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Characterization of carbapenem-resistant *Acinetobacter baumannii* in Brazil (2008–2011): countrywide spread of OXA-23–producing clones (CC15 and CC79) ☆

Thiago Pavoni Gomes Chagas [1], Karyne Rangel Carvalho [1], Ivson Cassiano de Oliveira Santos, Ana Paula D'Alincourt Carvalho-Assef, Marise Dutra Asensi *

Laboratório de Pesquisa em Infecção Hospitalar (LAPIH), Instituto Oswaldo Cruz/FIOCRUZ, Av. Brasil 4365, Manguinhos, Rio de Janeiro, Brazil

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ABSTRACT

The study investigated the genetic relationship of carbapenem-resistant *Acinetobacter baumannii* clinical isolated from inpatients during 2008–2011 from 11 Brazilian states. Antimicrobial susceptibility profile was determined by disc diffusion method and Etest. Polymerase chain reaction was applied for carbapenemase genes, and IS*Aba1*. Isolates were subjected to pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) for molecular typing. Most of the isolates showed high resistance rates to antibiotics tested. The $bla_{\text{OXA-51-like}}$ gene was found in all isolates, and 146 (94.2%) isolates were positive for $bla_{\text{OXA-23-like}}$. In the most OXA-23–producing isolates, the $bla_{\text{OXA-23-like}}$ gene was accompanied by IS*Aba1*. A total of 146 OXA-23–producing isolates were clustered into 28 genotypes by PFGE. Molecular analysis by MLST identified 13 sequence types (STs). The most prevalent PFGE profiles were designated as ST15 (CC15), ST1 (CC1), and ST79 (CC79). This study showed the widespread of clonal complexes of *A. baumannii* harboring the $bla_{\text{OXA-23-like}}$ gene in different Brazilian states.

1. Introduction

Acinetobacter baumannii is an opportunistic pathogen increasingly affecting severely ill patients. This microorganism is highly capable of surviving and spreading in the hospital environment and to develop resistance to antimicrobial agents. In the last years, carbapenem resistance has been reported among *A. baumannii* isolates and is often associated to infections with high morbidity and mortality rates. Carbapenem-hydrolysing class D β-lactamases of the oxacilinases are the most important cause of carbapenem resistance in *A. baumannii* worldwide (Poirel and Nordmann, 2005, Poirel and Nordmann, 2006 and Zarrilli et al., 2013).

In Brazil, *A. baumannii* has become particularly problematic because of its prevalence and the carbapenems resistance, usually related to oxacilinases, mostly involving OXA-23 producers, with reports of local outbreaks since the late 90s (Carvalho et al., 2009; Dalla-Costa et al., 2003). The first report of *A. baumannii* resistant to carbapenems was in 2003 with the description of 8 isolates collected from hospitalized patients hospitalized at 2 hospitals in the Southern Brazil (Curitiba – Paraná State). These isolates were OXA-23 producers and were part of the same clone, demonstrating the occurrence of inter-hospital spread in that city (Dalla-Costa et al., 2003). In 2009, we described the spread of multidrug resistance (MDR) OXA-23–producing *A. baumannii* clones in the city of Rio de Janeiro: 2 clones were prevalent, one being scattered at 7 hospitals and the second one in other 5 hospitals (Carvalho et al., 2009). In the following years, this microorganism has been identified in other Brazilian states.

Molecular epidemiological studies are important tools to clarify the dissemination of MDR *A. baumannii*, to understand epidemic dynamics and to identify the most efficient control measures. Thus, this study aimed to characterize carbapenem-resistant *A. baumannii* isolates recovered in 11 Brazilian states over a 3-year period (2008–2011), determining their genetic relationship by multilocus sequence typing (MLST) analysis.

2. Materials and methods

2.1. Bacterial isolates

A total of 155 *A. baumannii* clinical isolates representing 11 Brazilian states belonging to 5 different geographical regions of this country were included. They were collected at the Universidade Federal do Mato Grosso do Sul (UFMS)/Mato Grosso do Sul (MS) (n = 9) and Central Public Health Laboratories (LACENs) of Alagoas (AL) (n = 2), Amazonas (AM) (n = 1), Bahia (BA) (n = 1), Distrito

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* Corresponding author. Tel.: +55-2125621636; fax: +55-2125621634.
E-mail address: marise@ioc.fiocruz.br (M.D. Asensi).

[1] Authors contributed equally to this work.

