The Journal of Microbiology (2011) Vol. 49, No. 3, pp. 512-515
DOI 10.1007/s12275-011-1221-z

NOTE

Providencia Isolates Carrying *bla*$_{PER-1}$ and *bla*$_{VIM-2}$ Genes: Biofilm-Forming Capacity and Biofilm Inhibitory Concentrations for Carbapenem Antibiotics

Jungmin Kim, Shukho Kim, Hee Woo Lee[#], Sung Min Kim, and Sung Yong Seol[*]

Department of Microbiology, School of Medicine, Kyungpook National University, Daegu 700-422, Republic of Korea
[#]Present address: Department of Veterinary Internal Medicine, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Republic of Korea

(Received April 28, 2011 / Accepted June 8, 2011)

Multidrug-resistant clinical isolates of *Providencia* carrying *bla*$_{PER-1}$ and *bla*$_{VIM-2}$ were evaluated for the abilities to form biofilm and high biofilm forming capacity was demonstrated in them. Minimum biofilm inhibitory concentrations (MBICs), minimum biofilm eradication concentrations (MBECs), and minimum inhibitory concentrations (MICs) for imipenem and meropenem were also determined. In all tested strains, the MBICs were higher than the MICs for both drugs. Interestingly, the MBICs and the MBEC$_{50}$ for meropenem were lower than those for imipenem in the isolates producing high amounts of biofilm, suggesting that meropenem is superior to imipenem in the growth inhibition and eradication of biofilm forming *Providencia* strains.

***Keywords*:** multi-drug resistant *Providencia*, biofilms, PER-1, MBIC, MBEC

The genus *Providencia* is a member of the tribe *Proteeae* and consists of four species, *P. alcalifaciens*, *P. stuartii*, *P. rettgeri*, and *P. rustigianii*. Patients with long-term indwelling urinary catheters are prone to developing bladder colonization with these organisms (Peter, 1992). Recently, we identified three multidrug-resistant (MDR) *Providencia* isolates which were isolated from patients hospitalized in the same intensive care unit and catheterized with urinary catheter (Lee *et al.*, 2007). Two isolates carried *bla*$_{PER-1}$ and *bla*$_{VIM-2}$ genes and one isolate carried *bla*$_{PER-1}$, *bla*$_{VIM-2}$, and *armA* genes. The recruitment of *bla*$_{PER-1}$, *bla*$_{VIM-2}$, and *armA* genes within a single strain determined a resistance phenotype virtually almost of the available antimicrobial agents including β-lactams, monobactams, carbapenems, and aminoglycosides, which can be detrimental to the patients. Recently, MDR strains of *A. baumannii* carrying *bla*$_{PER-1}$ were found to have a significantly higher capacity for biofilm formation when compared to the *A. baumannii* strains without *bla*$_{PER-1}$ gene (Lee *et al*., 2008). Furthermore, the level of expression of *bla*$_{PER-1}$ was significantly correlated to the capacity of each isolate to form biofilm, suggesting the association of *bla*$_{PER-1}$ carriage and biofilm forming ability (Lee *et al*., 2008). Because cells growing in biofilms are highly resistant to the components of the human immune system and to numerous types of antimicrobial agent, biofilms endow opportunistic pathogens with enhanced survival capacities under stressed conditions, e.g., during host invasion or following antibiotic treatment (Costerton *et al.*, 1999; Donlan and Costerton, 2002). Therefore, the acquisition of high biofilm-forming ability endow MDR clinical isolates with one more threatening weapon, which enables them to colonize, persist, and spread more easily in hospital settings (Costerton *et al.*, 1999; Donlan and Costerton, 2002).

The objective of this study was to evaluate the biofilm forming ability of *bla*$_{PER-1}$ carrying *Providencia* isolates to see whether *bla*$_{PER-1}$ carriage of *Providencia* is also associated with biofilm forming ability. In addition, the antimicrobial susceptibilities of *Providencia* isolates in the biofilm state were evaluated by determining the minimum biofilm inhibitory concentrations (MBICs) and the minimum biofilm eradication concentrations (MBECs) for drugs and we compared with antimicrobial susceptibilities of these isolates in a planktonic state which were represented by the minimum inhibitory concentrations (MICs) for drugs.

