Detection of *Pseudomonas aeruginosa* isolates of the international clonal complex 11 carrying the \(\text{bla}_{\text{PER-1}}\) extended-spectrum \(\beta\)-lactamase gene in Greece

Kyriaki Ranellou¹, Kristina Kadlec², Aggeliki Poulou¹,³, Evangelia Voulgari¹, Georgia Vrioni¹, Stefan Schwarz² and Athanassios Tsakris¹*

¹Department of Microbiology, Medical School, University of Athens, Athens, Greece; ²Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt-Mariensee, Germany; ³Department of Microbiology, Serres General Hospital, Serres, Greece

*Corresponding author. Tel: +30-210-7462011; Fax: +30-210-7462210; E-mail: atsakris@med.uoa.gr

Received 5 July 2011; returned 31 August 2011; revised 7 October 2011; accepted 13 October 2011

**Objectives:** The extended-spectrum \(\beta\)-lactamase (ESBL) PER-1 initially disseminated among *Pseudomonas aeruginosa* strains in Turkey. Despite reports from other European countries, such strains have not been detected in Greece until now. We describe the first \(\text{bla}_{\text{PER-1}}\)-positive *P. aeruginosa* isolates from Greece and their genetic environment.

**Methods:** From January 2008 to December 2009, 287 consecutive non-duplicate *P. aeruginosa* isolates with reduced susceptibility or resistance to ceftazidime (MIC >8 mg/L) were screened for ESBL production with a modified boronic acid-based double-disc synergy test. Phenotypically ESBL-positive isolates were subjected to agar dilution, PFGE and multilocus sequence typing (MLST). Broad-spectrum \(\text{bla}\) genes were identified by PCR and sequencing. Plasmid analysis and conjugation experiments were performed. The location of the \(\text{bla}_{\text{PER-1}}\) gene was detected by Southern blotting and its genetic environment was characterized using inverse PCR.

**Results:** Five isolates were phenotypically positive for ESBL production, exhibited resistance to cefepime, ceftazidime, aztreonam and meropenem, and carried the \(\text{bla}_{\text{PER-1}}\) gene. MLST showed that they belonged to sequence type (ST) 235, which belongs to the international clonal complex 11. Four isolates had the same PFGE pattern. Southern blotting revealed the chromosomal location of the \(\text{bla}_{\text{PER-1}}\) gene. Analysis of the \(\text{bla}_{\text{PER-1}}\) flanking regions showed identity to transposon Tn1213 downstream and 1406 bp upstream of \(\text{bla}_{\text{PER-1}}\). Further upstream, an orfA gene and IS*Pa12* were identified; both were truncated by the insertion of IS6100.

**Conclusions:** This study confirmed the presence of PER-1-producing *P. aeruginosa* strains in Greece. The chromosomal location of \(\text{bla}_{\text{PER-1}}\), as part of a truncated transposon, suggests clonal expansion rather than horizontal gene transfer.

**Keywords:** PER-1, ESBLs, DDST, chromosomal location, transposon Tn1213

---

**Introduction**

PER-1 extended-spectrum \(\beta\)-lactamase (ESBL) is an Ambler class A \(\beta\)-lactamase that was first described in France in 1991 in a *Pseudomonas aeruginosa* isolate recovered from a Turkish patient.¹ Successive national surveys from Turkey have shown that the PER-1 enzyme is common among multidrug-resistant *P. aeruginosa* isolates from hospitalized patients and often coexists with OXA-type ESBLs or with the VIM-2 metallo-\(\beta\)-lactamase (MBL).²,³ PER-1 has been reported in European countries with no close geographical proximity to Turkey, such as France, Belgium, Italy and Poland,⁶–⁷ as well as in Asian regions.⁸,⁹ Epidemiological surveys have shown that a predominant *P. aeruginosa* sequence type (ST) and single-locus variants, all corresponding to the international clonal complex (CC) 11, are responsible for the dissemination of PER-1-producing *P. aeruginosa* isolates in Turkey, Belgium and Italy, as well as in several Eastern European countries.⁵,¹⁰

So far, other ESBL genes, such as \(\text{bla}_{\text{SHV-5}}, \text{bla}_{\text{IBC-2}}, \text{andbla}_{\text{GES-1}}\), have been detected in *P. aeruginosa* isolates from Greece,¹¹–¹³ but despite the geographical proximity to Turkey, *P. aeruginosa* isolates carrying the \(\text{bla}_{\text{PER-1}}\) gene have not yet...
been identified in Greece. In the present study, we describe the detection of multidrug-resistant \textit{P. aeruginosa} isolates in Greece carrying the \textit{bla}_{\text{PER-1}} gene and give detailed insight into its genetic environment.

