Molecular characterization of carbapenem-resistant Acinetobacter baumannii strains from a tertiary care center in South India

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Abstract

Objectives: Carbapenem resistant Acinetobacter baumannii is an important therapeutic and infection control challenge worldwide. In this study, we investigated the prevalence and distribution of molecular mechanisms of resistance among carbapenem resistant A. baumannii species at a tertiary care setting in South India.

Materials and Methods: A total of 89 non-duplicate clinical isolates of carbapenem-resistant A. baumannii were collected from critical care units of St. John’s Medical College Hospital, Bengaluru, India. Polymerase chain reaction (PCR) was performed to detect blaOXA-type carbapenemase blaOXA-51-like, blaOXA-23-like, blaOXA-24-like and blaOXA-58-like, MBL genes blaOXA, blagMP and blavIM genes. Molecular typing of carbapenem-resistant A. baumannii strains was performed by using Rep-PCR.

Results: Eighty-seven of the isolates were found to carry the blaOXA-51 gene and 81 (91%) isolates were found to have blaOXA-23-like gene and blaOXA-24-like gene. The blaOXA-24-like gene was detected in two isolates of which one also carried blaOXA-51-like gene and one isolate carried blavIM coding gene. The prevalence of blablaNDM, blablaVIM blablaOXA-51 was 12(13%), 14(16%) and 57(64%) respectively. Cluster analyses revealed a 90% similarity and were divided into 5 clusters. Most of the isolates containing carbapenemases coding genes grouped under cluster A, C and UC. Considerable heterogeneity was observed within UC cluster indicating circulation of multiple strains of A. baumannii within our institution.

Conclusions: Carbapenemase coding blablaOXA-23, blablaOXA-51 and blablaOXA-58 were more common than blablaNDM and blablaVIM. The presence of blablaNDM with other genes coding for carbapenemases indicate the ability of the strains to acquire novel genes despite having its share of the blablaOXA-51-like carbapenemase.

Keywords: Acinetobacter baumannii; carbapenemases; Genomic diversity; Rep-PCR; carbapenem resistance

Caracterización molecular de cepas de Acinetobacter baumannii carbapenem-resistente en un centro de atención terciaria en el sur de la India

Resumen

Objetivos: El Acinetobacter baumannii resistente a carbapenem es un reto importante en todo el mundo para su tratamiento y para el control de infecciones hospitalarias. Nosotros estudiamos la prevalencia y los mecanismos de resistencia en aislados de un centro de atención terciario, en el sur de la India.

Materiales y Métodos: Se estudiaron 89 aislados clínicos de A. baumannii recolectados en unidades de cuidado crítico del Hospital St. John’s Medical College en Bengaluru, India. Se realizó amplificación por PCR (Reacción en Cadena de Polimerasa) y luego tipificación molecular con la técnica Rep-PCR (PCR de elementos repetitivos palindrómicos) para detectar los genes de carbapenemasa blablaOXA, blablaOXA-51, blablaOXA-23, blablaOXA-24, blablaOXA-58, MBL, blablaNDM, blablaMP y blablaVIM.

Resultados: Se encontraron 87 aislados que llevaban el gen blablaOXA-51 y de ellos en 81 (91%) se encontró blablaOXA-51 y blablaOXA-23. El blablaOXA-24 se detectó en dos aislados de los cuales uno de ellos llevaba blablaOXA-51 y otro blablaMP. Los genes blablaNDM, blablaMP y blablaVIM se encontraron en 12 (13%), 14 (16%) y 57(64%) de los aislados, respectivamente. El análisis de agrupamiento reveló un 90% de similitud entre los aislados y que podían asignarse a 5 agrupamientos. La mayoría de los aislados llevaban genes de carbapenemasa de los grupos A, C y UC. Se observó mucha heterogeneidad dentro del agrupamiento UC indicando que existe circulación de múltiples cepas de A. baumannii dentro de nuestra institución.

Conclusions: Las carbapenemasas que codifican para blablaOXA-23, blablaOXA-51 y blablaOXA-58 son más comunes que blablaNDM y blablaVIM en nuestra institución. La presencia de NDM con otros genes codificando para carbapenemases indica la capacidad de los aislados que tienen este tipo de aislados para adquirir nuevos genes a pesar de contar con blablaOXA-51.

