UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Running title: Rapid phenotypic tests for carbapenemases

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ABSTRACT

Carbapenems are considered the last resort for the treatment of multidrug-resistant *Enterobacteriaceae.* The massive use of these agents, in addition to the horizontal transfer of resistance determinants, are responsible for the emergence and spread of carbapenemases. The rapid detection of these enzymes will allow early infection control measures, reducing the risk of therapeutic failure and avoiding the spread of the carbapenemases. The development of rapid tests for carbapenemase detection is mandatory. The aim of this study was to evaluate the performance of the rapid tests CARBA NP and BLUE-CARBA against carbapenemase-producing and non-carbapenemase-producing *Enterobacteriaceae*. A total of 90 isolates – 45 carbapenemase-producing- and 45 -non-producing-*Enterobacteriaceae* – were selected for this study. We performed both CARBA NP and BLUE-CARBA as previously described. We also added phenylboronic acid (PBA) in both assays to evaluate the presence of Class A carbapenemases. The sensitivity/specificity were 64.44%/97.78% and 91.11%/86.67% for CARBA NP and BLUE-CARBA, respectively. The modified assays with PBA did not provide any reliable results. Our results indicated that the BLUE-CARBA proved to be the best methodology (higher sensitivity) to be used as a screening test for carbapenemase detection.

Keywords: carbapenemase, *Enterobacteriaceae*, KPC, rapid tests.

INTRODUCTION

Nowadays, the emergence of carbapenem resistance is one of main concerns regarding antimicrobial resistance among *Enterobacteriaceae*. The problem is even more worrisome in the hospital environment, where resistant strains might be easily transmitted from patient to patient (Peleg *et al.*, 2010).With the emergence of extended-spectrum β-lactamase (ESBL) producing isolates, carbapenems have become the first therapeutic choice for serious infections due to *Enterobacteriaceae* (Pitout *et al*., 2008). However, in the early 2000s, the first carbapenem-resistant isolates due to the production of carbapenem-hydrolyzing enzymes (carbapenemases) have been reported in *Enterobacteriaceae* and rapidly spread around the globe (Yigit *et al.*, 2001) (Nordmann *et al.*, 2011).

The capacity of carbapenem hydrolysis varies according to different carbapenemases but even carbapenemases with low capacity of hydrolysis can confer high resistance levels whether combined with other resistant mechanisms, such as efflux pumps and porin loss (Paterson, 2006).

According to Ambler, the carbapenemases can be classified, based on molecular structure, in threeclasses: A, B and D (Ambler, 1980). Class A includes *Klebsiella pneumoniae* Carbapenemase (KPC) and Guiana Extended-Spectrum β-lactamase (GES).These enzymes present a serine residue in the active site and are usually inhibited *in vitro* by phenylboronic acid (PBA) (Pasteran *et al.*, 2009). Although both enzymes have already been reported in Brazil: KPC, first described in 2009 (Monteiro *et al.*, 2009), is endemic and GES, described in 2010 (Picao *et al.*, 2010) has only scattered reports in our country. Recently, a novel class A enzyme, denominated Brazilian *Klebsiella* Carbapenemase (BKC), was reported in Brazil; the latter, however, may not be inhibited by PBA (Nicoletti *et al.*, 2015).

Class B comprises theMetallo-β-lactamases (MBLs),which include, among others, the recently described New Delhi Metallo-β-lactamase (NDM)and the imipenemase (IMP). These enzymes have a zinc ion in their active site and, therefore, can be inhibited by chelating agents such as EDTA, a characteristic that is exploited in phenotypic assays (Lucena *et al.*, 2014; Shivaprasad *et al.*, 2014). Those enzymes have already been described in Brazil: IMP was first reported in2005, in São Paulo (Lincopan *et al.*, 2005) and NDM was detected for the first time in a *Providencia rettgeri* isolate from Porto Alegre (Carvalho-Assef *et al.*, 2013). While IMP is described only occasionally, it appears that the NDM is becoming widespread in Brazil. Class D includes the oxacilinases, which have a less pronounced carbapenem hydrolytic capacity, compared to other carbapenemases. The most relevant oxacilinase among *Enterobacteriaceae* is OXA-48, which was related with therapeutic failure when associated with other resistance mechanisms, such membrane impermeability and efflux (Jacoby *et al.,* 2005). Only a variant of the OXA-48 (OXA-370) was described in Brazil (Sampaio *et al.*, 2013).

