# *Burkholderia cepacia*: A problem that does not go away!

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## Abstract

Because of the genetic and metabolic capabilities to overcome environmental stresses during processing and manufacturing, *Burkholderia cepacia* is still the number one bacterial species found in contaminated pharmaceutical products. Nucleic acid based technologies are available to detect, identify, and quantify the numbers of *B. cepacia* cells in environmental samples such as pharmaceutical water, raw materials, and finished products. However, development and application of these technologies to pharmaceutical quality control have been rather slow. Rapid PCR detection of *B. cepacia* in pharmaceutical products contaminated with a mixed bacterial culture allowed faster detection times and higher resolution than standard microbiological methods that require time consuming and multiple procedures.

*Keywords:* Burkholderia cepacia; Pharmaceutical products; Biocides; PCR; Recalls

## Introduction

Microbial contamination is still a major reason for product recall in the United States. Several publications have reported the frequency of microbial contamination in non-sterile and sterile products from 1998 to 2011 [1,2] . When microbial contamination was found the number one species isolated were *Burkholderia cepacia* [1,2]. Looking at FDA recall data from a year after the last publication was reported, *B. cepacia* was still the number one microbial contaminant in non-sterile products (Table 1) [3]. Thirty nine percent of bacteria isolated from contaminated samples were identified as *B. cepacia* (Figure 1). The contaminated samples were sanitizers, oral pharmaceuticals, and gas relief drops.

 Furthermore, a recent outbreak of *B. cepacia* complex (Bcc) pseudo bacteremia was associated to contaminated antiseptic formulations [4]. During that outbreak *B. cepacia* was isolated from blood cultures of 40 patients and antiseptic formulations. The outbreak investigation determined that the formulation was misused as a skin antiseptic during blood culture. The contaminated product was discarded and the staff retrained. Another outbreak was reported at a private hospital where 13 cancer patients undergoing chemotherapy developed *B. cepacia* bacteremia due to a contaminated antiemetic drug [5]. The outbreak lasted 2 months and was controlled when hospital personnel were properly educated to optimize daily aseptic practices. Opened and unopened vials of the antiemetic drug grew *B. cepacia*. Environmental samples from water, surfaces, equipment, air, disinfectants, and antiseptics did not show the presence of *B. cepacia*.

## Discussion

The persistence of *B. cepacia* in pharmaceutical products can be explained by the lack of proper good manufacturing practices (GMP) and the use of compendial methods that do not provide the sensitivity and resolution to detect *B. cepacia* in pharmaceutical water, raw materials, and finished products [1,2,6]. Most companies relied on traditional cultivation and phenotypic methods to isolate and identify microbial contamination. These methods are time consuming (5-7 days) and laborious. They relied upon the growth of microorganisms on the specific substrates in the media. However, some bacteria do not grow on those substrates or grow extremely slow to be detected by the incubation times currently used. In some cases microbial cells undergo a physiological state by reducing metabolic reactions rates and

cell size which are affecting the protein and enzymatic profiles used to identify environmental isolates [1]. These microbial cell changes are triggered by physical processes and environmental systems implemented to reduce or eliminate microorganisms during manufacturing. If the processes and systems are not validated or properly implemented, microorganisms contaminated products, raw materials, and equipment [1,6]. *B. cepacia* genetic and metabolic diversity are severely underestimated by industrial operators. *B. cepacia* genome consists of more than one chromosome containing a wide variety of metabolic genes, which appear to be acquired by horizontal gene transfer [7]. *B. cepacia* is capable of growing on nitro aromatic and aromatic compounds by the action of different enzymes such as mono oxygenases and dioxygenases [8]. These enzymes not only oxidize aromatic structures but also breakdown halogenated compounds [9]. Therefore is not only the health hazard to patients that makes *B. cepacia* a real nightmare for quality control microbiologists but product stability and purity are compromised by the degradation of active ingredients and excipients resulting in sub potent formulations. Nitro aromatic compounds are major components of many pharmaceutical drugs [10]. For instance, antipsychotic and analgesic drugs are based upon aromatic structures sensitive to biodegradation attack by mono and dioxygenases from microbial contaminants.

*B. cepacia* populations grow and survive in biocides, pharmaceutical products, active ingredients, and excipients leading to the development of resistance to preservative systems used to protect formulations [11-13]. They have also intrinsic antibiotic resistance by the release of β-lactamases, the impermeability of the outer envelope to antimicrobial agents, and specific efflux pumps. Biofilm formation is another adaptation that provided physiological resistance to antimicrobial treatments. Recent studies showed that a wide variety of commercial products contain biocides concentrations that are insufficient to kill *B. cepacia* and other Bcc species [13]. Based upon their analysis, investigators calculated that Bcc bacteria might need more than 25 times more biocides over the MIC value to achieve killing. No correlation was found between susceptibility to biocides, susceptibility to antibiotics, and biofilm formation. Some strains showing very high biocide resistance exhibited vigorous biofilm formation.

