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One-Step Gram PCR Assay for Rapid and Accurate Bacterial Differentiation

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Gram differentiation through conventional Gram staining is not a reliable method due to high incidence of false positive results. Molecular techniques such as PCR provide a better alternative. A simple, rapid and accurate method has been developed that enables exact differentiation of Gram positive and negative bacteria using a one step PCR assay. The one-step PCR using Gram specific primers resulted in a single 1025bp amplicon in Gram negative bacteria and a 355bp amplicon in addition to 1025bp amplicon in Gram positive bacteria. All tested bacteria were identified correctly through a single PCR reaction and showed complete concordance with biochemical and Gram staining results. This protocol proves to be a rapid, powerful and sensitive tool in determining the Gram status of bacteria. This difference in banding patterns between Gram positive and negative bacteria can be exploited for their clear-cut differentiation.

Key words : Multiplex PCR, Nested PCR, Ribotyping, Gram staining.

The invention of Polymerase Chain Reaction (PCR) as a tool for DNA amplification has made it easier for quick & accurate bacterial typing eliminating the need for time consuming phenotypic and biochemical tests as a sole method for bacterial characterization. It is now possible to rapidly amplify specific regions of bacterial genomes by PCR and compare these at their sequence level[1, 2]. In addition to reproducibility and sensitivity, this technique has the added advantage of being independent of the state of the organism[3]. This technique is extremely sensitive, requiring only minute quantities of DNA for analysis. This sensitivity has been exploited as the basis for a number of tests, including the differentiation of bacteria based on their Gram character. Accurate identification of any bacterial isolate depends on a variety of biochemical tests, with the differentiation between Gram positive and Gram negative bacteria being one of the most important factors. Standard identification protocols depend on growth of culture and requires at least 24 to 72 hours for detection.

The most rapid phenotypic test is the Gram characterization by staining, but it has been hampered by the frequent incidence of false-positive results which further lead to erroneous bacterial identification. Not all species of bacteria respond to Gram reaction. Some species are Gram variable and some Gram indeterminate.[9] Endospore forming & non-sporulating Gram positive bacteria possess a thick cell wall and

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usually stain poorly with the usual stains. Most genera belonging to this group also show variable Gram staining characteristics, with younger cultures staining Gram positive while older cultures in later stages of growth staining Gram negative[10]. The bacteria belonging to the genus *Desulfotomaculum*, *Oscillospira*, *Sporohalobacter*, *Acetogenium*, *Butyrivibrio*, *Gardnerella*, *Lachnospira* etc have cell wall of Gram positive type but stain Gram negative.[11,12] Other bacteria such as *Caseobacter* and *Cellulomonas* stain Gram positive but are easily decolorized giving false Gram reaction. Spirochetes do not stain by the Gram method.[14] The results of Gram reaction is crucial in identification of bacterial cultures but are less sensitive than molecular methods.[8] Several alternatives to the classical Gram stain have appeared in the literature, but all have the disadvantage of being subjective and time consuming. The use of molecular techniques has, therefore, been investigated in order to improve the sensitivity and reduce the time needed to identify the Gram nature of a bacterial isolate.

The use of PCR primers that target DNA regions that are conserved in bacteria for the purposes of DNA sequencing and identification of bacteria has been described.[15] It is necessary to develop a reliable broad-range detection system for bacterial DNA that is rapid and easy to use and at the same time covers a wide range of bacteria. A recent study employed a universal bacterial broad-range PCR in combination with Southern blot hybridization with probes for differentiation of Gram-positive and Gram-negative species[17, 18]. Another study made use of a nested-PCR approach wherein the first-round of PCR employed universal bacterial primers based on conserved sequences of the 16S ribosomal gene, while a second round of multiplex PCR was able to differentiate between Gram-positive and Gram-negative pathogens[6].

Here, we report a one-step Gram type specific PCR for the differentiation of Gram positive and Gram negative bacteria, which is more rapid and less time-consuming than the nested PCR approach. This paper describes an integrated protocol for the direct detection of Gram nature of bacteria, confirmed through bacterial 16S rDNA sequencing.