Patients infected or colonized by carbapenem-resistant isolates are difficult to treat and tend to have a prolonged stay in healthcare facilities (Lee *et al*., 2013). Therefore, accurate, simple and rapid methodologies for the detection carbapenem resistance determinants are necessary in order to avoid the dissemination of carbapenemase-positive isolates. For many years, the Modified Hodge Test was considered by the Clinical and Laboratory Standards Institute (CLSI)as a reliable screening phenotypic method for carbapenemase detection, despite the fact that it may present reduced sensitivity and specificity and it was time-consuming (Girlich*et al.*, 2012).The gold standard method for carbapenemase detection is the genotypic test, including PCR and DNA sequencing. These methodologies present great sensitivity and specificity,

but they may be more expensive than the phenotypic methods. Moreover, the genotypic methods are also time-consuming and require special equipment to be performed (Bradford *et al.*, 2004; Monteiro *et al.*, 2012; Tenover *et al.*, 2013).

Two phenotypic methodologies (CARBA NP and BLUE-CARBA) have been recently described for the detection of carbapenemases, which are supposed to be rapid and non-expensive. These colorimetric methods are based on the pH reduction due to the hydrolysis of the β-lactam ring present in the carbapenems, which is detected by a pH indicator. CARBA NP, the first methodology described (Nordmann *et al*., 2012), uses phenol red as pH indicator. This methodology was included in the M100-S25 document of the CLSI (2015) as a screening method for carbapenemase detection. The other methodology, named BLUE-CARBA, uses bromothymol blue as pH indicator and it is supposed to have lower costs (Pires *et al.*,2013).

The aim of this study was to evaluate the performance of two phenotypic tests for carbapenemase detection (CARBA NP and BLUE-CARBA). We also proposed modifications in both methodologies in order to indicate the type of carbapenemase.

MATERIAL AND METHODS

Isolatesfrom a previous surveillance study (Rozales *et al.*, 2014) were identified by standard biochemical characterization and the susceptibility for carbapenems was determined by disk-diffusion and interpreted according to CLSI (2015).Isolates resistant to at least imipenem or meropenem were submitted to a Multiplex Real-Time PCR for the genotypic detection of the following carbapenemases: KPC, NDM, OXA-48-like, GES, IMP and VIM (Monteiro *et al.*, 2012).

The phenotypic methodologies evaluated in this study use imipenem as substrate and require colonies cultured overnight in Muller-Hinton agar. For CARBA NP (REF),

proteins were extracted using B-PERII (Bacterial Protein Extraction Reagent, Thermo Scientific Pierce, Rockford, USA).For each isolate, the protein extraction was incubated with a solution containing phenol red and imipenem. A negative control (only phenol red and imipenem) was included to help color interpretation. The test was considered positive according to the acidification of the solution, i.e., color changes from red to orange or yellow. The test was considered non-interpretable if the color of the negative control was more intense than the test (Nordmann *et al*.,2012).

The BLUE-CARBA test (REF) is a similar procedure as CARBA NP and the main differences are the use of bromothymol blue as pH indicator and the fact that a protein extraction is not required. This methodology uses one negative control (bacterial extract and a solution containing bromothymol blue) for each isolate tested. The test is considered positive if the color (initially blue) of test solution turns to green or yellow (Pires *et al.*, 2013).

Both methodologies (CARBA NP and BLUE-CARBA) were evaluated after two hours, with reads every 15 minutes and any change in the color was considered a positive result.

To improve the methodologies, an additional test was proposed: the use of phenylboronic acid as an inhibitor of Class A carbapenemases. This test contained the pH indicator, imipenem, and PBA 0,4 mg/mL, This test was performed in parallel with the original methodologies described above and was interpreted only when the isolate proved to be carbapenemase positive. The carbapenemase was considered of the Class A if the color of solution with PBA was the same as the negative control.

