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Sublethal Sodium Hypochlorite Exposure: Impact on Resistance-Nodulation-cell Division (RND) Efflux Pump Overexpression, and Cross-Resistance to Imipenem

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Abstract: Sodium hypochlorite (NaOCl) is widely used in public healthcare facilities; this exposure can result in the development of bacterial tolerance to disinfectant, which has known links to antibiotic cross-resistance. However, the mechanism through which cross-resistance to antibiotics and disinfectants develops remains ambiguous. Therefore, this study aimed to examine the phenotypic, and transcriptomic changes caused by disinfectant exposure in gram-negative bacteria and determine the cause of cross-resistance to antibiotics. The results demonstrated that the misuse of disinfectants plays an important role in the emergence of disinfectant resistance and in the increase in antibiotic resistance. Antibiotic resistance may occur from exposure of gram-negative bacteria to subminimal inhibitory concentrations (MICs) of NaOCl. Ten passages of gram-negative bacteria in increasingly higher subMICs of NaOCl disinfectant was sufficient to increase the MIC to >2,500 μg/ml NaOCl, particularly in *K. pneumoniae* and *P. aeruginosa*. To determine the development of cross-resistance to antibiotics due to NaOCl exposure, the MICs for each antibiotic before and after exposure of each strain to sublethal concentrations of NaOCl were compared. After overnight incubation with a sublethal concentration of NaOCl, a statistically significant increase in MIC was only observed for imipenem (P<0.01). Investigation of the mechanism of cross-resistance by means of transcriptome analysis revealed that 1,250 μg/ml NaOCl-adapted *K. pneumoniae* and *P. aeruginosa* strains increased resistance to imipenem due to increased expression of the resistance-nodulation-cell division (RND) efflux pumps, such as AcrAB-TolC and MexAB/XY-OprM. Therefore, we suggest that exposure to NaOCl can influence the expression of RND efflux pump genes, contributing to imipenem cross-resistance.

Keywords: cross-resistance; imipenem antibiotics; NaOCl disinfectant; resistance-nodulation-cell division (RND) efflux pump

1. Introduction

Due to the ongoing COVID-19 pandemic, the use of disinfectants has increased markedly. The most commonly used disinfectants for combating COVID-19 include quaternary ammonium compounds (QACs), sodium hypochlorite (NaOCl), hydrogen peroxide (H_2O_2), and ethanol [1]. The use of disinfectants has rapidly increased worldwide and has been associated with the accelerated emergence of antimicrobial resistance (AMR) in pathogenic microbes in the post-COVID-19 pandemic era [2–4]. The biocide tolerance and antibiotic resistance by one common mechanism exhibited by a certain microorganism can be attributed to cross-resistance between biocides and antibiotics [5]. In general, if a microorganism is intrinsically tolerant to certain QACs or acquires such tolerance after exposure, it is likely to exhibit cross-tolerance to various antimicrobial agents [3,6]. Among the 654 disinfectants listed by the Environmental Protection Agency of the USA for use against severe acute respiratory syndrome virus-2, QACs comprise 45.1% and chlorines (NaOCl and HOCl) comprise 17.3% of the active ingredients.

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Chlorine-based compounds, particularly sodium hypochlorite (NaOCl) or bleach in households (4–5%), are the most frequently used disinfectants. NaOCl is widely used in healthcare facilities in diverse settings for spot disinfection of surfaces, such as countertops and floors. Chlorination is widely used as a disinfection treatment for water or wastewater, to remove pathogens and potential antibiotic-resistant bacteria [7]. This general use of chlorine products is attributable to several factors: their broad-spectrum antimicrobial activity, absence of toxic residues, effectiveness even when used with hard water, cost-effectiveness, rapid action, and ability to remove dried or fixed organisms and biofilms from surfaces [8]. The exact mechanism by which free chlorine destroys microorganisms has not been elucidated; however, several factors have been proposed, including oxidation of intracellular content, inhibition of protein synthesis, decreased oxygen uptake, oxidation of respiratory components, decreased ATP production, breaks in DNA, and inhibition of DNA synthesis [9]. However, commonly used chlorine disinfectants are less effective against highly chlorine-resistant waterborne bacteria, such as pathogenic Pseudomonas aeruginosa, which can exist in drinking tap water and resist disinfection.

Several shared resistance mechanisms have been reported for disinfectants and antibiotics [10–12]. Cross-resistance between antibiotics and disinfectants may occur via cellular mechanisms that protect against multiple classes of antimicrobial agents, or by the selection of genetic determinants for resistance to non-antibiotic agents that are linked to genes for antibiotic resistance [9]. Previous studies have shown that many Proteobacterial species that are considered to be critical priority AMR-pathogens (e.g., *Enterobacteriales*, *Acinetobacter* spp., and *Pseudomonas* spp.) are intrinsically tolerant to higher concentrations of chlorine [13,14]. These species have demonstrated the ability to adapt to NaOCl upon prolonged or repeated exposure to sublethal NaOCl concentrations, ultimately leading to increased disinfectant tolerance, biocide cross-tolerance, and cross-resistance to clinically relevant antibiotics [2]. The annual global use of disinfectants being more prevalent than that of antibiotics remains a major concern [13,15]. Such overuse of disinfectants has made these compounds common pollutants in ecosystems.

In gram-negative bacteria, the most clinically relevant efflux pumps are resistance–nodulation–division (RND) family members, which recognize a broad range of substrates, including antibiotics and disinfectants such as QACs/chlorhexidine [16]. This family includes well-characterized members of the Enterobacteriaceae multidrug-resistant (MDR) efflux pumps AcrAB-TolC, MexAB-OprM from *Pseudomonas aeruginosa*, and AdeABC in *Acinetobacter baumannii*. These strains often demonstrate upregulation of MDR efflux pumps, such as AcrAB-TolC. While efflux is linked to increased biocide tolerance, little is known about the contributions of these individual efflux pumps to biocide tolerance.

