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Rapid High Specific Method for the Detection of *Pseudomonas fluorescens*

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Abstract The molecular detection *Pseudomonas fluorescens* has a vital importance for clinical and environmental microbiologist. Therefore this work aimed to establish a specific and sensitive method for diagnosis of this bacterium. To perform this aim ten *Ps. fluorescens*, eight closely related *Pseudomonas* isolates and another four common bacteria (*Escherichia coli, Proteus mirabilis, klebsiella pneumonia* and *Bacillus* sp) were collected and their detection were confirmed by 16S rRNA sequencing. Scanning the complete genome of *Ps. fluorescens* available in GenBank have denoted for the distinction of cumene dioxygenase. A couple of primer was designed to amplify 498 bp of cumene dioxygenase from *Ps. fluorescens*. The PCR using these primers resulted in single band when DNA purified from *Ps. fluorescens* isolates were used as template more over none of the of the DNA extracted from other isolates used in the study was resulting in any band at the experiment condition. The targeted band was amplified when serial dilution of *Ps. fluorescens* DNA (400, 200, 100, 50, 25, 12, 6, 3, 2, 1, 0.5 ng /µl) used as template in PCR pointing the high sensitivity of the method. Therefore this work is presenting a sensitive and specific method for the detection of *Ps. fluorescens*.

Keywords: *Ps. fluorescens, cumene dioxygenase, PCR*

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1. Introduction

Pseudomonas fluorescens is a member of genus *Pseudomonas*; this species was disregarded due to its tangency to the illustrious bacterium *Ps. aeruginosa* and considered to be of low level clinical significance [1]. Nevertheless it is known to cause bacteremia among oncology patients [2]. This bacterium involve in contamination of packed red blood cell unit [3]. Strains *of Ps. fluorescens* is responsible for the blue spoilage on fresh dairy products resulting in the so-called blue mozzarella event [4] and associated with the spoilage of poultry meat stored under chill conditions [5]. In Botany, *Ps. fluorescens* is a member of plant growth promoting rhizobacteria, increasingly appreciated for its contributions to primary productivity through promotion of growth and triggering of induced systemic resistance in plants [6]. On other hand it involves in massive soft rot of many vegetables due to production of pectate lyases enzyme [7]. This bacterium also known to be a good source for some enzymes of commercial value such as lipase [8] and proteinase [9].

Apart of conventional methods in detection of *Pseudomonas*, the molecular methods prove to be highly demanded to the diagnosis of *Pseudomonas* up to the level of species, due to its ease, rapidness and honesty. PCR emerged as a gold standard for the detection of many bacterial strains including *Pseudomonas* [10]. A successful PCR detection for the *Ps. aeruginosa* targeted the exotoxin A gene [11] while Investigating the extra cytoplasmic function gene by PCR has increased the specific detection of *Ps. aeruginosa* [12,13]. 23S rRNA gene amplification represents an ideal molecular method for the identification of *Ps. pseudomallei* [14]. *syrD* gene responsible for the syringomycin production, was utilized in specific detection of *Ps. syringae* PCR detection [15]. The plant pathogen *Ps. savastanoi* molecular diagnosis depended on the detection of a unique DNA sequence responsible for the expression of (indole-3-acetyl)-L-lysine synthethase [16]. *Ps. stutzeri* environmentally important bacterium for their denitrification activity, was detected through the PCR and Real-Time PCR depending on the nitrite reductase gene [17] Tolaasin gene dependant PCR protocol appeared to be effectively distinguish *Ps. Tolaasii* [18]. The 16S rRNA is one of the most important marker used in bacterial genotyping was used for the PCR detection of the fish pathogen *Ps. anguilliseptica* [19]. *Ps. fragi*, *Ps. lundensis*, and *Ps. putida* were also detected by PCR methods [20]. Although *Ps. fluorescens* by PCR was carried out depending on a central region of flagellin gene, however the close similarity of the fliagellin gene of *Ps.* fluorescens with that of *Ps. putida* caused cross reaction in some occasion which render the specific molecular detection for *Ps. fluorescens* [21]. The cumene dioxygenase is an enzyme that act on hydrocarbon degradation produced by *Ps.* fluorescens [22]. Therefore

the aim of this work was to develop a PCR protocol for the specific detection of *Ps.* fluorescens based on a unique sequence of cumene dioxygenase.