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Federal (DF) (n = 2), Espírito Santo (ES) (n = 26), Goiás (GO) (n = 58), Minas Gerais (MG) (n = 8), Rio de Janeiro (RJ) (n = 44), Rio Grande do Norte (RN) (n = 3), and Santa Catarina (SC) (n = 1) from February 2008 to January 2011 (1 per patient). The most frequent sites of isolation for these isolates were blood (18.7%), respiratory tract secretions (18.0 %), catheter (13.5%), and urine (3.9%).

2.2. Identification and antimicrobial susceptibility testing

A. baumannii isolates were identified by classical biochemical techniques, amplification of the *rpoB* (Gundi et al., 2009) and $bla_{\text{OXA-51-like}}$ gene, and by amplified ribosomal DNA restriction analysis (Vaneechoutte et al., 1995). The disc diffusion method was used to evaluate susceptibility to the antimicrobial agents according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). MIC values for imipenem, meropenem, polymyxin B, and tigecycline were determined by Etest (AB Biodisk, Solna, Sweden) (CLSI, 2012).

2.3. Molecular investigations

A multiplex polymerase chain reaction (PCR) was used to detect genes encoding the OXA-like carbapenemases OXA-23, OXA-24, OXA-51, OXA-58, and OXA-143 (Higgins et al., 2010). Additional screening of other carbapenemase encoding genes (bla_{KPC}, bla_{NDM}) and insertion sequence IS*Aba1* was performed by PCR (Naas et al., 2008, Nordmann et al., 2011; Turton et al., 2006). To determine whether IS*Aba1* was present upstream of $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-23-like}}$ gene, PCR mapping experiments using IS*Aba1* forward/OXA-51–like reverse or OXA-23–like reverse primers (IS*Aba1*F/OXA-51-likeR or OXA-23-likeR PCR) were performed (Pagano et al., 2013; Chaulagain et al., 2012).

Pulsed field gel electrophoresis (PFGE) typing was performed using *Apa*I restriction enzyme (Carvalho et al., 2009). DNA fragments were separated on 1% (w/v) agarose gels in 0.5% Tris-borate-EDTA buffer using a CHEF DRIII apparatus (Bio-Rad, Hercules, CA, USA). The patterns obtained were analysed by BioNumerics v.4.0 (Applied Maths, Sint-Martens-Latem, Belgium) with Dice similarity coefficient analysis. The unweighted-pair group method using average linkages was applied, and the bandwidth tolerance was set at 1.5%. Isolates clustering together with 85% level of similarity were considered to belong to the same genotype.

Isolates representing OXA-23–producing *A. baumannii* PFGE clones were further tested by the MLST scheme developed by the Institute Pasteur (www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html) (Diancourt et al., 2010). DNA sequencing was performed with Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed using an ABI Prism 3100 genetic analyser (Applied Biosystems) at the PDTIS-IOC DNA Sequencing Platform. To determine the CCs (complex clonal), eBURST software (http://eburst.mlst.net/) was used.

3. Results

3.1. Resistance patterns and screening of carbapenemase encoding genes and insertion sequence ISAba1

According to the results of the antimicrobial susceptibility testing, all antimicrobials tested showed elevated resistance rate, and the most of the isolates were resistant to more than 5 different classes of antibiotics. Ciprofloxacin, cefepime, and piperacillin/tazobactam showed the highest resistance rate (99.4%, n = 154), followed by ceftazidime (97.4%, n = 151), imipenem (95.5%, n = 148), meropenem (94.2%, n = 146), and ampicillin/sulbactam (93.5%, n = 145). Sulfamethoxazole/trimethoprim showed the highest susceptibility rate (23.9%, n = 37) followed by amikacin (11.6%, n = 18).

All isolates showed the presence of $bla_{\text{OXA-51-like}}$ gene, originally intrinsic to *A. baumannii*, and none were positive for $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-58-like}}$, and $bla_{\text{OXA-143-like}}$. The presence of $bla_{\text{OXA-23-like}}$ gene was confirmed in most carbapenem-resistant isolates (n = 146; 94.2%), and the IS*Aba1* element was detected upstream of the $bla_{\text{OXA-23}}$ gene in almost all OXA-23–producing isolates. By additional screening, genes encoding for KPC and NDM were not found.

