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Molecular Identification of the Antibiotic Producing Bacteria, *Burkholderia Cepacia* Complex, by Means of Real-Time PCR

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ABSTRACT

Molecular techniques, being fast and precise, have been used in the taxonomic research of many bacterial genera. The identification of a very heterogeneous group of species, such as the *Burkholderia cepacia* complex (Bcc) and related genera, through basic microbiology techniques, have been insufficient. In recent years, however, several studies have identified the biotechnological potential of (Bcc). Thus, generating techniques such as real-time PCR to be used as a definitive typing tool has been of utmost importance in research. The main objective of the present investigation was to identify bacteria producing (Bcc) antibiotics molecularly, through the analysis of the RecA region by means of real-time PCR. To fulfil with the objective, DNA extraction and purification methods were analyzed statistically: High Pure Template Preparation Kit-ROCHE, boiling and PCI, by means of ANOVA and Tukey, determining a significant difference ($p<0.001$); The ROCHE kit methodology presented the best DNA quality and concentration results. The boiling method is shown here, as a low-costalternative to show DNA with acceptable characteristics and quality for the real-time PCR technique. For molecular identification, amplification of the 16S regions rRNA-Bcc, *Burkholderia* sp. (RecA) and *B. cepacia*-genomovar I (RecA) using specific primers took place. The analysis of the amplification curves obtained by real-time PCR confirmed that BC1 belongs to the species *Burkholderia cepacia* (genomovar I); afterwards, it was confirmed that BC2 belongs to the genus *Burkholderia*, but the genomovar of the organism was not corroborated; however it is asserted that the strain belongs to some genomovar of Bcc based on the amplification of the 16S region for Bcc and its biochemical identification.

Keywords: *Burkholderia cepacia* complex; RecA gene, genomovar; molecular detection; Real-time PCR

Abbreviations: Bcc: *Burkholderia cepacia* Complex; PCI: Phenol-chloroform-isoamyl Alcohol; PCR: Polymerase Chain Reaction; P16S: 16S rRNA-Bcc Regions; PSPB: *Burkholderia* sp. (RecA); PBC1: *B. cepacia*-genomovar I (RecA); RFLP: Restriction fragment length polymorphism

INTRODUCTION

Burkholderia cepacia is a gram negative non-fermenting bacillus (BGNNF) identified as a phytopathogen and currently recognized as an important opportunistic pathogen. It comprises a very heterogeneous group of phenotypically similar but phylogenetically distinct species (genomovars) until now a total of 20 genomovars have been described that makeup what is called the *Burkholderia cepacia* complex (Bcc). The basic microbiology techniques have been insufficient to generate an accurate diagnosis in the identification of genomovars of Bcc and related genera, taking the PCR technique as a definitive typing tool [1,2].

The molecular identification techniques in bacteria have led to the search for candidate genes (5S, 16S, 23S rRNA) and their intergenic spaces to be used in the taxonomic research of many bacterial genera. However, the analysis of 16S rRNA to establish phylogenetic differences within the Bcc is limited. An alternativeis the amplification of the RecA gene fragments, which presents sufficient nucleotide variation to allow such discrimination between their variants [3,4].

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In recent years, genomovars belonging to the Bcc have been of great importance in studies relating to agriculture, as biological control agents with antifungal activity, improved crop yields, production of antibiotics, bioremediation of landfills, contaminated soils and groundwater; becoming organisms with a high biotechnological potential [1].

The present investigation identifies molecularly Bcc bacteria [*Burkholderia cepacia* (genomovar I)] through the analysis of the RecA-specific region by real-time PCR; isolated bacteria, characterized morphologically and biochemically from soils of the natural regions of Ecuador, capable of producing antibiotics [5].