2. Materials and Methods

2.1. Bacterial Isolates

The bacterial isolates used in the study where provided by the microbial store of Department of Biotechnology, College of Science, University of Baghdad and illustrated in Table 1. The isolates were cultured on brain heart infusion broth or brain heart infusion agar and incubated at 37°C and 25°C for the isolates from human and soil origin respectively.

Table 1. Names and sources of bacterial isolates used in the study

Name of the Isolates	Species	Origin
S1	*Ps. fluorescens*	Baghdad, Soil
S2	*Ps. fluorescens*	Baghdad, Soil
S3	*Ps. fluorescens*	Baghdad, Soil
S4	*Ps. fluorescens*	Baghdad, Soil
S5	*Ps. fluorescens*	Baghdad, Soil
S6	*Ps. fluorescens*	Baghdad, Soil
MD1	*Ps. fluorescens*	Baghdad, Human
MD2	*Ps. fluorescens*	Baghdad, Human
MD3	*Ps. fluorescens*	Baghdad, Human
MD4	*Ps. fluorescens*	Baghdad, Human
MD5	*Ps. cepacia*	Baghdad, Human
S7	*Ps. Putida*	Baghdad, Soil
S8	*Ps. pseudomallei*	Baghdad, Soil
AM1	*Ps. aeruginosa*	Baghdad, Human
AM2	*Ps. aeruginosa*	Baghdad, Human
AM3	*Ps. aeruginosa*	Baghdad, Human
AM4	*Ps. aeruginosa*	Baghdad, Human
AM5	*Ps. aeruginosa*	Baghdad, Human
WS 2	*Escherichia coli*	Baghdad, Human
WS4	*Proteus mirabilis*	Baghdad, Human
WS9	*klebsiella pneumoniae*	Baghdad, Human
YS16	*Bacillus* sp.	Baghdad, Soil

2.2. DNA Extraction

Genomic DNA extraction carried out based on automated method using ExiPrep 16 Plus (Bioneer, Republic of Korea). 0.2 ml of the fresh bacterial culture were predicated by centrifugation 6000 RPM for 10 min. The pellet was resuspended with the lysis buffer (Provided by manufacturing company) and incubated at 37°C for 30 min then loaded to extraction cartridge (Provided by manufacturing company). DNA was eluted by 50 µl elution buffer (Provided by manufacturing company). The DNA sample measured for their concentration and purity using Microvolume UV Spectrophotometer (ACTGene, USA).

2.3. PCR, Sequencing and *in seleco*

The primers cdsF:TTGAGCCCCGTTACATCTTC and cdsR:GGGGAACCCACCTAGGATAA were designed, based on the cumene dioxygenase gene sequence in the GenBank (accession no. D37828.1). Universal bacteria 16S rRNA gene forward and reverse primers (forward: TGGA GAGTTTGATCCTGGCTCAG, reverse: TACCGCGGCTGCTGGCAC) were used to amplify 16S rRNA gene in all the bacterial isolates in the study based on Hall *et al*. *[23]*. PCR was performed in a 50 µl mixture containing 1× PCR buffer (10 mM Tris–HCl, 1.5 mM MgCl2, 50 mM KCl [pH 9]) (Merck, India), 100 µM (each) deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Merck, India), 10 pM each of forward and reverse primers, and 100 ng of templet DNA. The program for PCR included an initial denaturation 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s, extension at 72 °C for 60 s and a final extension at 72 °C for 7 min. The PCR products were resolved on a 2% agarose gel, stained with ethidium bromide (5 ng ml−1) and bands observed using a gel documentation system (ATTA, Japan). PCR products were sent for sequencing at Scigenom, India. The generated sequences were compared and analyzed against the standard sequences in the GenBank by the BLAST resources from the NCBI to unravel the identity of our gene sequences