Other studies showed that after exposure to biocides, biofilm populations of *B. cenocepacia* expressed different genes related to membrane proteins, regulatory proteins, efflux pumps, oxidative stress response, and chemotaxis [14]. The transcriptional response to biocide treatment was ascertained by microarray analysis and real-time quantitative PCR. A wide variety of efflux pump systems were more predominant in biofilm-grown cells while planktonic cells relied on other resistance mechanisms. Many genes encoding for transport-related proteins were down regulated as a consequence of biocide treatment. All these studies indicate the need to improve and develop rapid detection methods for *B. cepacia* to implement control practices leading to the reduction of microbial contamination and morbidity reports.

However, the polymerase chain reaction (PCR) has been shown to detect and identify *B. cepacia* in artificially contaminated pharmaceutical samples within 27 hours [15]. The assay was validated using DNA primers targeting a specific and highly conserved 209 base pair (bp) 16S rRNA gene fragment. Adding 10 μl of the product suspension to 200 μl of the lysis buffer provided enough material for the PCR reaction to be performed [15]. The lysis buffer was based upon mild chemical ingredients. The samples were incubated at 37 °C for 20 minutes in Tris-EDTA buffer and Tween 20 followed by a Proteinase K treatment for 10 minutes at 95°C. The DNA extraction procedure was basically a two-step protocol that was easy to perform with minimal sample manipulation and no hazardous chemicals. The DNA primers targeting the 209 bp fragment were added to different aliquots of the extracted microbial DNA containing Ready-To-Go PCR beads. The beads provided all the reagents needed for the PCR reaction in a convenient, ambient-temperature-stable form. They contain buffer, nucleotides, and *Taq* polymerase. The use of the beads minimized sample handling and cross contamination. None of the tested products or raw materials inhibited the PCR reaction. No amplified DNA fragment was obtained with uninoculated samples. All contaminated samples, e.g., 10 products and raw materials, showed the presence of the fragment indicating a positive detection and identification [15].

However, microbial contamination of pharmaceutical products is not only caused by pure cultures of specific microorganisms. For example, Table 1 shows that microbial contamination can be a result of mixed cultures of microorganisms. PCR detection of a target bacterial species present in pharmaceutical samples contaminated with a mixed bacterial culture was previously reported [16]. A recent study showed that PCR was used to detect *Burkholderia* species in high purity water systems despite the high background with mixed microbial contamination [17]. After water samples were concentrated on membrane filters, microbial DNA was extracted and amplified by a Quantitative Real-Time PCR assay. Quantitative detection and identification were completed in less than 5 hours.

In our laboratory, we spiked two pharmaceutical products, an over the counter antihistamine medication (product A) and nausea medication (product B) with low levels, < 100 colony forming units (CFU), of *B. cepacia, Escherichia coli, Bacillus* sp., and Dietzia sp. Figure 2 shows the counts for the *B. cepacia* culture used during the study. *E. coli* is one of the indicators in the Microbial Limits testing of pharmaceutical products. Different types of *Bacillus* species are commonly found in environmental samples during pharmaceutical operations [1]. For instance, *B. cereus* was found in 17% of the recalled products (Table 1) (Figure 1). *Dietzia* is a member of the Actinobacteria phylum with slow growth rates in environmental samples.

The contaminated samples were incubated at 37°C for 24 hours with shaking, e.g., 200 rpm. After incubation, DNA was extracted as previously described [15]. Other DNA extraction protocols were analyzed but none of them provided enough material to optimize the PCR reaction. For optimal PCR detection of *B. cepacia* in pharmaceutical products contaminated with mixed bacterial cultures, an aliquot of 50 µl was necessary to obtain a positive reaction (Figure 3). Lanes 3 and 4 showed the presence of the 209 bp 16S rRNA fragment. Lanes 5 and 6 contained 10 µl aliquots of the extracted microbial DNA that evidently did not show the same band intensity.