Three clinical isolates of *Providencia* carrying *bla*$_{PER-1}$ and *bla*$_{VIM-2}$, which were identified in our previous study (Lee *et al*., 2007), were used for this study. One isolate of *P. rettgeri* (strain 22), which is susceptible to most antimicrobial agents and does not carry *bla*$_{PER-1}$ gene, was also included. The biofilm forming ability was analyzed by the method described by Heilman *et al.* (1997) and HB101 *E. coli* strain was used as a negative control strain for the biofilm assay. The inhibition effect of biofilm formation by EDTA was also assessed as described by Lee *et al.* (2008). Biofilm architecture was analyzed by a scanning electron microscope (SEM) as follows. Biofilms were formed on a plastic coverslip and then the coverslip was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. The biofilms on coverslips were sequentially dehydrated for 5 min in 50%, 70%, 90%, and

[*] For correspondence. E-mail: syseol@knu.ac.kr; Tel: +82-53-420-4842; Fax: +82-53-427-5664

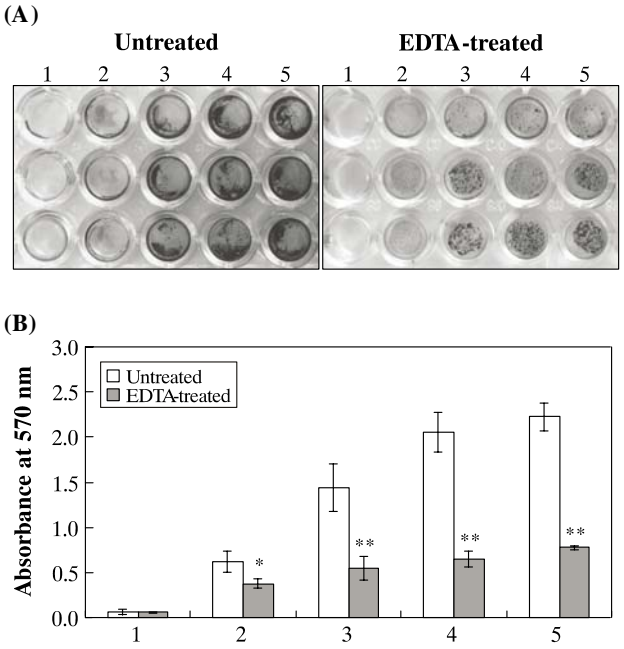

Fig. 1. Biofilm assay of *Providencia* isolates in the absence or presence of EDTA. Overnight culture of each *Providencia* strain was adjusted to an optical density at 600 nm of 2.0 and diluted 200-fold with LB medium. A 200 μl of bacterial suspension was inoculated into each well of a 96-well polystyrene plate in the absence or presence of EDTA (125 μg/ml) and incubated overnight at 37°C. Biofilms on each well were washed with PBS and stained with 0.1% gentian violet for 15 min (A). To measure the amount of the biofilms, dye was solubilized with 95% ethanol for 5 min, and the absorbance was determined at 570 nm. Each experiment was performed in triplicate and repeated three times. Data are expressed as Mean±SD. $^{*}P<0.05$ and $^{**}P<0.01$ compared with the corresponding control value as determined by *t*-test (B). Lanes: 1, HB101 *E. coli*; 2, *P. rettgeri* 22 strain; 3, *P. rettgeri* 852 strain; 4, *P. rettgeri* 1162 strain; and 5, *P. stuartii* 130 strain.

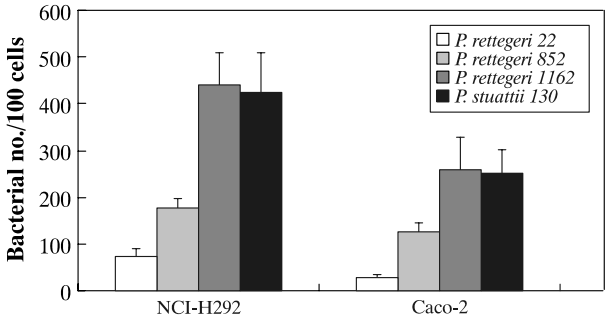

Fig. 3. Adhesion assay of *Providencia* isolates. The NCI-H292 and Caco-2 cells were prepared by seeding 2×10^5 cells on 13-mm diameter plastic coverslips in a four well plate containing RPMI 1640 medium. The monolayer was infected with 4×10^7 CFU of bacteria for 60 min at 37°C. Following infection, the nonadherent bacteria were removed by washing with PBS. The cells with the adherent bacteria were fixed in 100% methanol for 20 min and were stained for 30 min in a Giemsa stain solution. The coverslips were viewed under a light microscope at 400× magnification. Each experiment was performed in triplicate and repeated three times. Data are expressed as the Mean±SD.

