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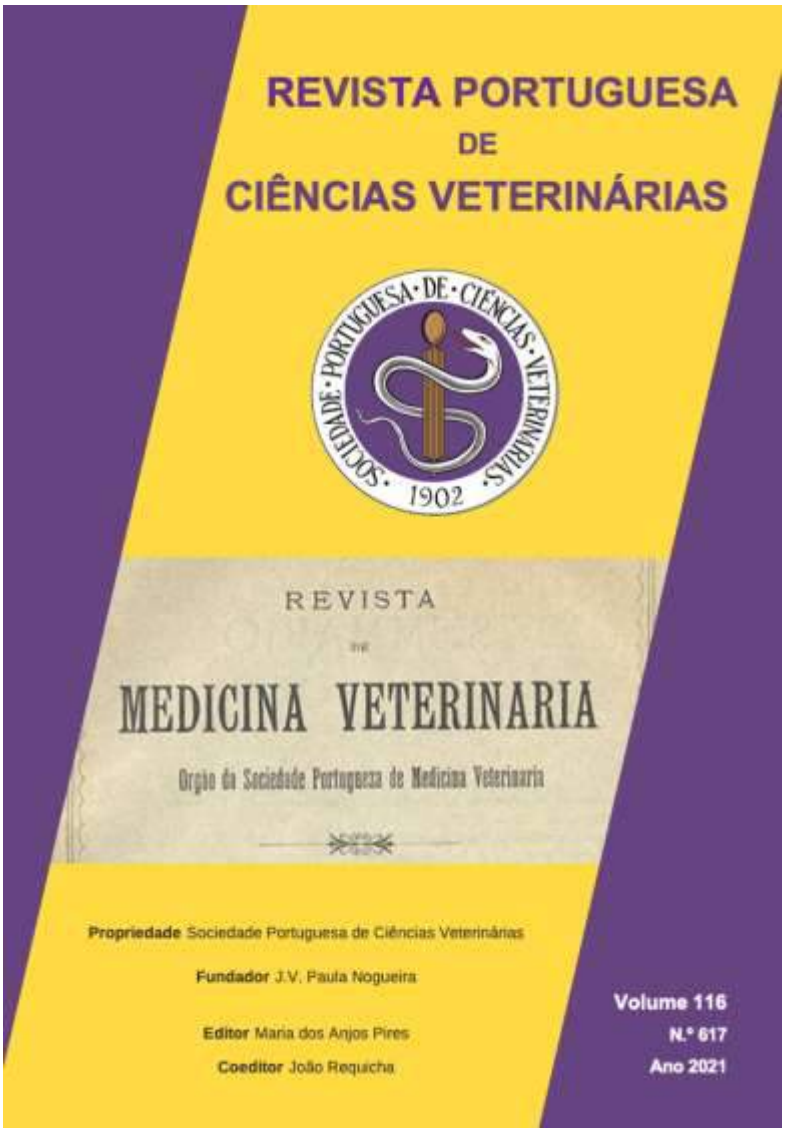

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Evidências de infeção por Mycobacterium avium subsp. paratuberculosis em ruminantes de uma região de produção leiteira intensiva

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Resumo

A paratuberculose é uma doença causada por *Mycobacterium avium* subsp. *paratuberculosis* (Map) que afeta negativamente a produção animal apresentando um grande impacto na indústria leiteira. Neste estudo, foram colhidas amostras de fezes de bovinos (n=58) e caprinos (n=17) com suspeita de infeção por Map de acordo com sinais clínicos e/ou dados epidemiológicos para deteção, identificação e genotipagem do agente etiológico. As amostras foram testadas por cultura bacteriológica e por PCR em tempo real baseado na deteção da sequência de inserção IS*900*, específica de Map, no DNA das amostras de fezes. Observou-se o crescimento bacteriano de Map e detectou-se o seu DNA em 78,7% e 73,3% das amostras, respectivamente. Um conjunto de 53 isolados foram genotipados usando um painel de oito locus MIRU-VNTR, tendo-se obtido dez perfis diferentes INMV, sendo INMV2 e INMV3 os mais predominantes em isolados de amostras bovinas e INMV16 em caprinas. Posteriormente, a análise de 13 SNPs foi usada para genotipagem de 21 isolados (11 de bovinos e 10 de caprinos), indicando que todos os isolados pertencem ao tipo C e subgrupo A, e permitido a diferenciação dos isolados de bovinos em duas linhagens, designadas por Clade4 e Clade11, e os isolados de caprinos em apenas uma linhagem, Clade3. Este resultado evidencia que os isolados de Map de origem caprina pertencem à mesma linhagem clonal, sugerindo a possibilidade de transmissão horizontal entre os animais. Este estudo focou-se no isolamento, identificação e caracterização de Map em explorações agrícolas portuguesas, suportando a necessidade de mais estudos de investigação de modo a ter acesso à real prevalência da infecção no país.