\section*{Materials and methods}

\textbf{Bacterial isolates, ESBL detection and susceptibility testing}

During January 2008 to December 2009, 287 consecutive non-duplicated \textit{P. aeruginosa} isolates with ceftazidime MICs of $>8$ mg/L were screened phenotypically for ESBL production by a modified double-disc synergy test (DDST) with the addition of boronic acid to the antibiotic discs. Boronic acid was used as an inhibitor of AmpC.\cite{6} Discs of aztreonam, cefepime, cefotaxime and ceftazidime (30 \textmu g each) were placed on cation-adjusted Mueller–Hinton agar at a distance of 20 mm (centre to centre) from a disc containing amoxicillin/clavulanate (20/10 \textmu g). A stock solution of boronic acid was prepared by dissolving phenylboronic acid (PBA, Sigma-Aldrich, Steinheim, Germany) in DMSO and water at a concentration of 40 mg/mL. From this solution 10 \textmu L containing 400 \textmu g of PBA was added to each disc. The modified DDST was considered positive for ESBL production when an enlargement of the zone diameter of any of the used \beta-lactams was observed in the presence of clavulanate.\cite{7} A combined-disc test employing discs of imipenem (Bachem, Luzern, Switzerland), meropenem, piperacillin/tazobactam, imipenem, meropenem and aztreonam and non-\beta-lactam antibiotics (amikacin, gentamicin, ciprofloxacin and colistin) were determined by agar dilution according to CLSI document M100-S21.

The ESBL-positive isolates were screened by PCR for the presence of the ESBL (\textit{bla}\textsubscript{\text{OXA-10}}, \textit{bla}\textsubscript{\text{PER}}, \textit{bla}\textsubscript{\text{VIM}}, \textit{bla}\textsubscript{\text{TEM}}, \textit{bla}\textsubscript{\text{GES}}, \textit{bla}\textsubscript{\text{CTX-M}}, \textit{bla}\textsubscript{\text{SHV}}, \textit{bla}\textsubscript{\text{KPC}}, \textit{bla}\textsubscript{\text{TEM}}\textsubscript{\text{I}}, \textit{bla}\textsubscript{\text{TEM}}\textsubscript{\text{II}}, \textit{bla}\textsubscript{\text{OXA-2}} and \textit{bla}\textsubscript{\text{OXA-10}}) and MBL (\textit{bla}\textsubscript{\text{PER}}, \textit{bla}\textsubscript{\text{VIM}} and \textit{bla}\textsubscript{\text{VIM}-}\text{I}) genes as previously described.\cite{10,13,14} Sequence analysis of the resulting amplicons was performed with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

\section*{PFGE and multilocus sequence typing (MLST)}

PFGE of SpeI-digested genomic DNA of the ESBL-producing isolates was performed to determine their clonal relationship using a CHEF III system (Bio-Rad, München, Germany). The pulse times were increased from 10 to 30 s during the first 11 h and from 30 to 50 s during the next 13 h. MLST was performed for each of the ESBL-producing isolates in accordance with the protocol available at http://pubmlst.org/paeruginosa/.

\section*{Conjugation experiments, Southern blotting and sequence analysis}

Plasmids were extracted by alkaline lysis and purified by affinity chromatography with Qiagen Midi columns (Qiagen, Hilden, Germany). The potential for conjugative transfer of ceftazidime resistance was examined using \textit{Escherichia coli} 20R764 ($\text{lac}^{\text{I-}}, \text{Rif}^{\text{R}}$) and \textit{P. aeruginosa} PU21 (\text{Rif}^{\text{R}}) strains as recipients. Selection for transconjugants was done on Mueller–Hinton agar containing ceftazidime (16 mg/L) and rifampicin (400 mg/L).

