Detection of quantitative and qualitative for \textit{p.aeruginosa} in contaminated and non-contaminated soil with oil residues targeting \textit{gyrb} gene by QPCR

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Abstract. Comparison of \textit{P. aeruginosa} counts is found in contaminated and non-contaminated soil with oil residue using a real-time quantitative PCR by Direct DNA extraction from soil samples as well as indirect DNA extraction from bacterial isolates. Sampling were collected randomly from ten different regions of the Ministry of Science &Technology during the period from October to December 2019. Twenty soil samples were investigated for hydrocarbon tolerance in selective broth containing hydrocarbon source, and then isolates diagnosed based on phenotype, microscopic, and biochemical tests, were used. Direct DNA extraction from soil samples using DNeasy Power Soil Pro Kit for the rapid detection of \textit{P. aeruginosa} from soil, as well as wizard DNA extraction method from bacterial isolation. Quantitative and Qualitative PCR was performed with the Magnetic Induction Cycler (Mic), DNA extraction from soil and bacterial isolates amplified and detected using fluorescent reporter dye probes specific for \textit{Pseudomonas aeruginosa} DNA and Internal Control IC. A total of twenty bacterial isolates were obtained from the different soil samples according to phenotyping tests, and also, we obtain 20 DNA samples from direct DNA extraction from soil, the results of purity were (1.70-1.95) for wizard method and (1.46-2.35) for direct extraction from soil. \textit{Pseudomonas aeruginosa} counts in contaminated soil samples (8 x 10\textsuperscript{7} Copies/μL) showed higher compared with the non-contaminated soil samples (2 x 10\textsuperscript{2} Copies/μL) at maximum use of qPCR and soil DNA. Rapid and specific test (less than 4 hours) using Rt-PCR by targeting \textit{gyrB} gene for \textit{Pseudomonas aeruginosa} detection in contaminated and non-contaminated soil samples, combined with correctly adjusted samples processing methods, as well as soil DNA extraction were made which were applied within the sensitivity required for methods.

1. Introduction

The most common environmental pollutants have contaminated soil with oil wastes which consider a great hazard to many ecosystems. Production, transportation, storage, processed oil, all that may arise of oil pollution, either accidentally or operationally, which causes big damage to the surrounding ecosystem it is considered a severe threat to the environment (Saadoun, I, et al., 2008). Biological treatments are more advantageous from the physicochemical treatments is related to clearing of oil pollution, and also an economical alternative effective to physicochemical treatment (Saadoun, I., 2002).

Numerous numbers of research have indicated that it is possible to isolate and diagnose different types of bacteria, which represent different types of hydrocarbon compounds, most of which are due to gram-negative bacteria like \textit{Pseudomonas aeruginosa}, Pseudomonads are ubiquitous bacteria in nature (Obuekwe, et al., 2008).

They possess considerable potential metabolic determinants which led to using a wide range of organic compounds, the dominance of the Pseudomonas genus in the representation and consumption of various aliphatic and aromatic hydrocarbons, given that these bacteria possess different enzymatic systems such as peripheral and peripheral oxidative systems (Monooxygenase, Dioxygenase) (van Beilen & Funhoff., 2007)

Different metabolic pathways that have biodegradable plasmids (Biodegradative plasmids) which are responsible for the representation and consumption of many polluting hydrocarbons.
Many of the inherent microbes don't grow in the laboratory, due to the availability of different cultural environment and growth conditions, thus, environmental microbiologists used molecular biology techniques rather than traditional culture techniques concerning enumeration and detection methods of the bacteria of soil (Roh, et al., 2006).

Therefore, the first objective of this study was to DNA extraction directly from soil samples, the second objective was to compare the number of P. aeruginosa between contaminated and non- contaminated soil with oil residue using the Magnetic Induction Cycler (Mic) through gyrB gene amplification.

2. Material and methods

2.1. Sampling site
Twenty contaminated and non-contaminated soil with oil residues samples were collected randomly from ten different sites in Ministry of science& technology from a surface layer (0-10 cm) during 2-months period from October to December 2019, all samples were collected in sterile bags, and transported immediately to the laboratory for immediate analysis or stored at 4°C.

