DETECTION OF OUTER MEMBRANE PORIN PROTEIN, AN IMIPENEM INFLUX CHANNEL, IN *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES

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Abstract. Decreased permeability to imipenem is the most frequent mechanism of imipenem resistance in *Pseudomonas aeruginosa*. We have determined the presence of OprD porin protein, an imipenem influx channel, in 70 carbapenem-resistant *P. aeruginosa* clinical isolates by Western blot analysis using rabbit anti-OprD polyclonal antibody. Ninety-eight percent (54 of 55 isolates) of imipenem-and meropenem-resistant *P. aeruginosa* clinical isolates were negative for OprD porin production. A small group of isolates resistant to imipenem but susceptible to meropenem (2 isolates) produced OprD protein but at a level 3-5 times lower than the wild type *P. aeruginosa* ATCC27853 strains. This study indicates that the loss of OprD porin protein was the main mechanism for imipenem resistance in *P. aeruginosa* clinical isolates. Determination of the status of OprD level in *P. aeruginosa* may help in the better selection of appropriate carbapenem antibiotics.

Key words: *Pseudomonas aeruginosa*, OprD porin protein, carbapenem resistance

INTRODUCTION

Pseudomonas aeruginosa is a clinically significant opportunistic human pathogen, which is a main cause of nosocomial infection worldwide (Stover *et al*, 2000; Pirnay *et al*, 2002). Infections by this pathogen are typically serious and difficult-to-treat infections, because this organism exhibits natural and acquired resistance to many structurally and functionally diverse antibiotics (Angus *et al*, 1982). This phenomenon is due partly to its relatively low outer membrane permeability, in conjunction with secondary resistance mechanisms like β-lactamase production and increased efflux pump activities (Köhler *et al*, 1999; Ochs *et al*, 1999). Subsequently, *P. aeruginosa* has the ability to develop multidrug resistance posing serious therapeutic problems and mortality associated with *P. aeruginosa* infection is high compared to that from other bacteria. Only a few antimicrobial agents, such as carbapenems and fluoroquinolones, show potent bactericidal activity against this species. In recent years, a number of clinical *P. aeruginosa* isolates have been reported to be resistant to some of these antibiotics, especially fluoroquinolones (Page

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and Heim, 2009). Therefore, carbapenem antibiotics, such as imipenem and meropenem, are among the last line of antibiotics used in the empirical therapy of severe infections due to multi-resistant *P. aeruginosa*.

Imipenem, a low molecular weight broad-spectrum carbapenem β-lactam antibiotic, is of particular interest because of its high potency against *P. aeruginosa*, and general lack of microbial cross resistance to other β-lactam antibiotics (Chen *et al*, 1995). However, prolonged treatment of *P. aeruginosa*-infected patients with imipenem has often allowed for the emergence of imipenem-resistant mutants (Yoneyama and Nakae, 1993). Recently, the incidence of imipenem-resistant *P. aeruginosa* is increasing. The National Healthcare Safety Network (NHSN) at the Centers for Disease Control and Prevention, 2006-2007 reported that *P. aeruginosa* causing device-associated infections are up to 25% resistant to imipenem *(*Hidron *et al*, 2008). For Thailand, according to a report from the National Antimicrobial Resistance Surveillance, Thailand (NARST) (2009), imipenem resistance rate increased from 11% in 1998 to 18% in 2007.

At least two types of imipenem-resistant mutants are observed in clinics (Köhler *et al*, 1999a,b; Ochs *et al*, 1999). The major type involves the loss of specific outer membrane porin protein, OprD (initially called D2 porin) (Ochs *et al*, 1999). Köhler *et al* (1999b) demonstrated that loss of OprD is the first mechanism of resistance, and such mutants are resistant only to zwitterionic carbapenem antibiotics. A second common type of resistance observed involves multiple antibiotic resistances to both imipenem and other unrelated classes of antibiotics. Such mutants have strongly reduced OprD levels due to an *nfxC*-type of quinolone-resistant mutation, which represses OprD and activates the *mexEF-oprN* multidrug efflux operon (Ochs *et al*, 1999). Additionally, production of carbapenem-hydrolyzing enzymes, such as acquired metallo-β-lactamases (MBLs), also plays an important role in carbapenem-resistant *P. aeruginosa* (Lagatolla *et al*, 2004, 2006).