Palabras clave: Acinetobacter baumannii; carbapenemases; diversidad genómica; Rep-PCR; resistencia a carbapenem

Introduction

Acinetobacter baumannii (A. baumannii) a non-glucose fermenting Gram-negative bacillus, is an important cause of nosocomial infections and widely isolated from healthcare facilities, especially in the intensive care units (ICUs). They are increasingly being associated with increased morbidity and mortality among hospitalized patients. The increased use of invasive procedures and exposure to antibiotics increases the risk of infections among immunocompromised patients admitted in critical care units. These organisms are known to colonize various surfaces of the environment and patients admitted to healthcare institutions. As they are known to survive in unfavorable environments and can contaminate other sites.
Carbapenems were considered the most powerful antibiotics because of their extremely effective antibacterial activity and low toxicity; however, with the emergence of carbapenem resistance, *A. baumannii* has become a growing therapeutic concern worldwide. Over the last 15 years, the resistance of *A. baumannii* to expanded-spectrum cephalosporins and carbapenems has grown rapidly. Carbapenem resistance, among these pathogens, is attributed to reduced expression of outer membrane proteins (29 kDa, 33–36 kDa), change of the penicillin-binding proteins, the specific drug efflux and carbapenemase production. Carbapenem-hydrolyzing Class D oxacillinsases (*bla_oxa-23* to 27, 40, 51, and 64 to 71) are the predominant carbapenemase has been reported globally. The *bla_oxa-51* is thought to be probably intrinsic to the genetic makeup of the organism. However, reports on the *bla_oxa-mp* or *bla_oxa-vmp* class Metallo-β-lactamase (MBL such as *bla_mpp*, 2, 4 and 5, and *bla_vmp, 1* and *bla_vmp, 2*) producing *A. baumannii* are also on the rise. Although most *bla_nmd* genes are frequently reported in Enterobacteriaceae, they have also been detected in *Acinetobacter* species, first of such case of *bla_nmd* in *Acinetobacter* was reported in 2010 from India. Expression of more than one type of carbapenemases has been documented in India. Due to the ability to survive in a harsh environment (presence of disinfectants, exposure to antibiotics), these organisms are known to persist and are able to be transferred to different healthcare facilities by colonizing or infecting patients. Prevention of infections by these organisms is a tough challenge. Outbreaks and persistent presence of these organisms have been documented in intensive care units for prolonged periods. The antibiogram patterns may not be able to suggest similarity among the different strains in circulation during a particular period.

*A. baumannii* strains have been typed using molecular methods such as ribotyping, amplified fragment length polymorphism, and repetitive extragenic palindromic sequence-based polymerase chain reaction (Rep-PCR). Due to limited gene set or technical limitations, most new tests are not suitable for clinical routine monitoring in low prevalence settings. Rep-PCR is simple, rapid test that can be highly discriminatory for the identification and differentiation of *A. baumannii*. The study was aimed to describe the presence of carbapenemases coding genes among Meropenem/Imipenem resistant *A. baumannii* obtained from clinical samples by phenotypic methods and to understand their clonal relationship using Rep-PCR.

**Materials and Methods**

A total of 89 non-duplicate isolates of meropenem/ Imipenem resistant *A. baumannii* were collected. These organisms were isolated from the samples received from patients in the critical care units (intensive care, burns, post-operative areas) of St. John’s Medical College Hospital, Bengaluru, India between May 2011 and January 2012. The isolates were collected from blood, respiratory secretions, sputum, urine, pleural fluid, ascitic fluid, and wounds, and were considered nosocomial if the specimens had been obtained more than 48 hours after the time of patient’s admission. More than one isolate from the same patient was not included. *A. baumannii* was identified in the clinical microbiology laboratory by standard biochemical tests as well as the Vitek 2 system (bioMérieux, Durham, NC), when necessary. Those identified *A. baumannii* complex were further confirmed *A. baumannii* using a multiplex PCR method by using the specific *Acinetobacter baumannii* primers (P-Ab-ITSF and P-Ab-ITSB) and the internal control primers (P-rA1and P-rA2) specific for the recA gene of all *Acinetobacter* spp. The study was approved by the Institutional Ethical Board, St. John’s Medical College, Bengaluru.