Palavras-chave: Paratuberculose, Map, IS*900*; VNTR, SNP.

Summary

Production-limiting diseases such as paratuberculosis, caused by Mycobacterium avium subsp. paratuberculosis (Map) have a great impact in the milk industry. In this study, 58 bovine and 17 caprine faecal samples were collected from animals suspected of being infected, based on clinical signs and/or epidemiological data, in order to detect, identify and genotype Map isolates. Samples were tested for bacteriological culture and direct nested IS900-targeted real time PCR assay. Map was isolated from 78.7% of samples and DNA from the agent was detected by PCR in 73.3% of total samples. Isolates were genotyped with a panel of eight MIRU-VNTR loci, revealing ten different INMV profiles, being INMV2 and INMV3 the most predominant in bovine isolates and INMV16 in caprine ones. A subset of 21 isolates (11 from bovine and 10 from caprine) was further genotyped by the analysis of 13 SNPs, which identified two subtypes in cattle isolates, designated Clade4 and Clade11, and one lineage in caprine strains, Clade3, all belonging to the type C and sub-group A. This confirmed that Map strains isolated from caprine samples belong to the same clonal lineage, suggesting the possibility of horizontal transmission. This study focused on the isolation, identification and characterization of Map in Portuguese farms, supporting the need for more investigation in order to access the real prevalence of the infection.

Keywords: Paratuberculosis, Map, IS900, VNTR, SNP.

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1. Introdução

Paratuberculosis is considered worldwide as one of the most important diseases for livestock industry, due to its considerable economic impact triggered by a progressive and fatal weight loss of the animals and a diminution of milk production (Atreya et al., 2014). This widely-recognized chronic enteric wasting disease of domestic ruminants is caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). Transmission is mainly fecal-oral during the animals first months of life, although infectious animals can also shed bacteria in colostrum and milk and in utero transmission has been shown to occur (Atreya et al., 2014; Salem et al., 2012).

In Portugal the true herd-level prevalence of paratuberculosis is unknown and the disease is considered under diagnosed. The seroprevalence of

paratuberculosis at flock/herd level in sheep may be high, with values ranging from 47% to 67% (Coelho et al., 2007). According to preliminary studies about paratuberculosis prevalence in cattle from São Miguel Island (SMI) at the Azores archipelago (Cruz, 2015; Oliveira, 2016), 39 out of 56 studied farms have infected animals and, with the exception of two counties in the Northeast of the Island, all counties have at least one infected farm.

Shedding animals can be detected by the isolation of Map from biological samples such as faeces using specific media supplemented with mycobactin, and by the detection of specific nucleic acid sequences of the agent by PCR assays (OIE, 2014; Salem et al., 2012). The multi-copy IS*900* and the single-copy F57 elements are the most used Map-specific genomic targets for molecular detection (Timms et al., 2011). Map strains with distinct phenotypes and genotypes have been isolated from different host species: type C (type II), associated with multiple host's infections, predominantly cattle; type S (type I and III), associated with primarily sheep and goats, and Bison type associated with buffalo, cattle, goats, humans and other hosts (Stevenson, 2015).

Since shedding contributes to the silent maintenance of the infection cycle of Map in the environment (Leão et al., 2015), the aim of this study was to appraise the presence of this agent in faecal samples of ruminants from a Portuguese dairy production region, in Azores, using culture and molecular approaches, and to characterize a set of isolates by MIRU-VNTR and SNP analysis.