RESULTS

A total of 90 isolates were evaluated in this study: 45 isolates with genotypic test HRM-PCR) negative for carbapenemases and 45 isolates carrying at least one of the carbapenemase as follows: 14bla_{KPC}, 13 bla_{NDM}, 12$bla_{OXA-370}$, 4 bla_{GES} and 2 bla_{IMP}.

In general both phenotypic tests presented positive results for the isolates bearing bla_{KPC}, bla_{IMP} and bla_{GES} but the CARBA NP failed to detect one isolate with bla_{KPC} and the BLUE CARBA failed to detect one isolate with bla_{GES}. CARBA NP failed to detect many isolates with bla_{NDM} and most isolates with blaOXA$_{370}$ (Table 1). False-positive results were more visualized on the BLUE CARBA than the CARBA NP (Table2). The CARBA NP test presented a sensitivity of 64% and a specificity of 98%, while the BLUE-CARBA presented a sensitivity of 91% and specificity of 87%. All positive results presented color change within 75 minutes of incubation (which did not change after 120 minutes).

We could not validate any results of the test using PBA, because we were not able to observe any difference in the color of the solutions with imipenem and the PBA and the color of the solution with imipenem only for all carbapenemase-producing isolates tested.

DISCUSSION

Different β-lactamases may present different hydrolytic capacity. Enzymes with high hydrolytic power, such as KPC and most MBLs, can be easily detected by the methodologies evaluated (Nordmann *et al.*, 2011)(Pires *et al.*, 2013).On the other hand, OXA-48 variants, which usually have a weak hydrolytic power of carbapenems, might be missed in clinical laboratories if the detection is guided solely by hydrolysis-based tests.

For highly hydrolytic enzymes, the results achieved were considered satisfactory: the percentage of positivity for KPC-, NDM-, and IMP-producers was92.86%; 69.23% and 100%, respectively, on CARBA NP, and 100% for all enzymes on BLUE-CARBA. For the weakly hydrolyzing enzymes, the methodologies presented different results: CARBA NP was able to detect only 8.33% of OXA-370-producing isolates, while75% of them were detected with the BLUE-CARBA.

Although CARBA NP presented lower sensitivity, that test had the highest specificity: as only 2.22% of the carbapenemase-non-producing isolates were considered positive by that test. The BLUE-CARBA presented 13.33% of false-positive results. No correlation was found between true false and false positive, and gender and specie of isolates.

Noteworthy the fact that the results of BLUE-CARBA were not easy to interpret because the solution containing the carbapenem and the inoculum (Figure 1 – wells marked with “I”) had the same color or was slightly different from the negative control (Figure1).That would be even harder to observe in CARBA NP test, whose methodology did not require a negative control for each sample (Figure 2).

Considering the tests using phenylboronic acid, we could not obtain interpretable results probably because of the acidic character of the PBA, which interfered with the pH of the solution regardless the activity of the carbapenemases.

In conclusion, considering the results of this study, the BLUE-CARBA can be considered the best screening test for carbapenemase detection as this methodology presented higher sensitivity than the CARBA-NP.

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273 **Table 1.** Results of rapid tests CARBA NP and BLUE-CARBA for carbapenemase- producing
274 *Enterobacteriaceae.*

