Emergence of *dhfr*XVb and *bla*$_{CARB-4}$ gene cassettes in class 1 integrons from clinical *Pseudomonas aeruginosa* isolated in Amazon region

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Integrons play a role in horizontal acquisition and expression of genes, as well as gene reservoir, contributing for the resistance phenotype, particularly relevant to bacteria of clinical importance. We aimed to determine the composition and the organization of the class 1 integron variable region present in Pseudomonas aeruginosa *clinical isolates from Brazil. Strains carrying class 1 integrons were resistant to the majority of antibiotics tested, except to imipenem and ceftazidime. Sequence analysis of the integron variable region revealed the presence of the* bla$_{CARB-4}$ *gene into two distinct cassette arrays:* aac*A4*-dhfr*XVb*-bla$_{CARB-4}$ *and* aad*B*-aac*A4*-bla$_{CARB-4}$. dhfr*XVb gene cassette, which is rare in Brazil and in* P. aeruginosa *species, was found in one isolate. PFGE analysis showed the spread of* bla$_{CARB-4}$ *among* P. aeruginosa *clones. The occurrence of* bla$_{CARB-4}$ *and* dhfr*XVb in Brazil may contribute for developing resistance to clinically important antibiotics, and shows a diversified scenarium of these elements occurring in Amazon clinical settings, where no study about integron dinamycs was performed to date.*

Key words: *dhfr* gene cassette - *bla*$_{CARB-4}$ - class 1 integron - *Pseudomonas aeruginosa* - Brazilian clinical setting

Integrons are genetic elements able to capture, excise, and rearrange genes, specially resistance gene cassettes, by a site-specific recombination mechanism, and express them under control of promoter P$_{ant}$ (Stokes & Hall 1989). Therefore, integrons are considered important tools for disseminating resistance among bacteria, acting as gene reservoirs.

The carbenicillin-hydrolysing β-lactamase CARB-4 gene encodes the sulbactam- and clavulanic acid-inhibited enzyme that belongs to Ambler class A β-lactamases, and is able to readily hydrolyse carbenicillin at high levels. A unique report showed the presence of the *bla*$_{CARB-4}$ gene in a transposon found in the plasmid pUD12 from the clinical strain of *Pseudomonas aeruginosa* P83372 isolated in France in the 1980s (Philippon et al. 1986, Sanschagrin et al. 1998). Descriptions of CARB-type genes in South America (Argentina) were restricted to *bla*$_{CARB-7}$ and *bla*$_{CARB-9}$, both from *Vibrio cholerae* super-integron (Melano et al. 2002, Petroni et al. 2004).

The major cause of trimethoprim resistance in Gram-negative bacteria is plasmid-born *dfr* genes, which are usually found in association with integrons (Grape et al. 2003). These genes are dispersed in various enterobacteria species from different geographic areas, mainly from Asian countries (Adrian et al. 1998, Gibreel & Sköld 1998, Poirel et al. 2000, Lee et al. 2001, Thungapathra et al. 2002, Navia et al. 2004, Yu et al. 2004), however, they are rare among *P. aeruginosa* isolates. The *dhfr*XVb (*dfr*A15b) gene cassette only found in a class 1 integron from a clinical *Klebsiella pneumoniae* isolated in French Guiana (Poirel et al. 2000), was recently identified in a *Salmonella enterica* subsp. *enterica* serovar Agona in Brazil (Michael et al. 2005), where *dfr* alleles have never been found before.

MATERIALS AND METHODS

Bacterial strains and antimicrobial susceptibility - *P. aeruginosa* isolates were recovered from different specimens of inpatients proceeding from clinical sources between February and October 2001. These strains were obtained from six hospitals in São Luís city, Maranhão, Brazil, and were screened for antibiotic resistance according to NCCLS recommendations (NCCLS 2003).

The MICs of the antibiotics were performed using the VITEK automated system (BioMerieux, Hazelhood, MO), and the MIC of imipenem were determined by the E-test method (AB Biodisk, Solna, Sweden) (Table I).

PCR amplifications and sequencing analysis - PCR reactions were performed using primers targeting the class 1 integrase, the integron cassette region, and the 3' conserved segment of class 1 integrons (Fonseca et al. 2005). The amplicons corresponding to the entire integron cassette regions were directly sequenced on both strands by the primer walking sequence strategy using the BigDye Terminator Cycle Sequencing Kit on an ABI Prism 377 Automated DNA Sequencer.

PFGE genome analysis - Genomic DNA of the four isolates carrying class 1 integrons was digested with *Xba*I and *Spe*I restriction enzymes. PFGE was performed using a Gene Navigator Pulsed Field System (Amersham). The λ DNA-PFGE marker (Amersham) was used as size marker. PFGE banding patterns were analyzed and compared visually. Isolates were considered to be clonal when the macrorestriction DNA patterns differed by fewer than three bands (Tenover et al. 1995).

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Nucleotide sequence accession numbers - The GenBank accession numbers of the class 1 integrons from isolates 83, 182, and 262, and from isolate 158 are AY913771 and AY913772, respectively.