**Susceptibility testing**

Antibiotic susceptibility was determined by the standard agar disc diffusion method using Mueller–Hinton agar plates (Himedia, India) using antimicrobial agents and interpreted according to the guidelines of the Clinical and Laboratory Standards Institute 2012 (CLSI, 2012)17. The following antibiotics Gentamicin (Gen), tetracycline (Tetra), trimethoprim-sulphamethoxazole ( Cotri), cephalexin (Ceph), ciprofloxacin (CFX), amikacin (Amik), Ceftriaxone and Tazobactum (Omna), cefotaxime, netilmicin (Net), ceftazidime (Fort), cefepime (Cep), piperacillin (Pip); piperacillin/tazobactam (PT), Meropenem and imipenem (Imi/Mero) were tested using discs obtained from Himedia labs, Mumbai, India. Meropenem and imipenem were obtained from BD India Ltd. Resistance to Meropenem was confirmed using agar dilution method. A minimum inhibitory concentration (MIC) of ≥16 μg/mL for imipenem and meropenem was used to define resistance. *Pseudomonas aeruginosa* ATCC 27853 was used as reference strain for quality control of the antibiotic discs and agar dilution.

**Isolation of bacterial DNA**

The bacterial genomic DNA was prepared by using the lysis buffer (1% Triton X-100, 10 mM Tris pH 8.0, 1 mM EDTA) and boiling method. Briefly, colonies were picked from a MacConkey plate and inoculated into Luria-Bertani broth (Hi-Media, Mumbai, India) and incubated at 37 °C in a shaker incubator for 18 – 24 hrs. After the incubation, cell pellet was prepared from the broth. The cell pellet was washed in molecular grade water and lysis buffer was added. The bacterial suspension was heated at 95 °C for 15 min, and then pelleted by centrifugation at 12,000 x g for 5 min. The supernatant was transferred to fresh sterile 1.5 ml tubes, for use as templates for PCR amplification.
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**PCR analysis of carbapenemases-encoding genes**

The genes detected included the integrase gene intI, OXA-type carbapenemase gene clusters bla\_OXA-51-\_IbV, bla\_OXA-23-\_IbV, and bla\_OXA-58-\_IbV, MBL genes bla\_VIM, bla\_NDM, bla\_IMP, and bla\_OXA-24. The negative control was run with every PCR. The primers used for the PCR are listed in Table 1 and PCR conditions are per the references quoted. The PCR products were separated on 2% agarose gels by electrophoresis, stained with ethidium bromide at a final concentration of 0.5 \( \mu \)g/mL, and then visualised under the gel documentation system (Biorad Life Science, CA, USA).

**Rep-PCR fingerprinting**

The repetitive extragenic palindromic polymerase chain reaction (Rep-PCR) fingerprinting was performed as previously described primer pair Rep-1 (5’-IIIGCGCCGICATCAGGC-3’) and Rep-2 (5’-ACGTCTTATCAGGCCTAC-3’) and are listed in Table 1. PCR products were analysed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide (5 mg/L). Strains were considered to be highly similar and belonging to the same DNA group when all visible bands of two isolates had similar migration distance. Within the same Rep-type, strains differing by no more than two bands were considered subtypes. Gel images were subsequently analysed by Bionumerics software (Applied Maths, Kortrijk, Belgium). A dendrogram was generated to analyse the similarity between the isolates, using the unweighted pair group with arithmetic averages (UPGMA) and the Dice similarity coefficient with 1% of tolerance and 0.5% of optimization.

**Results**

**Bacterial isolates and antimicrobial susceptibility pattern**

The isolates were predominantly from respiratory secretions (endotracheal aspirates or sputum) 60 (67.4%), followed by pus 25 (28.1%), urine 10 (11.2%) and three from other body fluids and two from blood samples. Forty-nine isolates (55%) were from male patients. As seen in Figure-1, most of the isolates were resistant to nearly all antibiotics tested except for netilmicin. Resistance to at least one of aminoglycoside or \( \beta \)-lactam antibiotics were observed in 80% and 97% of the strains. A total of 36 (40%) isolates out 89 are resistant to all the antibiotics tested and rest of the 53 isolates were multidrug resistant. Minimum inhibitory concentrations (MIC) for meropenem and imipenem determined using the agar dilution method (CLSI 2010).