Due to society's reliance on and overuse of disinfectants, understanding how sodium hypochlorite (NaOCl) may drive antimicrobial resistance is crucial. Sustained exposure to sublethal levels of disinfectants can lead to MDR; however, the mechanism through which cross-resistance to antibiotics and disinfectants develops remains ambiguous. Therefore, we hypothesized that disinfectant-induced tolerance mechanisms (i.e., specific efflux pumps) to antibiotic cross-resistance could be more clearly elucidated by transcriptomic analysis. Therefore, this study tested the phenotypic, and transcriptomic changes caused by disinfectant exposure in gram-negative bacteria and to determine the cause of cross-resistance to antibiotics.

2. Results

2.1. Effect of Exposure to Disinfectant on Gram-Negative Bacteria

The MICs for NaOCl in 121 wild-type isolates of gram-negative bacteria were determined using the broth microdilution method. The NaOCl disinfectant MIC values of *Enterobacteriaceae* (including carbapenem-resistant) and carbapenem-resistant *A. baumannii* ranged from 250–500 μg/ml and 250–1,000 μg/ml, respectively. In particular, the MICs for *P. aeruginosa* strains were above the median of that for other species and ranged from 250 to 1,000 μg/ml of NaOCl.

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Among the 121 strains (including reference strains), the MIC for NaOCl of 10 strains increased by a fold-change ≥ 2.5 after NaOCl exposure (Table 1). All *E. coli* and *A. baumannii* isolates showed a non-adaptive response (MIC increase < 2-fold). A strong and stable MIC change was observed for isolates of *K. pneumoniae* and *P. aeruginosa*. In the control experiment (passages without disinfectant exposure), no significant changes in the MICs were observed. As shown in Table 1, 10 passages of gram-negative bacteria cultured in gradually higher subMICs of the disinfectant was sufficient to increase the MIC for NaOCl up to 2,500 μg/ml. The stability of the NaOCl-adapted strains was tested in the absence of each disinfectant over five additional passages. The MIC of NaOCl was again tested at the end of these passages, and this value was still higher than the initial value.

Table 1. Evaluation of NaOCl minimal inhibitory concentration and antibiotic susceptibility changes with disinfectant exposure

Species		Strains	MICs (mg/l)		Antibiotic susceptibility (Exposure to 1,250 μg/ml NaOCl)	
			Before[1]	After	Increased	Reduced
K. pneumoniae	CRKP	Z0317KP0159	500	2,500	FOX, IMI, CHL	ND[2]
		Z0317KP0181	500	2,500	IMI	GEN
		Z0318KP0099	500	2,500	IMI	STR
		Z0318KP0107	500	2,500	IMI, CHL, AZI	ND
		Z0318KP0236	500	2,500	IMI, CHL	GEN, AZI
P. aeruginosa	CSPA	ATCC 27853	500	2,500	AXO, IMI, STR, COL	TAZ, CIP, NAL, TET, GEN, AMI, SXT
		Z0219PA0007	500	1,250	AXO, IMI, COL	CIP, NAL, STR, SXT
	CRPA	I0020PA0021 (HL-IMI)	500	2,500	X[3]	X
		I0020PA0028	500	1,250	IMI, CIP, NAL, TET, AMI, STR	FOT, TAZ, AXO, GEN
		Z0217PA0020 (HL-IMI)	500	2,500	FOT, TAZ, CIP, CHL, GEN, STR, SXT	NAL, AMI

[1] "Before" indicates no exposure to disinfectants (wild-type strain), and "After" indicates exposure to disinfectants (adapted-type strains). [2] ND: not detected. [3] X: Exposure to disinfectant does not alter antibiotic susceptibility profiles.* Red words: Antibiotic susceptibility profiles changed "susceptible" to "resistant" (S→R). * Blue words: Antibiotic susceptibility profiles changed "resistant" to "susceptible" (R→S). * CRKP, carbapenem-resistant *Klebsiella pneumoniae*; CSPA, carbapenem-susceptible *Pseudomonas aeruginosa*; CRPA, carbapenem-resistant *P. aeruginosa*; FOT, cefotaxime; AXO, ceftriaxone; FOX, cefoxitin; TAZ, ceftazidime; AMP, ampicillin; IMI, imipenem; HL-IMI, high-level imipenem resistant; CIP, ciprofloxacin; NAL nalidixic acid; TET, tetracycline, CHL, chloramphenicol; GEN, gentamicin; AMI, amikacin; STR, streptomycin; AZI, azithromycin; COL, colistin; SXT, trimethoprim/sulfamethoxazole.

2.2. *Antibiotic Susceptibility Was Reduced According to the NaOCl Exposure*

The MIC values before and after exposure to disinfectant were compared using the Mann–Whitney U test to determine the effect of overnight incubation of gram-negative bacteria with 1250 μg/ml of NaOCl on the MICs of antibiotics. As shown in Table 1, in isolates adapted to NaOCl, a statistically significant increase in MIC was observed for imipenem ($P = 0.010$, 83.3% of all the NaOCl-adapted strains). Particularly in the case of *Enterobacteriaceae*, NaOCl significantly increased the MIC for imipenem only ($P = 0.006$). We did not observe any changes in the MIC of *P. aeruginosa* with high levels of carbapenem resistance for imipenem.

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2.3. Cross-resistance between NaOCl disinfectant and β-lactams in Klebsiella pneumoniae

The KEGG pathway analysis was performed to explore the biological functions and pathways of DEGs. In the KEGG analysis of adapted and wild-type *K. pneumoniae* Z0318KP0159, 951 DEGs were identified and assigned to 93 pathways. Between adapted and wild-type *K. pneumoniae* Z0318KP0107, 1,472 DEGs in 110 pathways were identified. The significantly upregulated gene-enriched pathways included the two-component system (Bonferroni corrected P = 7.7E-16), phosphotransferase system (PTS, Bonferroni corrected P = 1.6E-16), and oxidative phosphorylation (Bonferroni corrected P = 9.2E-9), which includes energy metabolism (Figure S1). The most notable result involved the up-regulation genes annotated in the "β-lactam-resistance" pathway (Bonferroni corrected P = 0.01). The β-lactam-resistance pathway included 8 and 10 DEGs in Z0317KP0159 and Z0318KP0107, respectively (Figure S1).

