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# Heterogeneous production of proteases from Brazilian clinical isolates of *Pseudomonas aeruginosa*

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ABSTRACT

*Background:* *Pseudomonas aeruginosa* is an important human pathogen that causes severe infections in a wide range of immunosuppressed patients. Herein, we evaluated the proteolytic profiles of 96 Brazilian clinical isolates of *P. aeruginosa* recovered from diverse anatomical sites.
*Methods:* Cell-associated and extracellular proteases were evidenced by gelatin–SDS–PAGE and by the cleavage of soluble gelatin. Elastase was measured by using the peptide substrate *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide. The prevalence of elastase genes (*lasA* and *lasB*) was evaluated by PCR.
*Results:* Bacterial extracts were initially applied on gelatin–SDS–PAGE and the results revealed four distinct zymographic profiles as follows: profile I (composed by bands of 145, 118 and 50 kDa), profile II (118 and 50 kDa), profile III (145 kDa) and profile IV (118 kDa). All the proteolytic enzymes were inhibited by EDTA, identifying them as metalloproteases. The profile I was the most detected in both cellular (79.2%) and extracellular (84.4%) extracts. Overall, gelatinase and elastase activities measured in the spent culture media were significantly higher (around 2-fold) compared to the cellular extracts and the production level varied according to the site of bacterial isolation. For instance, tracheal secretion isolates produced elevated amount of gelatinase and elastase measured in both cellular and extracellular extracts. The prevalence of elastase genes revealed that 100% isolates were *lasB*-positive and 85.42% *lasA*-positive. Some positive/negative correlations were showed concerning the production of gelatinase, elastase, isolation site and antimicrobial susceptibility.
*Conclusion:* The protease production was highly heterogeneous in Brazilian clinical isolates of *P. aeruginosa*, which corroborates the genomic/metabolic versatility of this pathogen.



## La producción heterogénea de las proteasas a partir de aislamientos clínicos brasileños de *Pseudomonas aeruginosa*

*Palabras clave:*
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RESUMEN

*Antecedentes:* *Pseudomonas aeruginosa (P. aeruginosa)* es un importante patógeno humano que causa graves infecciones en diversos tipos de pacientes inmunodeprimidos. En este trabajo evaluamos los perfiles proteolíticos de 96 aislamientos clínicos brasileños de *P. aeruginosa* aislados de diferentes localizaciones anatómicas.
*Métodos:* Las proteasas extracelulares y de extractos celulares fueron analizadas por SDS-PAGE copolimerizada con gelatina y a través de clivaje de gelatina en solución. La elastasa fue medida usando

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el substrato peptídico *N*-succinil-Ala-Ala-Ala-*p*-nitroanilida. La prevalencia de genes codificantes para elastasa (*lasA* y *lasB*) fue evaluada por PCR.
*Resultados:* En primer lugar, los extractos de las bacterias fueron aplicados en geles de SDS-PAGE-gelatina, los cuales, después de revelados, revelaron 4 perfiles enzimográficos, así: perfil I (compuesto por bandas de 145, 118 y 50 kDa), perfil II (118 y 50 kDa), perfil III (145 kDa) y perfil IV (118 kDa). Todas las enzimas proteolíticas fueron inhibidas por EDTA, siendo, por tanto, identificadas como metaloproteasas. El perfil I fue el más detectado tanto en los extractos celulares (79,2%) como en los extracelulares (84,4%). Las actividades de gelatinasa y elastasa medidas en el medio de cultivo fueron significativamente más elevadas (cerca de 2 veces) que en los extractos celulares y el nivel de producción varió de acuerdo al sitio del cual fue aislada la cepa. Por ejemplo, cepas aisladas de secreción traqueal produjeron cantidades elevadas de gelatinasa y elastasa medidas tanto en el extracto celular como en los extractos extracelulares. La prevalencia de los genes de elastasa reveló que el 100% de los aislamientos fueron *lasB* positivos y 85.42% *lasA* positivos. En algunos casos se observó una correlación positiva/negativa respecto a la producción de gelatinasa, elastasa, sitio de aislamiento y susceptibilidad antimicrobiana.
*Conclusión:* La producción de proteasas fue altamente heterogénea en los aislamientos clínicos brasileños de *P. aeruginosa*, lo cual corroboran la versatilidad genómica/metabólica de este patógeno.