**Detection and characterization of antibiotic resistance coding genes**

As seen in Table 2, the majority of carbapenem-resistant isolates carried bla\_OXA-51 (98%) and bla\_OXA-23 (96%) genes followed by bla\_VIM gene (67%). Class-1 integrase genes were found in all of A. baumannii strains tested \( n=89/89, 100\% \) whereas class 2 integrase genes were found in 5 \( n=5/89, 6\% \) isolates. A total of 14 isolates out of 89 carries bla\_NDM-1 gene. The association of ISAba-1 along with bla\_OXA-23-\_IbV and bla\_OXA-51-\_IbV was present in 92% and 94% respectively.

**Rep-PCR**

Rep-PCR showed considerable diversity of genotypes among the isolates examined. The isolates were grouped into five different clusters A to E. However, there are many isolates which has not shown any bands or showed one or two bands categorized as unclassified (UC). The clusters A to C was defined by using a similarity threshold of 90% were marked in the dendrogram (Figure 2). Majority of the isolates from all the clusters demonstrated the presence of bla\_OXA-23\_IbV, bla\_OXA-51, and class-1 integron coding genes. Similarly, isolates in Cluster B carried bla\_VIM in few isolates, bla\_IMP coding gene coexisted with bla\_IMP and one isolate carried both bla\_NDM, and bla\_IMP coding genes. The Clusters D to E was defined by using a similarity threshold of 90% as marked in the dendrogram. Isolates in cluster D in addition to bla\_OXA-23\_IbV, bla\_OXA-51 and class-1 integrons coding genes, demonstrated only the presence of bla\_VIM gene. The isolates grouped under Cluster E carried genes code for bla\_VIM, bla\_IMP and bla\_NDM.

**Discussion**

Reports on carbapenemase- producing A. baumannii isolates are rising globally due to increased carbapenem use thereby creating a selection pressure. In the current study, we found that most of the carbapenem-resistant isolates (67%) were from respiratory secretions. Outbreaks caused by A. baumannii strains have been reported in the ICU due to cross-contamination or spread among patients\(^{1,26,27}\). It has been reported that carbapenem resistance rates in A. baumannii have mostly exceeded 40% throughout all of India. All of the isolates tested in the present study showed a high level of resistance to majority of the antibiotics except netilmicin. Prashanth et al. report the high effectiveness of netilmicin against netilmicin-resistant XDR A. baumannii from India\(^{38}\). However, Saranathan et al. also reported 48% of netilmicin resistance among A. baumannii clinical isolates from Southern India\(^{29}\). Infections like bacteremia and ventilator-associated pneumonia caused by XDR A. baumannii result in >50% mortality rates\(^{39}\). In the present study, 80% of A. baumannii isolates were detected as XDR. Rynga et al., report 78% XDR A. baumannii clinical isolates from two hospitals from New Delhi, India\(^{41}\). Similarly, Maspi et al., report 71% of XDR A. baumannii isolates from Iran\(^{52}\). Multi-drug resistant A. baumannii has caused many nosocomial outbreaks worldwide, and is considered as a difficult to treat nosocomial pathogen due to resistance to many antimicrobial agents and has been increasingly associated with mortality\(^{10,13}\).

The higher percentage of oxacillinases, bla\_OXA-23\_IbV and bla\_OXA-51 (most common resistance genes), than metallo-\( \beta \)-lactamas (bla\_VIM and bla\_IMP) detected from the tested A. baumannii isolates was in accordance with earlier published reports from India\(^{7,33}\). However, in the present study, bla\_OXA-51-\_IbV, Carba-penem resistance coding genes was not detected from any of the tested isolates. The bla\_OXA-58 like genes have been more commonly reported from Europe, North and South America, and West Asia, however low prevalence (2%) has been re-
It is important to note that IS elements such as ISAba1 can contribute to the spread of carbapenemases genes among different Acinetobacter species. The high prevalence of class 1 integrons among multidrug-resistant A. baumannii clinical isolates has been confirmed worldwide. The epidemic potential of A. baumannii may be linked to the presence of class-1 integrons and ISAba1. In our study, class-1 integrons was present in all of A. baumannii isolates. Whereas, class 2 integrons were present in four isolates and co-existed with class -1 integron. In the present study, 93% of A. baumannii isolates carried class-1 integrons coding genes and these isolates co-existed with ISAba1. Sun et al report 64% of the class -1 integrons in Acinetobacter spp. isolates in northeastern China. Amin et al reported the class -1 integron is often responsible for the dissemination of the MBL genes among A. baumannii isolates from Iran. In the present study, 83% (74/89) of A. baumannii isolates were positive for class-1 integrons coding genes.