MATERIALS AND METHODS

Selection of the sample

The present research study was carried out in the Life Sciences Laboratories of the Universidad Politécnica Salesiana Quito, Sede El Girón. Two bacterial strains capable of producing antibiotics were selected from a total of 27 isolated strains, from soils of the Insular-Galápagos region (Puerto Ayora) and the Sierra-Norte region (Quito-Pichincha) [5].

Analysis of bacterial concentration

Samples BC1 and BC2 were inoculated in TSB medium at pH 7 ± 0.2 for 24 hours at a temperature of 30 ± 2 ° C [5]. Using the McFarland turbidity standards the bacterial concentration of 9 x 108 CFU / mL was calculated.

Extraction of DNA from samples

Using three different extraction methods, genomic DNA was isolated from a total of 60 samples, 10 replicates for each strain and for each methodology.

DNA extraction and purification technique with "High Pure Template Preparation Kit" kit - ROCHE

200 µL of the sample material with 5 µL of lysozyme, was placed in a 1.5 mL Eppendorf tube free of nucleases; mixed and incubated at 37 ° C for 5 minutes to continue with the manufacturer's instructions.

The sample was transferred to a purification tube with its specific filter and centrifugation was performed for one minute at 8000 x g. Finally, 200 µL of pre-heated elution buffer at 70° C was added to the filter tube, centrifuged and stored at -20 ° C for subsequent analyzes [6].

DNA extraction technique by modified boiling

200 µL of the sample material was placed in a 1.5 mL Eppendorf tube free of nucleases; the sample was centrifuged at 10000 x g for 5 minutes and the supernatant was discarded, the sediment was suspended in 200 µL of sterile solution of sodium chloride 0.85%, the mixture was centrifuged at 10000 x g for 5 minutes and the supernatant was discarded.

The sediment was suspended in 200 µL of 1X TE Buffer and incubated at 95 ° C for 25 minutes. It was then centrifuged at 10000 x g for 3 minutes and 200 µL of the supernatant containing the DNA was transferred [7].

DNA extraction technique with organic solvents Phenol / Chloroform /Modified Isoamyl alcohol

200 µL of the sample material was placed in a 1.5 mL Eppendorf tube free of nucleases, the sample was then centrifuged for 2 minutes at 10000 x g. the supernatant was discarded. The obtained pellet was then mixed with 576 µL of TE 1X buffer, 30 µL of 10% SDS and 3 µL of Proteinase K (20 mg/mL), 100 µL of 5M Sodium Chloride and 80 µL of CTAB / NaCl. This was then incubated for 10 minutes at 65° C.

Subsequently, 710 µL of chloroform/isoamyl alcohol (24:1) was added, mixed and centrifuged for 5 minutes at 10000 x g. The aqueous phase was transferred to a new microcentrifuge tube and an equal volume of phenol/chloroform/isoamyl alcohol (25: 24: 1) was placed, centrifuged for 5 minutes at 10000 x g.

The supernatant was transferred to a new microcentrifuge tube and 0.6 volumes of absolute ethanol were added, the microcentrifuge tube was vortexed and the supernatant was removed. This was then followed by placing 400 µL of 70% ethanol, after a mild vortexing , it was centrifuged for 5 minutes at 10000 x g, the supernatant was carefully discarded and the pellet was allowed to dry briefly at 65 ° C, the dry contents of the tube were dissolved in 200 µL of TE 1X Buffer [8].

Quantification and purity of DNA

The DNA concentration was quantified in a Qubit 2.0 Fluorometer® kit, using the kits: Qubit dsDNA HS Assay Kit (0.2-100 ng) and Qubit dsDNA BR Assay Kit (2-1000 ng) [9]. The purity of the sample was examined by the absorbance ratio at 260 nm and 280 nm on a NanoDrop computer [10].

The values of DNA concentration (ng/mL) obtained were subjected to a logarithmic transformation of base 10. Logarithmic transformations are usually necessary and appropriate to analyze variables related to the growth of organisms [11].