MATERIAL AND METHODS

Bacterial cultures

The bacterial cultures used in this study are listed in Table 1. These were obtained in the form of streaked slants and stabs from Camson Biotechnologies Culture Collection (CBCC), and Project Directorate of Biological Control (PDBC), Bangalore, India. Of these 10 isolates were Gram positive, 10 were Gram negative and six were uncharacterized. Each isolate was streaked on nutrient agar and observed for purity and proper colony morphology. Prior to DNA isolation single colonies were grown in nutrient broth at 30°C for 18hrs with shaking.

Table 1. Cultures used in this study

Cultures	No. of cultures tested	Source[a]
Negative control		
Saccharomyces sp.	1	CBTL
Gram positive isolates		
Bacillus cereus	1	PDBC
B. megatherium	2	CBTL
Bacillus sp.	2	CBTL
B. thuringiensis	3	PDBC
Lactobacillus casei	1	CBTL
Streptomyces sp.	1	CBTL
Gram negative isolates		
Azotobacter paspali	1	CBTL
Erwinia sp.	1	CBTL
Escherichia coli	1	CBTL
Pseudomonas aeruginosa	1	CBTL
P. fluorescence	1	PDBC
P. putida	1	CBTL
Ralstonia solanacearum	1	CBTL
Rhizobium leguminosarum	1	CBTL
Serratia marcescens	1	CBTL
Xanthomonas sp.	1	CBTL
Uncharacterized isolates		
CBCC 176	1	PDBC
CBCC 832	1	CBTL
CBCC 1076	1	CBTL
CBCC 1103	1	CBTL
CBCC 1326	1	CBTL
CBCC 1793	1	CBTL

[a]PDBC - Project Directorate of Biological Control; CBTL – Camson Bio Technologies Ltd., Bangalore.

Biochemical and phenotypic tests

Phenotypic and biochemical tests like Gram stain, motility, oxidase, catalase, glucose and lactose fermentation, citrate utilization, gelatin liquefaction, indole, methyl red-Vogues Proskauer tests (MRVP), arginine utilization, urease production, nitrate reduction and starch hydrolysis were performed for all cultures as described by Bergey's manual of determinative bacteriology (9th edition)[21]. Gram staining was done for all the 26 isolates using standard Gram staining protocol[22].

DNA isolation

DNA extraction was done using method described by Rainey *et al.* (1992) with some modifications. Eighteen hour old cultures were pelleted by centrifugation and resuspended in 10ml of extraction buffer containing 100mM Tris-Cl (pH8), 100mM EDTA (pH8), 1.5M NaCl, 1%CTAB, 10mg/ml proteinase K and 0.5% sodium dodecyl sulfate (SDS) and incubated at 65°C for 30min with intermittent mixing. The supernatant was washed with Phenol: Chloroform: Isoamyl alcohol (25:24:1) followed by DNA precipitation using chilled Isopropanol. The DNA pellet was resuspended in TE buffer (10mM Tris[pH8] and 1mM EDTA). The DNA was quantified using 1% agarose gel and UV spectrophotometer and diluted to 50ng/µl concentration for PCR analysis.

PCR amplification

All DNA amplifications were carried out using multiplex PCR with a combination of 3 primers CBGM 01F, 01R and 02F as against 4 primers described previously.[6] The position of the primers within the 16S rRNA gene are indicated in table 2. Primer pair CBGM 01F and 01R are universal bacterial primers common to Gram positive and negative bacteria. The PCR reaction was carried out in a 20µl reaction volume containing 50ng of each template DNA, 5 pmol each of CBGM 02F and CBGM 01R and 1 pmol of CBGM 01F, 2µl of 10x PCR buffer (Chromous Biotech, Bangalore), 1µl of 0.25mM of each deoxynucleotide triphosphates (dNTPs) (Chromous Biotech, Bangalore) and 1U of Taq DNA polymerase (Chromous Biotech, Bangalore). PCR amplifications were conducted in a Mastercycler (Eppendorf) with initial denaturation of 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 58°C for 30 s, 72 °C for 1 min (10 min at 72 °C for the final extension). PCR products were resolved in 1.5% agarose gels prepared in 0.5x TAE buffer. The gels were stained with ethidium bromide and visualized under UV light using UViDOC V.99 (Uvitech) gel documentation system.

The 16S rDNA gene of six uncharacterized isolates were amplified separately and the bands were excised, eluted using the Gel extraction kit (Chromous Biotech, Bangalore), and sequenced. Sequences were subjected to Basic Local Alignment Search Tool (BLAST) search analysis for characterization[23].

