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Genetic and virulence characterization of colistin-resistant and colistin-sensitive *A. baumannii* clinical isolates

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ABSTRACT

Treatment of infections caused by *A. baumannii* is becoming a challenge due to the ability to develop multidrug-resistance, virulence, and high mortality. We described the colistin resistance and virulence genes present in six*A. baumannii* clinical isolates using WGS, expression by qPCR, and virulence in the *Galleria mellonella* model. The colistin-resistant isolates were assigned as ST233 and the colistin-susceptible isolates as ST236 and ST407. The colistin-resistant isolates contained mutations within PmrA/PmrB, and the *pmr*A showed up-regulation in all of them. Only one colistin-resistant isolate indicating virulence in *G. mellonella*. This particular isolate belonged to a different clone, and it was the only isolate that presented non-synonymous mutations in *pmr*B. Colistinresistance in *A. baumannii* isolates seems to be caused by up-regulation of *pmrA* gene. Only one isolate appeared to be virulent in the *G. mellonella* model. This finding indicating low virulence in isolates belonging to emerging clones circulating in our hospital.

Colistin is the last resort for treatment of infections caused by carbapenem-resistant *Acinetobacter baumannii* (Biswas et al. 2012; Dias et al. 2016). To date, the expression of several *A. baumannii* virulence genes has been linked to the persistence and survival of *A. baumannii* within the host (Zarrilli 2016). Moreover, the role of colistin resistance on *Acinetobacter* virulence remains controversial (Wand et al. 2015). *Galleria* is a non-vertebrate model that has been used as an alternative to traditional models of infection (López-Rojas et al. 2011). Therefore, we evaluated the virulence of *A. baumannii* isolates comparing colistin-resistant and -susceptible isolates in the *Galleria mellonella* model. In addition, we identified resistance and virulence genes present in MDR *A. baumannii* isolates, in order to observe the relationship between virulence and resistance.

Six *A. baumannii* clinical isolates were selected from the Laboratory of Bacteriology bacterial collection of the Department of Infectious Diseases, University of São Paulo, Brazil. They were chosen based on colistin susceptibility according to Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2013), carbapenems resistance mechanism, year of identification, sequence type (ST), and pulsed-field gel electrophoresis (PFGE) from a previous study (Supplemental Material – Table S1) (Leite et al. 2016). The whole genome sequencing (WGS) was performed by Ion Torrent Personal Genome Technology MachineTM (PGM) system using a 318 chip (Life Technologies, Foster City, CA). SNPs were identified using Samtools (Li et al. 2009) with *A. baumannii* str. AYE (CU459141.1). ResFinder 2.0, BLAST, and MAFFT (Katoh and Standley 2013) were used to identify antibiotic resistance gene. Colistin-resistant isolates were compared to the ColR28 isolated due to different behavior in the *Galleria* model. Gene expression studies were carried out on a StepOne® Real Time (Applied Biosystems, USA) using Quantifast® SYBR®Green RT-PCR (Qiagen, CA, USA) as directed by the manufacturer. Bacterial RNA was purified using the RNeasy mini kit (Qiagen, West Sussex, United Kingdom). The 16S rRNA gene was used as a reference endogenous gene for gene expression normalization and *A. baumannii* ATCC 19606 as reference isolates. Colistin-resistant isolates were cultured with and without colistin (4 mg/L). Negative controls without reverse transcriptase were included to detect DNA contamination. The level of expression was considering up-regulation >0.5, according to relative quantification using 16S for $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001; Pfaffl 2004). The *G. mellonella* larvae assay was performed to evaluated *A. baumannii* virulence as described by

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Table 1.
Virulence genes and protein function determined by WGS among the six multidrug-resistant *A. baumannii* isolates.

