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Comparason of extraction methods for PCR detection of *Burkholderia cepacia* complex (BCC) from cystic fibrosis patients

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Abstract: Direct detection of *Burkholderia cepacia* complex (BCC) and its genomovars from sputum by molecular tests emerges as a method for rapid identification. In this study, four DNA extraction methods were evaluated for the identification for BCC from sputum of CF patients. Sputa from 28 CF patients were aliquoted and spiked with BCC reference strain. Boiling, phenol-chloroform, CTAB methods and a commercial spin column kit was used for DNA extraction. Total DNA yields were determined by spectrophotometry and single-round *rec*A PCR was used for detection of BCC. No significant difference was observed in DNA yields from different extraction methods. Lower limit of detection for *rec*A PCR was determined as 10^6 cfu/ml. Amplification was observed in 7/16 (43.7%) of sputa for boiling, 8/16 (50%) of sputa for CTAB and 13/16 (81.2%) of sputa for phenol-chloroform method and spin column kit in the assay sensitivity range determined in the study. Phenol-chloroform and commercial spin column kit were found to be better suited for DNA purification from sputum of CF patients for BCC identification. Diagnostic impact of single-round *rec*A PCR directly from sputum was limited to chronically-infected patients.

Keywords: DNA extraction • PCR • Burkholderia • Burkholderia cepacia complex • Cystic fibrosis

1. Introduction

Burkholderia cepacia complex (BCC) is a group of closely related gram-negative bacteria that consists of at least nine genetically distinct members referred collectively as genomovars [1]. Originally identified as plant pathogens, species belonging to BCC have been recognised as important opportunistic bacterial agents not only in persons with cystic fibrosis (CF) but also for immunosuppressed patients. BCC infection has been shown to be an important threat for CF patients, owing to dramatical increase in symptoms and decline in pulmonary functions; besides causing a potentially fatal necrotizing pnemonitis with bacteremia, called as Cepacia Syndrome in 20% of infected persons. Patient to patient transmission (and potential for nosocomial spread) and multi-drug resistance are other possible consequences of BCC infection that contributes to increased morbidity and mortality in CF. Thus, reliable detection of organisms belonging to BCC is crucial for the optimal clinical management of CF patients, as well as for infection control purposes [1,2].

Closely related species like BCC are generally difficult to identify by conventional phenotypic and

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biochemical tests. It is reported that commercial systems lack sufficient sensitivity and specificity for accurate identification of bacteria belonging to BCC group [3,4]. Recently, PCR-based methods are emerging as useful diagnostic tools for rapid identification and typing of BCC, also enabling direct detection of bacterial DNA from patient's sputum [2,5,6]. The ability of these assays to detect BCC in sputum is dependent on both selected target sequence and efficiency of DNA extraction procedure. Sputum has previously shown to contain several PCR inhibitors and failure to remove these enzymatic inhibitors and adequately extract DNA could result in false-negative results [5,7]. In this study, we compare the efficiency of three commonly used standard DNA extraction procedures: boiling, phenol-chloroform and cetyltrimethylammonium bromide (CTAB) methods, and a commercial spin-column kit for the extraction for *Burkholderia cepacia* complex DNA for PCR from sputum of CF patients. Sensitivity and diagnostic impact of *rec*A PCR directly performed on sputum samples is also evaluated.

2. Material and Methods

2.1. Bacterial Strains

B.cepacia complex (KK 7394 Neqas, United Kingdom) was used as the reference strain for the study. Prior to spiking and analysis, *B.cepacia* complex reference strain was grown on 5% sheep blood agar and then further tested for growth on selective OFPBL agar (Oxidative, Fermentative, Polymyxin B, Bacitracin, Lactose) at 37°C. Fresh bacterial suspensions from overnight cultures were prepared in sterile saline and used to spike sputum specimens. DNA extracted from bacterial cultures by High Pure PCR Template Kit™ (Roche Diagnostics, Germany) was used as a positive control for *rec*A PCR.

A strain of *Pseudomonas putida,* identified previously from a clinical sample by PHOENIX™ (Becton-Dickinson, United Kingdom) automated identification system was used as negative control in the study. The strain was cultivated on sheep blood agar plates and bacterial DNA was extracted by High Pure PCR Template Kit™ (Roche Diagnostics, Germany). Purified DNAs were used as negative controls.

2.2. Study Population

Sputum samples of 28 cystic fibrosis patients that had attended to Hacettepe University Department of Pediatrics Chest Diseases Clinic were included in the study after informed consent and Hacettepe University Ethics Committee's approval. Sputum samples were collected in sterile screw-capped dishes and immediately transferred to the laboratory. Sputa were then randomly assigned to one of seven study groups for spiking with various amounts of bacteria.

