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Communication

Analysis of class 2 integrons as a marker for multidrug resistance among Gram negative bacilli

Cecilia Rodríguez[1,2], Marcelo H. Cassini[3], Gabriela del V. Delgado[4], Argentinian Integron Study Group, María S. Ramírez[1,5] and D. Centrón[1,*]

[1] Instituto de Microbiología y Parasitología Médica, Universidad de Buenos Aires-Consejo Nacional de Investigaciones Científicas y Técnicas (IMPaM, UBA-CONICET), Buenos Aires, Argentina
[2] Centro de Referencia para Lactobacilos (CERELA-CONICET), Tucumán, Argentina
[3] Grupo GEMA, DCB, Universidad Nacional de Luján, Buenos Aires, Argentina y Laboratorio de Biología del Comportamiento, IBYME, Buenos Aires, Argentina
[4] Hospital del Niño Jesús, Tucumán, Argentina
[5] Center for Applied Biotechnology Studies, Department of Biological Science, California State University Fullerton, Fullerton, California

* **Correspondence:** Email: dcentron@gmail.com; Tel: +54-115950-9500 x 2182.

Abstract: Class 1 and 2 integrons are considered the paradigm of multidrug resistant (MDR) integrons. Although class 1 integrons have been found statistically associated to *Enterobacteriaceae* MDR isolates, this type of study has not been conducted for class 2 integrons. *Escherichia coli* and 3 species that were found that harbored more than 20% of class 2 integrons in clinical isolates, were selected to determine the role of *intI2* as MDR marker. A total of 234 MDR/191 susceptible non-epidemiologically related isolates were analyzed. Seventy-four *intI2* genes were found by PCR and sequencing. An *intI2* relationship with MDR phenotypes in *Acinetobacter baumannii* and *Enterobacter cloacae* was found. No statistical association was identified with MDR *E. coli* and *Helicobacter pylori* isolates. In other words, the likelihood of finding *intI2* is the same in susceptible and in MDR *E. coli* and *H. pylori* strains, suggesting a particular affinity between the mobile element Tn*7* and some species. The use of *intI2* as MDR marker was species-dependent, with fluctuating epidemiology at geographical and temporal gradients. The use of *intI2* as MDR marker is advisable in *A. baumannii*, a species that can reach high frequencies of this genetic element.

Keywords: *IntI*2; genetic marker; Gram-negative bacilli; multidrug resistance; SXT

1. Introduction

The integron/cassettes system is a successful double component genetic mechanism associated to Lateral Genetic Transfer. The integron is composed by three elements: a gene encoding a site-specific recombinase (*intI*), a recombination site (*attI*), and a promoter (P_C) that enables the expression of the gene cassettes inserted in the variable region of the integron [1]. A gene cassette usually contains a single gene flanked downstream by a palindromic short sequence, the *attC* site, which is a specific recombination site for functional integron integrases. Most gene cassettes have unknown function. However, in nosocomial Gram-negative isolates, more than 130 antimicrobial resistance gene cassettes (ARGC) conferring resistance to several clinical relevant antibiotic families have been identified [2-7]. Although class 1 and class 2 integrons are the most frequent integrons among clinical samples [2-8], some genetic differences have been identified between both classes. Class 1 integrons are usually found in the 20% up to 80% of *Enterobacteriaceae* and *Pseudomonas aeruginosa* clinical isolates around the world [2-7]. They have been found with many arrays of ARGCs within the variable region [2-4]. Furthermore, more than 30 alleles have been described for *intI1* [9]. In contrast, class 2 integrons usually possess the typical ARGC array of *dfrA1-sat2-aadA1-ybeA-ybfA-ybfB-ybgA*. Most *intI2* have been found harboring the same allele with a stop codon at position 179 resulting in a nonfunctional IntI2 [8,10]. This phenomenon could be an explanation for the few arrays of ARGC documented in the literature. Geographical distribution of class 2 integrons varies substantially between countries [8,11]. For example, class 2 integrons were found in the 50% of *Acinetobacter baumannii* strains isolated in Argentina from 1982 to 2007, while no positive *intI2* isolate has been found in *A. baumannii* strains from 29 hospitals in United Kingdom [8,11]. Recent studies identified the emergence of class 2 integrons with novel ARGC arrays in *Enterobacteriaceae* and *A. baumannii* strains [5,8,12], suggesting that these species are active reservoirs.