Isolates	Year	Clusters	ST/CC	Biofilm maturation	Acinetobactin biosynthesis and transport Iron acquisition system	Phospholipase D	Outer membrane porin	Adherence Immune system evasion
ColS3	2003	C	236/103	*Bap*	*bau*DCEBA*bas*CD, *pbp*G, *ton*B	*plc*, *plcD*	*omp*A, *omp*33–36	*lps*B, *lpx*A, *lpx*B, *lpx*C, *lpx*D, *lpx*L, *lpx*M
ColS6	2002	C	236/103	*Bap*	*bau*DCEBA*bas*CD, *pbp*G, *ton*B	*plc*, *plcD*	*omp*A, *omp*33–36	*lps*B, *lpx*A, *lpx*B, *lpx*C, *lpx*D, *lpx*L, *lpx*M
ColS18	2004	D	407/st	NI	*bau*DCEBA*bas*CD, *pbp*G, *ton*B	*plc*, *plcD*	*omp*A, *omp*33–36	*lps*B, *lpx*A, *lpx*B, *lpx*C, *lpx*D, *lpx*L, *lpx*M
ColR23	2012	A1	233/113	*Bap*	*bau*DCEBA*bas*CD, *pbp*G, *ton*B	*plc*, *plcD*	*omp*A, *omp*33–36	*lps*B, *lpx*A, *lpx*B, *lpx*C, *lpx*D, *lpx*L, *lpx*M
ColR25	2011	A	233/113	*Bap*	*bau*DCEBA*bas*CD, *pbp*G, *ton*B	*plc*, *plcD*	*omp*A, *omp*33–36	*lps*B, *lpx*A, *lpx*B, *lpx*C, *lpx*D, *lpx*L, *lpx*M
ColR28	2011	B	233/113	NI	*bau*DCEBA*bas*CD, *pbp*G, *ton*B	*plc*, *plcD*	*omp*A, *omp*33–36	*lps*B, *lpx*A, *lpx*B, *lpx*C, *lpx*D, *lpx*L, *lpx*M

Abbreviations: MIC: minimum inhibitory concentration; ST - sequence type; CC - clonal complex; st - singleton.

(Kavanagh and Fallon (2010). All experiments were performed in triplicate.

The genetic and virulence characteristics of six *A. baumannii* isolates defined as MDR (Leite et al. 2016) and assigned as ST236, ST407, and ST233 are shown in Table 1.

Biofilm has been described as a virulence factor in *A. baumannii* and several authors suggested that the development of biofilm structure and intercellular adhesion were closely associated with Bap family proteins (Goh et al. 2013; Loehfelm et al. 2008). In our study, the quorum-sensing gene *abaI* was not found in ColR isolates and ColR28 isolate virulent in the *G. mellonella* model and ColS18 lost *bap* gene. Virulence regulator gene GacS showed one amino acid substitution (E510D) among ColR isolates, and in ColS isolates, the substitution was not observed. In *A. baumannii*, the pili involved in biofilm formation is produced by the *csuA/BABCDE* usher-chaperone assembly system, which is regulated by the two-component system *bfmRS* (Tomaras et al. 2008). In the present study, the regulator BfmS showed a single mutation (N453S) in six isolates, however, the substitution was not observed in the BfmR.

We observed that the expression of *pmr*A in colistin-resistant isolates occurred irrespective of the presence of colistin (Fig. 1A), indicating that the elevated expression of the gene appears to be constitutive and not induced by the presence of colistin (Park et al. 2011). The ColR23- and ColR25-resistant isolates showed mutations in the PmrA (D82G, S119T) and in the PmrB (G21V, V227A). whereas the ColR28-resistant isolate showed only a single mutation in the PmrB (P170L). It is consistent with increased *pmrA* expression described by Adams et al. (2009) and Arroyo et al. (2011). In addition, upregulation of *pmrB* was identified in ColR23 and upregulation of *pmrC* in ColR23 and ColR28 (Figure 1B). Both ColS3 and ColS6 showed the same mutations in the PmrC (V51A; V111G; R137P; S149V; Y341L; Q403P). Nonsynonymous mutations were not observed in the *lpx*ACD. Quantitative-PCR analysis demonstrated no overexpression with the exception of ColS3 *lpx*A (0.61), ColR23 *lpx*A (0.74), and ColR23 *lpxC* (0.73).

Low rates of mortality were found in the *G. mellonella* model in our casuistic (Supplemental Material Fig. 2). Only ColR28 demonstrated limited virulence, killing 50% of larvae 72 h postinfection. Although the 3 colistin-resistant isolates were assigned as ST233, only the ColR28 showed a single nonsynonymous mutation in *pmr*B and belonged to a different clone. Wand et al. (2015) observed that isolates carrying *pmr*B gene mutation retained virulence levels in the *Galleria* model. To investigate the genetic changes behind this ColR28, it was compared to the other 2 ColR isolates (ColR23 and ColR25). The ColR23 and ColR25 isolates showed synonymous mutations in *csu*D and *pga*C genes comparing to ColR28. A recent study showed that the potential to adapt to colistin and virulence differs between mutants of clonally distinct isolates and that mutations in *pmrB* do not always lead to loss of virulence; however, mutations in *lpxACD* show loss of virulence and increased susceptibility to several antibiotics (Wand et al. 2015). Nonsynonymous mutations were not observed for the 6 isolates in the *lpx*ACD.

An important limitation of our casuistic is that, apparently, the colistin-susceptible isolates studied were not the ideal isolates to conduct the *in vivo* model. Thus, further studies need to be conducted to confirm our findings.