2.3. Sample Processing

Sputum samples from each CF patient were homogenized by vigorous vortexing and separated into four 200 - 500 µl aliquots and the remaining amount were stored for bacteriological analysis. All four aliquots from each patient were spiked with BCC reference strain with approximate final concentrations of 10^9 to 10^3 cfu/ml according to study groups. A total of seven study groups containing 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 cfu/ml of BCC, each composed of sputa from four CF patients were prepared. All samples were then thoroughly mixed and an equal amount of freshly prepared Sputolysin (Sigma, United Kingdom) was added to each sample. The samples were then incubated at 37°C for 30 minutes as described previously [2,8].

2.4. DNA extraction methods
2.4.1. Boiling
Processed sputum samples were centrifuged at 10.000 g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 200 µl TE (10mM TRIS-HCL [pH:8.0], 1mM EDTA) and briefly mixed by vortexing. Samples were then kept at 100°C in a heat block for 10 minutes. After an incubation at 37°C for 1 hour with addition of 150 µg lysozyme (Sigma, United Kingdom) to each sample for achieving complete bacterial lysis, samples were centrifuged at 12.000 g for 5 minutes and the supernatant was used for PCR.

2.4.2. Phenol-chloroform and CTAB extraction
Following incubation at 55°C overnight in 500 µl digestion buffer (10 mM Tris-HCL, pH:8.0, 150mM NaCl, 25 mM EDTA, 0.5% SDS [v/v], 20 mg/ml Proteinase K), standart phenol-chloroform method was employed as previously described [9].

For CTAB extraction method, 30 µl of 10% SDS and 3 µl of proteinase K (20mg/ml) (Sigma, United Kingdom) was added to 500 µl sample, mixed thoroughly and incubated at 37 °C overnight. Then, standard procedure was performed as described before [10].

2.4.3. Commercial spin column kit
High Pure PCR Template Kit™ (Roche Diagnostics, Germany) was used according to the manufacturers' instructions.

2.4.4. Isolation and Identification of B.cepacia complex

Ten μl of remaining sputa from each patient were processed with Sputolysin as described above and inoculated simultaneously onto OFPBL agar (Oxidative fermentative, polymyxin B, bacitracin, lactose) and blood agar plates supplemented with 5% sheep blood and incubated at 37°C for 48-72 hours for the isolation of inherent BCC before spiking with bacteria. Colonies observed on OFPBL agar were tested for oxidase activity and identified using commercially available identification systems if required.

2.4.5. PCR Detection of B.cepacia complex

B.cepacia complex *rec*A gene was amplified using BCR-1 and BCR-2 primers, specific for BCC [2,8]. For the PCR, 5 μl of extracted DNA from each procedure were added to a master mix of 50 μl containing 750mM TRIS (pH:8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20 [v/v], 1.5 mM $MgCl_2$, 200 μM of each deoxyribonucleotides, 25 pmol of primers, 0.7 units of Taq DNA Polymerase. The thermocycling program consisted of an initial denaturation at 96°C for 7 minutes followed by denaturation at 96°C for 60 seconds, annealing at 56°C for 60 seconds and extention at 72°C for 90 seconds for 35 cycles. An extention of 10 minutes at 72°C were also performed after amplification. Thermocycling were performed in a PTC-200 Thermocycler (MJ Research, USA). Amplicons of 1040 bp were separated by electrophoresis in 1.5% agarose gel and visualised under ultraviolet light after staining with ethidium bromide. DNA extraction, PCR and electrophoresis were performed in separate laboratories in order to avoid contamination. All PCR amplifications were done in duplicate.

2.4.6. Spectroscopic determination of DNA amount

Absorbance values at 260 nm (OD $_{260}$) were used for calculating the amount of total nucleic acid yields from each extraction method for all samples.

2.4.7. Statistical Analysis

Mann-Whitney, Kruskal-Wallis and ANOVA tests were applied where appropriate. P values < 0.05 were assumed as statistically significant. Data analyses were performed by SPSS ® Version 12.0.

3. Results

3.1. Isolation of B.cepacia complex from sputa

BCC was not isolated in any of the patients' sputa evaluated prior to spiking in the study population.

3.2. DNA yields from extraction methods

Means of total DNA yield was calculated as 111.8 μg (median: 34.5, range: 0.00-620.0) for boiling; 44.4 μg (median: 23.6, range: 4.50-139.0) for phenol chloroform; 97.3 μg (median: 11.7, range:1.25-620.0) for CTAB and 17.8 μg (median: 10.5, range: 1.00-79.0) for spin column methods. The differences of purified nucleic acid amounts among extraction methods were not statistically significant (p:0.065). For sputa containing 10^9-10^6 cfu/ml bacteria, differences of total DNA yield were also not statistically significant (p:0.157). Nucleic acid yields and *rec*A PCR detection rates for this group are given in Table 1.

