In Vitro Evolution Predicts that the IMP-1 Metallo-β-Lactamase Does Not Have the Potential To Evolve Increased Activity against Imipenem

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In vitro evolution was used to predict whether the IMP-1 metallo-β-lactamase has the potential to evolve an increased ability to confer resistance to imipenem. Screening of eight libraries containing $9.8 \times 10^6 \pm 1.4 \times 10^6$ (mean ± standard error) variants per library, with an average of 1.2 mutations per variant, detected no increased resistance to imipenem. The results predict, with >99.9% confidence, that even under intense selection the IMP-1 β-lactamase will not evolve to confer increased resistance to imipenem.

Carbapenems are among the most potent agents for treatment of gram-negative bacterial infections (9, 12) and are hydrolyzed by a wide variety of metallo-β-lactamases (9). Widespread clinical use of carbapenems (8, 12) has led to several reports of resistance associated with the presence of metallo-β-lactamases (7). The IMP family of metallo-β-lactamases is a particular source of concern, because the IMP enzymes are typically plasmid-borne and typically found in integron cassettes (5, 10). IMP genes are therefore easily transferred among diverse bacterial species. A number of researchers have advised careful clinical use to prevent proliferation of carbapenem-resistant strains that produce metallo-β-lactamases (7, 12). Yano et al. (12) recently reported that IMP-6, which differs from IMP-1 by a single amino acid substitution, increases the MIC of meropenem 128-fold but does not increase the resistance to imipenem. If the rapid evolution of the class A extended-spectrum β-lactamases is typical, then we should indeed be concerned about the evolution of metallo-β-lactamases in response to the clinical use of imipenem and other carbapenems.

Instead of assuming that metallo-β-lactamases will evolve rapidly, it would be highly desirable to accurately predict their evolution in response to carbapenem selection.

The Barlow-Hall in vitro evolution model has been shown to accurately mimic the natural evolution of the TEM β-lactamases (4) and has been used to predict that both the class A TEM and class C CMY-2 β-lactamases will soon evolve to provide high levels of resistance to cefepime (2, 3). Here I have used that method to predict the evolution of the IMP-1 β-lactamase in response to clinical selection with imipenem.

The bla_{IMP-1} gene was amplified from genomic DNA of *Serratia marcescens* strain AK9373 (7) and cloned into the expression vector pACSE3 (4) where it is expressed from the plasmid's pTAC promoter upon induction with 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside). The sequence of the bla_{IMP-1} allele in the resulting plasmid, pIMP1, was determined to be identical to the published sequence (1) (GenBank accession no. AF416297) except for a silent T-to-C mutation at bp 15 of the coding sequence. Sequencing of the original amplicon

confirmed that the polymorphism is present in *S. marcescens* strain AK9373.

The IMP-1 gene of pIMP1 was mutagenized by amplification with the highly error-prone polymerase Mutazyme (Stratagene). The mutagenesis reaction yielded 9.85 μg of amplicon, or 1.2×10^{13} mutant molecules. The mutagenized amplicon was digested with restriction endonucleases *Bsp*HI and *Sac*I, and in eight separate reactions, the digested amplicons were ligated to plasmid pACSE3 and transformed into *Escherichia coli* strain DH5αE ([F⁻ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r⁻ m⁺) *deoR thi-1 phoA supE44* λ⁻ *gyrA96 relA1* Gal⁻) by selecting for tetracycline resistance to produce eight libraries containing $9.8 \times 10^6 \pm 1.4 \times 10^6$ (mean ± standard error) insert-bearing transformants per library. Because each library contained $<10^{-6}$ molecules of the original pool of mutant molecules, the libraries were essentially independent samples of the pool of mutant molecules, and the probability that sibling molecules were present in different libraries is negligible. The bla_{IMP-1} genes of plasmids extracted from 10 randomly chosen transformants were sequenced. There was an average of 1.2 mutations per gene.

In pIMP1, the bla_{IMP-1} gene is expressed under control of the inducible pTAC promoter that is regulated by the plasmid-borne *lacI*q repressor. In the presence of 100 μM IPTG, pIMP1 conferred an imipenem MIC of 2 μg/ml on the host strain DH5αE compared with the imipenem MIC of 0.125 μg/ml for DH5αE carrying only the pACSE3 vector. That is the same level of resistance that has previously been reported for IMP-1 in *E. coli* (12).

