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## **Direct detection of *Burkholderia cepacia* in susceptible pharmaceutical products using semi-nested PCR**

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## Abstract

*Burkholderia cepacia* (*B. cepacia*) has recently received a considerable attention as one of the major risks in susceptible pharmaceutical products. This microorganism can easily propagate and cause vast and severe contamination especially to the water supplies for pharmaceutical companies. Moreover, it proliferates within the products and can cause severe infections for humans. Therefore, fast and sensitive detection of these bacteria is of a great demand. The present study introduces improved application of PCR assay with relatively high sensitivity and specificity for the direct detection of *B. cepacia* from the aqueous pharmaceutical products. A semi-nested PCR (SN-PCR) approach using the primer set BCR1/BCR2 followed by BCR1/Mr yielding a 465-bp fragment of the *recA* gene was applied and tested using both crude lysate from isolated colonies and DNA directly extracted from artificially prepared and spiked syrup. The PCR assay showed no interference with other bacterial reference and environmental strains tested including: *Staphylococcus aureus* ATCC<sup>®</sup> 6538, *Pseudomonas aeruginosa* ATCC<sup>®</sup> 9027, *Escherichia coli* ATCC<sup>®</sup> 8739, *Salmonella abony* NCTC<sup>®</sup> 6017 and *Bacillus subtilis* ATCC<sup>®</sup> 6633, *Micrococcus luteus*, *Staphylococcus warneri*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Ralstonia pickettii*. Moreover, this semi-nested assay showed detection limit of around 10 cfu/sample and could detect *B. cepacia* strains isolated from municipal pre-treated potable water tank. Comparing the results for detection of *B. cepacia* in one hundred of randomly collected commercial syrup preparations using both conventional standard method and PCR assay revealed that, *B. cepacia* was detected in 2 samples using PCR assay while all samples showed negative results by conventional culturing and biochemical methods. These results highlights the advantage of using this PCR assay to detect *B. cepacia* in contaminated pharmaceutical products and even water for pharmaceutical purposes, without the need of culturing or pre-enrichment, where it may give false negative results and may be misidentified when biochemically tested.

**Keywords:** *Burkholderia cepacia*; semi-nested PCR; syrup preparation; conventional method; Vitek compact system; non-sterile preparations

## 1. Introduction:

*B. cepacia* is a pathogen that can infect immunocompromised populations which include elderly people, pregnant women, young children and people suffering chronic illness. However, *B. cepacia* is less frequently associated with illness in healthy non-immunocompromised patients (1).

*Burkholderia cepacia* complex (Bcc) comprises a group of related bacterial species widely distributed in nature and artificial habitats (2). They are Gram-negative, motile, aerobic bacteria with non-fermenting properties. They live in nature especially in soils, water and botanical products (3). They are multi-drug resistant organisms that can resist many disinfectants, cleansers, antiseptics and is not affected by many preservatives. Bcc has also a great ability to form biofilms and contaminate plastics, metals, water systems and consequently pharmaceutical facilities. There is a major risk on the pharmaceutical products and the patients when the process water used in pharmaceutical manufacturing is contaminated with Bcc (4).

Each pharmaceutical company is responsible for developing its own microbial specifications regarding the non-sterile products produced in its facility (5). The USP “Microbial Enumeration Tests” and “Tests for Specified Microorganisms” provide methodology for selected indicator microorganisms, but not all objectionable microorganisms (6, 7). It is well known that Bcc is objectionable if found in preparations to be inhaled or even administered locally using the nasal route as well as topicals used on broken skin, however, the USP chapters do not provide any detection or identification methods for *Burkholderia cepacia* (8).

According to the FDA findings, Bcc had contaminated products, even in presence of one or more antimicrobial preservatives. FDA faced an unusual case of contamination, in that ten lots of incoming

solution passed initial release testing for bioburden. Later samples taken from these same lots of bulk solution, showed failing levels of Bcc indicating that the bacteria was proliferating in the solution, therefore, reliance on conventional testing of finished products has not been successful for detecting and eliminating Bcc contamination hazards (9,1) However, the pharmaceutical manufacturer is responsible for controlling the drug manufacturing process to exclude potentially harmful microorganisms from entering the process. For this reason, research is needed to develop reliable methods for the detection of Bcc in pharmaceutical products; especially that Bcc has the potential for nutrient shock during conventional cultivation, giving false-negative results (1).

Although, the taxonomy studies of *B. cepacia* has improved its identification (10), differentiation of *B. cepacia* species from other related taxa, such as *Ralstonia*, *Cupriavidus*, *Pandoraea*, *Achromobacter*, *Brevundimonas*, *Comamonas* and *Delftia* species remains difficult (11).

Numerous advances have been made in the identifications of *B. cepacia* using traditional and molecular techniques; however the great diversity among *B. cepacia* strains limited the utility of the methods applied (12). Among the molecular methods used for identification of *B. cepacia* are the rec A gene based analysis, which was found to be effective for species identification based on 94-95% similarity of the rec A gene between different Bcc species and 98-99% similarity within Bcc species (13). Moore *et al.* (15) has previously developed a semi- nested PCR method based on rec A gene amplification for the direct identification of *B. cepacia* from sputum of patients with cystic fibrosis with a sensitivity reaching  $10^1$  and  $10^2$  cfu/g sputum for genomovars IIIa + b and genomovar II respectively.