By MIC results, all OXA-23–producing isolates were highly resistant to carbapenem (MIC >32 μg/mL) except for 1 isolate that showed an MIC of 2.0 μg/mL for meropenem. For polymyxin B, MIC range was 0.19–0.50 μg/mL, and MIC_{50}/MIC_{90} values were of 0.38/0.50 μg/mL, respectively. Considering tigecycline, MIC range was 0.19–6 μg/mL, and MIC_{50}/MIC_{90} values were of 3/6 μg/mL, respectively. Among the 9 isolates non-OXA-23-producing, 5 were resistant to imipenem and/or meropenem, and the IS*Aba1* element was found upstream of the $bla_{\text{OXA-51}}$ gene by PCR mapping.

3.2. Molecular typing

Based on PFGE analysis, the 146 OXA-23–producing isolates were clustered into 28 genotypes (Table 1). The most prevalent was Ab1 (37.4% of isolates; n = 58) found in 8 states belonging to different geographical regions (DF, ES, GO, MG, RJ, RN, SC, and MS), followed by Ab2 (18.7%; n = 29) found in GO and MS and genotype Ab3 (9.7%; n = 15) found in 5 states (DF, ES, MG, RJ, and RN).

MLST performed on 44 isolates representing PFGE OXA-23–producing clones, including isolates from each state, allowed us to identify 13 sequence types (STs) as shown in the Table 1. Four new STs were first described in our study and were deposited in the MLST database (ST316, ST317, ST318, and ST319). The most prevalent PFGE profiles Ab1, Ab2, and Ab3 were designated ST15, ST1, and ST79, respectively. The ST1 was found in Midwest (GO, MS), Northeast (RN), and Southeast (MG). In RJ, we also identified ST160 (genotype Ab21), ST162, (genotype Ab8 and Ab9), ST188 (genotype Ab6), and ST316 (genotype Ab20). In the North (AM), Midwest (MS), and Southeast (MG, RJ), we also observed the ST25 (genotype Ab27, Ab28, and Ab29).

By eBURST, we found 6 major complexes (CC1, CC15, CC25, CC33, CC79, and CC162), which included mainly single-locus variants or, less commonly, double-locus variants (Fig. 1). Some STs described in this study were singletons (ST107 and ST319). Phylogenetic analysis showed that ST79 and ST15 belong to the CC79 and CC15, respectively. The CC15 (ST15 and ST318) was described in 9 of 10 Brazilian states studied (AL, DF, ES, GO, MG, RJ, RN, SC, and MS) belonging to the 4 different geographical regions of the country. The OXA-23–producing isolates belonging CC79 (ST79) were found in 8 of 10 states (AL, DF, ES, GO, MS, MG, RJ, and RN). The ST1 and ST160 were grouped in CC1 observed in GO, MG, MS, RJ, and RN. Other CCs were found into our isolates (CC25, CC33, and CC162) as observed in Fig. 1, and the distribution of them may be observed in Fig. 2.

4. Discussion

This study described the resistance profiles and genetic relatedness of CRAB isolates collected in different Brazilian hospitals during 3 years beginning in February 2008. In recent years, *A. baumannii* has been commonly reported as resistant to multiple drugs, and the resistance rates to imipenem, meropenem, ceftazidime, piperacillin/tazobactam, ciprofloxacin, and gentamicin in Latin America seem to be among the world's largest (Peleg et al., 2008). Antimicrobial Surveillance Program involving Latin America, imipenem-resistant *Acinetobacter* spp. rates increased from 6.4%, 12.6%, and 0.0% in the 1997–1999 to 84.9%, 71.4%, and 50.0% in 2008–2010, respectively, in Argentina, Brazil, and Chile (Gales et al., 2012). *A. baumannii* MDR has also been reported in some countries in South America, such as Brazil, Argentina, and Colombia (Fiorilli et al., 2010; Villegas et al., 2007). All the *A. baumannii* clinical isolates included in this study were MDR,

Table 1
Molecular typing analysis and detection of IS*Aba1* of OXA-23–producing *A. baumannii* from different states in Brazil.