A previous analysis of class 1 integrons in 867 non-repeated isolates (619 strains resistant to at least two antimicrobial agents, and 248 completely susceptible or resistant to only one antimicrobial agent) comprising 8 species of *Enterobacteriaceae* from 23 European hospitals showed a significant relation between multidrug resistance (MDR) and the *intI1* gene, independent of species or origin [3]. To our knowledge, a similar study on the association of class 2 integrons with the MDR phenotype of Gram-negative bacilli has not been yet conducted. There are previous reports on *intI2* dissemination performed with MDR strains, but they did not investigate what happens simultaneously in several susceptible bacterial populations [6,8,13-17]. Since the use of MDR markers can be advisable during outbreaks and/or for epidemiological surveillance [18], our goal was to evaluate if *intI2* could be used to early detect MDR isolates.

In previous studies, we have analyzed the frequencies of *intI2* among 9 *Enterobaceriaceae* species, *Helicobacter pylori*, *P. aeruginosa* and *A. baumannii* [8,15,19]. As an epidemiological difference with class 1 integrons, we have found that only 3 species, *A. baumannii, Enterobacter cloacae* and *H. pylori,* harbored class 2 integrons in more than 20% of the isolates. In particular, a wide dispersion of class 2 integrons was found in *A. baumannii* strains, isolated from Argentinian clinical settings during 25 years (1982–2007) [15]. We hypothesize that, in this scenario, the *intI2* will behave as *intI1* for MDR phenotypes in species where it was frequent (>20%). The aim of this study was to analyze the relationship between the presence of the *intI2* within the MDR and the susceptible phenotypes in clinical isolates of the mentioned 3 species. We also include *E. coli*

isolates, since it has been already shown that this species harbors class 2 integrons in several geographical regions [8,16,20,21].

2. Materials and Methods

2.1. Bacterial isolates

A total of 425 Gram-negative clinical strains, including 195 *E. coli*, 44 *E. cloacae*, 137 *A. baumannii*, and 49 *H. pylori* isolates, that display susceptible or MDR phenotype (see below), were used. Isolates were obtained from 4 regions of Argentina, separated by more than 1000 km. Five out of 7 hospitals that provided strains for this study, have never been included in previous studies from our laboratory (Table 1). All isolates met the criteria of nosocomial infection. Until used, strains were frozen at −80 °C in Brain Heart Infusion (BHI) (Difco Laboratories, Detroit, USA), supplemented with 20% (v/v) glycerol.

Table 1. Multidrug resistant or susceptible antibiotypes of isolates analyzed in this study. The positive/negative amplification for the *intI2* gene done by PCR is shown.

Species	Susceptibility[a]	N	Positive (%)	Negative (%)	Region[b]	Years
E. coli	S	119	9.2	90.8	BA/US/CH/TU	2006–2010
	MDR	76	9.2	90.8	BA/US/CH/TU	2006–2011
E. cloacae	S	19	0	100.0	BA	2003–2010
	MDR	25	12.0	88.0	BA/US	2003–2011
A. baumannii	S	19	0	100.0	BA	1994, 1995, 1998, 2000, 2005
	MDR	118	43.2	56.8	BA	1982–2012
H. pylori	S	34	2.9	97.1	BA	2004–2006
	MDR	15	6.6	93.3	BA	2004–2006

[a] S, susceptible strains. The criterium of a susceptible strain was based on an antibiotype resistant up to two types of the following groups of antibiotics in *E. coli*, *E. cloacae* and *A. baumannii* species: three groups of β-lactams (i) ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, (ii) cefotaxime, ceftazidime, cefepime, and (iii) imipenem and meropenem, (iv) one group of aminoglycosides, amikacin, gentamicin, (v) sulfamethoxazole, (vi) trimethoprim, (vii) colistin, and (viii) fluoroquinolones (ciprofloxacin). *Helicobacter pylori* antimicrobial susceptibility was assayed using screening plate containing 0.125 μg/ml amoxicillin, 0.25 μg/ml clarithomycin or 8 μg/ml metronidazole according to epidemiological cut-off values (ECOFFs), which distinguish wild-type isolates from those with reduced susceptibility (http://www.eucast.org/setting_breakpoints/). In *H. pylori*, the profile of susceptibility was defined as strains exhibiting susceptibility to the 3 antibiotics tested (amoxicillin, clarithomycin, and metronidazole).

[b] Buenos Aires (BA), Ushuaia (US), Chaco (CH), Tucumán (TU).