Taking into consideration the low level of bioburden that might contaminate aqueous pharmaceutical products during manufacturing and the need of a sensitive method for detection of *B. cepacia* in susceptible products, the aim of the present work was to investigate and optimize the

applicability of a semi-nested PCR (SN-PCR) method based on *recA* gene amplification, that was previously applied for the detection of *Burkholderia cepacia* from sputum samples of cystic fibrosis patients (14), for the detection of *Burkholderia cepacia* in pharmaceutical aqueous syrup preparations without previous culturing or enrichment and to compare the optimized method with conventional methods that are mostly followed in pharmaceutical manufacturing microbiological control laboratories. *B. cepacia* ATCC 25416 and environmental *B. cepacia* isolates from municipal potable water tank were used as reference strains to represent the species that can most probably contaminate aqueous pharmaceutical preparations.

## **2. Materials and Methods:**

Unless otherwise specified, all tests were conducted under aseptic conditions and in triplicate.

### **2.1 PCR-based detection methods**

#### **2.1.1 DNA extraction and purification**

DNA extraction and purification steps were performed using QIAamp<sup>®</sup> DNA Mini kit except for sensitivity and commercial products testing, we used QIAamp<sup>®</sup> DNA Blood Midi kit. DNA purification from bacterial colonies or from inoculated syrup preparations was performed according to the manufacturer's instructions. All protocols followed were spin protocols and the centrifugation steps were carried out using Corning<sup>®</sup> LSE<sup>™</sup> high speed micro centrifuge device for QIAamp<sup>®</sup> DNA Mini kit and Corning<sup>®</sup> LSE<sup>™</sup> compact centrifuge QIAamp<sup>®</sup> DNA Blood Midi kit.

After extraction, the solution containing DNA was measured for DNA content using Thermo Scientific™ NanoDrop and the volume containing 100 – 200 ng (~150 ng) of DNA was used in PCR reaction.

### **2.1.2 PCR reactions mix**

The PCR reaction mix for all implemented PCR reactions was composed of: 12.5 µl Qiagen® Taq PCR master mix kit (equivalent to 1 X concentrate), 1 µl of 25 mM MgCl<sub>2</sub> (equivalent to 2.5mM MgCl<sub>2</sub> in the total reaction), 0.5 µl Primer 1, 0.5 µl Primer 2 (equivalent to 0.2 mM from each primer in the total reaction), 1-5 µl DNA template and complete to 25 µl using Qiagen® RNase free water.

### **2.1.3 Semi-nested PCR (SN-PCR) detection method**

A semi-nested PCR method employing the primer set BCR1/BCR2 followed by primer set BCR1/Mr (Table I) generating an amplicon of 465 bp as previously described (13,14) was carried out. This method was applied on the following standard microorganisms: *Staphylococcus aureus* ATCC® 6538, *Pseudomonas aeruginosa* ATCC® 9027, *Escherichia coli* ATCC® 8739, *Salmonella abony* NCTC® 6017, *Bacillus subtilis* ATCC® 6633 and *B. cepacia* ATCC® 25416, and the following environmental isolates recovered from environmental monitoring program of a pharmaceutical facility and identified using Vitek 2 compact identification system as:

*Micrococcus luteus*, *Staphylococcus warneri*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Ralstonia pickettii*. DNA was extracted from bacterial colonies of the previously mentioned microorganisms. The SN-PCR was carried out in two successive rounds, where 2 µl of the PCR product from the first round was employed as DNA template for the second round, and the thermal cycling conditions were as follows; 1<sup>st</sup> round was 96 °C for 5 minutes, followed by 25

cycles at 96 °C for 1 minute, 58 °C for 1 minute, 72 °C for 2 minutes, followed by final extension at 72 °C for 10 minutes. Second round was 96 °C for 5 minutes, followed by 30 cycles at 96 °C for 5 minutes, 60 °C for 1 minute, 72 °C for 1 minute, followed by final extension at 72 °C for 10 minutes using GeneAmp<sup>®</sup>-PCR system 9700 – Applied Biosystems. PCR products were detected by electrophoretic separation using Biometra<sup>®</sup> XS/S electrophoresis system on 1.5 % Ultrapure Invitrogen<sup>®</sup> agarose gel and Qiagen<sup>®</sup> GelPilot 100 bp Plus ladder at 100 Volt for 30 - 45 minutes. The gels were visualized and photographed using Life Technologies E-gel imager<sup>®</sup>. Also twenty three environmental isolates from municipal pre- treated water tank were tested using SN-PCR assay by extracting DNA from the bacterial cells and were biochemically identified using Vitek<sup>®</sup> 2 compact identification system.

## **2.2 Test samples**

Five litres form an aqueous syrup preparation was prepared as a placebo, sterilized using 0.22 µm membrane filters and stored refrigerated to simulate commercial aqueous syrup samples within the whole study. The preparation composed of the following: Purified water, Quinoline Yellow (D&C Yellow NO10), Ethyl Alcohol, Methyl Paraben, Plasdone K25 (PVP25) (Povidone K25), Propyl Paraben Base, Sorbitol 70/70 % Solution, Sucrose and a flavor (Orange Flavour E9904557). Besides, 100 randomly collected commercial aqueous preparations were included in order to be used during the application of the testing procedures. The commercial preparations were labeled from P1 to P100.