The location of the \textit{bla}_{\text{PER-1}} gene was detected by Southern blotting using a digoxigenin-labelled (Roche Diagnostics, Mannheim, Germany) \textit{bla}_{\text{PER-1}} PCR product of 925 bp as probe, and plasmid DNA as well as NdeI- or EcoRI-digested genomic DNA as targets. The genetic environment of \textit{bla}_{\text{PER-1}} was characterized in strain P66 by sequencing an inverse PCR product following a previously described approach.\cite{15} In brief, whole-cell DNA was digested with EcoRI, the fragments were re-ligated

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Patient\#} & \textbf{Date of isolation (month/year)} & \textbf{Ward} & \textbf{Reason for hospitalization} & \textbf{Previous consumption of antibiotics} & \textbf{Underlying disease/\textbf{predisposing factor}} & \textbf{Antibiotic regimen administered} \\
\hline
1 & 10/2008 & ICU & respiratory deficiency & levofloxacin & influenza, immunosuppression & piperacillin/tazobactam + colistin \\
2 & 12/2008 & surgical cholecystectomy & choledocholithiasis & cefuroxime & gallbladder empyema & piperacillin/tazobactam + colistin \\
3 & 05/2009 & pathology & hemorrhage & amikacin & permanent urinary catheter & piperacillin/tazobactam + colistin \\
4 & 10/2009 & PU21 (Rif\textsuperscript{R}) & fever of unknown origin & piperacillin + tazobactam & permanent urinary catheter & amikacin \\
5 & 12/2009 & outpatient clinic & bone fracture & piperacillin + tazobactam & permanent urinary catheter & amikacin \\
\hline
\end{tabular}
\end{table}
and an inverse PCR with the primers per1-inv1 (5’-GCTGGTTGCTTTTTGGTGA-3’) and per1-inv2 (5’-CGCTGAGGTTTCTGAACTGAAC-3’) was performed. The sequence of the resulting amplicon was deposited under the EMBL accession no. FR847979.

Results and discussion

**Identification and susceptibility testing of bla_{PER-1}-carrying isolates**

Five isolates (P56, P65, P66, P67 and P68) from five patients gave positive results in the modified DDST and were considered to express an ESBL. PCR and sequence analysis identified the presence of the bla_{PER-1} gene. The characteristics of the patients from which these PER-1-producing *P. aeruginosa* isolates originated are presented in Table 1. The bla_{PER-1}-carrying isolates were recovered from four males and one female patient with a mean age of 69.6 years. Four of the patients were hospitalized at different times and in distinct hospital wards, while the fifth was admitted to the outpatient clinic due to a healthcare-associated urinary tract infection. None of the patients had a history of travel to Turkey during the previous year. Turkey is the country from where the bla_{PER-1} gene has very likely emerged.1,2 It is commonly presumed that immigration and the transfer of colonized patients have contributed to

**Figure 1.** (a) Southern blot hybridization of whole-cell DNA digested with either NdeI or EcoRI. Lanes 1–5 represent the *P. aeruginosa* strains P67, P68, P56, P66 and P65, respectively. Lane M contains the marker (DNA molecular weight marker II, Roche Diagnostics, Mannheim, Germany) and lane + contains the bla_{PER-1} amplicon used as a positive control. (b) Schematic presentation of the bla_{PER-1} region of *P. aeruginosa* P66 (as deposited under EMBL accession no. FR847979) compared with the bla_{PER-1}-carrying transposon Tn1213.13,15 The arrows indicate reading frames, with the arrowhead giving the direction of transcription. Insertion sequences are displayed as boxes, with the inner arrows indicating the transposase genes tnp or tnpA. orfA encodes a putative sulphate permease, while orfB encodes a universal stress protein similar to UspA. A distance scale in kb is shown below each map.
the dissemination of this gene to several European regions. The present study confirms for the first time the presence of PER-1-producing P. aeruginosa isolates in Greece, a country neighbouring Turkey.