2. Materials and Methods

2.1. Collection of samples

Seventy-five faecal samples, from bovine (n=58) and caprine (n=17) suspected of being infected with paratuberculosis based on serology, histopathology and epidemiological data, were collected directly from each animal's rectum. All caprine samples were collected from the same farm while bovine samples were collected in farms and at the slaughterhouse from São Miguel Island (SMI), at the Azores archipelago, Portugal. Samples were kept at 4ºC and sent to the National Reference Laboratory for Animal Health in INIAV, I.P., in 48-72h, where they were stored at -20ºC until being processed.

2.2. Isolation and identification of Map by culture and F57-targeted real time PCR

Samples were processed for culture assays to detect Map according to the OIE manual (2014) with minor modifications, using 1 g of faeces and 0.9% of hexadecylpyridinium chloride (HPC). Briefly, after decontamination for 18 hours at room temperature, the sediment was carefully transferred to a new tube, washed with 10 ml of sterile distilled water and centrifuged at 900 ×g for 30 minutes. The pellet was resuspended in 500 µL of sterile distilled water and volumes of 100 µL were inoculated on Herrold's egg yolk medium (HEYM) slants with and without mycobactin J. Incubation was performed at 37ºC for up to 6 months. Isolated colonies were confirmed to be acid-fast bacilli by auramine-rhodamine staining (Bird and Madison, 2000). DNA from suspected colonies was extracted by a simple boiling method at 95ºC for 45 minutes.

A new F57-targeted TaqMan-based real time PCR assay for Map identification was designed in our laboratory, in the scope of a PhD thesis (Leão, 2015), using the forward primer F57_F (GCA GCT CCA GAT CGT CAT TC), reverse primer F57_R (GTC CAG TTC GCT GTC ATC GA) and probe TqF57b_FAM (AGC ACG CAG GCA TTC CAA GTC C-BHQ1). Primers and probe were tested for their *in silico* specificity using the Basic Local Alignment Search Tool from NCBI-GenBank (BLAST - http://blast.ncbi.nlm.nih.gov/Blast.cgi) showing 100% identity with the homologous F57 sequences in Map, confirming the specificity of this molecular procedure. To assess the analytical specificity of primers and probes, 19 reference clinical and environmental strains of *Mycobacterium avium* complex (MAC), non-MAC mycobacteria and non-mycobacterial species maintained at INIAV, I.P., were used. The analytical sensitivity of the assay was determined by the construction of a standard curve based on the analysis of 10-fold serial dilutions of Map ATCC 19698^T DNA. The limit of detection (LOD) of the real time PCR was also assessed using faecal Map spiked samples, with ten-fold dilutions of a suspension of either Map type C and type S strains, in a range of 104 to 101 cells per gram of faeces. Each sample was tested in triplicate and the LOD corresponds to the highest dilution at which the assay could not detect a positive result in at least one of the replicates (Leão, 2015). Reactions were carried out in a total volume of 20 µl containing 1× SSO Fast Super Mix (Bio-Rad), 0.4 µM of each primer, 0.15 µM of probe and 5 µl of DNA (5 ng/µL). Thermal cycling, fluorescent data collection, and data analysis were performed in a CFX96 (Bio-Rad) detection system real time PCR instrument under the following conditions: 1 cycle at 95 °C for 2 minutes, followed by 45 cycles at 95 °C for 5 seconds and 60 °C for 10 seconds. DNA extracted from *M. avium* subsp. paratuberculosis ATCC 19698^T was used as positive control.

2.3. DNA extraction from faecal samples and detection of Map by nested IS900-targeted real time PCR assay

DNA extraction from faecal samples was performed using the commercially available Invisorb® Spin Tissue Mini Kit (Stratec Biomedical AG), after previous steps of concentration and mechanical disruption of the cells. Five millilitres of the uppermost suspension obtained in the processing of samples for culture were centrifuged for 20 minutes at 3800 ×g and 4 ml of the supernatant was discarded. The pellet was

resuspended in the remaining 1 ml volume and 250 µL of the suspension was transferred to a new tube. Zirconium beads (1 mm) and 400 µl of the lysis buffer were added followed by mechanical disruption of cells using a FastPrep FP120 Bio101 bead shaker (Savant Instruments Inc., Holbrook, NY) two times at 6.5 msec-1 for 45 seconds. Disrupted samples were cooled on ice for 15 minutes, followed by the addition of 50 µL of proteinase K solution and incubation overnight at 52ºC. The procedure continued according to the manufacturer's instructions. The genomic DNA was eluted with 100 µL of elution buffer and stored at -20ºC until tested.