## **2.3 Conventional detection methods**

Conventional method suitability testing was carried out according to the procedures of the recovery in liquid medium by membrane filtration technique in the USP (16,17,18). Two



dilutions were prepared from the placebo preparation and from each of the commercial products (1:10 and 1:100) using USP phosphate buffer solution (PBS) + 4% Tween<sup>®</sup> 80 as a diluent.

The microorganisms used in conventional method suitability testing were as follows: *Staphylococcus aureus* ATCC<sup>®</sup> 6538, *Pseudomonas aeruginosa* ATCC<sup>®</sup> 9027, *Bacillus subtilis* ATCC<sup>®</sup> 6633, *Candida albicans* ATCC<sup>®</sup> 10231, *Aspergillus brasiliensis* ATCC<sup>®</sup> 16404 and *B. cepacia* ATCC<sup>®</sup> 25416, to ensure that the used methods are highly reliable in the recovery of various microorganisms.

Three groups each of 200 ml from the syrup preparation and from each of the commercial products were pooled in sterilized bottles.

For each preparation, 10 ml sample from each of the 1:10 and 1:100 dilution was inoculated with 0.1 ml containing <100 cfu of each microorganism, then samples were filtered through membrane filters of 0.45 µm pore size, rinsed once with 100 ml PBS 4%. The membrane filters were aseptically transferred to Tryptic Soy Agar (TSA) plates for recovery of bacteria and Sabouraud Dextrose Agar (SDA) plates for recovery of *Candida albicans* and *Aspergillus brasiliensis*. A negative control is carried out for each dilution from each bottle used from the preparations or commercial products. The microbial recovery of each dilution for each microorganism used was compared to a positive control containing the microorganism and the media without the product. The lowest dilution factor showing microbial count greater than or equal to 50% of the positive control of each microorganism was the accepted dilution. For recovery of *B. cepacia* membrane filter was aseptically transferred to 100 ml Tryptic Soy Both supplemented with 4% Tween<sup>®</sup> 80 (TSB 4%), incubated at 30 – 35 °c for 18 hours, and then 0.1 ml from the incubated TSB 4% bottles was streaked on TSA, Cefrimide and BBL™

Oxidation/Fermentation-Polymixin-Bacitracin-Lactose (OFPBL). After incubating the TSA and Cetrimide plates at 30 – 35 °c for 18 hours and OFPBL media at 30 – 35 °c for 4 days, growth was recorded and the lowest dilution showing growth and microbial count greater than or equal to 50 % of the positive control was chosen as the accepted dilution.

Each of the artificially prepared and of the commercial samples was tested later using the accepted dilution.

#### **2.4 Testing of filterable pharmaceutical preparations via SN-PCR**

One hundred ml of the artificially prepared syrup were spiked with *B. cepacia* ATCC 25416 (on triplicate basis) and with the environmental isolates that shown to be positive as *B. cepacia* either biochemically or by SN-PCR at an inoculum size of  $10^7$  cfu/100 ml. Bacterial DNA was extracted by filtering the whole spiked dilution through membrane filter, the membrane filter was soaked in 5 ml saline for 30 minutes, and then bacterial genomic DNA was extracted from the saline solution using QIAamp® DNA Mini Kit, following the manufacturer's instructions and was used as template in SN-PCR for identification of *B. cepacia*.

#### **2.5 Sensitivity of SN-PCR versus conventional methods**

*B. cepacia* ATCC 25416 was serially diluted to  $1000 \pm 200$  cfu/0.1ml,  $100 \pm 20$  cfu/0.1ml and  $50 \pm 10$  cfu/0.1ml. Aliquot of 0.1 ml from each serial dilution was used to spike 10 of the accepted dilution identified by the conventional method suitability testing of the prepared syrup. Each of the spiked dilutions was tested by membrane filtering of the whole volume, transferring the membrane filters on TSB 4%, incubated at 30-35 °c for 24 hours and then streaking 0.1 ml on TSA, Cetrimide agar and OFPBL agar.

Ten groups each of 100 ml of the prepared syrup (without being diluted) were spiked with 0.1 ml containing around 10 cfu (10 – 15 cfu) of *B. cepacia* ATCC 25416 as counted on OFPBL agar, then the bacterial DNA was extracted and purified directly from the product as previously described with the following modifications of using 5 ml Qiagen<sup>®</sup> tissue lysis (ATL) buffer for soaking the membrane filters, using Qiagen<sup>®</sup> proteinase K instead of Qiagen<sup>®</sup> protease, incubating the sample with ATL buffer and proteinase K at 56 °C for 3 hours before adding Qiagen<sup>®</sup> lysis (AL) buffer, warming Qiagen<sup>®</sup> elution (AE) buffer to 60 °C and following the steps of purification of DNA from whole blood using the QIAamp<sup>®</sup> Blood Midi Kit (Spin Protocol). During the elution phase, 100 µl of the warmed AE buffer was added to the QIAamp<sup>®</sup> Midi column, was left for 10 minutes before starting elution centrifugation, re-elution with the same eluate and finally elution with another 100 µl of the AE buffer. The final eluate was evaporated to around 50 µl using Thermoscientific<sup>®</sup> heat block at 60 °C with frequent vortexing to increase the DNA concentration in the eluate, and then SN-PCR method was applied as previously described.