OprD was first identified as a specific porin protein that is lost when *P. aeruginosa* clinical isolates become resistant to imipenem. The primary role of OprD porin is in the passive uptake across the outer membrane of basic amino acids and dipeptides containing a basic residue, but it forms pores that are also permeable to carbapenems (substrate analogues), though not to other β-lactams (Trias *et al*, 1990; Fukuoka *et al*, 1993; Huang *et al*, 1996; Ochs *et al*, 1999). Loss of OprD porin confers mainly resistance to imipenem but only a low degree of resistance to meropenem. Genetic analysis of laboratory derived mutants and clinical isolates has shown that the loss of OprD expression occur at the levels of transcription and translation (Köhler *et al*, 1997; Kolayli *et al*, 2004). Mutations (base transitions or deletions) in *oprD* structural gene generate a premature stop codon and early termination of translation. Deletions have also been shown to interfere with expression of *oprD* at the transcriptional level. Yoneyama *et al* (1993) observed a large deletion encompassing the promoter, initiation codon and putative Shine-Dalgarno sequence of *oprD* preventing transcription initiation. In addition to the deletion of *oprD* gene, Wolter *et al* (2004) have shown the first report of carbapenem resistance occurring through insertional inactivation of the *oprD* gene by insertion sequence (IS) elements. It was suggested that elimination of OprD porin from most imipenem-resistant *P. aeruginosa* isolates is due to

Table 1
Bacterial strains and plasmids used in the study.

Strain or plasmid	Relevant characteristics	Source of reference
Control strain		
Pseudomonas aeruginosa		
ATCC27853	Reference strain, OprD porin positive	ATCC
PAO1	Wide type, OprD porin positive	Dr Donald Woods
PAO1*oprD*	OprD porin-deficient mutant of PAO1	This study
Pseudomonas putida		
PP1, PP18, PP54, PP109	Clinical isolates (no *oprD* gene)	This study
Pseudomonas mendocina		
PM68	Clinical isolate (no *oprD* gene)	This study
Acinetobacter baumannii		
AB1	Clinical isolate (no *oprD* gene)	This study
***P. aeruginosa* clinical isolates**		
- Group 1 (imipenem-resistant and meropenem-resistant strains, 55 isolates)		
- Group 2 (imipenem-susceptible but meropenem-resistant strains, 13 isolates)		
- Group 3 (imipenem-resistant but meropenem-susceptible strains, 2 isolates)		
Cloning strain		
Escherichia coli		
One Shot® TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 araD139 Δ(ara-leu)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
BL21Star™ (DE3) pLysS	F$^-$, *omp*T, *hsdS*$_\beta$(r_β-m_β-), *dcm*, *gal*, (DE3), pLysS(Cm^R) *ton*A	Invitrogen
Plasmid		
pET100/D-TOPO® vector	TOPO cloning vector, 6x Histidine-tagged fusion	Invitrogen
pPN1	Carrying whole *oprD* gene of *P. aeruginosa* ATCC27853 in the cloning site of pET100/D-TOPO®	This study

efficient selection of *oprD* gene mutation.

Thus, imipenem resistance mechanism of *P. aeruginosa* results from a loss-of function mutation, and detection of the mutated structural gene alone is not always possible to determine this type of resistance. Therefore, the study at the protein level of OprD porin production is necessary in the investigation of such mutants. In this study we have produced an anti-OprD polyclonal antibody that should be a useful tool to evaluate the imipenem resistance mechanisms and facilitate characterization of imipenem-resistant clinical strains. Expression of *oprD* was studied by Western blotting with the aid of this antibody.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* ATCC27853 standard strain and wild type PAO1 were used as positive control strains, and clinical isolates of *P. putida*,

P. mendocina, *Acinetobacter baumannii*, and *P. aeruginosa* PAO1*oprD*$^-$ (spontaneous OprD-deficient mutant of PAO1 upon selection on 4 μg/ml imipenem containing agar plate) were used as the negative control strains. The study was performed on a total of 70 carbapenem-resistant *P. aeruginosa* clinical isolates that were collected from different patients who were admitted at Siriraj Hospital, Mahidol University, Bangkok, Thailand (69 isolates) during September 2001 to February 2002, and at Foothills Hospital (1 isolate), University of Calgary, Alberta, Canada in 2004. Identification of bacterial species and antimicrobial susceptibility test of these isolates were performed at the Clinical Bacteriology Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (2005). Two types of resistance determined in this study were intermediate and fully resistance. All isolates were stored at -20ºC in LB broth supplemented with 20% glycerol until used.