Genotype	Isolate	ST	Allelic profile	CC	States	Year	IS*Aba1*-upstream $bla_{\text{OXA-51-like}}$	IS*Aba1*-upstream $bla_{\text{OXA-23-like}}$
	CCBH4902				RJ	2009	+	+
	CCBH4971				GO	2009	−	−
	CCBH5233				ES	2009	−	+
Ab1	CCBH5471	15	6-6-8-2-3-5-4	CC15	MS	2010	−	+
	CCBH5881				MG	2010	+	+
	CCBH6006				DF	2010	+	+
	CCBH6890				RN	2011	+	+
	CCBH6959				SC	2011	−	+
Ab2	CCBH5343	1	1-1-1-1-5-1-1	CC1	GO	2009	−	+
	CCBH5468				MS	2010	−	−
	CCBH4808				RJ	2008	+	+
	CCBH5791				MG	2010	+	+
Ab3	CCBH6009	79	26-2-2-29-4-5	CC79	DF	2010	+	+
	CCBH6872				ES	2010	−	+
	CCBH6887				RN	2011	−	+
	CCBH4818				RJ	2008	+	+
Ab4	CCBH5231	79	26-2-2-29-4-5	CC79	ES	2009	−	+
	CCBH6554				AL	2010	−	+
Ab5	CCBH5164	107	34-35-37-1-5-6-36	–	ES	2009	−	−
Ab6	CCBH5405	188	1-3-6-1-3-4-4	–	RJ	2009	−	+
Ab7	CCBH5470	79	26-2-2-2-29-4-5	CC79	MS	2010	+	+
Ab8	CCBH4819	162	3-2-2-2-2-4-8	CC162	RJ	2008	−	+
Ab9	CCBH4900	162	3-2-2-2-2-4-8	CC162	RJ	2009	−	+
	CCBH5472				MS	2010	−	+
Ab10	CCBH5639	151	27-5-7-1-7-1-4	CC33	ES	2010	−	+
Ab11	CCBH5792	1	1-1-1-1-5-1-1	CC1	MG	2010	+	+
	CCBH6893				RN	2011	−	+
Ab12	CCBH5162	79	26-2-2-2-29-4-5	CC79	ES	2009	+	+
Ab14	CCBH5337	79	26-2-2-2-29-4-5	CC79	GO	2009	−	−
Ab15	CCBH5338	1	1-1-1-1-5-1-1	CC1	GO	2009	+	−
Ab16	CCBH5642	79	26-2-2-2-29-4-5	CC79	ES	2010	+	+
Ab17	CCBH5036	317	3-1-15-2-40-4-4	–	ES	2009	−	+
Ab19	CCBH5054	25	3-3-2-4-7-2-4	CC25	AM	2009	−	−
Ab20	CCBH4816	316	3-1-6-2-4-1-5	–	RJ	2008	+	+
Ab21	CCBH4800	160	1-1-1-1-5-1-41	CC1	RJ	2008	+	+
Ab22	CCBH5361	79	26-2-2-2-29-4-5	CC79	ES	2009	+	+
Ab24	CCBH5790	318	6-6-8-2-3-5-5	CC15	MG	2010	+	+
Ab25	CCBH4825	316	3-1-6-2-4-1-5	–	RJ	2008	−	+
Ab26	CCBH5258	319	3-1-2-2-7-1-5	–	MG	2009	−	+
Ab27	CCBH5255	25	3-3-2-4-7-2-4	CC25	MG	2009	+	+
Ab28	CCBH4797	25	3-3-2-4-7-2-4	CC25	RJ	2008	−	+
Ab29	CCBH5475	25	3-3-2-4-7-2-4	CC25	MS	2010	+	+
Ab32	CCBH5218	318	6-6-8-2-3-5-5	CC15	MG	2009	+	+
Ab33	CCBH6558	15	6-6-8-2-3-5-4	CC15	AL	2010	+	+

which constitute a therapeutic problem of serious concern and affect the clinical outcome of serious infections caused by such pathogens.

In this work, we noted that the carbapenem resistance mediated by the enzyme OXA-23 was found in most isolates from different Brazilian states, confirming several reports of outbreaks *A. baumannii* that have this mechanism of resistance (Carvalho et al., 2009). According to Clímaco et al (2013), the spread of OXA-23 is most likely due to mobile elements (i.e., plasmids, transposons, insertion sequence). In previous report, we showed that the $bla_{\text{OXA-23}}$ gene was consistently associated with transposon, Tn*2006*, and was chromosomally encoded in all carbapenem-resistant *A. baumannii* isolates studied (Grosso et al., 2011). Our results also indicated that some isolates carried the $bla_{\text{OXA-23-like}}$ gene with an IS*Aba1* upstream, and these findings are in accordance with the results of other studies. As previously demonstrated by European and Brazilian reports (Pagano et al., 2013), the degree of carbapenem resistance in *A. baumannii* isolates may be accentuated by the presence of promoter sequences provided by IS*Aba1*, leading to expression of the OXA-23.