## **2.6 Testing commercial products by SN-PCR and conventional method**

One hundred aqueous commercial products collected randomly from the market were tested by both conventional methods and SN-PCR for the detection of *B. cepacia*. One hundred ml of each product were used, half of the product's volume was diluted to the suitable dilution factor and was membrane filtered, membrane was rinsed with 100 ml 4% PBS. Ten ml of the membrane filtered solution was aseptically transferred to TSB 4%, incubated at 30 – 35 °C for 24 hours. One hundred microliter was then streaked on TSA, Cetrinide agar and OFPBL agar. All recovered colonies were identified via biochemical reactions using Vitek 2 compact system.

The other half of each product was filtered undiluted and the same steps of SN-PCR sensitivity tests were followed.

### **3. Results**

#### **3.1 SN-PCR detection method**

Only *B. cepacia* ATCC 25416 gave positive results and showed the characteristic band at 465 bp, while other reference strains and environmental isolates showed negative results (Figures 1 A and 1 B). Screening of 23 environmental isolates from municipal potable water tank using SN-PCR and Vitek<sup>®</sup> 2 compact identification system, revealed that, only 4 out of the 23 isolates were identified as *B. cepacia* using Vitek system, while 6 showed positive results for *B. cepacia* using SN-PCR (Table II) including those identified as *B. cepacia* using Vitek<sup>®</sup> 2 compact identification system.

#### **3.2 Conventional detection methods**

The triplicate samples of the 1:10 dilution of the prepared syrup showed successful count recovery for all the microorganisms tested and showed the expected positive growth on TSA, Cetrimide agar and OFPBL agar for *B. cepacia* which was successfully identified using Vitek<sup>®</sup> 2 compact identification system. Ninety eight of the 1:10 dilution of the commercial samples showed the expected recovery and *B. cepacia* positive results and only 2 preparations (P45 and P57) showed the accepted results with the 1:100 dilution.

### 3.3 Testing of filterable pharmaceutical preparations via SN-PCR

All the samples inoculated either with *B. cepacia* ATCC or with environmental *B. cepacia* isolates showed positive results using SN-PCR by directly from the products without the need for pre-enrichment steps (Figures 2 A & B).

### 3.4 Sensitivity of SN-PCR versus conventional methods

All the samples spiked with either  $1000 \pm 200$  cfu/0.1 ml or  $100 \pm 20$  cfu/0.1 ml showed positive results on TSA, Cetrimide agar and OFPBL agar (Table III A & III B). The samples spiked with  $50 \pm 10$  cfu/0.1 ml and tested conventionally via normal culturing methods showed 5 negative results as shown in (Table III C). No further dilutions were used due to the negative results shown.

The samples spiked with around 10 cfu showed positive results for *B. cepacia* upon testing via SN-PCR (Figure 3).

### 3.5 Testing commercial products by both conventional and SN-PCR

All aqueous commercial products did not show positive results for *B. cepacia* when tested conventionally, while 2 products namely; P57 and P82 showed positive results for *B. cepacia* when tested via SN-PCR (Figure 4).

## 4. Discussion

*Burkholderia cepacia* is an opportunistic pathogen that causes diseases primarily among immunocompromised populations. Among the most serious conditions caused by *B. cepacia*, are

pneumonia or bacterial infection that occurs in patients with impaired immune systems or chronic lung disease, particularly cystic fibrosis (CF) (18).

*B. cepacia* are among the most antimicrobial agent-resistant organisms. They have ability to grow in low-nutrient conditions (19), and in presence of chemical preservatives (20). Because the most common source of contamination is water, aqueous products are especially at risk because of *B. cepacia's* ability to remain viable in harsh conditions (1).

Since the organism may grow poorly or not at all when transferred from water (aqueous) systems to high-nutrient culture media, testing of finished product by conventional methods can lead to misleading, false-negative results (1). Although the use of certain specialized, low nutrient media could provide better results, the use of molecular methods for identification remains better.

In our study, we aimed at investigating & optimizing the detection of *B. cepacia* from aqueous pharmaceutical products using SN-PCR. We first examined the specificity of SN-PCR using DNA from selected panel of microorganisms, representing reference indicator strains specified in USP under “Tests for Specified Microorganisms” and environmental strains isolated from environmental monitoring program at a pharmaceutical facility and which would be likely to contaminate pharmaceutical products. Our results demonstrated that neither of the strains was amplified by SN-PCR. Previous studies have shown the specificity of the *recA* primers to amplify Bcc organisms (14). In addition, Moore et al. (15) confirmed the specificity of the primers against several species which may colonize the airways of cystic fibrosis patients. We further showed the ability of the SN-PCR to detect *B. cepacia* strains isolated from municipal pre-treated potable water tank that were not detected using Vitek 2 identification system. To