Real-time PCR technique

The DNA extracted from the sample BC1 and BC2 was amplified with a reaction volume for capillaries with a capacity of 20 µL; each reaction was carried out with 5 µL per sample and controls, 10.2 µL of Molecular Biology grade water, 0.4 µL of the First FW, 0.4 µL of the First RW

and 4 µL of the Master Mix SYBR Green I [12]. Two controls were used in the amplification run: a negative control PCR grade water and a positive control *Burkholderia cepacia* ATCC®25608.

The 16S rRNA Bcc (P16S) primers used were, F: 5'-GACTCCTACGGGAGGCAGCAG-3' and R: 5'-CTGATCCGCGATTACTAGCGATTC -3' [13].

Burkholderia sp. (RecA) (PSP), F: 5'-GTCGGGTAAAACCACGCTG -3' and R: 5'-TCCGCAGCCGCACCTTCA -3' [14]. *B. cepacia*-genomovar I (RecA) (PBC1), F: 5'-CAGGTCGTCTCCACGGGT -3' and R: 5'-CACGCCGATCTTCATACGA -3' [13].

The real-time PCR reactions were carried out in the LightCycler 2.0 device, according to the protocol of LC FS DNA MasterPLUS HY-Pb, 96 reactions. LightCycler (Roche Diagnostics), consisting of 35 cycles composed of four steps: Denaturation: 95 ° C, 10 min; Alignment: 62 ° C, 10 sec; Extension: 72 ° C, 7 sec; Cooling: 40 ° C, 30 sec [12].

STATISTIC ANALYSIS

DNA concentrations (ng/mL) were compared using the parametric statistical test DCA - ANOVA together with a Tukey analysis. The results were processed with the help of the statistical package InfoStat. Values of *p* less than 0.01 were considered significant.

RESULTS AND DISCUSSION

Quantification and purity of DNA

The total concentration and purity of the DNA for each method are described in **Table 1**. The three extraction methods resulted in a good amount of DNA. Bacterial DNA is worked with a sample of up to 1 to 10 ng, taking into account that the excess in the concentration of DNA mould in the PCR can lead to non-specific amplifications or failure to amplify [15]. A greater quantity of DNA was obtained by the Roche commercial method with an average of 6.205E+3 ng/mL, followed by the boiling method with 2.405E+3 ng/mL and in lesser quantity with the PCI method with 274.5 ng/mL.

Table 1. Concentration and purity of DNA obtained in strains BC1 and BC2 for each methodology

TREATMENTS	Time/sample	Sample	DNA Concentration (ng/mL)	DNA Purity (A $_{260}$ nm/A $_{280}$ nm)
Extraction Kit	30 min.	BC1	8,80E+3	1,70
		BC2	3,61E+3	1,71
Boiling extraction	45 min.	BC1	2,40E+3	1,72
		BC2	2,41E+3	1,68
PCI extraction	90 min.	BC1	2,45E+2	1,81
		BC2	3,04E+2	1,81

The analysis of variance together with the Tukey test, evaluated the best DNA extraction technique for each sample as shown in **Figure 1**, the commercial extraction method ROCHE tested in strains BC1 and BC2 showed a significant difference compared to the means of the boiling and PCI methods.