Rapid detection of *B. cepacia* was completed within 27 hours despite the presence of the other microorganisms in the product suspensions. Our laboratory is currently working on a Real Time Quantitative PCR protocol to detect, identify, and quantify the numbers of *B. cepacia* in pure and/or mixed cultures of contaminated pharmaceutical materials with a minimal incubation time, e.g., 3-5 hours. Rapid detection of *B. cepacia* in pharmaceutical products contaminated with mixed bacterial cultures demonstrated that nucleic acid based technologies can improve detection times leading to process optimization by early detection of potential problems with the possibility of rapid implementation of corrective actions. Pharmaceutical quality control is greatly improved when systems are developed to provide critical assessment of product quality and process control. Pharmaceutical manufacturing is a highly technical and detailed operation that requires specialized personnel and complex processes to control the continuity, reproducibility, and stability of the different systems put in place to guarantee the compliance to GMP regulations and provide safe, efficacious, and stable products. *B. cepacia* persistent presence in contaminated pharmaceutical samples indicates the inadequate application of control strategies and detection methods to optimize quality and process control.
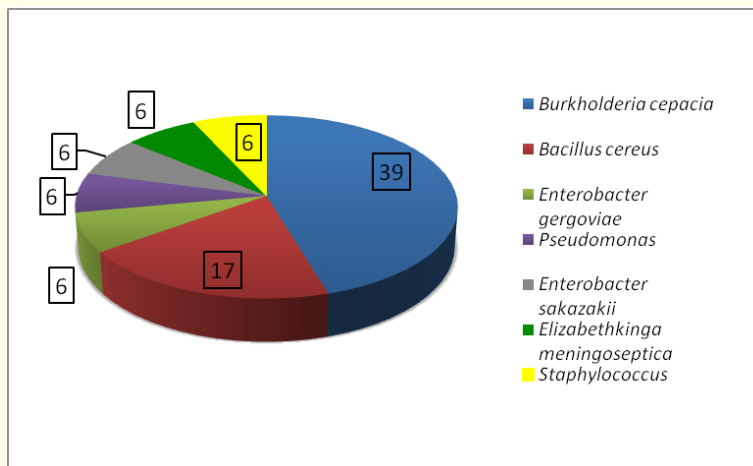
**Figure 1:** *Percentage of bacterial species isolated in pharmaceutical products recalled during the first 6 months of 2012. N=18.*

| Recall Number | Non Sterile | Reason |
|---|---|---|
| 1 | Alcohol Free Sanitizer | *B. cepacia* |
| 2 | Alcohol Free Sanitizer | *B. cepacia* |
| 3 | Oral pharmaceuticals | *B. cepacia* |
| 4 | Alcohols pads | *Bacillus cereus* |
| 5 | Alcohols pads | *Bacillus cereus* |
| 6 | Gelatin capsules | Microbial contamination |
| 7 | Alcohols pads | *Bacillus cereus* |
| 8 | Pharmaceutical cream | *Enterobacter gergoviae and Pseudomonas monteilii/plecoglossicida.* |
| 9 | Povidone iodine solution | *Elizabethkingia meningoseptica* |
| 10 | Pharmaceutical gel | Microbial contamination |
| 11 | Baby lotion | Microbial contamination, Staphylococcus |
| 12 | Pepto bismol | Microbial contamination |
| 13 | Gas relief drops (Simethicone) | Microbial contamination, *B. cepacia* |
| 14 | Gas relief drops (Simethicone) | Microbial contamination, *B. cepacia* |
| 15 | Pharmaceutical solution | LSA Antimicrobial preservative failure |
| 16 | Pharmaceutical solution | LSA Antimicrobial preservative failure |
| 17 | Hand sanitizer | *B. cepacia* |
| 18 | Hand sanitizer | *B. cepacia* |

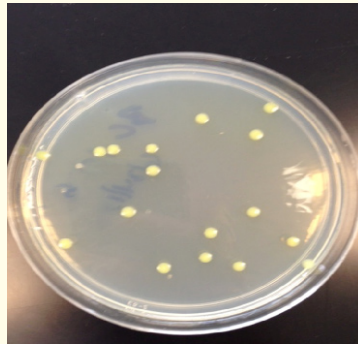**Table 1:** *Pharmaceutical product recall data from January 2012 to July 2012.*

*Figure 2: Inoculum counts for B. cepacia culture spiked into pharmaceutical product suspensions contaminated with a mixed bacterial culture.*
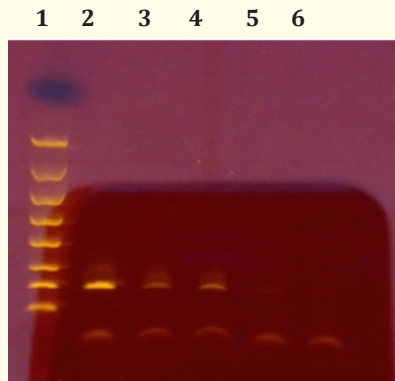


*Figure 3: Detection of B. cepacia in pharmaceutical products contaminated with a mixed bacterial culture. After microbial DNA was extracted from product suspensions, 50 μl and 10 μl aliquots were added to 3 Ready-To-Go PCR beads, sterile water, and B. cepacia DNA primers.*
*Lane 1. Molecular Weight Markers: 4000, 2000, 1250, 800, 500, 300, 200, 100 bp.*
*Lane 2. B. cepacia DNA, positive control*
*Lane 3. 50 μl of DNA from product A.*
*Lane 4. 50 μl of DNA from product B.*
*Lane 5. 10 μl of DNA from product A.*
*Lane 6. 10 μl of DNA from product B.*

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