investigate the applicability of SN-PCR to detect *B. cepacia* in pharmaceutical products, we spiked the accepted dilution of artificially prepared pharmaceutical preparation with an inoculum of  $10^7$  CFU/ml of *B. cepacia* ATCC strain and environmental *B. cepacia* separately with subsequent filtration and extraction of DNA from membrane filters using commercially available DNA extraction kit. The SN-PCR could detect *B. cepacia* in all spiked samples. For testing the detection limit of the method, we adopted a modified method for DNA extraction from biological fluids, that maximized the DNA yield from pharmaceutical preparation and we could detect up to 10 CFU/ preparation. In contrast, conventional method could detect *B. cepacia* in the accepted dilution factor of the preparations spiked with 1000 and 100 CFU but showed 5 negative results in those spiked with 50 CFU. We finally applied the conventional method and the optimized method for the testing of 100 randomly collected commercial preparations. The SN-PCR could detect *B. cepacia* in 2 pharmaceutical preparations that failed to be detected by conventional method.

### **Conclusion**

SN-PCR is a reliable method that can be used in microbiological quality control laboratories in pharmaceutical facilities for the detection of *B.cepacia* from aqueous filterable pharmaceutical preparations with high specificity and sensitivity, allowing the detection of the organism in small quantities equivalent to around 10 CFU in the whole preparation, which helps to prevent false negative results and possible proliferation of the microorganism in aqueous pharmaceutical preparations during shelf life. Further work will be done to test higher number of commercial preparations using the optimized method.

### **Conflict of interest**

The authors declare that they have no competing interests.

## References

- (1) Torbeck, L., D. Raccasi, D. E. Guilfoyle, R. L. Friedman and D. Hussong. "*Burkholderia cepacia*: This Decision Is Overdue." PDA J Pharm Sci Technol. 2011,65(5): 535-543.
- (2) Sousa, S. A., C. G. Ramos, J. H. Leitao. "*Burkholderia cepacia* Complex: Emerging Multihost Pathogens Equipped with a Wide Range of Virulence Factors and Determinants. Int J Microbiol. 2011, doi:10.1155/2011/607575.





- Burkholderia contaminans* sp. nov. and *Burkholderia lata* sp. nov. *Int. J. Syst. Evol. Microbiol.* 2002, 59: 102–111.
- (11) Henry, D.A., Mahenthiralingam, E., Vandamme, P., Coenye, T., Speert, D.P. Phenotypic methods for determining genomovar status of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* 2001, 39: 1073–1078.
- (12) Vandamme, P. and P. Dawyndt. Classification and identification of the *Burkholderia cepacia* complex: Past, present and future. *Syst Appl Microbiol.* 2011, 34(2): 87-95.
- (13) Mahenthiralingam, E., J.Bischof, S.K. Byrne, C. Radomski, J.E. Davies, Y. Av-Gay, and P. Vandamme. DNA- based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis* and *Burkholderia cepacia* genomovars I and III. *J.Clin.Microbiol.* 2000, 38: 3165-3173.
- (14) Moore, J.E., B. C. Millar, X. jiru, J. McCappin, M. Crowe and J. S. Elborn. Rapid Characterization of the genomovars of the *Burkholderia cepacia* complex by PCR- single stranded conformational polymorphism (PCR-SSCP) analysis. *J.Hosp. Infect.* 2001, 48: 129-134
- (15) Moore, J.E., J. Xu, B. C. Millar, M. Crowe and J. S. Elborn. Improved molecular detection of *Burkholderia cepacia* genomovar III and *Burkholderia multivorans* directly from the sputum of patients with cystic fibrosis." *J. Microbiol. Methods.* 2002, 49(2): 183-191.
- (16) United States Pharmacopoeia. USP <61> Microbiological Examination of Non-Sterile Products: Microbial Enumeration Tests. United States Pharmacopeial Convention: Rockville, MD, 2014.
- (17) United States Pharmacopoeia. USP <62> Microbiological Examination of Non-Sterile Products: Tests for Specified Microorganisms. United States Pharmacopeial Convention: Rockville, MD, 2014.

- (18) United States Pharmacopoeia. USP <1227> Validation of Microbial Recovery from Pharmaceutical Articles. United States Pharmacopeial Convention: Rockville, MD, 2008.
- (19) Vandamme, P. *Burkholderia cepacia: Pandora's Box redefined*. BCCM News, 2001, 9: p. 2-3.
- (20) Carson, L.; Favero, M.S.; Bond, W.W.; Peterson, N. J. Morphological, biochemical, and growth characteristics of *Pseudomonas cepacia* from distilled water. *Appl. Microbiol.* 1973, 25 (3): 476–483.
- (21) Luigi, C. *Burkholderia cepacia* complex species: health hazards and biotechnological potential. *Trends Microbiol.* 2006, 14 (6): 277–286.