Isolate ID	Bacteria gender and specie	Carbapenemase	CARBA NP	Time until Positive (min) in CARBA NP	BLUE-CARBA	Time until Positive (min) in BLUE-CARBA
1101F	*Morganella morganii*	KPC	-	NA	+	30
1345F	*Enterobacter cloacae*	KPC	+	15	+	15
1373F	*Enterobacter cloacae*	KPC	+	15	+	15
1388F	*Enterobacter cloacae*	KPC	+	15	+	15
1389F	*Klebsiella oxytoca*	KPC	+	45	+	15
1390F	*Serratia marcescens*	KPC	+	15	+	15
3401F	*Klebsiella pneumoniae*	KPC	+	15	+	15
3409F	*Klebsiella pneumoniae*	KPC	+	15	+	15
3436F	*Escherichia coli*	KPC	+	15	+	15
3440F	*Enterobacter aerogenes*	KPC	+	15	+	15
3443F	*Klebsiella pneumoniae*	KPC	+	15	+	15
3445F	*Klebsiella pneumoniae*	KPC	+	15	+	15
3446F	*Klebsiella pneumoniae*	KPC	+	15	+	15
3818F	*Klebsiella pneumoniae*	KPC	+	15	+	15
821F	*Enterobacter cloacae*	NDM	+	30	+	15
871F	*Enterobacter cloacae*	NDM	+	15	+	45
1233F	*Enterobacter cloacae*	NDM	+	15	+	30
2007F	*Klebsiella pneumoniae*	NDM	+	15	+	15
2130F	*Enterobacter cloacae*	NDM	+	15	+	15
2610F	*Escherichia coli*	NDM	-	NA	+	15
2612F	*Citrobacter freundii*	NDM	+	45	+	15
2748F	*Klebsiella oxytoca*	NDM	-	NA	+	15
3035F	*Klebsiella pneumoniae*	NDM	-	NA	+	15
3304F	*Enterobacter cloacae*	NDM	-	NA	+	15
3320F	*Enterobacter cloacae*	NDM	+	15	+	15
3763F	*Klebsiella oxytoca*	NDM	+	15	+	15
3768F	*Klebsiella oxytoca*	NDM	+	15	+	15

1888F	*Klebsiella pneumoniae*	IMP	+	15	+	15
3349F	*Enterobacter cloacae*	IMP	+	15	+	15
1047F	*Klebsiella pneumoniae*	GES	+	60	-	NA
1597F	*Kluyvera intermedia*	GES	+	15	+	15
2818F	*Providencia rettgeri*	GES	+	15	+	15
3691F	*Serratia marcescens*	GES	+	75	+	75
1534F	*Citrobacter freundii*	OXA-370	-	NA	+	15
1636F	*Escherichia coli*	OXA-370	-	NA	+	15
2169F	*Klebsiella pneumoniae*	OXA-370	-	NA	+	60
2246F	*Klebsiella pneumoniae*	OXA-370	-	NA	-	NA
2494F	*Klebsiella pneumoniae*	OXA-370	-	NA	+	60
2592F	*Enterobacter aerogenes*	OXA-370	-	NA	+	15
2729F	*Klebsiella oxytoca*	OXA-370	-	NA	+	15
2807F	*Enterobacter cloacae*	OXA-370	-	NA	+	30
3023F	*Providencia stuartii*	OXA-370	-	NA	-	NA
3149F	*Klebsiella pneumoniae*	OXA-370	+	30	+	15
3284F	*Enterobacter cloacae*	OXA-370	-	NA	+	30
3704F	*Klebsiella pneumoniae*	OXA-370	-	NA	-	NA

275 NA, not applicable.

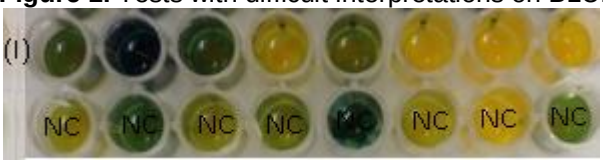
Table 2. Results of rapid tests CARBA NP and BLUE-CARBA for non-carbapenemase-producing *Enterobacteriaceae.*