PCR amplification and cloning experiments

Total genomic DNA of *P. aeruginosa* strain ATCC27853 was extracted using PUREGENE™ DNA Purification Kit (Gentra Systems) and was used as template for PCR amplification. The complete open reading frame of *oprD* genes encoding OprD porin was amplified using primers oprD-1 (5'-CACCATGAAAGTGATGAAG TGGAGC-3'), which contains CACC sequence at the 5' end for use in directional cloning, and oprD-2 (5'-TTACAGGAT CGACAGCGGATAGTC-3'), designed from the *oprD* nucleotide sequence of *P. aeruginosa* PAO1 (GenBank accession number AE004091.2). PCR amplicon of the whole *oprD* gene, 1,336 bp, was cloned into an expression plasmid, pET100/D TOPO® vector, using Champion™ pET Directional TOPO® Expression kit (Invitrogen) according to manufacturer's instructions, and used to transformed One Shot® TOP10 *Escherichia coli* (Invitrogen). Transformants were analyzed in order to find the positive clones that carried the pPN1 plasmid by using alkaline lysis miniprep of plasmid DNA (Ausubel *et al*, 2002), and the purified plasmids were analyzed by digestion with *Eco*RI (SibEnzyme). The recombinant plasmid containing *oprD* was sequenced to confirm the correct orientation, in-frame translation and gene sequence using T7 forward primer (5'-TAAT ACGACTCACTATAGGG-3') and T7 reverse primer (5'-CTAGTTATTGCTCA GCGGT-3') (ABI PRISM 310 Genetic Analyzer, 1st Base Sequencing, Singapore). The sequences were analyzed and compared to the homologous PAO1 *oprD* gene in GenBank.

Detection of *oprD* gene in clinical isolates by PCR

A single colony was picked, suspended in 100 μl of TE buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8.0) and heated at 95ºC for 5 minutes. Following sedimentation, 1 μl of the supernatant was subjected to PCR amplification using oprD-3 forward primer (5'-TGCTGCTCCGCAA CTACTATTTC-3') and oprD-4 reverse primer (5'-GTAGGCCAAGGTGAAAGT GTG-3'), designed from the *oprD* gene sequence of PAO1. PCR solution (50 μl total volume) contained 37.5 μl of sterile distilled water, 5 μl of 10x PCR buffer, 1 μl of a deoxynucleotide mixture (2 mM each), 2.5 μl of primer oprD-3 (10 μM), 2.5 μl of primer oprD-4 (10 μM), 1 μl of template DNA and 0.5 μl of *Taq* DNA polymerase (DyNazymeII). Amplification was performed in GeneAmp PCR system (Perkin Elmer). The amplification program was set at one cycle of initial denaturation at 94ºC

for 5 minutes, followed by 30 cycles of 94ºC for 45 seconds, 53ºC for 30 seconds, and 72ºC for 1 minute, followed by a final extension at 72ºC for 10 minutes. The reaction products were analyzed by 1.2% (w/w) agarose gel- electrophoresis and visualization after staining with ethidium bromide.

Expression of *oprD* gene and analysis

Expression of *oprD* gene was performed in BL21 Star™ (DE3) pLysS expression *E. coli* and induced by the addition of isopropyl-β-D-thiogalactoside (IPTG, United States Biological). A total 1 liter of induced culture was centrifuged at 9,000*g* for 10 minutes at 4ºC to pellet the cells. The cells were suspended in 20 ml of sodium phosphate buffer pH 7.4 and lyzed with 2 ml of 10 mg/ml lysozyme for 2 hours. Cells were sonicated on ice four times with 5 second pulses (Sonicator®, Heat Systems-Ultrasonics, New York, USA). Following centrifugation at 16,000*g* for 20 minutes at 4ºC, the pellet was resuspended in solubilization buffer (6 M urea, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, 20 mM Tris-HCl, pH 8.0) and shaken for 60 minutes at room temperature. The solution was centrifuged at 16,000*g* for 15 minutes at 4ºC and supernatant was analyzed by 12% SDS-PAGE together with the supernatant from sonicated cell samples.

Protein bands were stained with Coomassie brilliant blue R-250 and compared to Rainbow molecular weight markers (Amersham Biosciences).