100% ethanol, and then affixed to aluminum stubs using carbon tape. After coating the biofilms with a layer of gold 7 nm thick, they were examined by using a scanning electron microscope (Hitachi S-4300, Japan) at 15 kV. A cell adherence assay was performed as described in the previous study (Lee *et al.*, 2008) and NCI-H292 and Caco-2 cells were used for the assays. Determination of MBICs and MBECs was performed by the method described in the previous study (Kim *et al.*, 2010) and the MBECs were assessed with $MBEC_{50}$ and $MBEC_{90}$, which were termed as the concentration of drugs that eradicate 50% and 90% of bacteria in preformed biofilms, respectively. The MICs were determined by a broth microdilution method

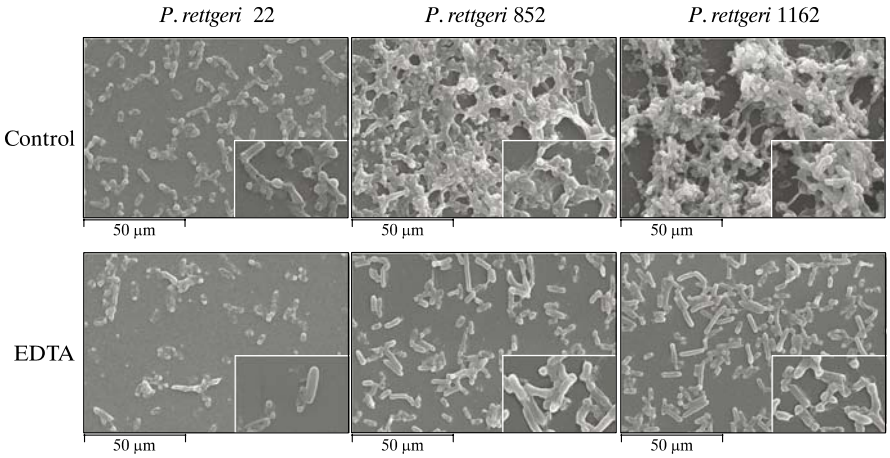

Fig. 2. SEM analysis of biofilms formed by *Providencia* isolates in the absence or presence of EDTA. The bacterial suspension was inoculated into each well of a four-well polystyrene plate containing a coverslip and incubated overnight at 37°C. The coverslip was washed with PBS and fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. The coverslip was sequentially dehydrated for 5 min in 50%, 70%, 90%, and 100% ethanol, and affixed to aluminum stubs using carbon tape. They were coated with a layer of gold 7 nm thick and were examined by using a scanning electron microscope.

Table 1. Antimicrobial susceptibility of *Providencia* isolates under a planktonic state and a biofilm state

Strains	Antimicrobial susceptibility of *Providencia* isolates against;							
	Imipenem (μg/ml)				Meropenem (μg/ml)			
	MIC	MBIC	$MBEC_{50}$	$MBEC_{90}$	MIC	MBIC	$MBEC_{50}$	$MBEC_{90}$
P. rettgeri 22	1	8	64	>512	0.5	4	32	>512
P. rettgeri 852	8	16	64	>512	4	4	8	>512
P. rettgeri 1162	8	64	>512	>512	4	8	>512	>512
P. stuartii 130	8	64	256	>512	4	8	32	>512

Abbreviations; MIC, minimum inhibitory concentration; MBIC, minimum biofilm inhibitory concentration; $MBEC_{50}$ and $MBEC_{90}$, concentration of drugs that eradicate 50% and 90% of bacteria in preformed biofilms, respectively.

following CLSI guidelines (Clinical Laboratory Standards Institute, 2008).

Three bla_{PER-1} carrying *Providencia* strains showed significantly higher biofilm-forming ability compared to the *P. rettgeri* 22 without bla_{PER-1} (Fig. 1). SEM analysis also showed that the biofilm-forming capacities were higher in bla_{PER-1} carrying *P. rettgeri* isolates than in the *P. rettgeri* 22 strain (Fig. 2). The structure of the biofilms formed by the *P. rettgeri* 1162 strain was characterized with compact conglomerates separated by channel-like spaces. In all *Providencia* strains, biofilm formation was effectively inhibited by EDTA treatment (Figs. 1 and 2).

The ability of the *Providencia* isolates to adhere to epithelial cells was also evaluated. As shown in Fig. 3, *P. rettgeri* 1162 and *P. stuartii* 130 showed significantly increased adherence to NCI-H292 and Caco-2 cells compared with the *P. rettgeri* 22. All four *Providencia* strains adhered more efficiently to NCI-H292 cells than to Caco-2 cells (Fig. 3).

Table 1 showed MICs, MBICs, $MBEC_{50}$, and $MBEC_{90}$ for carbapenem antibiotics in the four *Providencia* strains. The values of the MBIC for imipenem and meropenem were higher than those of MIC in all four strains. The bla_{PER-1} carrying *P. rettgeri* strains showed 2-8 fold higher values of MBIC than those of MIC for imipenem but 1-2 fold higher values of MBIC than those of MIC for meropenem. In case of *P. rettgeri* 22, which formed less biofilm than the three bla_{PER-1} carrying *P. rettgeri* strains, the values of MBIC and $MBEC_{50}$ for both imipenem and meropenem were 8 fold and 64 fold higher than those of MIC, respectively.