2.4. Minimal Sensitivity.

In order to determine the lowest DNA concentration required for PCR identification for the *Ps. fluorescens* using our protocol, DNA extracted from *Ps. fluorescens* (S2) was diluted in distilled water to give concentration of 400, 200, 100, 50, 25, 12, 6, 3, 2, 1, 0.5 ng /µl. 2 µl of template DNA was added from each dilution to the PCR reaction.

3. Results and Discussion

All the bacterial isolates gave high growth after 18 h of incubation at the optimum temperature. DNA purification through the described method, provide high DNA concentration range between 295 and 1091 ng /µl, with average concentration 575.3 ng /µl. The purity f the samples were acceptable as shown in Table 2 and suitable for further PCR steps.

Table 2. The concentration and purity of nucleic acid prepared from bacterial isolates

Name of the Isolates	DNA concentration ng /µl	Purity (A260/A280)
S1	671	1.9
S2	678	1.8
S3	690	1.6
S4	683	1.8
S5	643	1,9
S6	488	1.9
MD1	338	1.6
MD2	1091	1.5
MD3	732	1.9
MD4	498	1.8
MD5	172	2
S7	820	1.9
S8	749	1.7
AM1	585	1.8
AM2	693	1.7
AM3	588	1.7
AM4	489	1.8
AM5	372	1.7
WS 2	583	1.8
WS4	379	2
WS9	421	2
YS16	295	1.6

The *Ps. fluorescens* isolates examined in study were detectable by producing a significant PCR product of 498 bp (as shown in Figure 1) using the amplification conditions in our experiment. On contrary other *Pseudomonas* isolates used in the study and the other bacterial isolates were negatively reacted with cdsF primers (Figure 2). The sensitivity of the PCR in the detection of diluted concentration of *Ps. fluorescens* was the similar to the use of high concentration as in Figure 3. The amplification of 16S rRNA gene resulted in single band of 542 bp. Sequencing of PCR product reveals the sequence of 498bp. Sequence alignment results using BLAST tool of NCBI confirm 99% of similarity of our sequence with sequences of cumene dioxygenase gene from *Ps. fluorescens* available in GenBank of NCBI. The sequencing and alignment of *16S rRNA* gene have confirmed the biochemical diagnosis.

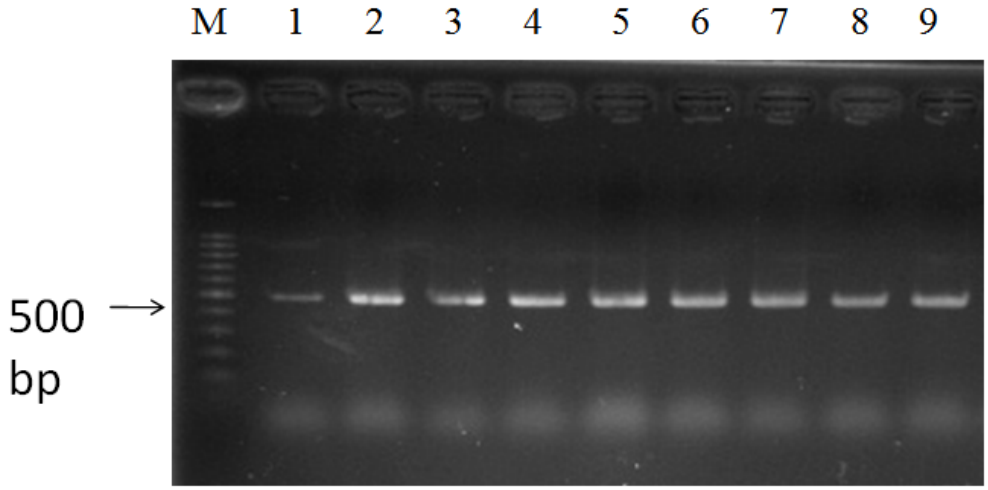

Figure 1. Detection of different isolates of *Ps. fluorescens* by PCR. Lane M, 100bp DNA ladder; lane 1 to 10, (S1,S2, S3, S4, S4, S5, S6, MD1, MD2, MD3, MD4 respectively)