Purification of $(His)_6$-tagged OprD fusion protein

$(His)_6$-tagged OprD fusion protein was purified using HisTrap HP chromatography and HisTrap™ HP Kit (Invitrogen) as described by the manufacturer. The recombinant protein was eluted by using a linear gradient starting with solubilization buffer and ending with the elution buffer (0.5 M NaCl, 0.5 M imidazole, 1 mM 2-mercaptoethanol, 20 mM Tris-HCl, pH 8.0), and monitored using Prime View Evaluation software. The eluted protein fractions (1 ml) were analyzed by SDS-PAGE as described above. The protein band of interest was cut from the gel and sent to BSU facility (BIOTEC), Thailand for protein sequence determination using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and Mascot (Matrix Science, London, UK) software.

Generation of anti-OprD polyclonal antibody

Before being used as an immunogen, the purified $(His)_6$-tagged OprD fusion protein was desalted and concentrated using Centricon® centrifugal Filter YM-10 (Millipore Corporation) according to manufacturer's instructions. Protein concentration was determined by Bio-Rad Protein Assay using bovine serum albumin as standard. Male New Zealand white rabbit was immunized with approximately 100-150 μg of 1:1 antigen and Freund's incomplete adjuvant (Sigma Chemical) mixture. Approximately 300 μl of the mixture was injected intramuscularly into the hamstring muscles (hind legs). The rabbit was given booster injections two times using the same protein concentration in Freund's incomplete adjuvant at intervals of 7 and 15 days. Blood (1-4 ml) was incubated at 37ºC for 2 hours to inactivate the complement and allowed to clot. Serum was purified by absorption with crude lysate of the plasmid-free *E. coli* strain BL21 star™ (DE3) pLysS and used as anti-OprD polyclonal antibodies.

Western blot analysis

For the OprD quantitative assay, the clinical isolates were estimated for the numbers of cells by measurement OD_{600}

(1 OD_{600} = approximately 3 x 10^8 cell/ml). For whole-cell lysate preparations, cells were grown in 3 ml LB broth at 37ºC overnight and 500 µl of cultures were microcentrifuged for 1 minute. Cell pellets were dissolved in 100 µl of 1X SDS-PAGE sample buffer. After heating at 95ºC for 5 minutes, 5 µl of the solution were separated by electrophoresis in 12% SDS-PAGE. Gels were stained with Coomassie brilliant blue R-250 dye. For Western blot analysis, proteins were electrotransferred to a polyvinylidine difluoride membrane (Hybond-P PVDF membrane, Amersham Biosciences) using a Tank transfer system (Scie-Plas, UK). Membrane was incubated overnight in a blocking solution containing 5% non-fat dried milk, 0.1% (w/v) Tween-20 in Tris-buffered saline (TBS-T). Then the membrane was washed 3 times in TBS-T and incubated with anti-OprD polyclonal antibody diluted in TBS-T (final dilution = 1:10,000) for 1 hour at room temperature. After being washed three times in TBS-T, the PVDF membrane was incubated with horseradish-conjugated anti-rabbit immunoglobulin G for 1 hour at room temperature. After four washes, the detection was performed using the enhanced chemiluminescence method employing luminol-H_2O_2-based ECL system (Amersham Bioscience). For quantitation of OprD, *P. aeruginosa* ATCC27853 OprD protein band was used as the 100% control and OprD protein bands from clinical isolates were compared and analyzed using GeneTools software.

RESULTS

Cloning of the *P. aeruginosa* ATCC27853 *oprD* gene

P. aeruginosa ATCC27853 *oprD* gene encoding the outer membrane OprD porin protein was selected for cloning and expression. The 1,336 bp of the whole *oprD* gene was successfully cloned into pET100/D-TOPO® expression vector, designated pPN1, and established in One Shot® TOP10 *E. coli* and BL21 Star™ (DE3) pLysS expression *E. coli*. *Eco*RI analysis and sequencing of pPN1 insert showed the correct orientation and in-frame translation of the inserted *oprD* gene (data not shown).