The overexpression of RND efflux pump genes in *K. pneumoniae* Z0317KP0159 and Z0318KP0107 after exposure to 1,250 μg/ml NaOCl promoted resistance to β-lactams (Table 2). Expression of AcrAB-TolC genes, associated with the RND efflux pump, was increased by at least two-fold, except for tolC in K. pneumoniae Z0318KP0107. Moreover, the β-lactam-resistance pathway of Z0317KP0107 was significantly upregulated (P < 0.001). Treatment of *K. pneumoniae* Z0318KP0107 with NaOCl induced overexpression of the MarRAB operon (67.7-fold increase in marA expression), a global antibiotic-resistance regulator involved in the production of the AcrAB-TolC efflux pump that extrudes antibiotics. As shown in Table 1, antibiotic phenotypic characteristics after exposure to 1,250 μg/ml NaOCl also significantly reduced susceptibility to imipenem by using Mann-Whitney U test (P = 0.006). This implied that increased resistance to β-lactam antibiotics (including carbapenem) and overexpression of AcrAB-TolC were associated with NaOCl exposure. In *K. pneumoniae*, β-lactamase and carbapenemase genes showed strain-specific differences in expression after NaOCl exposure.

Table 2. Fold-change of expression of genes related to β-lactam antibiotic-resistance in *Klebsiella pneumoniae* after exposure to 1,250 μg/ml NaOCl

Group	Function	Gene locus tag (Gene name)	Gene expression fold-change*	
			Z0317 KP0159	Z0318 KP0107
Enzymatic degradation	Class A carbapenemase Kpc-2	KPHS_p200360 (*bla*KPC-2)	-2.26	3.40
	β-lactamase	KPHS_25220 (*bla*SHV)	2.43	-1.44
		KPHS_13880	-	2.71
RND efflux pump	Repression of porin OmpF (*mar-sox-rob* regulon activator)	KPHS_25470 *(marA)*	-1.44	**67.73**
	Multidrug efflux membrane fusion protein	KPHS_11890 *(acrA)*	1.46	**5.18**
	Multidrug efflux transporter	KPHS_11880 *(acrB)*	2.19	2.30
	Multidrug efflux transporter (permease EefB)	KPHS_52090 *(acrB)*	2.07	2.65
	Outer membrane channel protein	KPHS_45760 *(tolC)*	2.83	1.30
	Multidrug efflux transport outer membrane protein EefC	KPHS_52100 *(adeK)*	2.13	-1.16
Porin	Outer membrane protein 1A/OmpK35 porin	KPHS_18370 *(ompF)*	-2.45	1.52

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	Peptidoglycan synthetase, penicillin-binding protein 2	KPHS_27710 *(mrdA)*	2.54	4.02

* Difference in fold-change to antibiotic-resistance gene expression between before and after exposure to 1,250 μg/ml of NaOCl.

2.4. Cross-Resistance between NaOCl Disinfectant and β-Lactams in Pseudomonas aeruginosa

In the KEGG pathway analysis of adapted and wild-type *P. aeruginosa* ATCC 27853, 1,328 DEGs were identified and assigned to 107 pathways, while 1,332 DEGs, assigned to 110 pathways, were identified in *P. aeruginosa* Z0219PA0007, and 1,309 DEGs assigned to 104 pathways were identified in *P. aeruginosa* Z0217PA0020. As shown in Figure S2, the expression of genes was significantly related to biofilm formation in *P. aeruginosa* Z0219PA0007, in which the upregulated gene-enriched pathways were associated with the two-component system (Bonferroni corrected $P = 2.3E-25$), biofilm formation (Bonferroni corrected $P = 2.2E-23$), bacterial secretion system (Bonferroni corrected $P = 8.2E-23$), and quorum sensing (Bonferroni corrected $P = 5.5E-5$). Likewise, the most notable change in *Pseudomonas*, as shown in Figure S2, was the upregulation of the pathway related to β-lactam-resistance (Bonferroni corrected $P = 9.7E-4$ in *P. aeruginosa* Z0219PA0007). β-lactam-resistance was associated with many DEGs: ATCC 27853, Z0219PA0007, and Z0217PA0020 showed upregulation of 12, 16, and 17 DEGs, respectively.

Overexpression of the RND efflux pump genes in *P. aeruginosa* ATCC 27853, Z2019PA0007, and Z0217PA0020 after exposure to 1,250 μg/ml NaOCl also promoted resistance to β-lactam antibiotics (Table 3). The expression of MexAB-XY efflux pump genes associated with the RND efflux pump was increased by at least two-fold, except for mexAB-oprM in *P. aeruginosa* ATCC 27853. As shown in Figure S2, the β-lactam-resistance pathways of Z2019PA0007 and Z0217PA0020 were significantly upregulated ($P < 0.001$). In particular, NaOCl treatment of the three adapted *P. aeruginosa* strains commonly induced overexpression of *armZ-mexXY-oprM* (2.09–21.02-fold increase), which encode a global antibiotic-resistance regulator/modulator involved in the production of the MexXY-OprM efflux pump that extrudes antibiotics. In *P. aeruginosa* ATCC 27853, MexEF-OprN efflux pump gene expression increased by at least 2-fold, whereas expression of ParRS regulator genes decreased.