Table 1: List of primers used for SN-PCR

<b>Primer</b>	<b>Sequence</b>	<b>Target gene</b>	<b>Reference</b>
BCR1 (f)	TGA CCG CCG AGA AGA GCA A	RecA	(13)
BCR2 (r)	C TTC TTC GTC CAT CGC CTC	RecA	(13)
Mr (r)	CGA TTT CGG CCT TCG GC	RecA	(14)

**Table 2. Identification of environmental *B. cepacia* isolates by SN-PCR and Vitek 2 compact identification system**

Isolate N0#	Vitek 2 Compact system	SN-PCR
Isolate 1	<i>B. cepacia</i> group	<i>B. cepacia</i>
Isolate 2	<i>B. cepacia</i> group	<i>B. cepacia</i>
Isolate 3	<i>B. cepacia</i> group	<i>B. cepacia</i>
Isolate 4	<i>B. cepacia</i> group	<i>B. cepacia</i>
Isolate 5	Un-identified	<i>B. cepacia</i>
Isolate 6	Un-identified	<i>B. cepacia</i>
Isolate 7	<i>Enterobacter cloacae</i> complex	No bands
Isolate 8	<i>Kocuria rosea</i>	No bands
Isolate 9	<i>Kocuria varians</i>	No bands
Isolate 10	<i>Methylo bacterium</i> species	No bands
Isolate 11	<i>Micrococcus luteus</i> / <i>lylae</i>	No bands
Isolate 12	<i>Niesseria animaloris</i> / <i>zoodegmatis</i>	No bands
Isolate 13	<i>Pantoea</i> species	No bands
Isolate 14	<i>Pseudomonas aeruginosa</i>	No bands

Isolate 15	<i>Pseudomonas alcaligenes</i>	No bands
Isolate 16	<i>Pseudomonas putida</i>	No bands
Isolate 17	<i>Pseudomonas fluorescens</i>	No bands
Isolate 18	<i>Ralstonia Pickettii</i>	No bands
Isolate 19	<i>Ralstonia mannitolilytica</i>	No bands
Isolate 20	<i>Rhizobium radiobacter</i>	No bands
Isolate 21	<i>Roseomonas gilardii</i>	No bands
Isolate 22	<i>Staphylococcus hominis</i> ssp. <i>hominis</i>	No bands
Isolate 23	<i>Sphingomonas paucimobilis</i>	No bands

**Table III. Sensitivity of conventional methods for detection of *B. cepacia* using inoculum sizes ranging from 1000- 50 CFU/ 0.1ml****Table III A. *B-cepacia* inoculum size 1000  $\pm$  200 cfu/0.1ml**

<b>TSB + 4% Tween 20 (24 h incubation)</b>				
<b>Sample Number</b>	<b>TSB Appearance</b>	<b>Media</b>		
		<b>TSA</b>	<b>CET</b>	<b>OFPBL</b>
<b>1</b>	Clear	+ve	+ve	+ve
<b>2</b>	Clear	+ve	+ve	+ve
<b>3</b>	Clear	+ve	+ve	+ve
<b>4</b>	Clear	+ve	+ve	+ve
<b>5</b>	Clear	+ve	+ve	+ve
<b>6</b>	Clear	+ve	+ve	+ve
<b>7</b>	Clear	+ve	+ve	+ve
<b>8</b>	Clear	+ve	+ve	+ve
<b>9</b>	Clear	+ve	+ve	+ve
<b>10</b>	Clear	+ve	+ve	+ve

**Table III B.** *B-cepacia* inoculum size  $100 \pm 20$  cfu/0.1ml

Sample Number	TSB Appearance	TSB + 4% Tween 20 (24 h incubation)		
		TSA	CET	OFPBL
1	Clear	+ve	+ve	+ve
2	Clear	+ve	+ve	+ve
3	Clear	+ve	+ve	+ve
4	Clear	+ve	+ve	+ve
5	Clear	+ve	+ve	+ve
6	Clear	+ve	+ve	+ve
7	Clear	+ve	+ve	+ve
8	Clear	+ve	+ve	+ve
9	Clear	+ve	+ve	+ve
10	Clear	+ve	+ve	+ve



**Table III** *C. B. cepacia* inoculum size 50 + 10 cfu/0.1ml

Sample Number	TSB + 4% Tween 20 (24 h incubation)			
	TSB Appearance	Media		
		TSA	CET	OFBBL
<b>1</b>	Clear	+ve	+ve	+ve
<b>2</b>	Clear	+ve	+ve	+ve
<b>3</b>	Clear	<b>-ve</b>	+ve	+ve
<b>4</b>	Clear	+ve	+ve	+ve
<b>5</b>	Clear	<b>-ve</b>	<b>-ve</b>	<b>-ve</b>
<b>6</b>	Clear	+ve	+ve	+ve
<b>7</b>	Clear	+ve	+ve	+ve
<b>8</b>	Clear	+ve	+ve	<b>-ve</b>
<b>9</b>	Clear	+ve	+ve	+ve
<b>10</b>	Clear	+ve	+ve	+ve

## Figure Captions

### Figure 1 A: Semi-nested PCR (SN-PCR) detection method using reference strains

Lane 1: Qiagen® GelPilot 100 bp Plus ladder, Lane 2: *Burkholderia cepacia* (ATCC 245416), Lane 3: *Staphylococcus aureus* ATCC® 6538, Lane 4: *Pseudomonas aeruginosa* ATCC® 9027, Lane 5: *Escherichia coli* ATCC® 8739, Lane 6: *Salmonella abony* NCTC® 6017, Lane 7: *Bacillus subtilis* ATCC® 6633, Lane 8: Negative control

