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Clonal Expansion of *Acinetobacter baumannii* Strains Displaying Elevated Tigecycline MIC Values Responsible for Increasing Resistance Rates in Latin America

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SENTRY ANTIMICROBIAL SURVEILLANCE

ABSTRACT

Objective: To evaluate the high prevalence of *Acinetobacter* spp. (ASP) displaying elevated tigecycline MICs (>2 mg/L) in Latin American (LATAM) hospitals surveyed by the SENTRY Program. We recently noted a significant difference in the percentage of ASP with tigecycline MICs >1 mg/L in LATAM compared to other geographic regions of the world (12.5% vs. 3.9-6.6%).

Methods: 1,950 ASP were received from LATAM during 2005-2011-period (9 hospitals from 2005 to 2010 and 20 in 2011). Isolates were susceptibility tested according to CLSI guidelines. *A. baumannii* (ACB) isolates from 2011 displaying tigecycline MIC values >2 mg/L were molecular typed by PFGE. Expression of *adeA* and *adeF* encoding the efflux pumps AdeABC and AdeFGH was determined for 18 unique isolates from 2011 using high quality DNA-free RNA preparations and measured by quantitative RT-PCR, normalized using *rpoB* and compared to ACB ATCC 19606.

Results: ASP displaying tigecycline MIC values >1 mg/L varied from 6.9 to 32.2% in the study period and showed an increase from 14.6% in 2010 to 32.2% in 2011 ($p< 0.0001$; OR=0.16[0.09-0.28]). Isolates with confirmed tigecycline MIC values >2 mg/L were 49 *A. baumannii* and 1 *A. pittii* (formerly genomic species 3; by MALDI-TOF). Isolates were mainly from Sao Paulo, Brazil (SP; 29 isolates) and Guadalajara, Mexico (14), but also from Durango, Mexico (3), Florianopolis, Brazil (1), Panama City, Panama and Santiago, Chile (1). PFGE showed that 15/29 isolates from SP belonged to a single clone. Three other clusters were noted in the same hospital (5, 3 and 2 isolates). Ten strains from Guadalajara belonged to a major clone and the remaining 4 strains belonged to two other PFGE types. All three strains from Durango were genetically related. Expression results of AdeABC and AdeFHG tested for 18 strains with MIC values ranging to 4 to 8 mg/L showed that only two isolates had significantly greater expression of AdeFGH (>10-fold difference from the control ATCC strain) both from clonal groups from SP and displaying tigecycline MIC values of 4 mg/L. All strains had AdeABC expression similar to the control strain.

Conclusions: We documented the recent increase of ASP displaying elevated tigecycline resistance in LATAM hospitals, dominantly due to the clonal expansion of isolates in Brazil and Mexico. Control of tigecycline usage in those countries and more strict infection control practices in the involved centres will be needed to contain these ACB outbreaks.

INTRODUCTION

Acinetobacter baumannii is a nosocomial pathogen that can cause various types of opportunistic infections in patients suffering of underlying conditions that have been hospitalized for extended periods. These organisms that are commonly isolated worldwide have the ability to acquire resistance to several antimicrobial agents and *A. baumannii* populations display high resistance rates to virtually all antimicrobial agents that are clinically available. Mechanisms that lead to multidrug resistance are particularly important for this species; and it may occur due to horizontal acquisition of genetic elements carrying several resistance genes or overexpression of chromosomally encoded efflux systems that can lead to the extrusion of compounds from several antimicrobial classes.

Tigecycline resistance in *A. baumannii* has been related to the overexpression of two resistance-nodulation-cell division (RND) multidrug transporters, AdeABC and AdeFGH. AdeABC overexpression contributes to resistance to various antimicrobial classes, including ß-lactams, aminoglycosides, quinolones and tigecycline. Additionally, this efflux system is regulated by *adeRS* operon that is a two-component regulation system and mutations on the components *adeS* and *adeR* have also been associated with tigecycline resistance in single-step mutants. AdeFGH overexpression confers high-level resistance to quinolones, chloramphenicol, trimethoprim and clindamycin as well as decreased susceptibility to tetracycline, tigecycline and sulfamethoxazole without affecting ß-lactams and aminoglycosides.