Table 3. Fold-change in β-lactam-resistance gene expression in 1,250-μg/ml NaOCl-adapted *Pseudomonas aeruginosa*

Group	Function		Gene locus tag (Gene name)	Gene expression fold-change*		
				ATCC 27853	**Z0219 PA0007**	**Z0217 PA0020**
β-lactamase	AmpC beta-lactamase		PA4110 *(ampC)*	-	-2.32	-1.07
	Beta-hexosaminidase		PA3005 *(nagZ)*	4.30	-	-
	Transport of degraded muropeptides (GlcNAc-anhMurNAc)		PA4218 *(ampG)*	**-6.95**	**13.74**	1.92
RND efflux pump	MexAB-OprM	MexR antirepressor ArmR	PA3719 *(armR)*	-	2.29	3.87
		Transcriptional regulator AmpR	PA4109 *(ampR)*	-	2.31	2.62
		Transcriptional regulator	PA3721 *(nalC)*	-2.65	-	-
		Transcriptional regulator	PA3574 *(nalD)*	-2.39	-2.33	-1.75

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	Multidrug resistance protein MexA	PA0425 *(mexA)*	-	2.50	2.03
	Multidrug resistance protein MexB	PA0426, PA4375 *(mexB)*	-	2.75	3.09
	Outer membrane protein OprM	PA0427 *(oprM)*	-	2.09	3.06
MexEF-OprN	Multidrug efflux membrane fusion protein MexE	PA2493 *(mexE)*	**11.88**	-	-
	Multidrug efflux transporter MexF	PA2494 *(mexF)*	4.11	-	-
	Transcriptional regulator AmpR	PA2495 *(oprN)*	**9.75**	-	-
MexXY-(OprM)	Transcriptional regulator	PA2020 *(mexZ)*	-	2.84	-2.21
	Two-component response regulator ParR	PA1799 *(parR)*	**-7.73**	-2.53	-2.06
	Multidrug efflux membrane fusion protein	PA2019 *(mexX)*	4.04	**20.45**	**8.88**
	Multidrug efflux transporter	PA2018 *(mexY)*	2.32	**21.02**	**8.00**
	Outer membrane protein	PA4144 *(oprM)*	2.64	1.11	-2.13
	MexZ antirepressor	PA5471 *(armZ)*	**9.59**	4.59	**10.34**
MexVW-OprM	Multidrug efflux membrane fusion protein MexV	PA4374 *(mexV)*	-	-	-
	Multidrug efflux membrane protein	PA4375 *(mexW)*	-	1.31	2.62
	Outer membrane protein OprM	PA4974 *(oprM)*	-	-2.63	**5.65**
Porin	Porin D (imipenem)	PA0958 *(oprD)*		-1.19	2.91

* The difference in fold-change to antibiotic-resistance gene expression between before and after exposure to 1,250 μg/ml of NaOCl.

NaOCl exposure (adaptation) in *P. aeruginosa* also altered the gene expression related to the AmpC-AmpR-AmpG pathway. However, AmpC overexpression for β-lactamase production was not observed in this study. The genes associated with β-lactamase resistance in *P. aeruginosa* also exhibited strain-specific differential expression upon exposure to NaOCl exposure. This confirmed that increased resistance to β-lactams (including carbapenem) and overexpression of the RND efflux pump were linked to NaOCl tolerance in *P. aeruginosa* and *K. pneumoniae*.

3. Discussion

Authors Household items that contain disinfectants may be used "inadequately" by consumers, and diluted products and/or residues may allow for the growth of multidrug efflux pump-hyper-expressing strains that are concomitantly multidrug-resistant, which may pose a pressing

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epidemiological issue. Processes that are demonstrated in a laboratory may also be reproduced by humans and in the environment. Thus, in this study, the initial MIC values of unadapted strains for disinfectant were compared with the MIC values of NaOCl-adapted strains. As shown in Table 1, NaOCl-adapted gram-negative bacteria were not killed by sublethal or recommended disinfectant concentrations (500–5,000 μg/ml). We demonstrated that 10 passages of gram-negative bacteria in increasingly higher sublethal MICs of NaOCl disinfectant was sufficient to increase the MIC for NaOCl to > 2,500 μg/ml, particularly in *K. pneumoniae* and *P. aeruginosa*. Moreover, the MICs of each of the tested strains for a range of antibiotics before and after exposure to sublethal concentrations of NaOCl were compared. A statistically significant increase in MIC was only observed for imipenem ($P < 0.010$).

In a previous study, 5,000 μg/ml of NaOCl showed a lethal effect on 94.1% of *P. aeruginosa* isolates [17]. Ni et al. recommended that disinfectant concentrations of chorine-containing disinfectants for carbapenem-resistant *K. pneumoniae* (CRKP) disinfection be set at 2,000–5,000 μg/ml in China [18]. In addition, Kanamori et al. demonstrated that disinfectants commonly used in healthcare facilities would likely be effective (> $3\log_{10}$ reduction) against carbapenem/colistin-resistant *Enterobacteriaceae* when used at appropriate concentrations, such as ≥ 5,000 μg/ml NaOCl [19]. These results indicated that NaOCl should not be used at sublethal concentrations in order to lower the risk of development of bacterial tolerance and resistance to antibiotics.

Whole genome sequencing (WGS) was conducted to elucidate the gene differences among wild and NaOCl-adapted *K. pneumoniae* and *P. aeruginosa* strains, respectively (data not shown). The average nucleotide identity (OrthoANI) analysis of the draft genomes (>99.9% similarity) suggests that wild and NaOCl-adapted *K. pneumoniae* or *P. aeruginosa* strains are the same, respectively. The draft genomes of wild- and NaOCl-adaptive *K. pneumoniae* contained genes conferring resistance to β-lactam (*bla*$_{SHV}$) and disinfectant (*qacC*) on the chromosome. In addition, wild and NaOCl-adapted *K. pneumoniae* strains carried carbapenem resistance genes (*bla*$_{KPC-2}$) on the IncX3 plasmid as well as various β-lactam resistance genes (*bla*$_{TEM}$,/*bla*$_{CTX-M-1}$/*bla*$_{OXA-1}$) on the IncFIIK plasmid. Wild- and adaptive-strains in *Pseudomonas* spp., multidrug resistance genes (*bla*$_{OXA-133}$, *cat*, *van*, etc.) were only detected on the chromosomes. As a result of comparative genomic analysis of the wild and the NaOCl-adapted strain, mutations were observed only in the cording region of the almost hypothetical proteins and the partial ribosomal RNA. However, transcriptome analysis revealed that 1,250 μg/ml NaOCl-adapted *K. pneumoniae* and *P. aeruginosa* strains increased resistance to β-lactam antibiotics (particularly imipenem) due to increased expression of the RND superfamily efflux pumps, such as AcrAB-TolC and MexAB/XY-OprM. In the case of NaOCl exposed, only *bla*$_{KPC-2}$ gene was overexpressed among the β-lactam resistance genes on the plasmid of *K. pneumoniae* Z0318KP0107 (Table 2). Besides, all wild- and adaptive-strains of *Pseudomonas* don't have any plasmid. Therefore, our data might mean that disinfectant (NaOCl) raised cross-resistance more than co-resistance with antibiotics (β-lactams).