In our study the ceftazidime-resistant isolates represented 29% of the total P. aeruginosa isolates recovered. The incidence of ESBL-producing P. aeruginosa among them was low (1.7%), but comparable to those of other countries, such as Hungary (1.3%) and Belgium (2%). The five blaPER-1-positive isolates from Greece did not carry additional blaOXA-type ESBL genes and had elevated carbapenem MICs without the co-production of MBLs or KPC carbapenemase. Detection of ESBL genes in P. aeruginosa isolates is especially challenging due to the likelihood of additional co-existing β-lactam resistance mechanisms such as MBL production or AmpC overexpression. In this study we introduced a modified DDST for the phenotypic detection of ESBLs in accordance with a previously described test for P. aeruginosa. A boronic acid compound was employed to inhibit the possible overexpression of AmpC enzyme, and an aztreonam disc was used to reveal the presence of an ESBL in case of MBL co-production. Given the high prevalence of VIM-producing P. aeruginosa isolates in Greece, there is always the fear of PER-1 and VIM-type co-production as has been reported in Italy, Turkey, Belgium and, recently, France. Infection control, enforcement of hygiene measures and early, accurate detection by the clinical laboratory (as these strains may co-produce additional β-lactamases masking the presence of PER-1) are essential. In this regard, the modified DDST should be further evaluated for the detection of ESBLs among P. aeruginosa isolates.

Agar dilution susceptibility testing showed that the five blaPER-1-positive isolates were resistant to most β-lactams, with MICs for cefepime >128 mg/L, ceftazidime and aztreonam >256 mg/L, and meropenem >64 mg/L, while they exhibited imipenem MICs of 8 or 16 mg/L and were susceptible to piperacillin/tazobactam with a MIC of 32/2 mg/L. Susceptibility testing to non-β-lactam antibiotics showed that all isolates were non-susceptible or resistant to amikacin (MICs 32–64 mg/L), gentamicin (MICs >256 mg/L) and ciprofloxacin (MICs >16 mg/L), but remained susceptible to colistin (MICs 1–2 mg/L). Piperacillin/tazobactam alone or in combination with colistin was the antibiotic regimen that was administered successfully to all five patients.

**Molecular typing, location and genetic environment of blaPER-1**

PFGE showed that isolates P65, P66, P67 and P68 had very similar patterns, whereas isolate P56 differed in its pattern (Figure S1, available as Supplementary data at JAC Online). MLST assigned the isolates to sequence type ST235, which according to the eBURST analysis is the founder ST of the international CC11. Conjugation experiments failed to transfer ceftazidime resistance. Southern blot hybridization experiments showed that the blaPER-1 gene was located on similar-sized NdeI fragments (~7.8 kb) and EcoRI fragments (~5.3 kb) of the chromosomal DNA in the five P. aeruginosa isolates (Figure 1a). The clonal dissemination of the blaPER-1 gene is attributed to the fact that it is commonly located on the chromosome. However, its potential for horizontal interspecies dissemination has been documented as it may also be located on conjugative plasmids.

Detailed analysis of this blaPER-1-carrying 5309 bp EcoRI fragment showed that it differed from the blaPER-1 gene regions previously identified in P. aeruginosa (Figure 1b). The blaPER-1 upstream part comprised 3939 bp, of which only 1406 bp corresponded to the previously described transposon Tn1213. This part included a truncated insertion sequence ISPa12 and a 13 bp spacer sequence between the blaPER-1 gene and the ISPa12 relic. Further upstream was an open reading frame, orfB, coding for a hypothetical protein of 283 amino acids, and the 3′-terminal 1348 bp of an orfA coding for a putative sulphate permease. Both orfA and ISPa12 were truncated by the insertion of an intact IS6100 element (as deposited in the NCBI database under accession no. GU475050) (Figure 1b). This IS6100 element was 981 bp in length, had perfect 14 bp terminal inverted repeats and encoded a transposase of 264 amino acids. The 443 bp in the blaPER-1 downstream part corresponded exactly to the sequence of transposon Tn1213. The observed differences might indicate a genetic diversity in the evolution of the blaPER-1 genetic environment among P. aeruginosa strains of CC11 in Greece.

**Acknowledgements**

We thank Kerstin Meyer and Roswitha Becker for excellent technical assistance and Anne-Kathrin Schink for helpful discussions.

**Funding**

This study was supported by internal funding.

**Transparency declarations**

None to declare.

**Supplementary data**

Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**