Map direct detection in biological samples was performed by a novel nested IS*900*-targeted real time PCR assay targeting IS*900*, developed at INIAV, I.P. in the scope of a PhD thesis (Leão, 2015). This system combines a conventional PCR and a real time PCR based on the work of Sidoti et al. (2011) in order to increase diagnostic sensitivity in animal samples (Cunha et al., 2020; Leão, 2015; Leão et al., 2017). For the first amplification the forward EXT-IS*900*-FW (TGA TCT GGA CAA TGA CGG TTA CGG A) and reverse EXT-IS*900*-RV (GGC GTT GAG GTC GAT CGC CCA CGT GAC) primers were designed flanking the Map specific 67 bp IS*900* sequence (Sidoti et al., 2011). For the real time PCR, primers and probe were selected from literature (Sidoti et al., 2011): IS*900*Q F (CCGGTAAGGCCGACCATTA), IS*900*Q R (ACCCGCTGCGAGAGCA), IS*900*Q P (FAM-CATGGTTATTAACGACGACGCGCAGC-TAMRA) and the 224 bp fragment from the first reaction was used as target.

The first amplification reaction was carried out in a 25 µl reaction mix containing 200 µM of each dNTP (Applied Biosystems), 2.0 mM of $MgCl_2$ (Life Technologies), 0.4 µM of each primer, 1 U of Taq DNA polymerase, 1× of the respective buffer (Life Technologies), and 5 µl of the extracted DNA solution. Initial denaturation was made at 94ºC for 3 min, followed by 40 cycles at 94ºC for 45 s, 55ºC for 30 s and 72ºC for 90 s, with a final step at 72ºC for 10 min. The second real time amplification reaction, using as DNA template the 224 bp amplified product from the first PCR, was carried out in a total volume of 20 µl containing 1× SSO Fast Super Mix (Bio-Rad), 0.4 µM of each primer, 0.15 µM of probe and 5 µl of the PCR product obtained in the first reaction. As internal PCR control β-actin gene-targeted probe/primers were used (Costa et al., 2013). Thermal cycling, fluorescent data collection, and data analysis were performed as described above.

2.4. MIRU-VNTR characterization of Map isolates

For the molecular discrimination of a set of 53 isolates (n = 43 bovine and n = 10 caprine), eight VNTR loci were tested: VNTR-292, MIRU-3 (alias X3), VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, VNTR-32 (Thibault et al., 2007). PCR reactions were carried out in a total volume of 25 µl containing 1× buffer (Promega), 200 µM of each dNTP (Promega), 2.0 mM of $MgCl_2$ (Promega), 1 µM of each forward and reverse primers, 2.5 U of GoTaq® DNA polymerase and 1 µl of DNA. Amplification was performed in a thermal cycler with an initial step at 95ºC for 10 minutes, followed by 38 cycles at 98ºC for 10 seconds, 60ºC for 30 seconds and 72ºC for 1 minute, ending with a step at 72ºC for 7 minutes. DNA from Map ATCC 19698^T was used as positive control and amplicon size reference. Allele calling tables available (Castellanos et al., 2010) and the INMV database (http://mac-inmv.tours.inra.fr) were used to assign the estimated size of each PCR product to their correspondent tandem repeat copy number.

2.5. SNP-based genotyping of Map isolates

A set of 21 Map isolates (n = 11 bovine and n = 10 caprine), were randomly selected for SNP-based genotyping, according to Leão et al. (2016). Using a decision tree, the combined results of 14 SNPs can distinguish type C from type S strains; within type S, type I from type III; and within type C, Byson group from sub-group A and from sub-group B. Sub-group A can also be distinguished in 10 clades.

After the purification of amplified fragments containing each SNP, with the NZY Gelpure (NZYTech) kit, sequencing was achieved by GATC Biotech, Germany, with the same primers used in the amplification reaction. Verification of the presence or absence of the SNP in the expected position of the genome was made using BLAST tool. The phylogenetic profile for each isolate was obtained by combining the results for all SNPs.