Bacterial efflux pumps with inherent/acquired biocide tolerance can reduce susceptibility to other biocides and induce cross-resistance to specific antibiotics [12]. On the contrary, mechanisms of tolerance to biocides, such as permeability, degradation, and mutation can also result in an increase in tolerance or lead to cross-resistance. This report also mentioned that increased resistance to other biocides and cross-resistance to certain antibiotics is possible if phenotypic changes and induction occur due to biocide exposure. Some efflux pumps are upregulated by cationic disinfectants, which contributes to the antimicrobial resistance phenotype, and the role of these efflux pumps in cross-resistance to other disinfectants and antibiotics has been explored [16]. This study aimed to investigate the tolerance of gram-negative bacteria isolated from humans and the environment to NaOCl and evaluate cross-resistance to antibiotics after exposure to this disinfectant. Adaptation and tolerance to QACs and chlorhexidine are attributed to the presence and upregulation of specific efflux pumps, such as the small multidrug resistance pump [2,20], while NaOCl induces the expression of many functional gene families, including those associated with responses to oxidative stress, DNA repair, energy metabolism, membrane damage, and efflux pumps [21].

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In particular, the RND family of efflux pumps strongly promote inherent antibiotic-resistant gram-negative bacteria. These pumps are composed of three components spanning the inner and outer membranes and export a broad spectrum of antibiotics and biopharmaceuticals [22,23]. The components of the RND efflux pump include an inner membrane pump protein specific to a particular substrate, an outer membrane protein, and a periplasmic accessory protein that binds to both the inner and outer membrane proteins, allowing extrusion of substrates from the cell. AcrAB-TolC and MexAB-OprM are the major RND efflux systems present in *E. coli* and *P. aeruginosa* and are essential for their survival, colonization, and virulence [24]. In the present study, we revealed the effect of NaOCl disinfectant on the promotion of microbial tolerance to disinfectant and antibiotic-resistance in *K. pneumoniae* and *P. aeruginosa*.

Among RND pumps in the *Enterobacteriaceae*, AcrAB-TolC is the most clinically important antibiotic efflux pump [25]. Our results, summarized in Figure 1/S1 and Tables 1/2/S1, show that 1,250 μg/ml NaOCl-adapted *K. pneumoniae* Z0317KP0159 and Z0317KP0107 showed increased resistance to β-lactam antibiotics (particularly imipenem) due to increased expression of the AcrAB-TolC efflux pump system. The AcrAB-TolC efflux pump has three global regulators: MarA, SoxS, and Rob [25]. Multiple regulators play important roles in promoting the expression of *acrA/B, tolC,* and *micF*, which are genes in the *marA-soxS-rob* regulon. The *micF* transcript inhibits the translation of *ompF* porin mRNA, which provides an entry channel for small hydrophilic antibiotics (β-lactams, aminoglycosides, and colistin). The multiple antibiotic-resistance (*mar*) locus mediates resistance primarily by up-regulating efflux of some antibiotics, disinfectants, and organic solvents via the AcrAB-TolC efflux pump and down-regulating influx through the outer membrane protein F [26]. Encoded by a mar locus containing *marR/A/B*, MarA positively regulates the expression of *marR/A/B* and many other genes (*acrA/B, tolC, micF*, etc.). SoxS and the Rob activator also stimulate the expression of many genes under the mar regulon. In addition, SoxR is activated by superoxide compounds, such as NaOCl and H_2O_2 [11]. Oxidation of SoxR induces activation of a second redox sensor, SoxS, which induces the transcription of several genes (manganese superoxide dismutase, ferredoxin, *micF*, etc.).