This study was also aimed to determine the distribution of resistant genes in genetically similar and dissimilar carbapenem-resistant A. baumannii strains by using Rep-PCR. All the isolates were classified into different clonal clusters A to E according to genetic similarity based on banding pattern obtained by Rep-PCR. Resistance coding genes detected from these isolates were not associated with a particular group; rather each group confined more than one resistance gene type. Similar to other study, most of the strains showed to harbor multiple carbapenemases genes possibly indicate that there are multiple clones in circulation in our hospital. In this study, it has been shown that genetic determinants like blaOXA-23, intI1, ISAba1, blaVIM, blaNDM and blaIMP were widely distributed among all the carbapenem resistant A. baumannii isolates grouped under different clonal clusters A to E. These genetic determinants are capable of possessing the potential horizontal gene transfer between susceptible A. baumannii or any other bacterial species. Rep-PCR uses amplification of intervening fragments between highly conserved sequences by means of consensus primers. It is a rapid, simple and low-cost method for molecular typing, which has been applied previously to identify genetic differences among the isolates of A. baumannii. It is challenging to discriminate a clear pattern among some isolates because rep-PCR bands are faint, and some profiles have few bands. The rep-PCR is less discriminatory than PFGE in identifying unique strains. However, sometime it requires isolates sharing the same pattern to be tested with a more discriminatory method, like PFGE or multilocus sequence typing whenever appropriate. Vijayakumar et al reported the carbapenem-resistant A. baumannii isolates were grouped under clonal complex 208 which belongs to the international clonal lineage 2 and high occurrence of ST-848 carrying blaOXA-23-like gene from India.

There has been increasing concern regarding the rise of Acinetobacter infections in critically ill patients. Our study also showed that Acinetobacter infections most frequently involve the respiratory tract of intubated patients as also reported.

Figure 1. In-vitro antibiotic susceptibility profile of Acinetobacter baumannii species clinical isolates

Note: Antibiotics tested is Gentamicin (Gen), tetracycline (Tetra), trimethoprim-sulphamethoxazole (Cotri), cephalixin (Ceph), ciprofloxacin (CFX), amikacin (Amik), Ceftriazone and Tazobactum (Omna), cefotaxime, netilmicin (Net), ceftazidime (Fort), cefaperazone (CpZ), piperacillin (Pip); piperacillin/tazobactam (PT), Meropenem and imipenem (Imi/Mero).
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Figure 2. Repetitive sequence-based polymerase chain reaction (rep-PCR) similarity analysis and gel images for multidrug-resistant Acinetobacter baumannii isolates.
Table 1. Primers used for amplification of resistance genes by polymerase chain reaction

| Genes     | Primer sequence                  | Reference |
|-----------|----------------------------------|-----------|
| OXA51-LIKE F | TAATGCTTGTGATGGCCCTTG             | 21        |
| OXA51-LIKE R | TGGATGGACACTCATCGTGG              | 21        |
| OXA23-LIKE F | GATCGGATGGGAAACCCGA              | 21        |
| OXA23-LIKE R | ATTTGCAACGGATTTCCAG              | 21        |
| OXA24-LIKE F | GGTAGTTGGCCCTCCCTAA             | 21        |
| OXA24-LIKE R | AGTTGACGGGAAAAGGGCAT             | 21        |
| OXA58-F | AAGTATGGGCTGTGTGTCG             | 21        |
| OXA58-R | CCCCCTCTGGCTCTACATAC            | 21        |
| IMP F | CACACCTTTATCAAGCGGACC           | 21        |
| IMP R | GAGAATTAAGCCACTCTATGGC           | 21        |
| VIM F | TCACGATAGTATACGAC               | 21        |
| VIM R | TTGCGGATTCGCTGGCACC             | 21        |
| NDM F | GGTGGGCGATCTGTTTC               | 21        |
| NDM R | CGGAATGGCATCAGAATAC              | 22        |
| ISAba-F | CACGAATACGAGAAGTTG               | 22        |
| ISAba-R | CGACAAATCACATGAC                 | 22        |
| Inta1-F | CAGTGGACATAAGCGGTTC             | 22        |
| Inta1-R | CCCGGGCGATAGAATCGTA             | 22        |
| Inta2-F | TGGCGTATACCCCTACTCTG             | 22        |
| Inta2-R | TTACCTGCTAGTATACGG              | 22        |
| Rep-1 | IIIIGGCGCATGACGAC               | 25        |
| Rep-2 | AGGTCTTATAGGGCTAC              | 25        |