3.3. *B.cepacia* complex *rec*A PCR

BCC DNA was detected in 7 out of 28 (25%) samples by boiling; in 8 out of 28 (28.5%) by CTAB; 13 out of 28 (46.4%) by phenol-chloroform and spin column kit. BCC concentrations lower than 10^6 cfu/ml in 12 sputum samples could not be detected by PCR with any of the extraction method employed. When calculated for 10^9-10^6 cfu/ml concentration range, detection rate was 7/16 (43.7%) for boiling, 8/16 (50%) for CTAB and 13/16 (81.2%) for phenol-chloroform method and spin column kit. Results of BCC *rec*A PCR according to the final concentration of bacteria in sputum and extraction method above 10^6 cfu/ml were given in Table 1.

Boiling method could detect BCC nucleic acids in 3 out of 4 samples in 10^9 cfu/ml group and 2 out of 4 in 10^8 and 10^7 cfu/ml groups, giving negative results for all samples containing 10^6 cfu/ml of bacteria. CTAB methods was also unable to identify any samples with 10^6 cfu/ml of BCC, but detecting 50% and 75% of the samples in 10^7 cfu/ml and 10^8-10^9 cfu/ml groups respectively (Table 1). All PCR positive samples purified by boiling and CTAB methods were also positive by phenol-chloroform method and spin column kit.

Phenol-chloroform extraction method identified BCC nucleic acids in 3 out of 4 samples for sample groups with 10^6, 10^7 and 10^8 cfu/ml of bacteria and detecting all samples with a concentration of 10^9 cfu/ml (Table 1). Amplification of two samples having 10^6 cfu/ml of BCC was only accomplished by phenol-chloroform method. Commercial spin column kit identified target sequences in all samples for sputa with a final bacterial concentration of 10^9-10^7 cfu/ml. For sputa containing 10^6 cfu/ml of BCC, amplification was successful in 1 out of 4 samples (Table 1).

Comparason of extraction methods for PCR
detection of *Burkholderia cepacia* complex
(BCC) from cystic fibrosis patients

Table 1. Nucleic acid yields and *recA* PCR detection rates of various nucleic acid extraction methods for sputa containing ≥106 cfu/ml of BCC.

Extraction Method	Number of Samples	Total nucleic acid (µg)			# of recA PCR positive samples			
		Mean	Median	Range	10^9 cfu/ml	10^8 cfu/ml	10^7 cfu/ml	10^6 cfu/ml
Boiling	16	56.20	34.50	2.00 - 191.00	3 / 4 (75%)	2 / 4 (50%)	2 / 4 (50%)	0 / 4
Phenol- chloroform	16	24.80	15.25	7.25 - 71.50	4 / 4 (100%)	3 / 4 (75%)	3 / 4 (75%)	3 / 4 (75%)
CTAB	16	15.20	10.63	1.75 - 40.50	3 / 4 (75%)	3 / 4 (75%)	2 / 4 (50%)	0 / 4
Spin Column Kit	16	12.00	8.00	1.00 - 44.00	4 / 4 (100%)	4 / 4 (100%)	4 / 4 (100%)	1 / 4 (25%)

4. Discussion

Bacteria belonging to BCC, with their considerable impact on morbidity and mortality of CF patients, require special care for accurate isolation and identification. Laboratory identification of BCC up to genomovar level is also crucial since certain genomovars are reported to be highly transmissible between CF patients and stringent infection control policies should be undertaken to prevent transmission to other patients [1,8,11]. The discrepancy between results obtained by phenotypic and molecular detection techniques is also of importance for the identification of BCC. It has been reported that certain bacterial strains can be misidentified as members of the BCC by commercial identification systems [3,4,12]. It was generally accepted that because of low specificity, differences in methodologies and phenotypic variations that can occur within species, the confirmation of BCC identification by phenotypic tests should be made by a reference laboratory [13,14]. Molecular methods are emerging as reliable tools for accurate BCC identification [15]. Previously, we have noted that the identification of BCC isolates via commercial phenotypic systems need to be confirmed by molecular methods due to discrepant results obtained from phenotypic tests [16].

Direct detection of pathogens by molecular methods in clinical specimens offer a useful alternate diagnostic tool in cases where phenotypic methods exhibit reduced sensitivity and / or specificity; such as BCC infections in CF. The main objective of this study was to evaluate the commonly used nucleic acid extraction techniques for PCR detection of BCC in sputum.

Diagnostic sensitivity of PCR from sputum may be influenced by various factors, namely the copy number of target sequence in the sample, size of the target amplicon, quality of the clinical specimen and the presence of PCR inhibitors. It is not clear whether sputa from CF patients have altered characteristics that have to be taken into consideration when performing molecular tests directly from these clinical specimens. In this study, phenol-chloroform and CTAB as standard manual extraction methods, a commercial spin column based

extraction kit and boiling method that is very practical and effective in some applications, were evaluated for PCR amplification of BCC directly from experimentally-spiked sputum samples of CF patients.