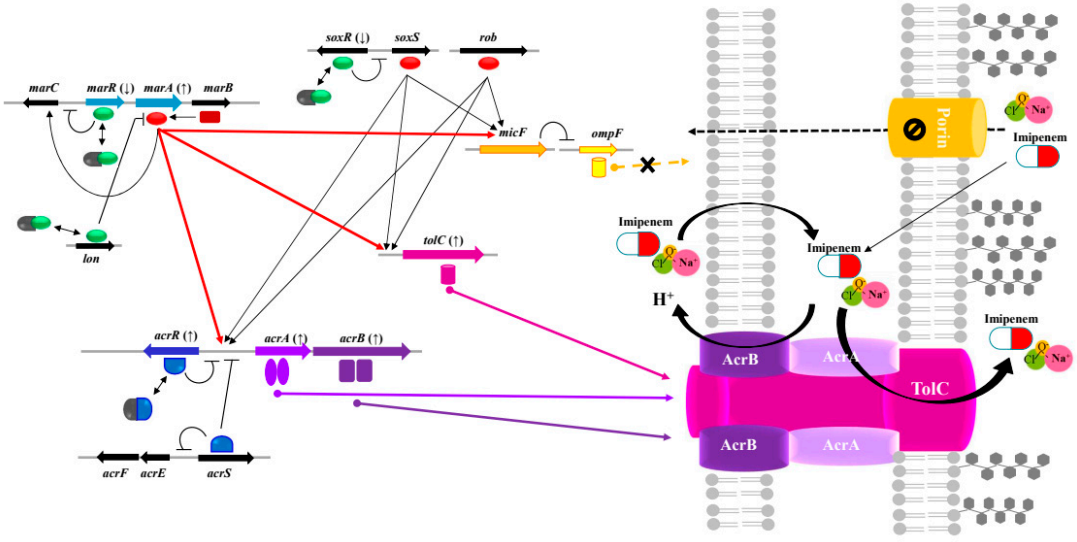

Figure 1. Acquisition of cross-resistance to imipenem antibiotic and NaOCl disinfectant by ArcAB-TolC efflux pump-related gene expression in NaOCl-exposed *Klebsiella pneumoniae*. Gene expression prediction based on RNA-Seq and quantitative reverse-transcription polymerase chain reaction datasets of *K. pneumoniae* strains (Z0318KP0107 and Z0317KP0159). The depiction of the AcrAB-TolC efflux system was modified from Li et al., Jia et al., and Li et al. [25,27,28]. AcrAB-TolC is a tripartite complex formed by AcrA, a membrane fusion protein, AcrB, a cytoplasmic-membrane protein, and TolC, an outer membrane protein. *acrA* and *acrB* are part of the same operon, which is negatively regulated by the local repressor (blue semicircle) AcrR. The genes are represented as arrows, and their translated proteins are represented as green ovals (repressors) and red ovals (activators). The

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activation of *acrAB-tolC* and *micF* transcription occurs primarily because of the global regulatory proteins MarA, SoxS, and Rob (red ovals). The arrows in parenthesis represent down/up-regulation of gene expression. The bold red arrowed lines indicate that MarA increases its own transcription and activates the expression of *acrAB-tolC* and *micF* by NaOCl exposure in our study. Overall, under conditions of NaOCl exposure, these multiple regulation mechanisms can create cross-resistance by simultaneously allowing both decreased influx (via OmpF porin) and increased efflux (via AcrAB-TolC) of imipenem antibiotics.

We confirmed that AcrAB-TolC efflux pump-related genes were overexpressed in 1,250-μg/ml NaOCl-adapted *K. pneumoniae*, by using qRT-PCR (Table S1). In particular, the regulatory gene *marR* showed decreased expression, whereas the expression of the positive regulatory gene *marA*, which serves downstream of these regulators, was increased. Consequently, the expression of genes encoding multidrug efflux pumps (*acrA*/*B, tolC*) was increased. In contrast, expression levels of *acrR* and *soxR* were upregulated or had different regulatory levels, depending on the strain. Therefore, we concluded that the overexpression of *marA* in *K. pneumoniae* strains made the adapted strains less sensitive to the effects of NaOCl. Our results also confirmed that the overexpression of *marRAB* led to cross-resistance between NaOCl and imipenem. These results are similar to the previous hypothesis by Randall and Woodward and Chetri et al. [26,29]. Although the level of antibiotic resistance conferred by *marRAB* is relatively low, increasing evidence suggests that *marRAB* and related systems are important for clinical antibiotic resistance, likely serving as a 'stepping stone' to achieve higher levels of resistance such as those of carbapenems.

Under various conditions, these multiple regulatory mechanisms can induce cross-resistance to NaOCl and imipenem by allowing simultaneously decreased influx (via the OmpF porin) and increased efflux (via AcrAB-TolC) of antimicrobial agents. Recent studies have suggested that chlorhexidine-adapted strains of *K. pneumoniae* are cross-resistant to other biocides and antibiotics, presumably because upregulation of *acrAB* and *ramA* in turn activate the AcrAB-TolC efflux pump [30,31]. In both these studies, the activation of AcrAB-TolC resulted in reduced susceptibility of *K. pneumoniae* to several antibiotics and biocides, including chlorhexidine, triclosan, and QACs. This result is consistent with our findings. In summary, we hypothesized that NaOCl exposure could influence gene expression, particularly those related to the AcrAB-TolC efflux pump of the RND family in *K. pneumoniae*, contributing to imipenem resistance. Based on this hypothesis, we described the gene expression levels of NaOCl–imipenem cross-resistance involving (1) a regulator (especially MarA), (2) a drug transporter and efflux pump, (3) cell membrane structure and transporter protein, and (4) loss of porin, in Figure 1.

Among the RND pumps in *P. aeruginosa*, the most clinically important antibiotic efflux pumps are MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN [25]. Verdial et al. described the overexpression of RND efflux pump systems as a common intrinsic or acquired resistance trait in *P. aeruginosa* [32]. They reported that overexpression of MexAB-OprM and MexXY-OprM results in *P. aeruginosa* resistance to aminoglycosides and β-lactams, and that *mexAB-oprM, mexCD-oprJ,* and *mexEF-oprN* are among the most studied genes encoding regulators of QACs, chlorhexidine, and trichlosan tolerance. As summarized in Figure 2 and Tables 1 and 3 our results showed that NaOCl-adapted *P. aeruginosa* Z0219PA0007 and Z0217PA0020 had reduced sensitivity to β-lactam antibiotics (particularly imipenem), due to increased expression of the MexAB-OprM and MexXY-OprM efflux pump systems. In the case of 1,250 μg/ml NaOCl-adapted *P. aeruginosa* ATCC 27853, decreased sensitivities to β-lactam antibiotics were due to an increase in the expression MexEF-OprN and MexXY-OprM. Hou et al. showed that chlorine-injured *P. aeruginosa* cells that were exposed to sublethal concentrations (4 μg/ml) of NaOCl developed increased resistance, by 1.4–5.6 fold, to ceftazidime, ampicillin, and chloramphenicol [33]. These results were confirmed by quantitative PCR, which showed that genes related to the MexEF-OprN efflux pump were overexpressed. Bubonja-Sonje et al. revealed that approximately 30% of 62 isolates (mostly obtained from intensive care unit patients, and with reduced carbapenem susceptibility) showed increased production of transcripts related to MexEF-OprN (from 4- to 19-fold in *mexF* mRNA transcripts as compared with a wild-type reference isolate) [34]. Li et al. reported that the MexEF-OprN efflux system was not well-expressed