2.2. Antimicrobial susceptibility test

The disk diffusion method was performed in agar as recommended by the CLSI [22]. The antimicrobial agents tested for *E. coli*, *E. cloacae* and *A. baumannii* isolates were ampicillin (AMP), amoxicillin-clavulanic acid (AMC), piperacillin-tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), gentamicin (GEN), amikacin (AMK), ciprofloxacin (CIP), trimethoprim-sulfamethoxazole (SXT), imipenem (IPM) and meropenem (MEM) (Oxoid Laboratories, Dartford and Perth, UK). For *E. coli*, *E. cloacae* and *A. baumannii*, resistance to 7 types of antibiotics were grouped as follows: (i) AMP, AMC, TZP, (ii) CTX, CAZ, FEP, (iii) MEM, IPM, (iv) AMK, (v) GEN, (vi) SXT, and (vii) CIP. The first three types corresponded to β-lactams antibiotics while groups (iv) and (v) corresponded to aminoglycosides. The MDR phenotype for these 3 species was defined as suggested previously [23], i.e., a strain harboring resistance to one antibiotic among three different types of antimicrobial agents. Regarding *H. pylori*, the antimicrobial susceptibility was assayed using screening plate containing 0.125 μg/ml AMX, 0.25 μg/ml clarithromycin (CLT) or 8 μg/ml metronidazole (MTZ) according to epidemiological cut-off values (ECOFFs), which distinguish wild-type isolates from those with reduced susceptibility (http://www.eucast.org/setting_breakpoints/). In *H. pylori*, the profile of susceptibility was defined as strains exhibiting susceptibility to the 3 antibiotics tested (AMX, CLT, and MTZ).

2.3. Detection of intI2

Polymerase chain reaction (PCR) amplification using total DNA was performed using specific primers as previously described [24,25]. DNA products were analyzed by conventional agarose gel electrophoresis and confirmed by sequencing. Multiple sequence alignment analysis, using the CLUSTAL O (1.1.0) tool, was performed with sequences of the *intI2* from an isolate and sequences available in the GenBank accession No. AJ001816.

2.4. Detection of dfrA1

PCR amplification was used to detect the *dfrA1* gene cassette as previously described [8].

2.5. Statistical analysis

Frequencies of *inti2* in both susceptible and MDR strains were compared [3]. In order to determine if there were significant differences in occurrence of *intI*2 between isolates with and without MDR strains, two statistical methods were applied, using STATISTICA 8.0 package. Assuming that occurrence of MDR followed a stepwise function with susceptibility adopting a 0-value and multidrug resistance a 1-value, we applied Gamma statistics [26], which is a non-parametric correlation test that it is especially suitable for 0–1 data. A 2 × 2 Fisher exact test was used as additional test for *E. coli* because the sample size allows us to perform it [26].

3. Results

3.1. Class 2 integrons distribution among the studied isolates

The *intI2* was detected by PCR with specific primers in 74/425 of total tested isolates. Taking into account only the MDR isolates, slight changes in the epidemiology of *intI2*-positive isolates of *E. coli* have been found (8.3 vs 9.2%) compared to our previous study [8]. In contrast, the MDR isolates of *H. pylori*, *E. cloacae* and *A. baumannii* showed a decrease in the frequency of *intI2*-positive isolates (37.5 vs 6.6%, 33.0 vs 12.0% and 50.0 vs 43.2%, respectively) [8,19].

3.2. intI2 association to MDR phenotype

Two scenarios were found concerning the association of *intI2* positive-strains with MDR phenotype. Positive amplifications of *E. cloacae* and *A. baumannii* for *intI2* were detected only in MDR isolates (Table 1). *IntI2* was present in both susceptible and MDR isolates (Table 1) of *E. coli* and *H. pylori*. However, there were non-significant differences in occurrence of *intI2* between isolates with and without MDR phenotype in this two species. Gamma correlations were non-significant ($p > 0.05$) for *E. coli* ($b = 0.0019$) and *H. pylori* ($b = -0.404$), and non-significant differences ($p > 0.05$) were found when the frequencies of occurrence of the *intI2* were compared between susceptible and MDR *E. coli* isolates using a Fisher Exact test ($\chi^2 = 0.0001$).

3.3. Analysis of SXT resistance in E. coli

We conducted an additional analysis with the aim of disentangling the responses of *E. coli* to SXT related to the presence of *intI2*. We were especially interested in this antibiotic because we found by PCR with specific primers [8] that the *dfrA1* gene cassette was located in first place in the variable region of class 2 integrons in 17 out of our 18 *intI2*-positive *E. coli* isolates. Fisher exact test was applied to a 2 × 2 matrix composed by the absolute frequencies of isolates with and without *intI2*, resistant and susceptible to SXT. A striking presence of *intI2* in SXT resistant isolates compared with susceptible isolates was observed. Therefore, response to SXT phenotype alone was different to MDR phenotype in *E. coli* in regards to the presence of the *intI2*.