3. Results

3.1. Isolation and identification of Map by culture and F57-targeted real time PCR

From the 75 faecal samples, 59 (78.7%) showed growth of colonies after two to three months of incubation, suggesting to belong to type C Map strains according to growth characteristics. Fourteen samples (18.7%) were considered culture negative, after six months of incubation, and two (2.6%) were discarded due to the heavy contamination. All the 59 cultures were confirmed to be acid-fast bacilli by auramine-rhodamine staining and identified as Map by the newly designed F57-targeted real-time PCR assay.

The F57 PCR system showed 100% sensitivity and specificity with a limit of detection of one cell per reaction mixture. None of the MAC members, with exception of Map strains, non-MAC mycobacteria or non-mycobacteria yielded any amplification (results not shown).

3.2. DNA extraction from faecal samples and detection of Map by nested IS900-targeted real time PCR assay

When using the nested IS*900* PCR approach 73.3% (55/75) of samples gave a positive result. There was agreement between culture and nested IS*900* real-time PCR in 62 samples (51 positive and 11 negative in both tests). Additionally, 11 samples showed discrepant results between culture and nested IS*900* PCR: three samples were positive in PCR, but negative in culture and eight samples were negative in PCR but positive in culture. One of the two samples that were heavily contaminated and discarded in the culture assay was positive in nested IS*900* real-time PCR.

To discard the presence of inhibitors, co-amplification of the β-actin gene was used, as an internal control in the real time PCR reactions. Amplification of this target was observed in all samples confirming the absence of inhibition.

3.3. MIRU-VNTR characterization of Map isolates

Based on the size of the amplified fragments analysed by agarose gel electrophoresis, the number of repeats and INMV profiles were assessed (Table 1). Ten different profiles were obtained for all 53 tested strains, according to the INMV database, being the INMV3 (28%), INMV2 (24%) and INMV16 (19%), the most common ones in isolates from bovine samples. Therefore, INMV16 was obtained in all isolates from caprine faeces, collected from the same farm.

3.4. SNP-based genotyping of Map isolates

To evaluate the performance of SNP-based genotyping for the discrimination of Map strains, 11 isolates from bovine and 10 from caprine samples, randomly selected, belonging to seven INMV profiles (INMV3, INMV74, INMV2, INMV117, INMV141, INMV14 and INMV16), were further characterized according to Leão et al. (2016). Since snp3842359 can distinguish between type I and III, and all Map isolates belong to type II, this SNP was not tested. The 11 bovine isolates from six INMV profiles were distributed in two clades: two isolates belong to Clade 11 and the remaining to Clade 4. All the caprine isolates were characterized in the same INMV profile (INMV16) and in the same SNPs clade (Clade3), suggesting to have the same clonal origin (Table 1).

Tabela 1 - Genotyping results based on VNTR and SNPs analysis of, respectively, 53 and 21 Map isolates.

N. of Isolates	VNTR Loci								INMV profile	SNPs assay*	
	Number of repeats										
	292	X3	25	47	3	7	10	32		N. of Isolates	SNP Clade
15	3	2	3	3	2	2	1	8	INMV3	4	Clade 4
8	3	3	3	3	2	2	2	8	INMV74	2	Clade 4
13	3	2	3	3	2	2	2	8	INMV2	2	Clade 11; Clade 4
1	3	2	3	2	2	2	2	8	INMV117	1	Clade 4
2	3	3	3	3	2	2	1	8	INMV140	0	ND
1	2	2	3	3	2	2	2	8	INMV13	0	ND
1	4	2	3	2	2	2	2	8	INMV141	1	Clade 11
1	3	2	3	3	2	1	2	8	INMV6	0	ND
1	4	3	3	3	2	2	2	8	INMV14	1	Clade 4
10	3	2	3	3	2	5	2	8	INMV16	10	Clade 3#

Legend: ND – not determined; * within seven INMV profiles a set of 21 isolates was randomly selected for the SNPs assay; # previously published by Leão et al. (2016).