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in wild-type *P. aeruginosa*, and thus, its inactivation led to little or no change in antibiotic susceptibility [25]. In *P. aeruginosa*, where even small antibiotics must slowly diffuse across the outer membrane (OM) via its slow porin, the active efflux of its major RND pump is very effective in increasing the MICs of antibiotics [28]. In addition, imipenem can penetrate the OM much more rapidly than can other antibiotics, by utilizing a specific channel, OprD. Dulyayangkul et al. reported that hypochlorite triggers overexpression of major facilitator superfamily (MFS) pumps in *Pseudomonas aeruginosa* [22]. They also reported that increasing the production of MexXY-mediated by ArmZ reduces antibiotic susceptibility. Our results did not indicate loss of OprD or other porin proteins related to porin transcription and β-lactam-resistance (Table 3). Based on our results, we suggest that the MexXY-OprM efflux pump of the RND family is involved in the cross-resistance to NaOCl and imipenem. In addition, as shown in Figure 2, NaOCl and imipenem cross-resistance involved local regulators and gene expression related to the RND (MexAB, MexXY, and MexEF) efflux pumps of *P. aeruginosa*. Taken together, our results suggest that NaOCl disinfectant exposure influences expression of genes that contribute to β-lactam (carbapenem, particularly imipenem) cross-resistance, and particularly those related to the RND efflux pump in gram-negative bacteria. This can provide useful information to identify efflux pump-related gene mutations and elucidate the molecular mechanism of cross-resistance to NaOCl and imipenem.

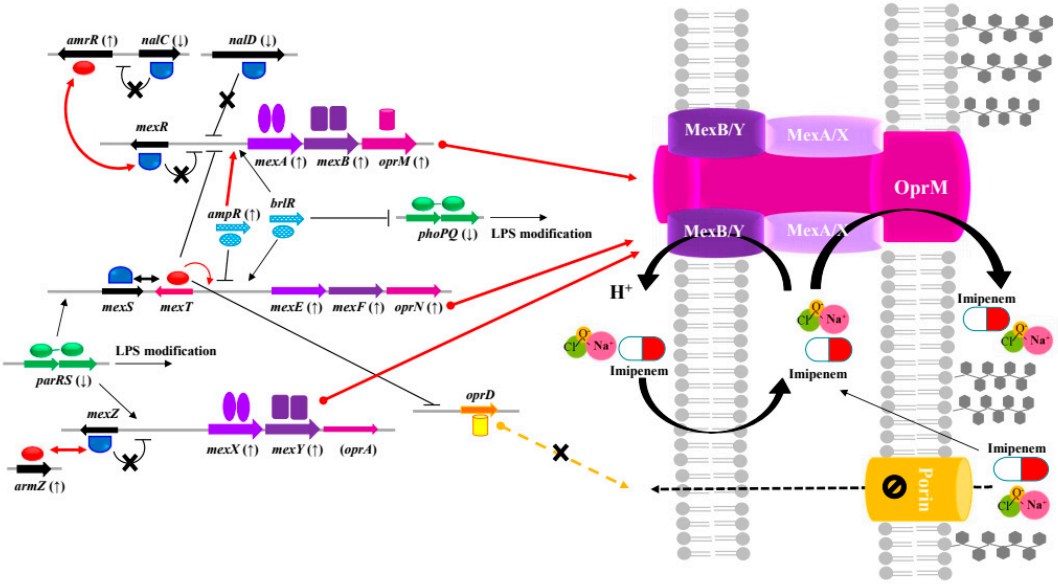

Figure 2. Induction of cross-resistance to imipenem antibiotics by overexpression of the Mex-Opr efflux pump system in NaOCl-exposed *Pseudomonas aeruginosa*. Gene expression prediction was based on transcriptome analysis of *P. aeruginosa* (ATCC 27853, Z0219PA0007, and Z0217PA0020). The depiction of the efflux system was modified from Li et al. and Moradali et al. [25,28,35]. The genes are represented as arrows, and their translated proteins are represented as blue semicircles (repressors) and red ovals (activators). The arrows in parenthesis represent down/up-regulation of gene expression. The bold red arrowed lines indicates that AmrR and ArmZ increase their own transcription and activate the expression of *mexAB/XY-oprM* upon NaOCl exposure. Overall, under conditions of NaOCl exposure, these multiple regulation mechanisms can create cross-resistance by simultaneously allowing increased efflux of imipenem antibiotics.

4. Materials and Methods

4.1. Isolation and Identification of Bacterial Strains

Overall, 117 bacterial strains were included in this study (Table S2). Of them, 91 isolates were obtained from humans, including 41 *Escherichia coli*, 9 *Klebsiella pneumoniae*, 26 *A. baumannii*, and 15 *P. aeruginosa strains*. Further, 26 isolates were obtained from hospital and livestock environments: 7

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E. coli, 4 *K. pneumoniae*, 11 *A. baumannii*, and 4 *P. aeruginosa*. All isolates from humans were MDR, including resistance to carbapenems. Environmentally obtained strains of *K. pneumoniae* and *A. baumannii* are known to be MDR, including resistance to carbapenems. The standard strains included *E. coli* ATCC 25922 and ATCC 10536, *A. baumannii* ATCC 19606, and *P. aeruginosa* ATCC 27853.

4.2. Determination of Antibiotics and Disinfectant Minimum Inhibitory Concentrations (MICs)

Antimicrobial susceptibility testing was performed using the broth microdilution method with a customized Sensititre KRCDC2F panel (TREK Diagnostic Systems, East Grinstead, United Kingdom) in accordance with the guidelines established by the Clinical and Laboratory Standards Institute [36]. The following antimicrobial agents were tested: amikacin, ampicillin, azithromycin, cefotaxime, cefoxitin, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, colistin, imipenem, gentamicin, nalidixic acid, streptomycin, tetracycline, and trimethoprim/sulfamethoxazole. All experiments in this study were carried out with independent biological replicates at least 3 times.