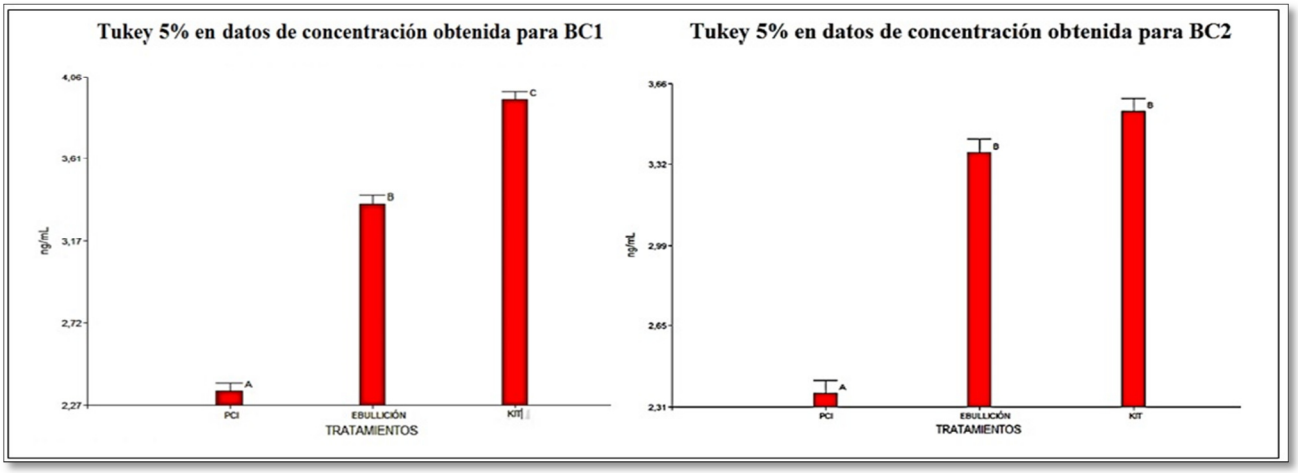

Figure 1. Statistical analysis of DNA concentrations obtained in each extraction methodology for BC1 and BC2

The purity range of the DNA (A260/A280) was between 1.81 and 1.68. For strain BC1, the three methodologies allowed the obtention of good quality DNA; for strain BC2, the commercial methodology ROCHE and PCI allowed to also obtain good quality DNA, however, it is observed that in the boiling technique there is a slight contamination with proteins. The use of traditional methods has advantages in terms of low costs of reagents and materials, as well as obtaining a DNA with high performance, however, sometimes the material obtained is fragmented because the methods can be susceptible to contamination, variations and errors due to the multiple handling steps [16].

In order to carry out new molecular techniques such as real-time PCR, the obtention and usage of a high-quality DNA template sequence (integral and pure) is essential. Therefore, making the correct selection and application of an appropriate methodology is an integral part of DNA extraction [17-18].

Molecular identification of *Burkholderia cepacia* by real-time PCR

Figure 2. shows the amplification products for the strain BC1, strain identified biochemically as *Burkholderia cepacia* with an 88.79% probability. The amplification showed hybridization with the first primer PSP, specific for *Burkholderia* sp., corroborating the genus of the species and with the first primer PBC1, specific for *Burkholderia cepacia* (Genomovar I), confirming the species.