Expression and purification of $(His)_6$-tagged OprD fusion protein

Expression of $(His)_6$-tagged OprD fusion protein in BL21 Star™ (DE3) pLysS *E. coli* resulted in insoluble inclusion bodies. This protein could be solubilized in 6 M urea and the fusion protein then was purified on a HisTrap™ HP column. The $(His)_6$-tagged OprD fusion protein was eluted as one sharp peak (data not shown). The concentration and purity of eluted proteins were evaluated by 12% SDS-PAGE, showing an approximately 52 kDa band (Fig 1). This protein was analyzed by matrix assisted desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and Mascot search database showed that it matched the outer membrane porin protein OprD precursor of *P. aeruginosa* PAO1 (data not shown). After desalting and concentrating, the $(His)_6$-tagged OprD fusion protein of 0.6 mg/ml was obtained.

Evaluation of anti-OprD polyclonal antibody

The rabbit anti-OprD polyclonal antibody showed specificity against purified $(His)_6$-tagged OprD fusion protein (52 kDa) and native OprD protein from *P. aeruginosa* ATCC27853 and PAO1 (46 kDa) (data not shown). Conversely, this antibody did not react with the relevant protein from *E. coli* BL21 Star™ (DE3) pLysS, PAO1*oprD*$^-$ (OprD-deficient mutant), *P. putida*, and *A. baumannii* (data not shown).

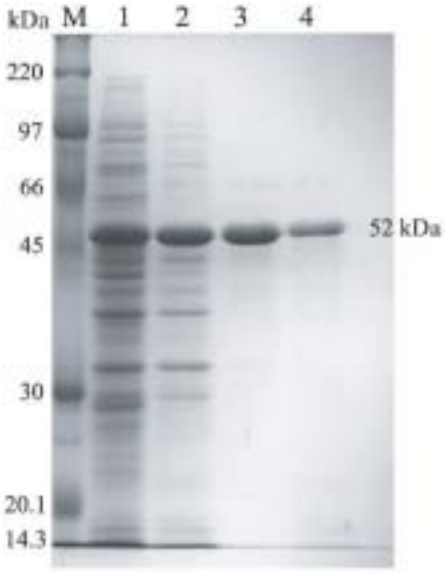

Fig 1–SDS-PAGE analysis of $(His)_6$-tagged OprD fusion protein from different steps of purification. $(His)_6$-tagged OprD fusion protein was expressed in BL21 Star™ (DE3) pLysS expression *E. coli* induced by isopropyl-β-D-thiogalactoside (IPTG). The protein was obtained from *E. coli* by sonication and was purified by HisTrap HP chromatography. Lane M, Rainbow molecular weight marker; lane 1, total cell protein fraction; lane 2, solubilized protein fraction; lane 3, purified $(His)_6$-tagged OprD fusion protein; lane 4, purified $(His)_6$-tagged OprD fusion protein after concentration and desalting step.

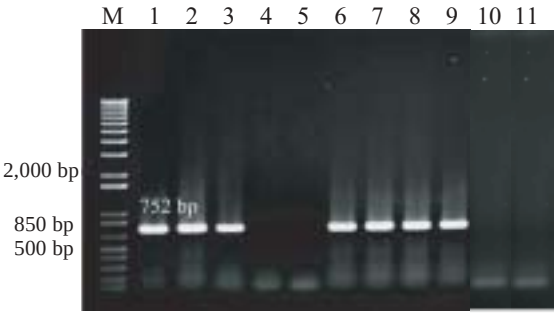

Fig 2–*oprD* gene detection by PCR in *P. aeruginosa* ATCC27853, PAO1, PAO1*oprD*$^-$, and clinical isolates. PCR amplification was performed with the *oprD* specific primers designed from *oprD* gene of PAO1 *P. aeruginosa*, and amplicons were analyzed by gel electrophoresis in 1.2% agarose. Amplicon of *oprD* gene is 752 bp. Lane M, DNA markers; lane 1, *P. aeruginosa* ATCC27853; lane 2, *P. aeruginosa* PAO1; lane 3, *P. aeruginosa* PAO1*oprD*$^-$; lane 4, *P. putida* PP1; lane 5, *A. baumannii* AB1; lane 6, *P. aeruginosa* FH1; lane 7, *P. aeruginosa* PA1; lane 8, *P. aeruginosa* PA2; lane 9, *P. aeruginosa* PA3; lane 10, *P. aeruginosa* PA42; lane 11, *P. mendocina* PM68.