Worldwide dissemination of the $bla_{\text{OXA-23}}$ carbapenemase gene has been associated with specific clones. The study revealed that the most prevalent PFGE profiles Ab1 (n = 58 isolates) and other genotypes (Ab1, Ab24, Ab32, and Ab33) belonged to CC15 (ST15 and ST318) from 9 Brazilian states. According Diancourt et al. (2010), *A. baumannii* belonging to ST15 have experienced evolutionary success and almost universally exhibit MDR phenotypes, which facilitated their rapid clonal expansion during recent years. This complex has been identified in several European countries, including Italy, Spain, Greece, and Turkey (Di Popolo et al., 2011, Gogou et al., 2011; Villalon et al., 2011).

In Latin America (Argentina and Brazil), OXA-23–producing *A. baumannii* strains have been commonly reported as belonging to CC79 (Grosso et al., 2011, Martins et al., 2013; Stietz et al., 2013). We characterized the CC79 in 8 Brazilian states representing the second major clonal complex in the country, followed by CC1. This CC1 is designated as the second major *A. baumannii* clone with a broad international distribution (Karah et al., 2012). We and other previous reports described the ST79 and ST15 in public and private hospitals in RJ (Southeast) (Grosso et al, 2011; Martins et al., 2013). In the current study, we showed that these STs were also disseminated along the country over the past 3 years.

Despite of non-OXA-23-producing, we found 5 carbapenem-resistant isolates, and these results may be explained by the presence of the *ISAba*1 element upstream the $bla_{\text{OXA-51}}$ gene. As reported by other studies, the reduced susceptibility to carbapenems in non–OXA-23 isolates may be mediated by gene $bla_{\text{OXA-51-like}}$, which is normally little expressed. However, the presence of the insertion sequence IS*Aba1* or IS*Aba9* is required for increased expression associated with other mechanisms such as reduced permeability of the outer