Isolate ID	Bacteria gender and specie	CARBA NP	Time until Positive (min) in CARBA NP	BLUE-CARBA	Time until Positive (min) in BLUE-CARBA
3310F	*Morganella morganii*	-	NA	-	NA
3314F	*Enterobacter cloacae*	-	NA	+	30
3316F	*Klebsiella pneumoniae*	-	NA	+	60
3318F	*Klebsiella pneumoniae*	-	NA	+	30
3326F	*Enterobacter aerogenes*	-	NA	-	NA
3328F	*Enterobacter cloacae*	-	NA	-	NA
3330F	*Klebsiella pneumoniae*	-	NA	-	NA
3331F	*Enterobacter cloacae*	-	NA	-	NA
3332F	*Morganella morganii*	-	NA	-	NA
3336F	*Proteus mirabilis*	-	NA	-	NA
3340F	*Klebsiella pneumoniae*	-	NA	-	NA
3348F	*Klebsiella pneumoniae*	-	NA	-	NA
3362F	*Enterobacter cloacae*	-	NA	-	NA
3363F	*Enterobacter cloacae*	-	NA	+	15
3370F	*Enterobacter cloacae*	-	NA	-	NA
3372F	*Klebsiella pneumoniae*	-	NA	-	NA
3373F	*Klebsiella pneumoniae*	-	NA	-	NA
3376F	*Klebsiella pneumoniae*	-	NA	-	NA
3394F	*Morganella morganii*	-	NA	-	NA
3404F	*Morganella morganii*	-	NA	-	NA
3421F	*Klebsiella pneumoniae*	-	NA	-	NA
3423F	*Morganella morganii*	-	NA	-	NA
3424F	*Enterobacter cloacae*	-	NA	-	NA
3428F	*Providencia stuartii*	-	NA	-	NA
3437F	*Enterobacter cloacae*	-	NA	-	NA
3442F	*Enterobacter cloacae*	-	NA	-	NA

3444F	*Klebsiella pneumoniae*	-	NA	-	NA
3445F	*Klebsiella pneumoniae*	-	NA	-	NA
3452F	*Morganella morganii*	-	NA	-	NA
3457F	*Klebsiella oxytoca*	-	NA	-	NA
3459F	*Klebsiella pneumoniae*	-	NA	-	NA
3460F	*Klebsiella pneumoniae*	-	NA	-	NA
3479F	*Enterobacter cloacae*	-	NA	-	NA
3481F	*Enterobacter cloacae*	-	NA	-	NA
3485F	*Klebsiella pneumoniae*	+	15	+	15
3495F	*Enterobacter cloacae*	-	NA	-	NA
3497F	*Klebsiella oxytoca*	-	NA	+	30
3498F	*Klebsiella pneumoniae*	-	NA	-	NA
3510F	*Klebsiella pneumoniae*	-	NA	-	NA
3511F	*Klebsiella pneumoniae*	-	NA	-	NA
3548F	*Klebsiella pneumoniae*	-	NA	-	NA
3560F	*Enterobacter aerogenes*	-	NA	-	NA
3571F	*Serratia marcescens*	-	NA	-	NA
3597F	*Klebsiella pneumoniae*	-	NA	-	NA
3599F	*Klebsiella pneumoniae*	-	NA	-	NA

278 NA, not applicable.

Figure 1. Tests with difficult interpretations on BLUE-CARBA assay.



NC, negative control, bromothymol blue solution containing bacterial/protein extract .I, bromothymol blue solution containing bacterial/protein extract and imipenem;

Figure 2. Tests with difficult interpretations on CARBA NP assay.

NC, negative control, fenol red solution containing bacterial/protein extract. I, fenol red solution containing bacterial/protein extract and imipenem:

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If you have concerns about the level of English in your submission, you may choose to have your manuscript professionally edited by a native English speaker or a scientific editing service prior to submission to improve the English. All services are to be arranged and paid for by the author, and use of one of these services does not guarantee acceptance or preference for publication. In the case of the author being a native English speaker, please replace the certificate of English editing service by a justification letter.

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• American Journal
Experts: http://www.JournalExperts.com?rcode=BSM1

SECTIONS

Industrial Microbiology:
Bacterial Fermentation

- biosynthesis and bioconversion of natural products, including antibiotics, xenobiotics, and macromolecules produced by bacteria.
- molecular aspects of bacterial biotechnology

Fungal Fermentation

- biosynthesis and bioconversion of natural products, including antibiotics, xenobiotics, and macromolecules produced by fungi
- molecular aspects of fungal biotechnology