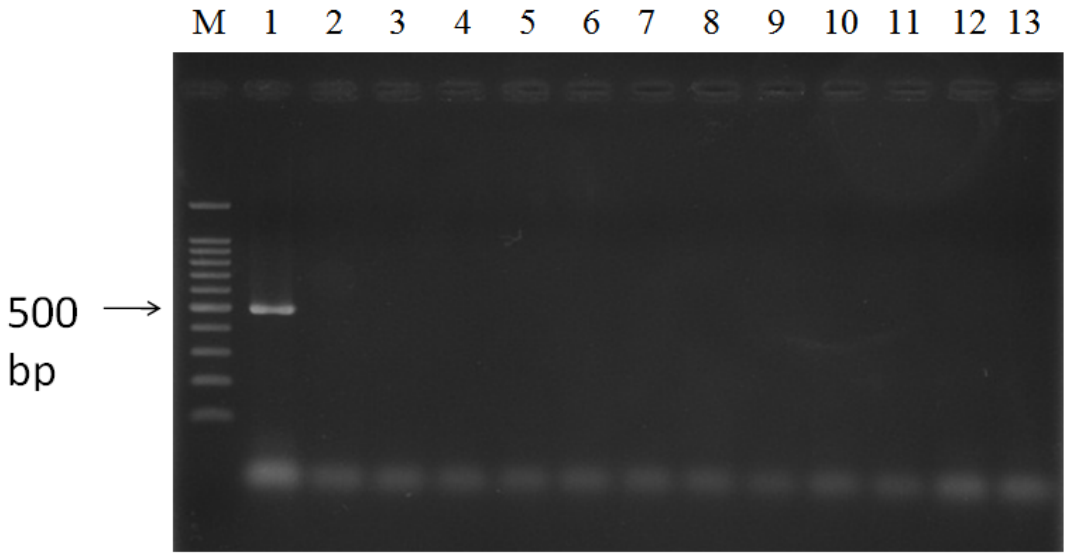

Figure 2. PCR product using specific primers cdsF and cdsR with DNA from different *Pseudomonas*. Lane M 100bp DNA ladder; lane1, *Ps. fluorescens* S2; lane 2, *Ps. cepacia*; lane 3, *Ps. Putida;* lane 4 *Ps. pseudomallei*, lane 5to9, *Ps. aeruginosa* (AM1, AM2, AM3, AM4 and AM5 respectively); lane 10, *Escherichia coli*; lane11, *Proteus mirabilis*; lane12, *klebsiella pneumonia*; lane13, *Bacillus* sp

In spite of the enthusiastic shift to the rapid methods in the detection of microorganisms, however there are very limited trials have been carried out to develop a rapid method for the detection of *Ps. fluorescens*. Therefore till 2012, there were seen papers depending on biochemical characters alone for the detection of *Ps. fluorescens* [24]. Denning *et al.*, *[21]*, used flagellin gene for the fast detection of *Ps. fluorescens* in a PCR dependant method. However this method had limited application in the specific detection of the species due to the variability of the flagellin gene in different strains and the close relatedness of the gene with flagellin gene of other *Pseudomonas* especially *Ps. putida*. To solve this matter this work came to point out a unique gene or DNA sequences that can be utilized in the specific detection of *Ps. fluorescens* b y a molecular mean. Therefore the starting tip of this work was the search for unique genes in *Ps. fluorescens* from the sequences available in the GenBank of NCBI (http://www.ncbi.nlm.nih.gov), the genetic particularity was examined through by scanning the function of each gene and the sequence's percentage of similarity with other genes procured by the use of BLAST tool [25]. The cumene dioxygenase gene from *Ps. fluorescens* GenBank (accession no. D37828.1) reveals significant particularity through its sequence distinction (data not shown). A pair of PCR primers were designed