2.2. Isolation of Ps.aeruginosa
In aseptic conditions twenty soil samples was investigated for hydrocarbon tolerance in selective broth containing hydrocarbon source and shaking incubation at 37°C for five days (Patel and Desai, 1997), then bacterial suspension transfer to asparagine medium incubated at 37°C (Stolp and Gadkari, 1984), and then isolates diagnosed based on phenotype, microscopic, and biochemical tests, were used (McFadden, 2000).

2.3. Molecular methods
Direct DNA extraction from 20 soil samples use DNeasy Power Soil Pro Kit, as well as wizard DNA extraction method from 20 bacterial isolates. DNA purity and concentrate were determined using a Nano Drop instrument.

Quantitative and Qualitative PCR was performed in a The Magnetic Induction Cycler (Mic PCR), amplified and detected gyrB gene in Pseudomonas aeruginosa, with Pseudomonas aeruginosa Real-TM Quant kit, the following PCR protocol was applied in accordance with the manufacturer’s instructions.

JOE(Yellow)/HEX/Cy3 channel is used to detection of DNA amplification of Pseudomonas aeruginosa, while FAM (Green) channel is used to detection of IC DNA amplification, the relationship between the cycle threshold (Ct) and the initial concentration of DNA target is linear which is basic for Quantitative DNA analysis.

3. Results and discussion

3.1. Isolation of bacteria
A total of 20 bacterial isolates were obtained from the soil samples. Bacterial isolates were revealed fluorescent pigmentation in asparagine medium and released a specific odor. This was an indication that the isolated bacteria were potentially Pseudomonas.

The results of Gram staining and biochemical tests are indicating that the organism is Pseudomonas aeruginosa and confirmed by morphology and biochemical examinations (Brenner et al., 2005). Typically, colonies are appeared with brownish to greenish-black centers, and non-lactose fermenting on MacConkey agar (MacFaddin, J.F. 2000). They grow on another selective medium (cetrimeide agar) for pyocyanin pigment production (Forbes et al., 2007), capable to grow at 42°C and also at 4°C (Jawetzet et al., 2007), the negative result for H2S, Indole, glucose fermentation and positive result for oxidase and Simmon's citrate (Guerra-Santos, et al., 1986).
3.2. Genotyping method
DNeasy Power Soil Pro Kit for Soil DNA extraction DNA purity range from (1.46 -2.35) which was assessed by (A260/A280), and DNA concentrate on in the range from (16.22-90.61 ng/μl).as well as Wizard DNA extraction from bacterial isolates give the purity level of DNA range from (1.49-2.21) which was assessed by (A260/A280), and DNA concentrate on in the range from (23.11-152.30 ng/μl). table-1-

| Soil samples | using DNeasy Power Soil Pro Kit (Direct DNA extraction from soil) | using Wizard DNA extraction (DNA extraction from Pseudomonas.aeruginosa) |
|--------------|---------------------------------------------------------------|---------------------------------------------------------------------|
|              | DNA purified | DNA concentrate (μg/μl) | DNA purified | DNA concentrate (μg/μl) |
| 1            | 2.06        | 22.65                  | 1.96        | 23.11                  |
| 2            | 1.53        | 63.12                  | 1.58        | 120.60                 |
| 3            | 1.73        | 43.75                  | 1.67        | 90.63                  |
| 4            | 1.89        | 17.73                  | 1.70        | 96.37                  |
| 5            | 1.63        | 31.35                  | 1.82        | 70.42                  |
| 6            | 1.00        | 51.35                  | 2.07        | 65.60                  |
| 7            | 2.76        | 77.66                  | 1.63        | 78.90                  |
| 8            | 2.35        | 49.15                  | 1.67        | 44.36                  |
| 9            | 2.30        | 63.17                  | 1.70        | 80.38                  |
| 10           | 2.07        | 48.67                  | 2.21        | 110.25                 |
| 11           | 1.46        | 90.61                  | 1.51        | 152.30                 |
| 12           | 1.50        | 62.15                  | 1.66        | 89.22                  |
| 13           | 2.23        | 25.51                  | 1.94        | 34.32                  |
| 14           | 2.04        | 45.21                  | 1.57        | 63.24                  |
| 15           | 2.01        | 35.36                  | 1.49        | 53.24                  |
| 16           | 1.72        | 19.37                  | 1.85        | 48.67                  |
| 17           | 1.65        | 29.45                  | 1.75        | 70.61                  |
| 18           | 1.00        | 12.45                  | 1.69        | 62.25                  |
| 19           | 1.25        | 16.22                  | 1.62        | 55.31                  |
| 20           | 1.89        | 25.24                  | 1.95        | 35.31                  |