Food Microbiology:
Food Technology

- applications of microorganisms (bacteria and fungi) for food production

Food Safety and Quality

- food borne diseases
- food spoilage
- microbial ecology in foods

Medical Microbiology:
Bacterial Pathogenesis

- genetic, biochemical, and structural basis of bacterial pathogenesis

Fungal Pathogenesis

- genetic, biochemical, and structural basis of pathogenesis of fungi

Clinical Microbiology:
Micology

- studies of medically-important fungi

Bacteriology

- studies of medically-important bacteria

Virology

- studies of medically-important virus

Environmental Microbiology:
Microbial Ecology

- ecology of natural microbial assemblages, microbial diversity of natural environments such as water, soil, sediments and higher organisms
- microbial interactions

Biotechnology

- environmental aspects of public health
- biodegradation
- bioremediation
- environmental considerations for genetically engineered microorganisms

Fungal Physiology

- fungal biochemistry, biophysics, metabolism, cell structure, stress response, growth, differentiation and other related process

Bacterial Physiology

- bacterial biochemistry, biophysics, metabolism, cell structure, stress response, growth, differentiation and other related process

Genetics and Molecular Biology of Fungi

- fungal genetics, molecular biology, gene regulation, DNA replication and repair, genomics, proteomics, transcriptomics

Genetics and Molecular Biology of Bacteria

- bacterial genetics, molecular biology, gene regulation, DNA replication and repair, genomics, proteomics, transcriptomics

Genetics and Molecular Biology of Viruses

- viral genetics, molecular biology, gene regulation, DNA replication and repair, genomics, proteomics, transcriptomics

Veterinary Microbiology

- diseases of animals
- control and/or treatment of animals
- animal pathogen diagnostics
- veterinary or zoonotic pathogens

Education in Microbiology

- Teaching strategies in microbiology
- New teaching tools in microbiology

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The suggestions and recommendations of the reviewers and Editors will be forwarded electronically to the corresponding author, who should return the reviewed manuscript to the Editors within the stipulated date, via online system. Whenever applicable, the corresponding author should explain or comment each modification introduced in the text.

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- Title
- Authors and Affiliations
- Abstract (200 to 250 words)
- Three to five key-words
- Introduction
- Materials and Methods
- Results
- Discussion
- Acknowledgements (optional)
- References

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- Three to five key-words
- Text
- Acknowledgements (optional)
- References

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Research papers and *mini-reviews* consist of 20 pages, including references, tables and figures.

Abbreviations of terms and symbols should follow the recommendations of IUPAC-IUB Commission (*Comission on Biochemical Nomenclature, Amendments and Corrections*) and the units are to be used according to SI (*International Systems of Units*).

SUGGESTED REVIEWERS

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USE OF PLANT EXTRACTS IN MICROBIOLOGICAL EXPERIMENTS

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ORGANIZATION

The **Title** should be a brief as possible, contain no abbreviations and be truly indicative of the subject of the paper.

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The **Results** section should, by means of text, tables and/or figures, give the results of the experiments. If a *Discussion* section is to be included, avoid extensive interpretation of results but do so in the *Discussion* section.
If *Results* and *Discussion* are combined, then results should be discussed where,

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The **Discussion** section should discuss the results in relation to the literature cited.

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b. **Paper or chapter in a book**
Franco BDGM, Landgraf M, Destro MT, Gelli DS, (2003) Foodborne diseases in Southern South America. *In*: Miliotis, M.D., Bier, J.W.(eds). International Handbook of Foodborne Pathogens. Marcel Dekker, New York, USA, 733-743.
c. **Book**
Montville TJ, Matthews KR (2005) Food Microbiology - an introduction. ASM Press, Washington, D.C.
d. **Patent**
Hussong RV, Marth EH, Vakaleris DG. January 1964. Manufacture of cottage cheese. U.S. Pat. 3, 117, 870.
e. **Thesis and Dissertations**
Santos MVB (2005) O papel dos anticorpos contra os componentes da parede celular de Paracoccidioides brasiliensis na evolução da doença experimental. São Paulo, Brasil, 110p. (M.Sc. Dissertation. Instituto de Ciências Biomédicas. USP).
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