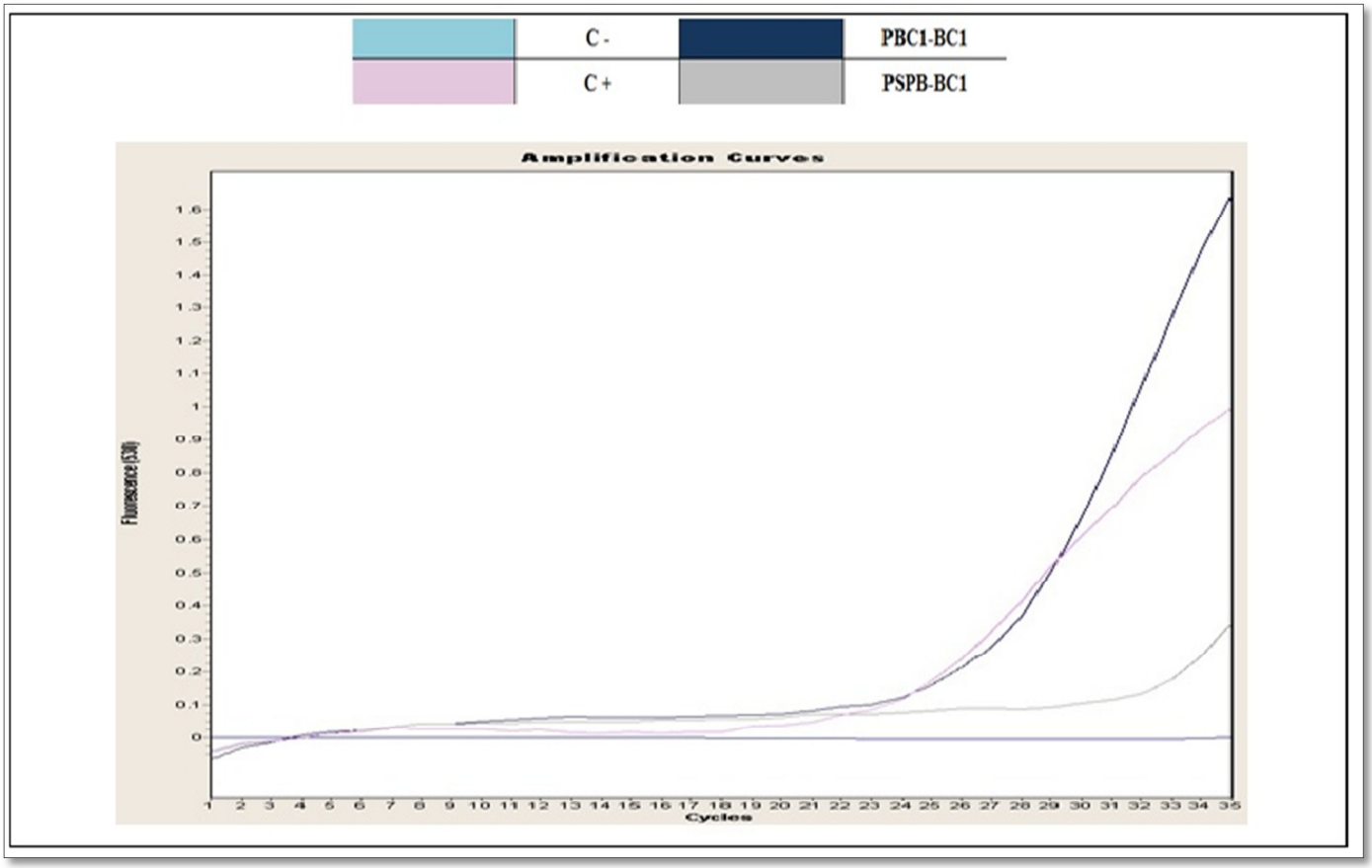

Figure 2. Real-time PCR amplification curve - SYBR Green with strain BC1: fluorescence vs. cycles. C- .: negative control; C +: positive control.

In **Figure 3.** We see the amplification products for the strain BC2, strain identified biochemically as *Burkholderia cepacia* with a 99.51% probability. The amplification did not show hybridization with the first specific primer PBC1 for *Burkholderia Cepacia* (Genomovar I), however, the amplification with the first primers PSPB and P16S, based on these results, was corroborated by the technique of PCR in real time that the strain BC2 It belongs to the genus *Burkholderia*, however, it is not identified as the subspecies *Burkholderia cepacia* (genomovar I), so it is believed that this strain could be located at a different genomovar belonging to the heterogeneous group of Bcc.