A well-defined *recA* based PCR protocol was used for the detection of BCC. *B.cepacia* complex can be detected and distinguished by sequence variations in 16S rRNA and *recA* gene; but very high specificity of *recA* gene for pathogens in BCC and possibility of accurate genomovar identification by Restriction Fragment Length Polymorphism (RFLP) or other methods make this locus a good target for diagnostic PCR [2,5].

Although no statistically significant difference in DNA yields obtained from different extraction techniques determined by spectroscopy was observed ; phenol-chloroform extraction and spin column kit performed better for *recA* PCR, identifying 13 of 28 (46.4%) of the experimentally-spiked samples. This detection rate is a result of presence of $\leq 10^6$ cfu/ml BCC in twelve of the samples, which were below the limit of detection for *recA* PCR observed in this study. Boiling and CTAB methods could only detect target sequences in a total of 6 and 8 sputum samples respectively, which also gave positive results via other extraction methods. For sputa spiked for a final concentration of 10^6 cfu/ml of BCC, target nucleic acids were detected in 75% of samples after purification by phenol-chloroform method, from which only one could be amplified after purification by spin column kit. Interestingly, all samples in 10^7-10^9 cfu/ml concentration range were successfully amplified by PCR after purification by spin column kit (Table 1). Decreased performance of the commercial kit observed in samples with 10^6 cfu/ml concentration could be attributed to the lower initial amount of sputum used in the process. All manual techniques employ a starting amount of 500 µl of sputum, whereas a 200 µl of material is used for the spin column kit as recommended by the manufacturer. Thus, for samples close to the assay sensitivity, this difference possibly affects PCR results. Modifications and/or additional column binding steps might be incorporated when spin columns. For pure cultures, waste water, artificially-infected EDTA-blood and lung tissue, it has been shown that a lysis buffer with proteinase K was

sufficient to extract DNA for BCC DNA [17,18].

Bacterial concentrations lower than 10^6 cfu/ml could not be amplified by any of the purification techniques applied, thus the detection limit of *rec*A PCR was 10^6 cfu/ml in the study. It was previously reported that regular *rec*A PCR could detect 10^6 cfu of BCC and DMSO significantly enhanced amplification of *rec*A gene [2]. This observation was not verified in our study, where the most efficient amplification for the 1040 bp *rec*A target was obtained by using 200 mM $(NH_4)_2SO_4$ and 0.1% Tween 20 in the PCR master mix (data not shown).

Importance of this relative insensitivity of single round *rec*A PCR is of debate in patients with chronic BCC infection or colonization, where bacterial loads of 10^7 - 10^9 cfu in sputum was previously noted [5]. Reducing the target amplicon size from 1040 bp to 465 bp is reported to provide only a 10-fold increase in sensitivity (2,5). For accomplishing a sensitivity of 1.2×10^1 to 1.3×10^2 cfu per gram of sputum, a semi-nested PCR is required, where consecutive genomovar identification must then be performed by direct sequencing, excluding RFLP analysis [5]. Drvinek et al. reported a nested PCR method with a first round of *rec*A PCR, followed by a genomovar–specific amplification step with a sensitivity of 10^3 cfu/ml [6]. FISH technique employed via a 16S rRNA probe was reported to have 4×10^5 cfu/ml detection sensitivity when applied directly on sputum samples, but genomovar identification was not possible [19]. In a study by Vonberg et al., *rec*A gene was targetted by a novel rapid-cycle PCR and genomovar-specific Flourescence Resonance Energy Transfer (FRET) probes, offering another molecular method for BCC identification and typing [20]. Pyrosequencing and *rec*A real-time PCR had also been employed for BCC typing [21,22]. Recently, sensitivity of an optimized commercial fluorescent in situ hybridization (FISH) assay performed directly on sputum samples was determined as 8×10^5 cfu/ml, whereas a novel rRNA-based PCR assay with 100% sensitivity and specificity for all BCC species could detect 10^4 cfu/ml of bacteria in sputa [23]. Diagnostic impact of molecular detection of BCC directly from patients' sputa and the most suitable techniques to be used for this purpose need to be determined.

In conclusion, the overall sensitivity of *rec*A PCR from sputum was 10^6 cfu/ml in our study which is appropriate for detecting chronic BCC infections in CF patients. Of the extraction methods evaluated; phenol-chloroform and commercial spin column kit performed better than other manual methods for extraction of experimentally-spiked BCC DNA from sputum for *rec*A PCR.

Acknowledgements

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Comparason of extraction methods for PCR
detection of *Burkholderia cepacia* complex
(BCC) from cystic fibrosis patients

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