In this study, three MDR *P. rettgeri* isolates carrying bla_{PER-1} genes were able to produce higher amounts of biofilms and adhere more to Caco-2 urinary bladder epithelial cells compared with the antimicrobial susceptible *P. rettgeri* isolate. Although the observation should be proved through further studies with an acceptable number of such isolates, a strong association between bla_{PER-1} carriage with high biofilm forming ability and cellular adherence was revealed again in the *Providencia* species as well as in MDR strains of *A. baumannii* carrying bla_{PER-1} (Lee *et al.*, 2008). As in the case of *A. baumannii*, the high biofilm-forming ability combined with the multidrug-resistance of *Providencia* may contribute to their colonization and persistence on patients' urinary catheters leading to small outbreaks. We are currently investigating how bla_{PER-1} carriage is associated with the high biofilm forming ability in *Providencia* spp. and *A. baumannii*.

Biofilm has been known to play three roles in the spread of antibiotic resistance; 1) long-term antibiotic therapy is required to treat biofilm-related infections and thereby exposes bacteria to prolonged antibiotic selection pressure, 2) biofilm physiology enables embedded bacteria to survive antibiotic exposure long enough to develop a specific resistance to the drug, and 3) biofilms provides an ideal niche for the exchange of extrachromosomal DNA such as plasmid, a major source of antibiotic resistance genes (Costerton *et al.*, 1999; Donlan and Costerton, 2002; Stewart, 2002). Due to the reasons, biofilm is considered as one of the major risk factors for the spread of antibiotic resistance. Therefore, it is very important to know and it should be further investigated whether the high biofilm-forming capacity of *Providencia* isolates in this study is associated with the creation of the threatening combination of bla_{PER-1}, bla_{VIM-2}, and *armA* genes through horizontal transfer of such resistance determinants within biofilms.

It has been known that bacteria enclosed within biofilms can be resistant to antimicrobial agents at concentrations of 10-1,000 times MIC that is needed to kill genetically equivalent planktonic bacteria (Stewart, 2002). In this study, the values of MBIC for imipenem and meropenem were higher than those of MIC in all four strains. The difference between MIC and MBIC was the greatest in the *P. rettgeri* 22 strain which showed the lowest level of MICs for imipenem and meropenem due to the absence of bla_{VIM-2} gene. Among three strains carrying bla_{VIM-2} gene, the difference between MIC and MBIC in each strain was correlated with the strain's capacity to form biofilm. The $MBEC_{90}$, the concentration of drugs that eradicate 90% of bacteria in preformed biofilms, was higher than the highest concentration of drugs we tested (512 μg/ml) in all four strains, showing the limitation of antimicrobial agents for the removal of biofilmed bacteria. Interestingly, the values of MBICs and $MBEC_{50}$ for meropenem were lower than those for imipenem in the three bla_{PER-1} carrying *Providencia* strains, suggesting that meropenem is superior to imipenem in the growth inhibition of biofilm forming *Providencia* strains and the removal of preformed biofilms.

Acknowledgements

This work was supported by BioMedical Research Institute grant, Kyungpook National University Hospital (2008).

References

Clinical Laboratory Standards Institute. 2008. Methods for dilution antimicrobial susceptibility tests for bacteria and grow aerobically, approved standard, 8th ed. M07-A7, CLSI, Wayne, PA, USA.

Costerton, J.W., P.S. Stewart, and E.P. Greenberg. 1999. Bacterial biofilms: A common cause of persistent infections. *Science* 84, 1318-1322.

Donlan, R.M. and J.W. Costerton. 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15, 167-193.

Heilman, C., M. Hussain, G. Peters, and F. Götz. 1997. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. *Mol. Microbiol.* 29, 1013-1024.

Kim, S., M.J. Kim, H.Y. Kang, S.Y. Seol, D.T. Cho, and J. Kim. 2010. A simple colorimetric method for testing antimicrobial susceptibility of biofilmed bacteria. *J. Microbiol.* 48, 709-711.

Lee, H.W., H.Y. Kang, K.S. Shin, and J. Kim. 2007. Multidrug-resistant Providencia isolates carrying bla_{PER-1}, bla_{VIM-2}, and *arm*. *J. Microbiol.* 45, 272-274.

Lee, H.W., Y.M. Koh, J. Kim, J.C. Lee, Y.C. Lee, S.Y. Seol, D.T. Cho, and J. Kim. 2008. Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. *Clin. Microbiol. Infect*. 14, 49-54.

Peter, Z. 1992. Opportunistic *Enterobacteriaceae*, pp. 551-552. *In* W.K. Joklik, H.P. Willett, D. Bernard Amos, and C.M. Wilfert (eds.). Zinsser Microbiology, (20th ed.) Appleton & Lange, Norwalk, Conneticut/San Maeto, CA, USA.

Stewart, P.S. 2002. Mechanisms of antibiotic resistance in bacterial biofilms. *Int. J. Med. Microbiol.* 292, 107-113.