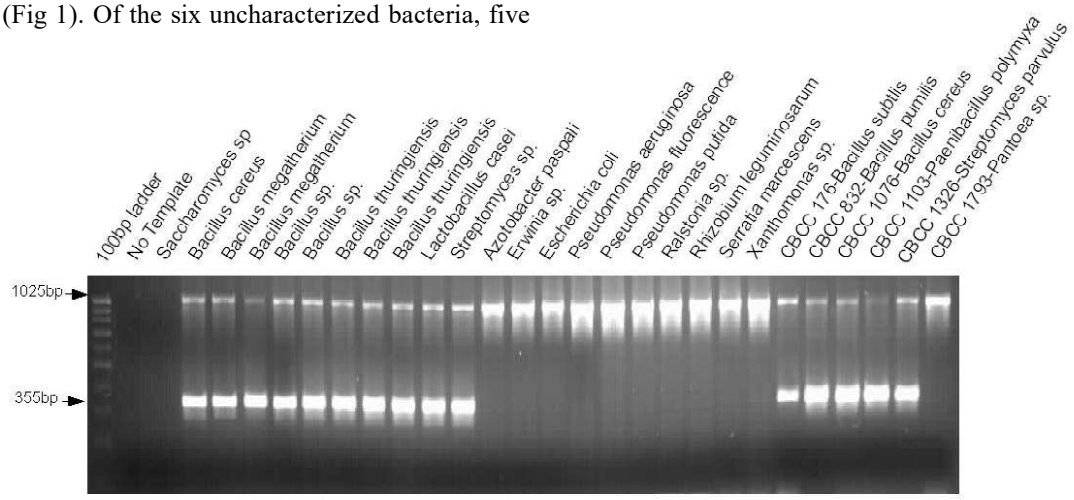
Table 2. Oligonucleotide primers used in this study

Primer	Sequence	Position on *E. coli* rRNA gene sequence (bases)
CBGM 01F	5' GGCGGCAKGCCTAAYACATGCAAGT 3'	42-66
CBGM 01R	5' GACGACAGCCATGCASCACCTGT 3'	1044-1067
CBGM 02F	5' GCGRCTCTCTGGTCTGTA 3'	712-729

RESULTS AND DISCUSSION

The main aim of this study was to develop a simple, rapid and accurate one-step PCR based assay for the differentiation of bacteria based on their Gram nature. This was accomplished by a multiplex PCR approach described previously with some modifications. The specificity of the Gram specific primers were evaluated on a range of bacterial isolates as detailed in Table 2. In both Gram positive and negative cultures a 1,025 bp amplicon was observed which is the product of the primer pair CBGM 01F and 01R, and is common for Gram positive and Gram negative bacteria (Fig 1). The 355 bp amplicon observed is the product of the primer pair CBGM 02F and

01R which is specific for only Gram-positive bacteria. All Gram negative bacteria showed a single amplicon of 1,025 bp molecular weight, whereas all Gram positive bacteria showed a 355bp product in addition to 1025bp amplicon (Fig 1). Of the six uncharacterized bacteria, five showed amplification pattern similar to Gram positive bacteria and one showed similar to Gram negative bacteria. Bacteria can be classified into Gram positive or Gram negative using this approach based on their banding patterns.



Lane 1-2: Negative controls; Lane 3-12: Gram Positive bacteria
Lane 13-22: Gram Negative bacteria; Lane 23-26: Test cultures

Fig. 1. One-step gram PCR for bacterial differentiation

Multiplex PCR was applied to a total of 26 bacterial samples. These isolates were also extensively tested using Gram staining, phenotypic and biochemical tests. Six of the isolates were characterized through 16S rDNA sequencing. Sequencing results of the six uncharacterized bacteria were concordant with the Gram specific PCR results (data not shown). The results obtained from the Gram specific PCR was correlated with the Gram staining results. In all 20 characterized bacterial samples the results of PCR were concordant with the Gram staining results. Also, the results of biochemical tests and subsequent DNA sequencing of six uncharacterized cultures matched the identity of the bacterium (Table 3).