from the most distinctive sequences in the cumene dioxygenase gene of *Ps. fluorescens*. The primers show excellent ability to detect all the ten *Ps. fluorescens* isolates used in the experiment as it seen in Figure 1. To prove the potency of the method in specific detection of the targeted species the experiment usually repeated using the same PCR conditions with DNA template of another closely related species and some of highly distributed bacteria [26]. Hence eight *Pseudomonas* other than *Ps. fluorescens* and 4 non *Pseudomonas* bacterial isolates were procured from the department of Biotechnology, University of Baghdad, as described in Table 1. All the isolates were previously diagnosed to the level of species based on extensive biochemical tests. The results (Figure 2) reveal that no PCR product were noticed in all bacterial isolates except.

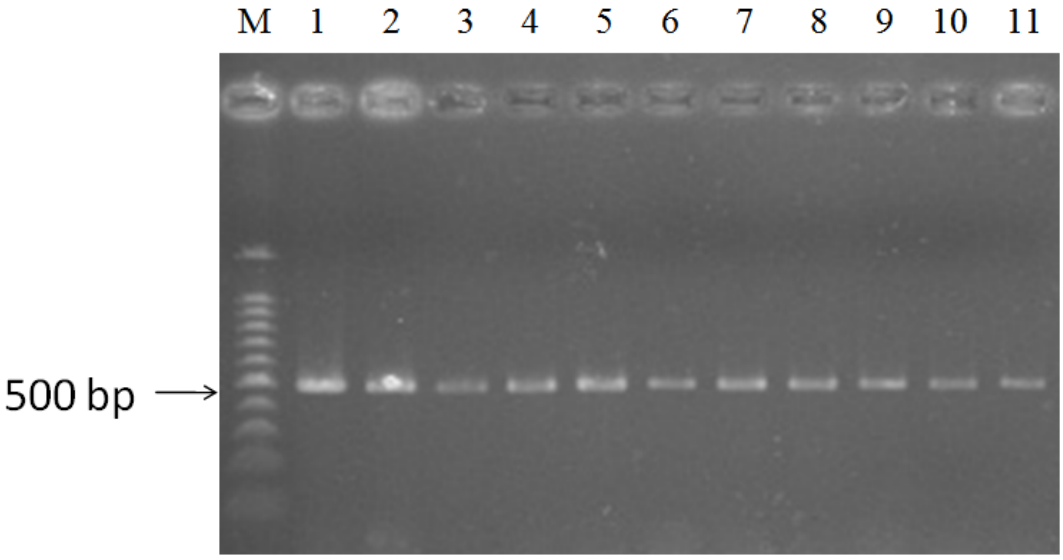

Figure 3. Detection of *Ps. fluorescens* by PCR using different concentration of DNA purified from S2. Lane M 100bp DNA ladder; lane1 to 11, different concentration of DNA template (400, 200, 100, 50, 25, 12, 6, 3, 2, 1, 0.5) ng /µl respectively

Ps. fluorescens, increasing the value of this method in the specific rapid detection of *Ps. fluorescens*. To confirm the previous diagnosis of the bacterial isolates used in this investigation the most conserved gene in prokaryotes (*16SrRNA*) was amplified by PCR using previously evaluated primers [23]. The product sequenced and aligned and the results confirmed the biochemical diagnosis in all the cases. The positive result of detection cumene dioxygenase gene in series of DNA dilution (Figure 3) raising the possible application of this work in the rapid and accurate detection of *Ps. fluorescens*.

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