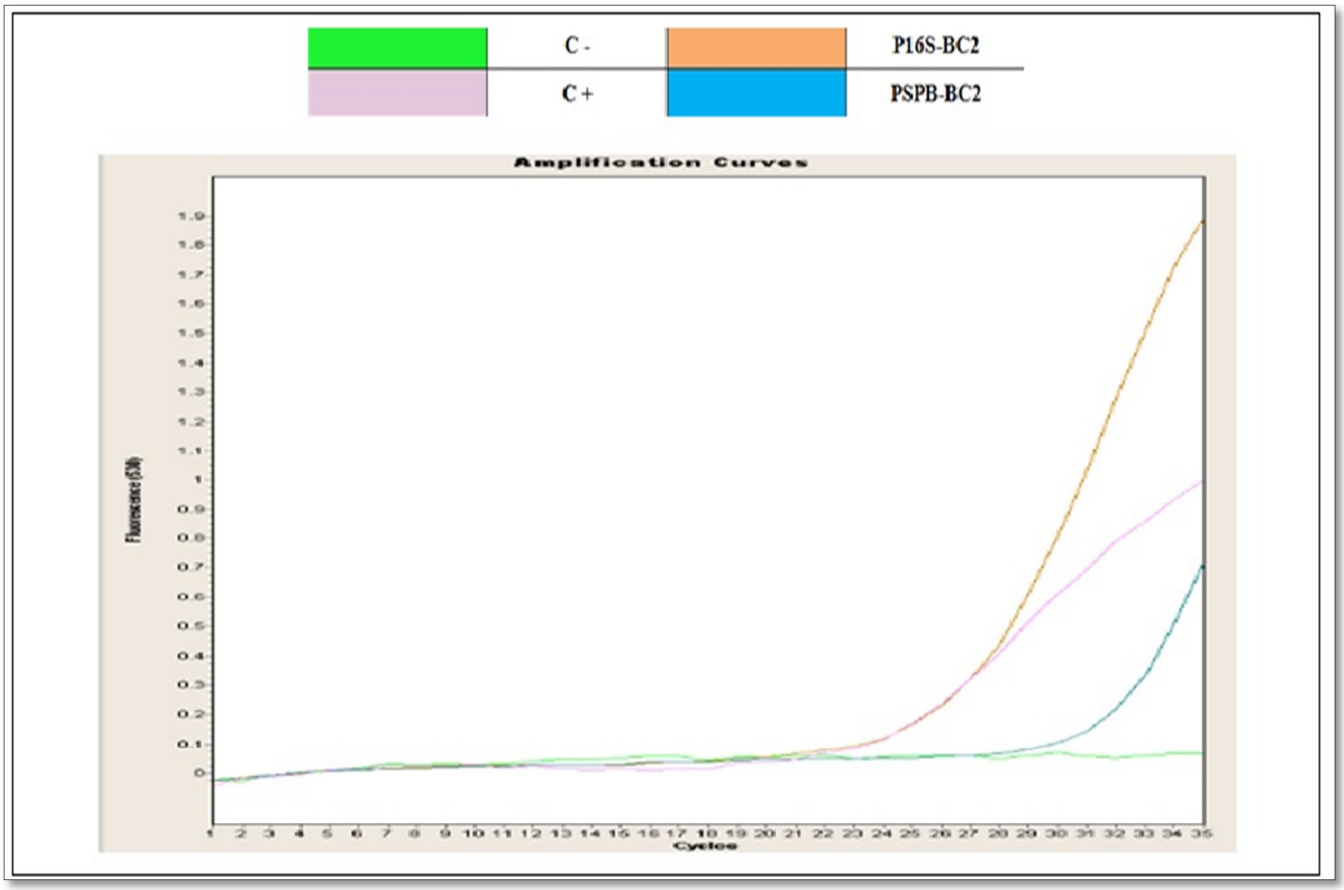

Figure 3. Real-time PCR amplification curve - SYBR Green with strain BC2: fluorescence vs. cycles. C- .: negative control; C +: positive control.

The identification of *B. cepacia* is a complex task. This complexity is due to the close phylogenetic relationship of *B. cepacia* with other genera of BGNNF since it is not a single species, but a heterogeneous group [1,2] . In the study carried out by Canale [3], he argues that commercial systems of phenotypic identification have significant variations in their ability to accurately identify Bcc since they can not differentiate the different genomovars, which is why several studies have made erroneous identifications of *B. cepacia*. In their study, only 1 of 23 phenotypically analyzed samples tested positive for *B. cepacia* (4%), by molecular analysis (PCR-RFLP) 11 of the 23 samples gave positive results for *B. cepacia* (48%).