In this study, we evaluated a total of 1,950 *A. baumannii* isolates collected in Latin American hospitals as part of the SENTRY Antimicrobial Surveillance Program to document an increase in tigecycline resistance in the most recent years. Isolates from 2011 were evaluated for clonality and 18 unique strains were submitted to further assays to determine the expression of AdeABC and AdeFGH.

MATERIALS AND METHODS

Bacterial isolates. A total of 1,950 *Acinetobacter* spp. isolates collected in nine (2005-2010) to twenty (2011) Latin American hospitals were analysed. Only one isolate per patient from documented bloodstream infections were included in the study. Species identification was confirmed for all isolates displaying tigecycline MIC values >2 mg/L from 2011 by Matrix-Associated Laser Desorption Ionization-Time Of Flight mass spectrometry (MALDI-TOF MS) using the Bruker Daltonik MALDI Biotyper (Billerica, Massachusetts, USA) by following the instructions of the manufacturer.

Antimicrobial susceptibility testing. All isolates were susceptibility tested using the broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI, M07-A9) with freshly made Mueller-Hinton broth. Quality control (QC) was performed using *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. All QC results were within specified ranges as published in CLSI documents (M100-S23).

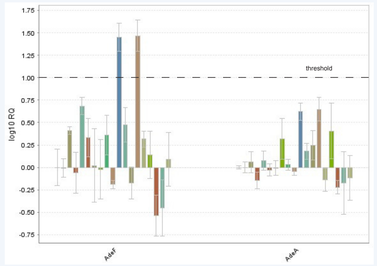
Molecular typing. *A. baumannii* isolates showing tigecycline MIC values at >2 mg/L, collected in 2011 (n=49) were epidemiologically typed by pulsed-field gel electrophoresis (PFGE) using previously described procedures. Genomic DNA was prepared in agarose blocks and digested with SmaI (New England, Beverly, Massachusetts, USA) and resolved in the CHEF-DR III (BioRad, Richmond, California, USA) using running conditions described elsewhere. Results were analyzed by GelCompar II software (Applied Math, Kortrijk, Belgium). Percent similarities were identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.2% and 0.5%, respectively.

Expression of RND systems. The expression of *adeA* and *adeF* was determined by quantitative real-time PCR (qRT-PCR) using DNA-free RNA preparations for 18 tigecycline-non-susceptible *A. baumannii* unique isolates from 2011. Total RNA was extracted from mid-log-phase bacterial cultures (cell density at OD_{600} of 0.3-0.5) using RNA Protect Reagent and RNeasy Mini Kit (Qiagen, Hilden, Germany) in the Qiacube workstation (Qiagen) and residual DNA was eliminated with RNase-free DNase (Promega, Madison, Wisconsin, USA). Quantification of mRNA and sample quality was assessed using the RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) according to manufacturer instructions. Only preparations with RNA integrity number (RIN) >6.5 that showed no visual degradation were used for experiments. Relative quantification of target genes was performed in triplicate by normalization to an endogenous reference gene (*rpoB*) on the StepOne Plus instrument (Life Technologies, Carlsbad, California, USA) using *Power* SYBR® Green RNA-to-CT™ (Life Technologies) and custom designed primers showing efficient >98.0%. Transcription levels were considered significantly different if at least a 5-fold difference was noted compared with. *A. baumannii* ATCC 19606.

RESULTS

- A total of 244 (12.5%) of the *Acinetobacter* spp. isolates from Latin America hospitals displayed tigecycline MIC values of >1 mg/L. Tigecycline non-wildtype (WT) population MIC values (>1 mg/L) varied from 6.9 to 32.2% with and increasing trend in more recent year: 32.2% in 2011 and 14.6% in 2010 ($p< 0.0001$; OR=0.16[0.09-0.28]); see EUCAST ECOFF tables <http://mic.eucast.org/Eucast2/SearchController/search.jsp?action=performSearch&BeginIndex=0&Micdif=mic&NumberIndex=50&Antib=345&Specium=-1>.

Figure 1. Relative quantification of transcriptional levels for *adeA and adeF* genes compared to those obtained from *A. baumannii* ATCC 19606.