### Figure 1 B: Semi-nested PCR (SN-PCR) detection method using environmental strains

lane 1: Qiagen® GelPilot 100 bp Plus ladder, lane 2: *B. cepacia* (ATCC 245416), lane 3: *Micrococcus luteus*, lane 4: *Staphylococcus warneri*, lane 5: *Pseudomonas fluorescens*, lane 6: *Pseudomonas putida*, lane 7: *Ralstonia picketti*, lane 8: Negative control

### Figure 2 A: Testing artificial filterable pharmaceutical preparation via SN-PCR using *B. cepacia* (ATCC 245416) at an inoculum size of $10^7$ CFU/100 ml. DNA was extracted from the preparation.

Test was repeated 3 times.

lane 1: Qiagen® GelPilot 100 bp Plus ladder, lane 2: pharmaceutical preparation containing *Burkholderia cepacia* (ATCC 245416)  $10^7$  CFU/100 ml, lane 3 and 4: repeated test, lane 5: Negative control

### Figure 2 B: Testing artificial filterable pharmaceutical preparation via SN-PCR using 6 environmental strains of *B. cepacia*. DNA was extracted from the preparation

lane 1: Qiagen® GelPilot 100 bp Plus ladder, lane 2: pharmaceutical preparation containing *Burkholderia cepacia* (ATCC 245416)  $10^7/100$ ml, lane 3- 8: environmental isolates (1-6), Lane 9: Negative control

**Figure 3. Sensitivity of SN-PCR for testing artificial filterable pharmaceutical preparations using *Burkholderia cepacia* (ATCC 245416) at an inoculum size of 10 CFU/ preparation. Test was repeated 6 times.**

lane 1: Qiagen<sup>®</sup> GelPilot 100 bp Plus ladder, lane 2: *Burkholderia cepacia* (ATCC 245416) DNA extracted from colony, lane 3: pharmaceutical preparation containing *Burkholderia cepacia* (ATCC 245416) 10 CFU/preparation, lane 4- 9: test repeated 6 times.

**Figure 4. Testing commercial products by SN-PCR**

lane 1: Qiagen<sup>®</sup> GelPilot 100 bp Plus ladder, lane 2: *Burkholderia cepacia* (ATCC 245416) (DNA extracted from colony), lane 3: Product No#57, lane 4: negative *B. cepacia* product, lane 5: Qiagen<sup>®</sup> GelPilot 100 bp Plus ladder, lane 6: *Burkholderia cepacia* (ATCC 245416) (DNA extracted from colony), lane 7: Product No#82, lane 8: negative *B. cepacia* product