Conclusion

A. baumannii previously thought to be contaminants have now gained notoriety as multidrug resistant, hospital-acquired pathogens due to the acquisition of a number of resistance genes. Analysis of the genes that codes for carbapenem resistance among randomly selected isolates has confirmed the presence of multiple resistant genes such as, blaOXA-23, blaOXA-51 and blaVIM, and isolates that showed considerable genetic heterogeneity. Presence of blaNDM was documented along with other Metallo-β-lactamases genes. In most of isolates the combination of genes demonstrates the ability of the organism to acquire novel genes from other bacteria (such as blaNDM) and increase its own repertoire of resistant genes, thereby markedly reducing treatment options with β-lactam antibiotics.

Acknowledgement

We gratefully acknowledge the grant support of the Research Society of St. John’s Medical College Hospital for their generous support to this study.

Financial support and sponsorship

Funded by the grants for internal faculty from the Institutions Research Society, St. John’s Medical College Hospital, Bengaluru, India.

Ethical disclosures

Protection of human and animal subjects. This research do not used human nor animal material.

Right to privacy and informed consent. The authors declare that no data that enables identification of the patients appears in this article.

Conflict of interest. None declared

Footnotes

This study was partially presented at the 17th International Congress on Infectious Diseases (Abstract no. 41.081).

Table 2. Distribution of resistance-associated genes among clinical isolates of multidrug-resistant Acinetobacter baumannii

| Clonal groups | Inta I | Inta II | ISAba1 | blaOXA-51-like | blaOXA-23-like | NDM | VIM | IMP | ISAba1 + blaOXA-51-like | ISAba1 + blaOXA-23-like | ISAba1 + NDM | ISAba1 + VIM | ISAba1 + IMP |
|---------------|--------|---------|--------|----------------|----------------|-----|-----|-----|------------------------|------------------------|--------------|-------------|--------------|
| A (n=16)      | 16(100)| 1(6.2)  | 15(94) | 15(94)         | 15(94)         | 3(20)| 10(67)| 2(13)| 15(94)                  | 15(94)                  | 3(20)| 10(67)     | 2(13)       |
| B (n=9)       | 9(100) | 1(11)   | 9(13)  | 9(100)         | 8(13)          | 1(11)| 7(64) | 3(33)| 9(100)                  | 9(100)                  | 1(11)| 7(64)      | 3(33)       |
| C (n=14)      | 14(100)| 1(7)    | 14(100)| 14(100)        | 14(100)        | 4(28)| 10(71)| 2(14)| 14(100)                 | 14(100)                 | 4(29)| 10(71)     | 2(14)       |
| D (n=11)      | 11(100)| 1(9)    | 11(100)| 11(100)        | 9(82)          | 0   | 7(64) | 0   | 11(100)                 | 9(82)                  | 0   | 7(64)      | 0           |
| E (n=6)       | 6(100) | 0       | 5(83)  | 6(100)         | 6(100)         | 2(33)| 3(50) | 1(17)| 5(83)                  | 6(100)                 | 1(17)| 3(50)      | 1(17)       |
| UC (n = 33)   | 33(100)| 1(3)    | 30(91)| 32(97)         | 30(91)         | 2(6)| 20(61) | 6(61)| 30(91)                 | 29(88)                 | 2(6)| 18(54)    | 6(18)       |
| Total (n=89)  | 89(100)| 5(6)    | 84(93)| 87(98)         | 85(96)         | 12(13)| 57(64)| 14(16)| 84(94)                 | 82(92)                 | 11(12)| 55(62)    | 14(16)      |
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**Author's contribution**

Concept and design (SN, RM), data collection and laboratory work (BSK, SPC), data analysis, interpretation and drafting of the manuscript (BSK, SPC, SN), critical review of the manuscript (RY, RM). All authors read and approved the final version of the manuscript.

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