- Confirmed tigecycline MIC values at >2 mg/L were observed among 49 *A. baumannii* and 1 *A. pittii* (formerly genomic species 3). These isolates were collected from: Sao Paulo, Brazil (29 isolates), Guadalajara, Mexico (14), Durango, Mexico (3), Florianopolis, Brazil (1), Panama City, Panama (1) and Santiago, Chile (1).
- PFGE showed that 15/29 isolates from Sao Paulo belonged to a single clone. Three other clusters were noted in the same hospital (5, 3 and 2 isolates). Ten strains from Guadalajara belonged to a major clone and the remaining four strains belonged to two other PFGE types. All three strains from Durango were genetically related. Single isolates from other hospitals had unique patterns.
- At least one unique isolate from each hospital was selected for AdeABC and AdeFHG expression experiments. Genetic related strains showing differences in the susceptibility profile were also selected for study. Tigecycline MIC values ranged from 4 to 8 mg/L and the isolates were all resistant to tetracycline (MIC, >8 mg/L), but doxycycline and minocycline MIC results showed generally lower values (**Table 1**).
- Two isolates had significantly greater expression of AdeFGH (>10-fold difference compared to the control ATCC strain), both from clonal groups in Sao Paulo, and displaying tigecycline MIC values of 4 mg/L (**Table 1**). All strains had AdeABC expression considered similar to the baseline ATCC strain.

Table 1. Susceptibility testing results, PFGE patterns and efflux pump expression results for 18 *A. baumannii* isolates displaying elevated tigecycline MIC values (≥2 mg/L) selected for follow up studies.

		MIC in mg/L:									Relative Expression of efflux pumps[a]	
Country	City	Tigecycline	Doxycycline	Tetracycline	Minocycline	Ceftazidime	Imipenem	Levofloxacin	Colistin	PFGE	AdeABC	AdeFGH
Brazil	Florianopolis	8	>8	>8	2	>32	>8	>4	1	046B	0.600	0.290
	Sao Paulo	4	2	>8	2	>32	>8	>4	1	048A1	0.986	1.049
		8	4	>8	2	>32	>8	>4	1	048B	0.996	0.982
		8	4	>8	4	>32	>8	>4	>4	048B	1.084	2.293
		4	2	>8	2	32	>8	>4	1	048C	0.675	0.352
		4	4	>8	2	32	>8	>4	1	048D	0.935	2.136
		4	1	>8	1	>32	>8	>4	2	048E1	4.238	**28.237**[b]
		4	1	>8	0.5	>32	>8	>4	0.5	048F	4.510	**29.464**[b]
		4	1	>8	1	>32	>8	>4	1	048G	1.527	2.988
		8	2	>8	1	>32	>8	>4	2	048I	2.621	1.373
Chile	Santiago	4	4	>8	2	>32	>8	>4	>4	043A	1.760	0.673
Mexico	Durango	4	>8	>8	4	32	2	>4	0.5	126A1	0.770	1.228
		4	>8	>8	>8	>32	0.5	>4	2	126A2	0.735	0.865
	Guadalajara	4	4	>8	2	>32	>8	>4	1	115A1	1.233	4.819
		4	2	>8	2	>32	>8	>4	0.5	115B1	0.729	2.065
		4	>8	>8	>8	>32	>8	>4	0.5	115B1	0.940	0.647
		4	1	>8	1	>32	>8	>4	0.5	115C	1.148	2.576
Panama	Panama City	4	>8	>8	8	>32	>8	>4	2	346A	2.127	0.951

a. Relative expression level that corresponds to the critical threshold (C_T) numbers determined by the detection system software as the amount of target was given as $2^{-\Delta\Delta T}$, where $\Delta\Delta T$ is the difference between the target (*adeA* and *adeF*) and reference gene (*rpoB*) C_T values and was determined in comparison to the *A. baumannii* ATCC 19606 (relative expression = 1.000).
b. Values in bold were considered significant upregulation of the gene expression (>10-fold difference from the control ATCC strain).

CONCLUSIONS

- Increased tigecycline resistance rates were secondary to the dissemination of clonal strains in two hospitals that participate in the surveillance program over all years sampled. Two isolates were recovered in hospitals only surveyed in 2011.
- We did not detect strains with elevated expression of AdeABC and only two sampled organisms had upregulation of AdeFGH. Additional studies are being carried out in an attempt to further explain the elevated tigecycline MIC values resulting from these Latin American isolates.
- More stringent infection control and antimicrobial stewardship practices appear needed to control increasing tigecycline resistance rates in these Latin American hospitals, where tigecycline and colistin might be prescribed for multidrug-resistant *Acinetobacter* infections.

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