The colistin resistance in our *A. baumannii* isolates assigned as ST233 seems to be caused by upregulation of *pmrA* gene. The expression of the gene was probably constitutive and not induced by the presence of colistin. Although *A. baumannii* isolates contained several virulence genes, only 1 colistin-resistant isolate appeared to be virulent in the *G. mellonella* model. This finding indicates low virulence in isolates belonging to emerging clones circulating in our hospital.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2019.04.013.

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by the Research Ethics Committee of Faculty of Medicine–University of Sao Paulo (FM-USP) according to reference number 9877 on 09/09/2012. All data were analyzed anonymously and confidentially, with approval by the Research Ethics Committee. It was a retrospective study; thus, it was not possible to apply the consent to participate.

Consent to publish

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the repository "Teses e Dissertações" from Biblioteca Digital USP, http://www.teses.usp.br/teses/disponiveis/5/5134/tde-09032017-113321/pt-br.php.

Competing interests

The authors declare that there is no conflict of interest.

Authors' contributions

GCL, SFC, and ASL conceived and designed the experiments. GCL, PN, and CR performed the experiments. GCL, RAS, RCRM, and SFC analyzed the data. GCL, RAS, PN, LVPN, RCRM, CR, FR, ASL, and SFC contributed reagents/materials/analysis tools. GCL, RAS, and SFC wrote the paper. RAS, CR, and RCRM provided technical support.

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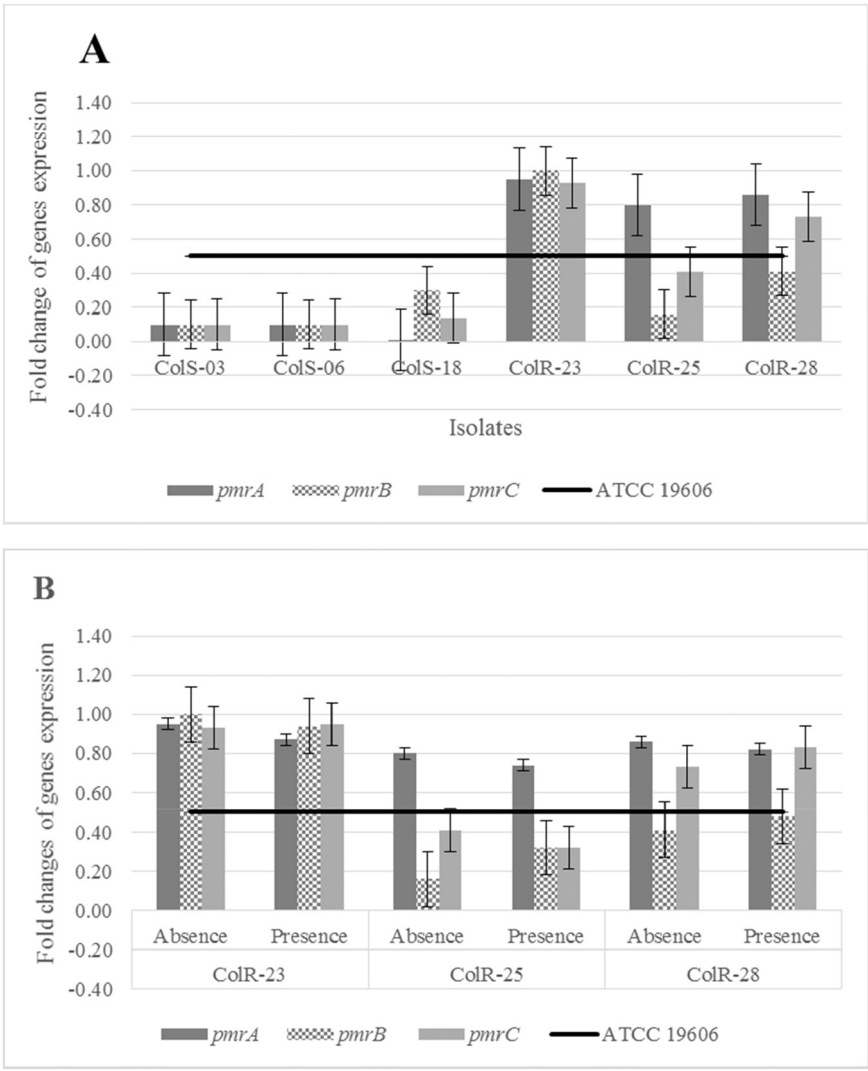

Fig. 1. Expression of *pmrCAB* operon for all *A. baumannii* isolates. (A) Expression observed in the absence of colistin for all isolates. (B) Expression observed in the presence of colistin (4 mg/L) for colistin-resistant isolates. ATCC 19606–standard isolate, basal expression 0.5 according to relative quantification using 16S for $2^{-\Delta\Delta Ct}$.

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