For NaOCl, a household chlorine bleach (Clorox ≥ 4%, Yuhan Corp, Seoul, Korea) was used. To determine the initial exposure concentrations of the disinfectant, the MIC of NaOCl was tested. Briefly, an overnight bacterial culture was diluted with sterile 0.85% NaCl solution to a 0.5 McFarland standard. Then, 96-well plates were prepared and 90 μl of bacterial suspension (1.5×10^6 CFU/ml), containing 10 μl of serially, two-fold diluted NaOCl disinfectant in cation-adjusted Mueller–Hinton Broth (CA-MHB) medium, was added to each well. For the growth control group, 90 μl bacterial suspension was added to 10 μl MHB medium without NaOCl disinfectant. For the negative control group, 90 μl sterile 0.85% NaCl solution was used instead of bacterial suspension.

4.3. NaOCl Disinfectant Exposure and Bacterial Subculturing

To determine the adaptation to disinfectant, the strains were exposed to increasing sublethal concentrations of NaOCl. Each disinfectant concentration was continuously increased two-fold and the cells were subcultured for 2 weeks, using 96-well plates. Specifically, 10 μl overnight culture of wild-type gram-negative bacteria was first added to each of at least eight wells containing in 90 μl of CA-MHB medium with 2× sublethal concentration of NaOCl. These plates were incubated at 37 °C with shaking at 150 rpm for 24 h. When growth was observed at the highest inhibitory concentration, this culture suspension was inoculated into a well containing medium with a NaOCl concentration two-fold higher than the previous concentration. Finally, we selected disinfectant-adapted strains that increased the disinfectant MIC of the wild-type strain by at least two-fold and maintained the highest MICs for 10 continuous subcultures.

4.4. RNA Extraction, Transcriptome Sequencing, and Quantitative reverse Transcription Polymerase Chain Reaction

Wild-type strains were cultured in CA-MHB for 16–18 h at 37 ℃. Adapted isolates were cultured in CA-MHB supplemented with 1,250 μg/ml NaOCl for 24 h at 37 °C. The adapted strains included *K. pneumoniae* Z0317KP0159, *K. pneumoniae* Z0318KP0107, *P. aeruginosa* ATCC 27853, *P. aeruginosa* Z2017PA0020, and *P. aeruginosa* Z2019PA0007. Cell pellets derived from exponential phase cultures of each bacterial strain was stored at -80 °C in a 10X volume of the stabilization reagents RNALater (ThermoFisher Scientific, Waltham, MA, USA). DNA and RNA were extracted using the methods described below and sequenced.

Total RNA was extracted using the TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. RNA samples were purified using a RNeasy Mini Kit (Qiagen, Munchen, Germany), including on-column DNase digestion, according to the manufacturer's protocol. RNA purity was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The RNA concentration was measured using a Qubit RNA BR Assay Kit and a Qubit 4 Fluorometer (Invitrogen). The extracted RNA was used for strand-specific cDNA library construction and Illumina paired-end sequencing (HiSeq 2500; Illumina Inc., San Diego, CA, USA) at Macrogen Co. (Seoul, Korea). mRNA expression levels were normalized to fragments per kb of transcript per

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million mapped reads (FPKM). The Database for Annotation, Visualization, and Integrated Discovery (DAVID) database analysis used edgeR to identify genes that were differentially expressed (DEGs) between the wild-type and NaOCl-adapted strains. Potential targets of the DEGs were analyzed using the Gene Ontology term (GOTERM) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps.

AcrAB-TolC pump-related gene expression (regulator/transporter) was quantified by modification of the quantitative reverse transcription polymerase chain reaction (qRT-PCR) method described by Jia et al. [27]. A significant effect on gene expression was deduced when the corresponding ratios exceeded 2.0. All reactions were performed in triplicate.

4.5. Statistical Analysis

The statistical significance of the MIC data (disinfectant-wild versus -adapted strain upon exposure to a specific antibiotic) was evaluated using the nonparametric Mann–Whitney U test. Significance was set at $P < 0.05$. KEGG enrichment analysis of the disinfectant-adapted strain was performed using Fisher's exact test with the Bonferroni correction ($P < 0.05$). All statistical calculations were performed using SPSS version 24 (IBM Corp., Armonk, NY, USA).

5. Conclusions

Disinfectants have seen a rapid increase in use, particularly in recent years, due to the ongoing COVID-19 pandemic. The most used disinfectants for combatting COVID-19 include NaOCl, QACs, H_2O_2, and ethanol. The mode of action of these disinfectants, except for triclosan that has a single specific target, is nonspecific. The mechanism of resistance to disinfectants involves several transcriptional regulators, and no single gene has been linked to NaOCl resistance. NaOCl often leads to the overexpression of the efflux pump, which may confer resistance to multiple antimicrobials. Although RND efflux pumps are overexpressed in the presence of NaOCl, they are associated with increased cross-resistance to some β-lactam antibiotics (carbapenems, particularly imipenem) and other disinfectants.

In general, if recommended guidelines for the use of disinfectants are followed in a way that limits the exposure of bacteria to sublethal doses of disinfectants, the risk of developing resistance and cross-resistance could be eliminated. Therefore, the use of disinfectants that carry a high risk of antimicrobial resistance, such as NaOCl and QACs, in household products and over-the-counter medications should be reevaluated. The use of the disinfectant has increased substantially since the onset of the COVID-19 pandemic. Our findings highlight a need for monitoring the cross-resistance between different disinfectants (e.g., chlorhexidine, H_2O_2) and clinically important antibiotics (e.g., tigecycline, colistin). Additionally, the findings emphasize the importance of disinfectants in the development and spread of antibiotic-resistant bacteria.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Kyoto Encyclopedia of Genes and Genomes enrichment histogram of *Klebsiella pneumoniae* of genes differentially expressed after exposure to 1,250 μg/ml NaOCl; Figure S2: Kyoto Encyclopedia of Genes and Genomes enrichment histogram of *Pseudomonas aeruginosa* of genes differentially expressed after exposure to 1,250 μg/ml NaOCl; Table S1: Fold-change in expression of genes related to the AcrAB-TolC efflux pump in 1,250 μg/ml NaOCl-adapted *Klebsiella pneumoniae*; Table S2: Results of antibiotic susceptibility tests of 117 strains of Gram-negative bacteria.

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