Detection of *oprD* gene in clinical isolates by PCR

Normal *oprD* gene is situated in the chromosome of all *P. aeruginosa* strains. A mutated *oprD* gene might affect OprD porin production and carbapenem susceptibility. In this study, 70 carbapenem-resistant *P. aeruginosa* clinical isolates were studied for the existence of the *oprD* gene. Sixty-nine clinical isolates (98%) except PA42, were positive for *oprD* gene by PCR amplification of 752 bp product the same as in *P. aeruginosa* ATCC27853, PAO1, and PAO1*oprD*$^-$ (Fig 2). In clinical isolates of *P. putida*, *P. mendocina*, and *A. baumannii*, no PCR product was detected indicating that the *oprD* gene was specific only for *P. aeruginosa*.

Detection of OprD protein expression by Western blot analysis

Seventy carbapenem-resistant *P. aeruginosa* clinical isolates were tested for outer membrane OprD porin production by using 12% SDS-PAGE and an ECL Western blotting analysis system (Amersham Bioscience). The results from the total cell proteins analysis on 12% SDS-PAGE could not distinguish the presence of OprD protein band among the clinical isolates. On

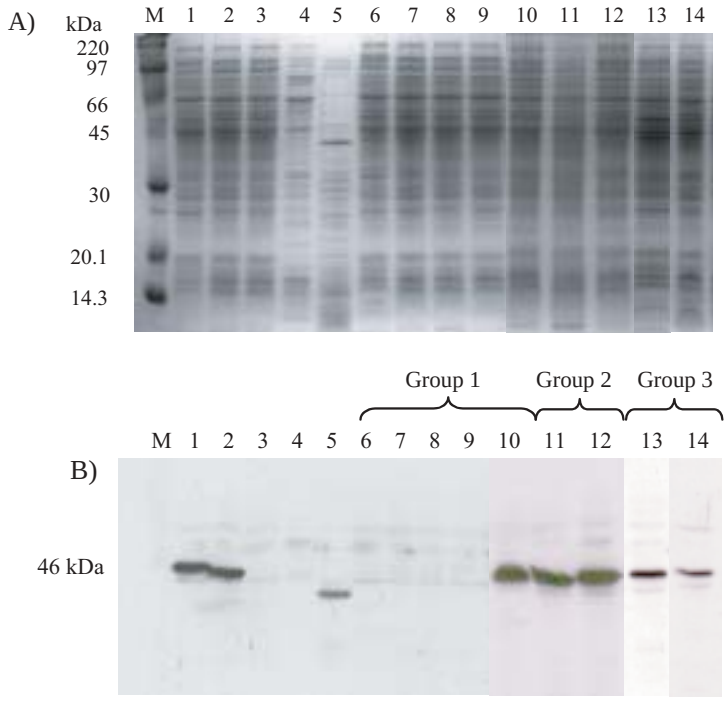

Fig 3–SDS-PAGE analysis of total cell proteins of *P. aeruginosa* strains used in the study. (A) Coomassie stained gel and (B) Western blot analysis using rabbit anti-OprD polyclonal antibody. The band position of 46 kDa of OprD protein is indicated in panel B. For each lane, 5 µl of crude cell lysate was applied. Lane M, Rainbow molecular weight markers; lane 1, *P. aeruginosa* ATCC27853; lane 2, *P. aeruginosa* PAO1; lane 3, *P. aeruginosa* PAO1*oprD*$^{-}$ (OprD-deficient mutant); lane 4, *P. putida* PP1; lane 5, *A. baumannii* AB1; lane 6, *P. aeruginosa* FH1; lane 7, *P. aeruginosa* PA1; lane 8, *P. aeruginosa* PA2; lane 9, *P. aeruginosa* PA3; lane 10, *P. aeruginosa* PA56; lane 11, *P. aeruginosa* PA65; lane 12, *P. aeruginosa* PA67; lane 13, *P. aeruginosa* PA91 and lane 14, *P. aeruginosa* PA105.

the other hand, Western blot analysis showed the clear presence of OprD porin production (Fig 3). Imipenem- and meropenem-resistant group (group 1, 54 of 55 isolates, 98%) was negative for OprD porin production, and only one isolate, *P. aeruginosa* strain PA56, was positive by exhibiting the 46 kDa positive band as seen in the *P. aeruginosa* ATCC27853 and PAO1. All isolates of the imipenem-susceptible but meropenem-resistant group (group 2, 13 isolates) showed OprD porin production. Finally, the imipenem-resistant but meropenem-susceptible group (group 3, *P. aeruginosa* strain PA91 and PA105) were positive for OprD porin production. Interestingly, these isolates showed detectable OprD protein band from Western blot analysis when the amounts of OprD protein in these isolates were compared with the OprD protein present in *P. aeruginosa* ATCC27853 by a quantitative assay, the level of OprD produced by *P. aeruginosa* strain PA91 and PA105 was approximately three and five times lower respectively, than that of *P. aeruginosa* ATCC27853 (Fig 4).

