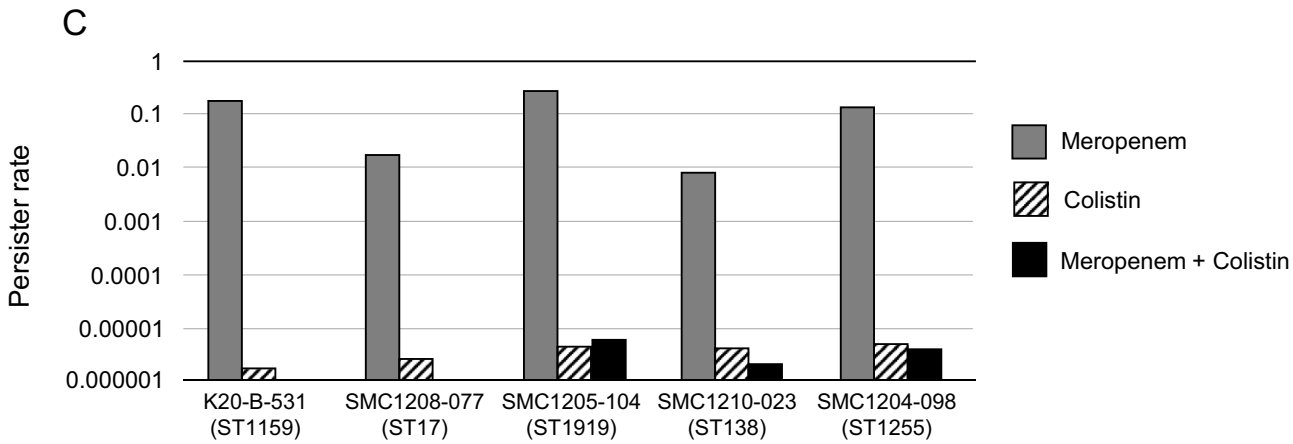

Fig. 4. (continued)

antibiotic combination based on meropenem is not effective for removal of *K. pneumoniae* persister cells.

In this study, we identified variable persister formation rates against meropenem in *K. pneumoniae* clinical isolates. While they were not associated with clinical outcomes and underlying diseases, they were high in some genotypes, including CC15 and CC23. The persister formation rates were low when treated with amikacin and colistin, and combination treatment based on meropenem showed no synergistic effect to remove persister cells. Our study provides the basis for further study of persister cell formation and investigation of strategies to remove persister cells in *K. pneumoniae*.

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Conflict of interest

None to declare.

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