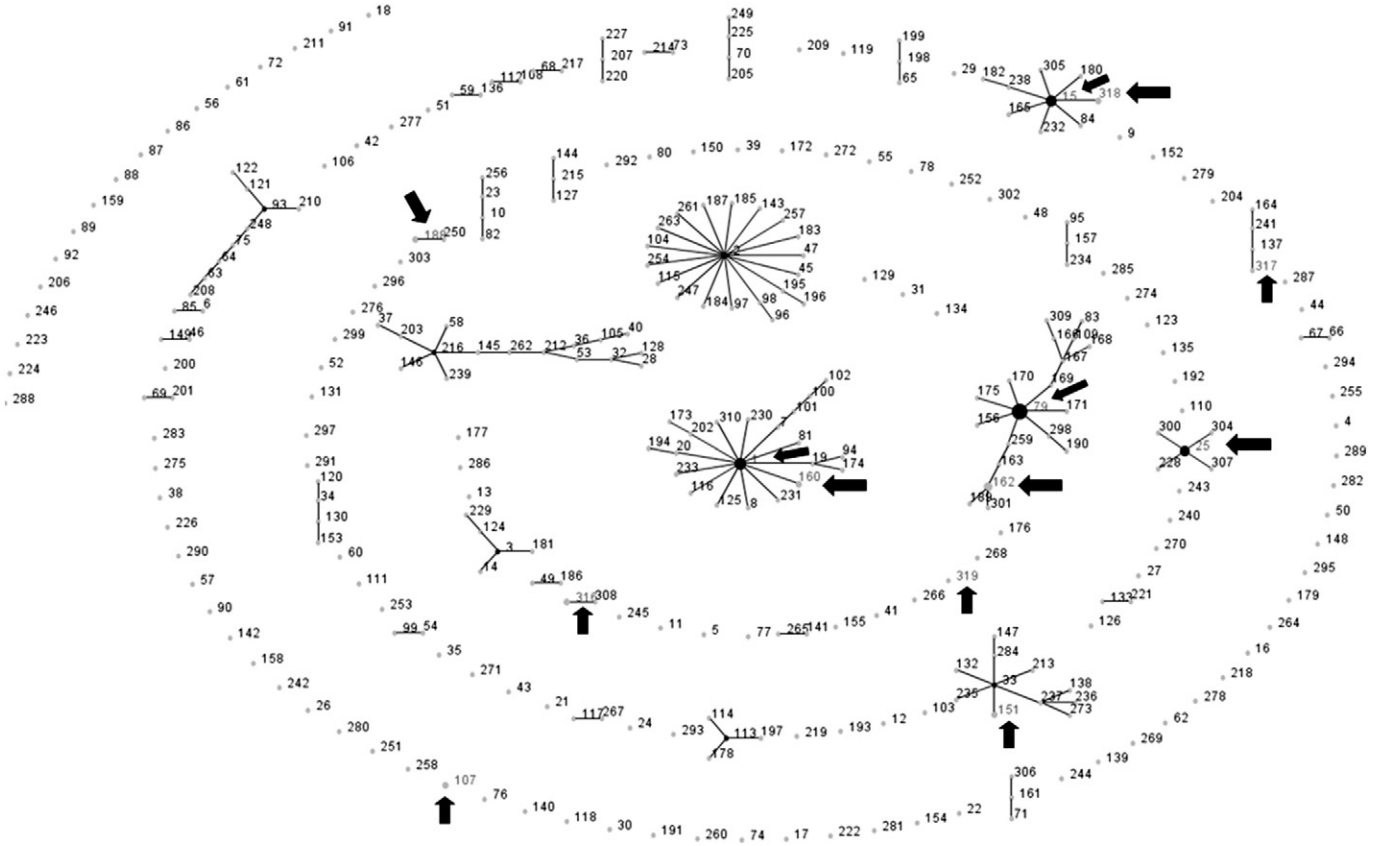

Fig. 1. Relatedness of OXA-23–producing *A. baumannii* STs by eBurst analysis. Each ST is represented by a dot in proportional size to the number of *A. baumannii* involved, considering all data in the Pasteur database. STs related in this study are indicated by arrows.

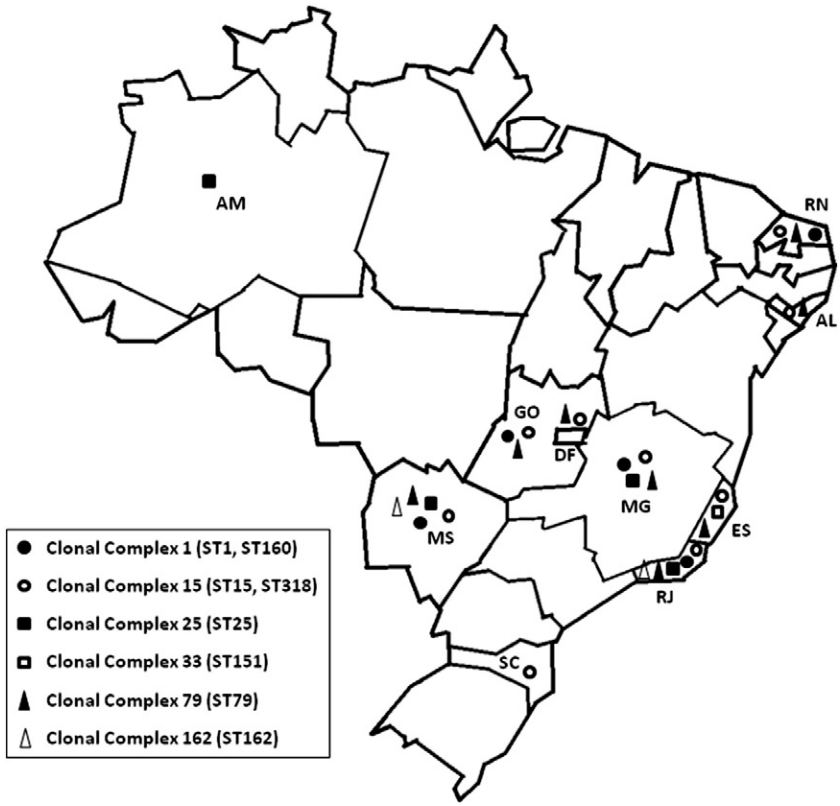

Fig. 2. Geographical distribution of CCs detected in different Brazilian states.

membrane or over expression of efflux pumps (Figueiredo and Poirel, 2009; Nordmann et al., 2011).

In conclusion, we report here the presence of MDR *A. baumannii* carrying the same carbapenem-resistance determinant (bla_{OXA-23} gene) with clonal country widespread dissemination of CC15 and CC79. These results confirmed the wide geographical distribution of OXA-23 among clinical carbapenem-resistant *A. baumannii* isolates in Brazil. The dissemination of these major clusters of MDR *A. baumannii* in different states harboring bla_{OXA-23} gene illustrated the success that this organism has to acquire carbapenem resistance and emphasizes the importance of having effective control measures. Until now, few reports have focused on *A. baumannii* in all geographical regions of Brazil, mainly descriptions of local outbreaks. This is the first study that attempt to determine the Brazilian countrywide spread of MDR *A. baumannii* harboring the bla_{OXA-23} gene in different regions of Brazil.

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