4. Discussion

In 2018, the global Portuguese milk production reached 19.8 millions of hectolitres, 95% of which was from bovine origin. According to the National Statistics Institute (INE), 34% of the global milk production was from Azores region, being SMI the one with the highest production. During the past decades, the economic impact of paratuberculosis in cattle industry has been attributed to direct and indirect losses in dairy herds (EFSA, 2017; Garcia and Shalloo, 2015; Kirkeby et al., 2019; McAloon et al., 2016). Despite the decrease of milk production, paratuberculosis also affects milk quality by increasing the incidence of mastitis and by changing the protein and fat contents.

According to Garcia and Shalloo (2015), the majority of countries do not report the true prevalence of paratuberculosis at animal and herd level,

suggesting that the disease, although endemic all over the world, is largely underreported. A recent study, spanning from 2012 to 2018 (Whittington et al., 2019), comparing the control of paratuberculosis in 48 countries, in different animal species, concluded that in 60% of them the prevalence was underestimated (29/48 countries). In Portugal, paratuberculosis is not a notifiable disease and, for that reason, the real prevalence is not known. However, this scenario is likely to change soon. In fact, the EU Animal Health Law, to be implement in 2021 (EU Regulation No 2016/429), advocates the evaluation of paratuberculosis in each country and in different animal species, based on disease profile and impact, prevention and control rules. This will enable the eligibility for categorisation and notification of the disease (EFSA, 2017; Whittington et al., 2019). To be prepared for these challenges it is essential to establish a specific, sensitive, rapid and cost-effective approach for paratuberculosis diagnosis.

Aiming to implement an efficient and rapid approach to detect Map in animal faeces, we previously optimized a real time PCR assay (Cunha et al., 2020; Leão, 2015) using a published IS*900*-targeted hydrolysis (TaqMan) probe, and respective flanking primers (Sidoti et al., 2011), fully validated for use with human biopsy samples. However, faeces are challenging biological matrices for molecular detection of Map due to the presence of PCR inhibitors, such as phytic acid and polysaccharides, and to the large amounts of nucleic acids from other bacteria and host cells (Leite et al., 2013). When using an optimised DNA extraction procedure associated with the nested IS*900*-targeted real time PCR approach it allowed an increase in the diagnostic sensitivity and specificity (Leão, 2015). The IS*900* target was selected since it exists in multi-copy sequences in the Map genome, which further increases the sensibility of the method. However, some reports describe the occurrence of IS*900*-like sequences in non-Map mycobacteria (Cousins et al., 1999; Englund et al., 2002) what could raise some concerns about the specificity of IS*900*-targeted molecular assays for detecting Map. Nonetheless, as far as we know, the occurrence of these IS*900*-like elements among non-Map mycobacteria seems to be very rare (Rindi et al., 2014), with only very few sequences disclosed in public databases such as GenBank-NCBI. We believe that the rare occurrence of these IS*900*-like sequences does not pose significant specificity concerns when using IS*900*-targeted assays for the detection of Map in faeces. Furthermore, alignments of the 67 bp amplified fragment with IS*900* sequences available in NCBI database, using BioEdit software and BLAST tool, confirmed 100% homology with Map IS*900* sequence, while with IS*900*-like sequences the homology was lesser.

According to our results, Map is widespread in cattle farms from SMI and is being shed in faeces, perpetuating the infection among animals sharing geographically close grazing regions. It was found that the most prevalent INMV profile in isolates from bovine samples was INMV2 and INMV3. All isolates from caprine origin have been characterized as belonging to INMV16 profile, suggesting the existence of a clonal lineage and a horizontal transmission of the microorganism. However, in this work data from only a single farm is reported and more studies are needed in order to characterize the presence of Map in goat farms. These results were corroborated by SNPs analysis that enabled the characterization of isolates in three phylogenetic profiles. In the future, systematic confirmatory diagnosis is needed to assess the real prevalence of paratuberculosis in Portugal, especially in regions of relevant milk industry, enabling impact studies and implementation of control programs.

This study focused in the isolation and characterization of Map in Portuguese ruminants from Azores archipelago, showing that paratuberculosis is insidiously present in Portugal and that a throughout evaluation of its prevalence is needed.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Author Contributions

CP and AB worked in the conception, design of the study and interpretation of data; CL, AO and CC contributed with the lab work and acquisition, analysis and interpretation of data; all authors contributed in the analysis and interpretation of data; CL and AB contributed in drafting the article. All authors contributed and approved the final version of the article.

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