Each of the eight libraries was expanded by growth overnight in L broth containing tetracycline (in 1 liter, 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 1 g of glucose, 15 mg of tetracycline). Bottles containing 50 ml of L broth with 100 μM IPTG, with twofold serial dilutions of imipenem from 8 μg/ml down to 0.0625 μg/ml, were inoculated with 1.1×10^8 cells (10 times the largest library size) of the libraries. For a control, a similar imipenem dilution series was inoculated with 1.1×10^8 DH5αE carrying the unmutagenized plasmid pIMP1.

After 48 h of incubation at 37°C, the control series and each of the library series grew at imipenem concentrations of 2 μg/ml and below, but none grew at concentrations in excess of

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2 μg/ml. Plasmid was prepared from each of the cultures growing in the presence of 2 μg of imipenem per ml and was transformed into *E. coli* strain DH5αE by selection for tetracycline resistance. The resulting populations of transformants and *E. coli* DH5αE/pIMP1 after imipenem selection were used to measure the MIC of imipenem in Muller-Hinton broth (Difco) containing 100 μM IPTG using an inoculum of 10^5 cells per ml by broth serial dilution as previously described (4). For seven of the eight mutant populations and for the control, the imipenem MIC was 2 μg/ml, and for one mutant population, the MIC was 1 μg/ml. Thus, neither the initial selections nor the MIC measurements provided any evidence of mutants that exhibited increased resistance to imipenem.

Because MICs can detect only twofold increases in resistance, a more sensitive disk diffusion test was used to detect small improvements in resistance. Forty cultures were grown overnight in L broth containing tetracycline. The cultures were grown from individual colonies of transformed library 8 after imipenem selection. For a control, 10 cultures were grown from individual colonies of *E. coli* DH5αE/pIMP1. One hundred microliters of each culture was spread on a Muller-Hinton broth plate containing 100 μM IPTG, a BBL antibiotic disk containing 10 μg of imipenem was placed onto the center of each plate, and after 24 h of incubation at 37°C, the zone of inhibition was measured. The diameters of the zones of inhibition were 17.2 ± 0.2 mm for pIMP1 and 17.1 ± 0.1 mm for cells carrying plasmids from library 8 after imipenem selection. The results of the disk diffusion test thus confirm that the mutants failed to confer increased resistance to imipenem, leading to the strong prediction that the IMP-1 metallo-β-lactamase does not have the potential to evolve increased activity against imipenem.

Confidence in that prediction is based on a simulation of the in vitro evolution process using the program In vitro Evolution Simulator (6, 11). The program simulates the random mutation of the input sequence and determines the fraction of possible single and double amino acid substitutions that are obtained in a library of a given size. It is important to consider the effects of only one or two independent amino acid substitution mutations, because in nature mutations almost always arise one at a time, and each mutation must be fixed into microbial populations by selection. The input sequence was the IMP-1 sequence, the mutation frequency was 1.2 mutations per molecule, and the fraction of possible single and double amino acid substitutions obtained was calculated separately for each library. The mean fractions per library were 0.897 ± 0.009 of the single amino acid substitutions and 0.670 ± 0.01 of the double amino acid substitutions (mean ± standard error). For the eight libraries taken together, the probability of having failed to screen any particular single amino acid substitution enzyme is 1.0×10^{-8}, and the probability of having failed to screen any

particular double amino acid substitution enzyme is 1.3×10^{-4}. These results predict, with >99.9% confidence, that bla_{IMP-1} will not evolve to confer increased resistance to imipenem. That prediction depends on the sensitivity with which we can detect increased resistance in the laboratory. I cannot eliminate the possibility that increased resistance, below the level of laboratory detection, could be selected in nature.

It is clear from this study that the risks associated with the presence of bla_{IMP-1} do not include the risk of evolving increased activity against imipenem. This study, alone, is not sufficient to justify reconsideration of policies concerning the use of imipenem. In order to understand the risks posed by metallo-β-lactamases, it will be necessary to conduct similar studies on representative members of each of the three metallo-β-lactamase subfamilies and to include all clinically relevant carbapenems in those studies.

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