Araque et al. [1] concludes that the use of conventional biochemical methods, complementary biochemical tests, and non-commercial systems do not allow for correct and clear species-level discrimination within the Bcc, even though the analysis of the methods showed moderate sensitivity and specificity ranges compared to other studies previously reported. However, the use of these methods allow a good identification of *B. cepacia* between isolates of BGNNF, it is also mentioned that to achieve a correct identification of the strains it is necessary to accompany the phenotypic methodology with molecular techniques such as real-time PCR (PCR-RFLP), technique that with the use of specific primers allows a high discriminatory range. Therefore the relationship between the biochemical methods (conventional tests and galleries) with the results of PCR, demonstrates the high level of resolution of this methodology.

The study carried out by Dalmastri et al. [19] concludes that the RecA species-specific PCR technique for the identification of Bcc environmental isolates, as *B. cenocepacia*, may lead to an underestimation of the organisms belonging to the complex. The use of isolates from different microbial populations, different geographical locations and sampling time can present differences in the sensitivity of the method, causing a bias in the analysis of the populations. However, it explains that this decrease in sensitivity is not sufficient to establish a deficiency of the method since it can depend also on the variability of the RecA sequence. In addition, it mentions that the application of the RecA species-specific technique associated with different patterns of the RFLP technique increases the sensitivity of the method. Therefore it considers the analysis RecA-RFLP useful, fast and precise in the identification of

the complex in clinical isolates. Therefore, the evaluation and optimization of new identification tests for Bcc species should be performed not only in clinical isolates but also in environmental isolates in order to improve the detection of these strains in natural habitats.

CONCLUSIONS AND RECOMMENDATIONS

The comparison of three methods of extraction of bacterial genomic DNA concluded that the best results were achieved through the kit protocols and boiling. In this case, boiling is presented as a viable alternative to the more expensive commercial protocols: besides presenting DNA of good quality (pure DNA) and adequate concentration values, the extraction time is relatively short. Current molecular biology techniques such as real-time PCR do not demand large amounts of DNA because it is a sensitive methodology, but it does demand integrity and purity [20].

The molecular technique of PCR in real time, through the analysis of the amplification curves of the region RecA-specific in *Burkholderia cepacia*, confirmed that strain BC1 belongs to the species *Burkholderia cepacia* (Genomovar I). In turn, it was confirmed that the BC2 strain belongs to the genus *Burkholderia*, but not corroborated the genomovar of the organism. However, it was asserted that the strain belongs to the group of the complex *Burkholderia cepacia* based on the amplification of the 16s region for Bcc and in its biochemical identification.

It was determined that the real-time PCR technique, through the analysis of the region, allows the molecular identification of bacterial strains, confirming with precise data biochemical tests that do not allow a clear discrimination.

It is recommended to evaluate and standardize the technique of DNA extraction by boiling in Gram-negative bacteria. In this case, the results obtained from DNA quality in bacteria of the genus *Burkholderia* were optimal and suitable for molecular analyses as real-time PCR, in addition, it is presented as a viable alternative against high-cost commercial methods.

It is necessary to evaluate the strains identified as *Burkholderia cepacia* (Genomovar I) and *Burkholderia* sp., belonging to the Bcc, by using the polymorphism technique in the length of the restriction fragments (RFLP) of the RecA gene. This is a fast and precise technique, which increases the sensitivity of the species-specific PCR RecA, in order to identify and corroborate with the results obtained, and in turn, improve the detection of isolated strains in natural habitats.

Finally, the antibiotics produced by the strains belonging to the *Burkholderia cepacia* complex must be analyzed and chemically identified, since these bacteria have proven to possess a high biotechnological potential.

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