3.4. Diversity of intI2 alleles

A putative functional IntI2 protein was found in one *E. coli* isolate, the MDR E106 strain (GenBank accession No. JN987180). The *intI2* from E106 did not possess the common internal stop codon at position 179 [8,10]. Our partial DNA sequence of the *intI2* (GenBank accession no. JN987180) has 4 nucleotide changes compared to the *intI2* sequence that harbors the internal stop codon, being identical to part of the *intI2* from GenBank accession no. EU780012. The remaining *intI2* (34/35), showed an internal stop codon at position 179, as it was previously described worldwide [8,10].

4. Discussion

Class 1 and class 2 integrons are usually detected among MDR clinical isolates, so they are usually referred as MDR integrons [27]. However, several differences among both classes of integrons have been described, such as geographical distribution, level of spread among pathogenic species, and diversity of ARGC in variable regions [2,8,10,11,28]. We report further differences between the two classes of integrons, both in regard to the epidemiology of class 2 integrons over time (in 3 out of 4 species tested), and in the behavior of the *intI2* as genetic marker for MDR phenotypes. While frequencies of class 1 integrons remain stable over time [2,7], our study confirmed previous works which suggested that class 2 integrons showed a fluctuating epidemiology [14,19]. We observed a slight increase of *intI2* presence in *E. coli* isolates, and a decrease in the frequency of *intI2*-positive isolates in *H. pylori*, *E. cloacae* and *A. baumannii*. In particular, the change in the epidemiology of *intI2*-positive isolates of *A. baumannii* in Buenos Aires City has been explained by the progressive replacement of the prevalent *A. baumannii* lineages belonging to $CC113^{B}/CC79^{P}$ and $CC103^{B}/CC15^{P}$ [29], usually harboring class 2 integrons in their genomes, by new emerging clonal complexes as the widespread $CC109^{B}/CC1^{P}$ and the world-wide emerging CC110/ST25 [30]. *A. baumannii* strains isolated in 2005–2009 from 6 hospitals from Rosario, Argentina, reached 68% of *intI2*-positive [14], evidencing alternating decreases and increases of this genetic element over time in the same geographical region.

We showed that class 1 and class 2 integrons differed in their behavior as MDR markers. Until now, the role of the *intI2* as a MDR marker among Gram negative bacilli has not been established. In Argentina, several bacterial species exhibited low frequency of class 2 integrons [8]. For that reason the study was restricted to *E. coli*, *H. pylori*, *E. cloacae* and *A. baumannii* strains isolated since 1982 to 2012. We found that the likelihood of finding an *intI2* in *E. coli* and *H. pylori* isolates was independent of the phenotype of susceptibility or MDR. Instead, there was an evident association of this element with MDR *E. cloacae* and *A. baumannii* isolates, taking into account that *intI2* was never found in susceptible strains. Since *A. baumannii* can reach high frequencies (50% and 68% from two independent studies) of class 2 integrons in our geographical region [8,14], the *intI2* may be useful as MDR marker in this species.

E. coli showed a particular behavior concerning the presence of class 2 integrons. On one hand, a significant relationship between SXT resistant isolates and the presence of the *intI2* was found, probably due to the presence of the *dfrA1* gene cassette in the variable region of class 2 integrons, and a putative selection exerted over *intI2*-positive isolates. On the other hand, as described above, there are similar probabilities of encountering *intI2* in susceptible or in MDR *E. coli* strains. Both features show the different behavior of the two components of the integron/gene cassettes system: antimicrobial resistance gene cassettes (*dfrA1* in this case) are selected under antibiotic pressure, while the integron integrase genes are not selected alike by antibiotics. Taking all results as a whole, it is likely that the finding of the *intI2* in susceptible or in MDR *E. coli* and *H. pylori* isolates is not due to the antimicrobial pressure exerted in the nosocomial or community environment, but to be related to a particular affinity between the mobile element Tn*7* and the respective genomes.

We identified one different *intI2* allele in an *E. coli* isolate among the 74 *intI2*-positive strains. The emergence of this allele is in concordance with previous findings from our geographical region, since at least three *intI2* alleles were described in our neighboring country Uruguay [8,12,13].

5. Conclusion

We found that the use of *intI2* as MDR marker is species dependent, a main difference with *intI1* gene [3]. Among gram-negative bacilli, our results showed a clear association between the *intI2* and MDR *A. baumannii* or *E. cloacae* clinical isolates. Although phenotypic susceptibility tests are routinely used for clinical samples, MDR markers based on PCR can provide early data that can be essential for patient therapy. The *intI2* gene can be helpful as MDR marker in *A. baumannii* or *E. cloacae* outbreaks, and also for epidemiological surveillance.

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Conflict of interest

The authors declare that they have no conflict of interest.

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