Figure 1A

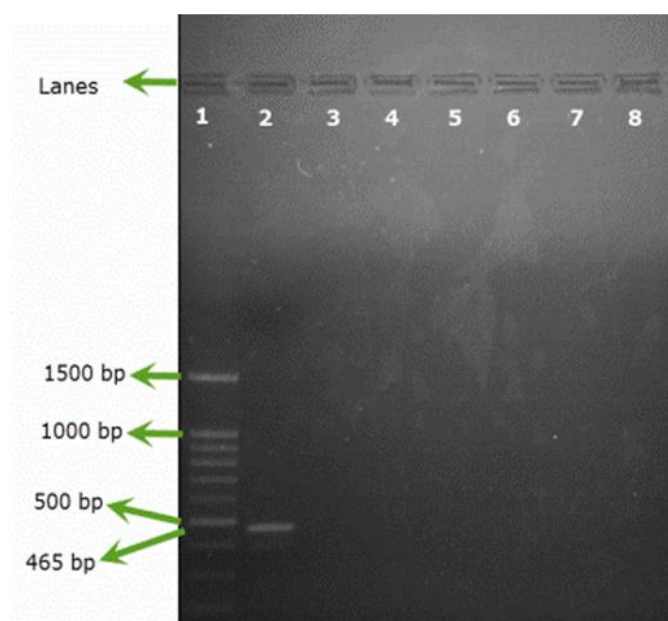


Figure 1B

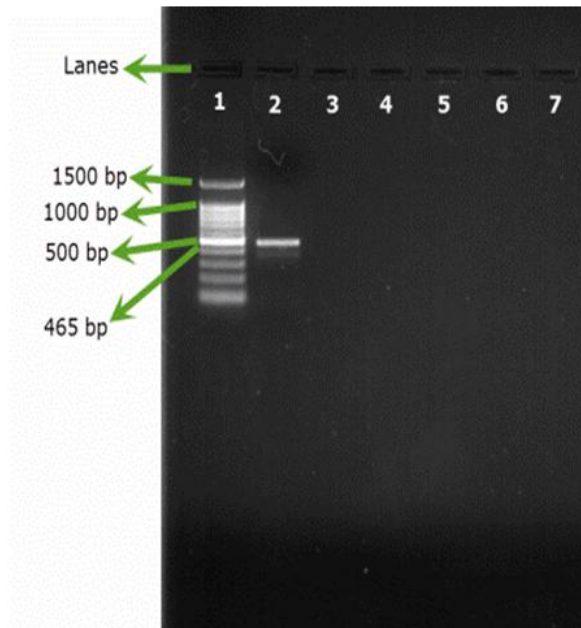


Figure 2A

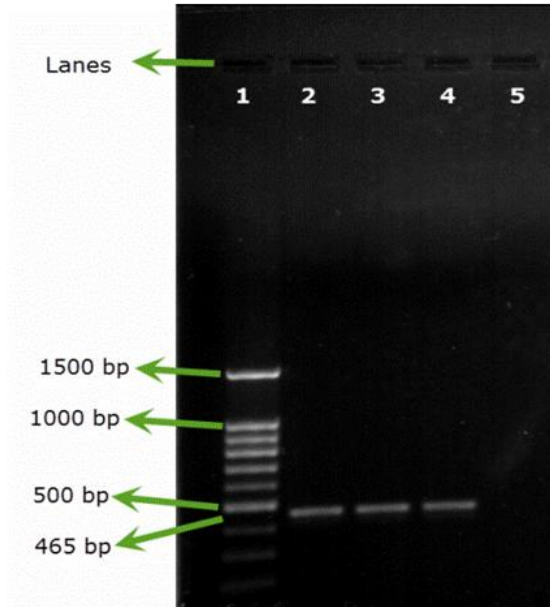


Figure 2B

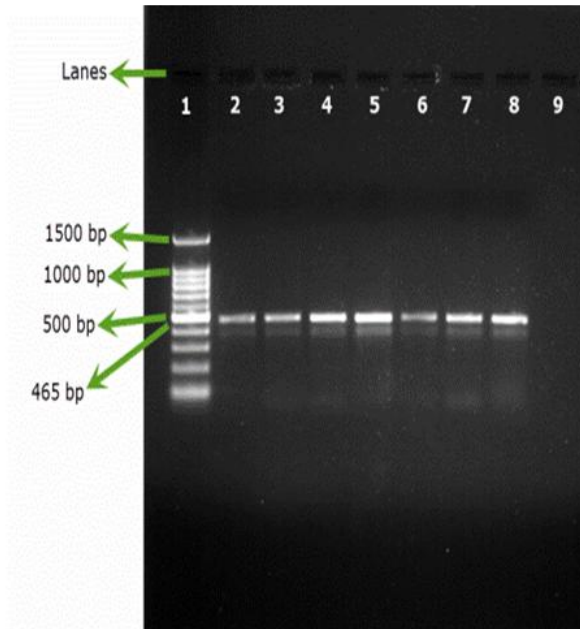


Figure 3

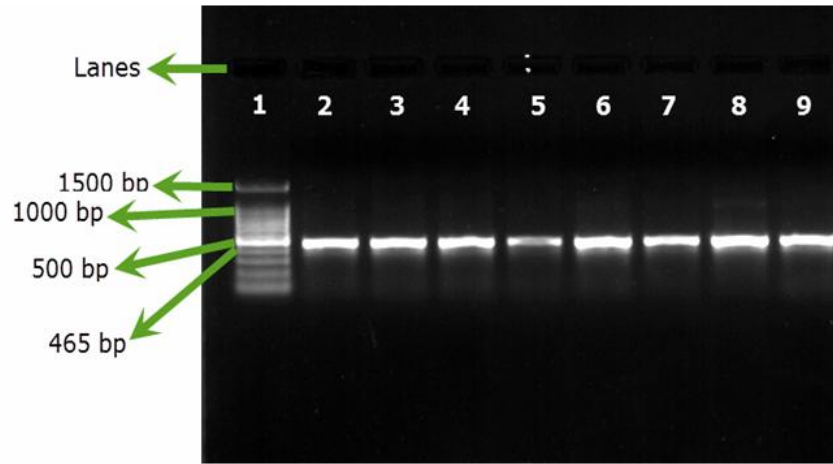
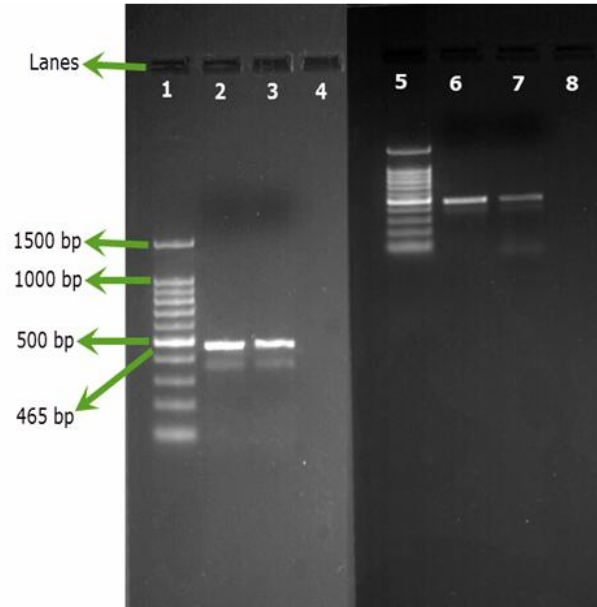




Figure 4



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