Previous studies have used specific primers for PCR based rapid detection and identification of bacteria in environmental samples.[24, 25] However, single-step PCR for the initial classification of bacteria has not been reported so far. The Gram type-specific broad-range PCR could form the basis for the development of a rapid and sensitive procedure for the detection and preliminary classification of bacteria in any sample. Real time PCR-based protocols for the rapid detection of bacteria and exact Gram stain classification by means of fluorescence hybridization probes have also been reported[26, 27]. However, the use of real time PCR involving fluorescent probes becomes cumbersome and expensive. Multiplex PCR is a variant of PCR which enables simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. Nested PCR using two sets of primers specific to either Gram positive or Gram negative bacteria have been reported, requiring two step PCR.[6] These protocols involve amplification of 16S rDNA region of the bacterial DNA using 16S rDNA specific primers, followed by a nested PCR of this 16S rDNA amplicon. The nested PCR step requires more than one reaction vial per bacterial sample, one using Gram positive specific primers and other using Gram negative specific primers. This study obliterates the need for the two-step nested PCR, giving a simple one-step bacterial Gram nature identification technique combining 16S rDNA amplification and nested PCR together. Here, the initial step of 16S rDNA amplification

Table 3. Characterization of bacteria using one-step PCR amplification

Cultures (No. of cultures tested)	Biochemical test	Gram stain result	Gram specific PCR result
Controls			
Negative control - *Saccharomyces sp.* (1)	+	-	No amplification
Blank (no template)	-	-	No amplification
Gram positive isolates			
Bacillus cereus (1)	+	Gram positive	Gram positive
Bacillus megatherium (2)	+	Gram positive	Gram positive
Bacillus sp. (2)	+	Gram positive	Gram positive
Bacillus thuringiensis (3)	+	Gram positive	Gram positive
Lactobacillus casei (1)	+	Gram positive	Gram positive
Streptomyces sp. (1)	+	Gram positive	Gram positive
Gram negative isolates			
Azotobacter paspali (1)	+	Gram negative	Gram negative
Erwinia sp. (1)	+	Gram negative	Gram negative
Escherichia coli (1)	+	Gram negative	Gram negative
Pseudomonas aeruginosa (1)	+	Gram negative	Gram negative
Pseudomonas fluorescence (1)	+	Gram negative	Gram negative
Pseudomonas putida (1)	+	Gram negative	Gram negative
Ralstonia (1)	+	Gram negative	Gram negative
Rhizobium leguminosarum (1)	+	Gram negative	Gram negative
Serratia marcescens (1)	+	Gram negative	Gram negative
Xanthomonas sp. (1)	+	Gram negative	Gram negative
Uncharacterized isolates			
CBCC 176 - *Bacillus subtlis**	NC	Gram positive	Gram positive
CBCC 832 - *Bacillus pumilis**	NC	Gram variable	Gram positive
CBCC 1076 - *Bacillus cereus**	+	Gram positive	Gram positive
CBCC 1103 - *Paenibacillus polymyxa**	NC	Gram variable	Gram positive
CBCC 1326 - *Streptomyces parvulus**	NC	Gram positive	Gram positive
CBCC 1793 - *Pantoea sp.**	+	Gram negative	Gram negative

*Identified through 16S rDNA sequencing; NC - Not characterized; + - Biochemically characterized

is eliminated and the multiplex PCR directly amplifies genomic DNA so that previously reported two sets of PCR is reduced to a single step PCR in this study. Also, four primers required by other nested PCR approaches[6] has been scaled down to three. Some of the cultures used in this study failed to be characterized by standard Gram stain method but were successfully differentiated into Gram positive or negative using this protocol. Thus this technique was able to predict the Gram nature of bacteria that cannot be successfully determined by traditional Gram staining protocols. The protocol reported here enables analysis by multiplex PCR in a single-tube PCR assay. The initial step in phenotypic characterization of bacteria is aided by the Gram staining results. The Gram stain status of any bacteria is therefore very important because it determines the subsequent tests to be performed for further identification using Bergey's manual and plays a crucial role in the final outcome of bacterial typing[28]. In actual practice however, this test sometimes shows erroneous results as many bacterial genera exhibit variable Gram staining results even though they fall under a definite group and encapsulated bacteria fail to take up the stain. Molecular techniques, on the other hand, are not only rapid but also efficient in definite classification of bacteria. In conclusion, this protocol has demonstrated potential as an accurate and rapid one-step PCR based technique in determining the Gram status of bacteria with high specificity. Furthermore, new molecular approaches for better, more specific and faster identification of microbes should be looked into.

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