## Introduction

*Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium able to grow and survive in almost all environments, living primarily in water, soil, vegetation and both human and animal sewages.[1,2] Moreover, *P. aeruginosa* is a human pathogen frequently isolated in worldwide hospital settings, causing numerous debilitating nosocomial infections, especially in immunocompromised individuals and patients with cancer, cystic fibrosis and burns.[1,3] The pathogenesis of *P. aeruginosa* is a complex and multifactorial event, which can be faced as a typical war battle: on one hand, the production of attributes of bacterial virulence and, on the other hand, the host's ability to plan a vigorous counterattack by generating a powerful immune response.[4,5]

A multitude of virulence factors are produced by *P. aeruginosa* cells, including: adhesins (e.g., flagella and type 4 pili), endotoxin (e.g., lipopolysaccharide), exotoxins (e.g., exotoxin A), pigments (e.g., pyocyanin), siderophores (e.g., pyoverdine), rhamnolipids, alginate-formed biofilm and a plentiful of extracellular hydrolytic enzymes (e.g., phospholipases, esterases, lipases and proteases).[4–6] Collectively, all these bacterial virulence attributes act by increasing the tissue damage and/or protecting this pathogen against the host immune system recognition, contributing to the establishment and maintenance of the infectious process.[4–6] Among all the virulence factors described in *P. aeruginosa*, proteases seem to play central physiological roles and particularly important functions in different stages of the bacteria-host interplay. Corroborating this statement, of the 5568 open reading frames encoded in the genome of the *P. aeruginosa* type strain PAO1, 155 (2.8%) are listed as proteases and, in addition, the manifold secretory systems synthesized by this bacterial pathogen churn out vast quantities of proteases.[7] The foremost well-characterized proteases produced by *P. aeruginosa* are: elastases A (Las A) and B (Las B), alkaline protease and protease IV.[8,9] *Pseudomonas* elastases and alkaline protease are metallo-type proteases that can degrade a variety of proteins, including a huge range of extracellular matrix components, defense molecules and proteinaceous surfactants encountered in several cells, tissues and fluids of the host.[9,10] Also, this pathogen secretes protease IV, a serine-type protease, widely found in *P. aeruginosa* clinical isolates from ocular infections.[11,12]

Despite the importance of proteases in both physiological and pathological events of *P. aeruginosa* cells, very little is known on the production/expression of these host-damaging enzymes taking into consideration the anatomical site of infection. In this context, the aim of the present work was to evaluate the cellular and extracellular proteases produced by 96 clinical isolates of *P. aeruginosa*, which were recovered from distinct anatomical sites of patients attended at Brazilian hospitals.

## Methods

### *Clinical strains*

The present study was conducted with 96 non-duplicated clinical strains of *Pseudomonas aeruginosa* isolated from rectum ($n = 20$), tracheal aspirate ($n = 19$), mouth ($n = 18$), blood ($n = 8$), urine ($n = 7$), central venous catheter ($n = 6$), pleural secretion ($n = 5$), eschar ($n = 4$), cystic fibrosis lungs ($n = 4$), sputum ($n = 3$) and nasal secretion ($n = 2$) of patients hospitalized in intensive treatment units of four hospitals located in the Southeast States of Brazil.[13–15] Each *P. aeruginosa* isolate used herein came from only one patient; consequently, each isolate was recovered from only one anatomical site regarding each patient. Regarding the isolation site, we separated our sample collection in two major groups: (i) isolates from sites (urine, eschar, blood, pleural secretion, sputum, venous catheter tip, nasal secretion and cystic fibrosis lung) linked with bacterial infection, in which the patients presented signs and symptoms (e.g., fever, pus from wound, pneumonia, etc.) and (ii) isolates from sites (tracheal secretion, mouth and rectum) associated to bacterial colonization, in which the patients had no signs and/or symptoms.[15] The genetic variability and antimicrobial susceptibility profiles of the *P. aeruginosa* isolates used in all parts of the current study were recently published by our research group.[15] The reference strain of *P. aeruginosa* ATCC 27853 was used as a control in all experiments.

### *Growth conditions and bacterial extracts*

*P. aeruginosa* strains were grown on trypticase soy agar (TSA; Merck, Darmstadt, Germany) for 18 h at 37 °C. Subsequently, bacterial cells were subcultured in tryptone soy broth (TSB) supplemented with 1% glycerol, 50 mM glutamate, 10 mM $CaCl_2$ and 10 mM $ZnCl_2$ and incubated for 24 h at 37 °C under constant agitation.[9] Cultures were centrifuged (4000 rpm, 20 min, 4 °C) and bacterial cells were washed three times in phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.2). Then, bacteria were suspended in 500 μl of PBS supplemented with 0.1% Triton X-100 and lysed in a cell homogenizer (Braun Biotech International, Germany) by alternating 2 min shaking periods and 2 min cooling intervals (total of 5 cycles). The mixtures were centrifuged (4000 rpm, 20 min, 4 °C) and the obtained supernatants were considered as bacterial cellular extracts.[16] In parallel, the