RESULTS

The strains positive for the presence of class 1 integrons were resistant to all antibiotics tested, except for ceftazidime. PS 83, 158, and 182 were also susceptible to imipenem (MIC, 0,75 and 1 µg/ml, respectively), while PS 262 showed resistance to this antibiotic (MIC, > 32 µg/ml) (Table I).

Sequence analysis of the integron variable regions revealed that PS 83, 182, and 262 harboured a class 1 integron with the same cassette composition and arrangement: *aad*B, *aac*A4 and bla_{CARB-4} (Fig. 1A), while PS 158 presented a class 1 integron carrying *aac*A4, *dhfr*XVb, and bla_{CARB-4} (Fig. 1B). *dhfr*XVb was identical to that previously reported in a integron found in a *K. pneumoniae* strain from French Guiana (Poirel et al. 2000), including the non-coding regions, but presented an amino acid substitution compared with the *dfr*A15b identified in a Brazilian *S.* Agona isolate (Michael et al. 2005). All signatures of class 1 integrons, which include the integrase gene (*int*I1), the recombination site (*attI*), the promoter region (P_{ant}) and the *qac*EΔ1 and *sul*I genes of 3' CS, were found in the four integrons analyzed. The promoter P_{ant} seemed to be identical to a weak version described previously (Lévesque et al. 1994, Collis & Hall 1995) with a single point mutation in regions –35 and –10 (Fig. 1A).

Despite the identical integron composition and similar susceptibility profiles, the PFGE analysis revealed that isolate 83 is distinct from 182 and 262, which belong to a unique clone. Isolate 158 also presented a unique and distinct PFGE profile (Fig. 2, Table II).

TABLE I

Minimum inhibitory concentrations (MICs) of antibiotics for *Pseudomonas aeruginosa* isolates harbouring class 1 integrons

	MIC (µg/ml) for			
Antibiotics	PS 83	PS 182	PS 262	PS 158
Amikacin	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
Aztreonam	≥ 32 (R)	≥ 32 (R)	≥ 32 (R)	≥ 32 (R)
Cefepime	≥ 32 (R)	≥ 32 (R)	≥ 32 (R)	16 (I)
Cefotaxime	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
Cefoxitin	≥ 32 (R)	≥32 (R)	≥ 32 (R)	≥ 32 (R)
Ceftazidime	≤ 8 (S)	≤ 8 (S)	≤ 8 (S)	≤ 8 (S)
Cephalothin	≥ 32 (R)	≥ 32 (R)	≥ 32 (R)	≥ 32 (R)
Ciprofloxacin	≥ 4 (R)	≥ 4 (R)	≥ 4 (R)	≥ 4 (R)
Gentamicin	≥ 16 (R)	≥ 16 (R)	≥ 16 (R)	≥ 16 (R)
Imipenem	0.75 (S)	1 (S)	>32 (R)	0.75 (S)
Pip/Taz	≥ 128 (R)	≥ 128 (R)	≥ 128 (R)	≥ 128 (R)
Tic/Cla	≥ 256 (R)	≥ 256 (R)	≥ 256 (R)	≥ 256 (R)
Trim/Sulfa	≥ 320 (R)	≥320 (R)	≥ 320 (R)	≥ 320 (R)

R: resistant; S: susceptible; I: intermediary resistance; Pip/Taz: piperacillin/tazobactam; Tic/Clav: ticarcillin/clavulanic acid; Trim/Sulfa: trimethoprim/sulfamethoxazole.

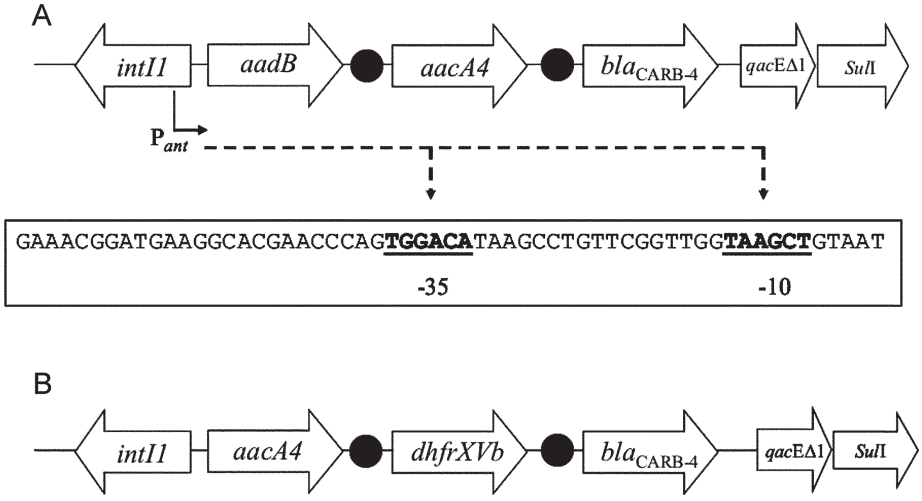

Fig. 1: schematic representation of class 1 integrons carrying bla_{CARB-4} gene found in *Pseudomonas aeruginosa* isolates 83, 182, 262 (A) and 158 (B). The weak promoter P_{ant} found in 5'CS of the four class 1 integrons determined in this work is underlined. Direction of transcription is indicated by arrow orientation and the 59-be is represented by closed circles.