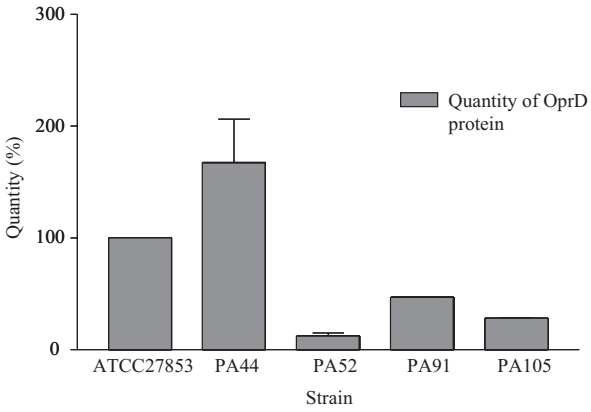

Fig 4–Quantitative analysis of OprD production from Western blot analysis. Equal amounts (1.5 x 10^7 cells) of *P. aeruginosa* ATCC27853, PA44, PA52, PA91, and PA105 were separated by SDS-PAGE and subjected to Western blot analysis as described in legend of Fig 3. The 46 kDa band intensity was measured using GeneTools software.

DISCUSSION

The problem of carbapenem resistance in gram-negative bacteria is gradually worsening due to the frequent use of this antibiotic class and its consequent selective pressure. *P. aeruginosa* is among the pathogens that can become resistant rapidly due to at least 4 different resistance mechanisms, namely, changes in drug targets, drug impermeability, increased drug efflux, and production of carbapenem hydrolyzing enzymes (Poole, 2002). In this study we tried to identify the most common mechanism used by this organism, reduced OprD porin production. The reduction in OprD production may be from mutations in the *oprD* gene or from reduction in protein translation (Gotoh *et al*, 1990; Yoneyama *et al*, 1993; Ochs *et al*, 1999). PCR amplification showed that the open reading frame of this gene in almost all clinical isolates (98%) was intact. However, we did not check all the *oprD* sequences of these isolates to determine nonsense or missense mutations. We then used the detection of OprD porin by using Western blot analysis. $(His)_6$-tagged OprD fusion protein was produced for the generation of rabbit OprD specific antibody. The polyclonal antibody showed specificity and adequate sensitivity in detecting OprD porin in clinical *P. aeruginosa* isolates, identifying a minority of imipenem/meropenem resistant isolates (2% of group 1). However, there was a reduction of OprD protein shown by quantitative analysis of Western blot band intensity in two isolates of group 3 (imipenem-resistant and meropenem-sensitive). This study demonstrated that the mechanism commonly used in imipenem-resistant *P. aeruginosa* was the absence or reduction in OprD porin production. Mutations in the promoter region can be the cause of reduction in protein production (Yoneyama *et al*, 1993; Köhler *et al*, 1999; Ochs *et al*, 1999). Recently, OprD null mutation has been shown to be the major carbapenem resistance mechanism (Farra *et al*, 2008). However, other acquired mechanisms should be studied further, such as the production of carbapenem hydrolyzing enzymes, and efflux pump proteins. Screening test for metallo-β-lactamase production showed positive results in 4 isolates of 69 Thai isolates tested (data not shown). The detection of OprD porin level by Western blot analysis would be the most convenient technique to detect the null or transcription mutation. We are now developing OprD detection technique based on the use of mouse monoclonal antibody. If the infection-causing strains produce OprD porin, it is most likely that the strains would be susceptible to the imipenem. For the strains that give negative or reduced OprD production, the physicians then can

provide alternative antibiotic treatment and also initiate infection control measures to confine or isolate these cases.

In summary, the majority of imipenem-resistant *P. aeruginosa* isolates collected in this study were OprD-non-producing isolates, indicating that reduction in OprD production is the major mechanism of imipenem resistance.

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