TABLE II

Features of *Pseudomonas aeruginosa* clinical strains carrying bla_{CARB-4}

Strains	Patient	Pulsotype	Hospital/Isolation date	Gene cassette array
83	1	A	H4 [a]/Feb – 2001	*aad*B – *aac*A4 – bla_{CARB-4}
182	2	B	H4 [a]/Aug – 2001	*aad*B – *aac*A4 – bla_{CARB-4}
262	3	B	H4 [a]/Oct – 2001	*aad*B – *aac*A4 – bla_{CARB-4}
158	4	C	H3 [b]/June – 2001	*aac*A4 – *dhfr*XVb – bla_{CARB-4}

a: University hospital, UFMA; *b*: Nova Aliança hospital.

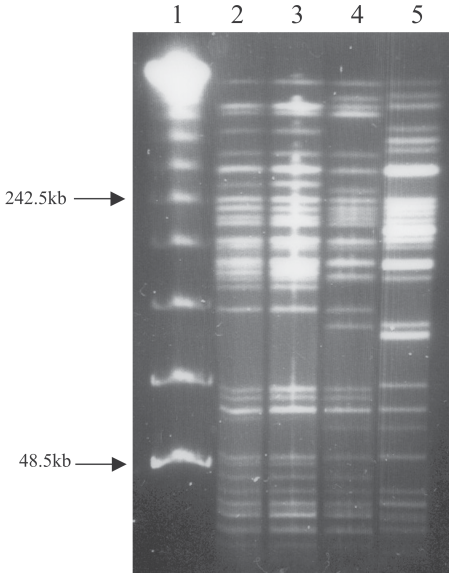

Fig. 2: pulsed-field gel electrophoresis patterns of *Spe*I-digested genomic DNAs from *Pseudomonas aeruginosa* isolates carrying bla_{CARB-4} gene cassette in class 1 integrons. λ DNA-PFGE marker, lane 1; PS 182, lane 2; PS 262, lane 3; PS 83, lane 4; PS 158, lane 5.

DISCUSSION

The bla_{CARB-4} gene sequence had been previously identified as part of a gene cluster present in a plasmid from a French *P. aeruginosa* clinical strain. In this study, we characterized the bla_{CARB-4} as a gene cassette widespread in functional class 1 integrons disseminated in hospitals from the Brazilian Amazon region. Although CARB genes have already been observed in association with class 1 integrons, the majority of these genes were not found in resistance gene cassette arrays (Petroni et al. 2004). Here, the bla_{CARB-4} gene cassette was identified in a class 1 integron genetic environment distinct from that characterized by Sanschagrin et al. (1998) in France. Four versions of promoters exhibiting different strengths were identified in class 1 integrons (Lévesque et al. 1994). Despite of the presence of a weak promoter in the Brazilian integrons, all strains presented high levels of resistance to β-lactams and amikacin (Table I). The lack of β-lactamase inhibitors activity plus the imipenem resistance may indicate that this phenotype is consequence of additional resistance mechanisms present in the analyzed strains, such as overexpression of AmpC β-lactamase, efflux pump systems and/or membrane impermeability (Livermore 2002), and not of the bla_{CARB-4} expression. This hypothesis was also reinforced by the fact that clonal strains (PS 182 and PS 262) presented differences between their resistance profile. In fact, bla_{CARB-4} not only hydrolyses carbenicillin, but also other β-lactams like oxacillin and piperacillin, which are used for treating infections caused by *Staphylococcus aureus* and *P. aeruginosa*. Therefore, the occurrence of this gene cassette in Brazil may contribute for developing resistance to clinically important β-lactams.

The PFGE results indicated the spread of a clone among patients, as well as the transmission and/or selection of an integron carrying the *aad*B-*aac*A4-bla_{CARB-4} gene cassette array among non-related isolates. Moreover, the identification of bla_{CARB-4} in class 1 integrons harbouring different cassette array compositions (*aac*A4-*dhfr*XVb-bla_{CARB-4} and *aad*B-*aac*A4-bla_{CARB-4}) among *P. aeruginosa* clones is a strong evidence of its mobility and, therefore, of the possibility of its spread to other genera, as has been demonstrated to occur with various gene cassettes.

In this work we also reported the emergence of the *dhfr*XVb (*dfr*A15b) allele in a Brazilian *P. aeruginosa* isolate, which is the first description of a *dfr* gene cassette in this species in all South America. Despite of the P_{ant} weak configuration, PS 158 displayed high levels of resistance to trimethoprim. In fact, Dubois et al. (2002) have found a *P. aeruginosa* strain highly resistant to β-lactams, which harboured a class 1 integron containing a weak promoter version and an ESBL gene cassette.

The emergence of bla_{CARB-4} and *dhfr*XVb in Brazil and in *P. aeruginosa* isolates demonstrates, once more, the dynamics and the mobility of gene cassettes. Moreover we showed a considerable gene cassette diversity occurring in Amazon clinical settings, where no previously study focusing this issue had been performed.

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