3.3. Pseudomonas aeruginosa detection by Rt-PCR
Establishment of Standard curves for Ps. aeruginosa was done with the Magnetic Induction Cycler (Mic) is a compact rotary based 48-well qPCR instrument that applies magnetic induction to achieve heating and forced airflow for cooling.

We used a negative control in each run to detect any contamination and also to determine the uniqueness of primers, whenever increased reporter fluorescence above the baseline we can determine the threshold cycle (Ct) (Motorhome M, et al., 2007).

qPCR detected P. aeruginosa in forty soil samples showed higher bacterial counts contaminated soil with oil residue (8 x 10^7 Copies/μL) compared to the non-contaminated soil with oil residue samples (2 x 10^2 Copies/μL) at maximum. (Table 2)
### Table (2) Absolute Quantification for Pseudomonas aeruginosa using MIC-Rt-PCR run.

| NO. | ID samples | Cq | Calculated Concentration (Copies/µL) | Efficiency R² |
|-----|------------|----|--------------------------------------|---------------|
| 1-   | OB         | 11 | $4 \times 10^6$                      | 0.99842       |
| 2-   | OB         | 18 | $1 \times 10^7$                      | 0.99854       |
| 3-   | OB         | 11 | $8 \times 10^6$                      | 0.99849       |
| 4-   | OB         | 25 | $3 \times 10^6$                      | 0.99844       |
| 5-   | OB         | 10 | $3 \times 10^7$                      | 0.99873       |
| 6-   | OS         | 24 | 560                                  | 0.99870       |
| 7-   | OS         | 12 | 112                                  | 0.99883       |
| 8-   | OS         | 12 | 173                                  | 0.99915       |
| 9-   | OS         | 17 | 82                                   | 0.99879       |
| 10-  | OS         | 27 | 347                                  | 0.99887       |
| 11-  | OB         | 28 | $2 \times 10^5$                      | 0.99885       |
| 12-  | OB         | X | $1 \times 10^4$                      | X             |
| 13-  | OS         | 27 | 3456                                 | 0.99805       |
| 14-  | OS         | 31 | 2357                                 | 0.99845       |
| 15-  | OS         | 13.02 | 6940                          | 0.99778       |
| 16-  | AB         | 16.11 | $1 \times 10^3$                     | 0.99721       |
| 17-  | AB         | 9.52 | $1 \times 10^5$                     | 0.99726       |
| 18-  | AB         | 33.16 | $2 \times 10^7$                     | 0.99785       |
| 19-  | AB         | 26.82 | $3 \times 10^5$                     | 0.99753       |
| 20-  | AB         | 32.17 | $2 \times 10^6$                     | 0.99807       |
| 21-  | AS         | 25 | 485                                  | 0.99678       |
| 22-  | AS         | 23 | 23                                   | 0.99821       |
| 23-  | AS         | 27 | 2                                    | 0.99526       |
| 24-  | AS         | 30 | 4131                                 | 0.99985       |
| 25-  | AS         | 25 | 450                                  | 0.99453       |
| 26-  | AB         | 16 | $3 \times 10^6$                      | 0.99307       |
| 27-  | AB         | 20 | $2 \times 10^5$                      | 0.99850       |
| 28-  | AS         | 24 | 1800                                 | 0.99873       |
| 29-  | AS         | 17 | 2100                                 | 0.99945       |
| 30-  | AS         | 22 | 2470                                 | 0.99839       |
| 31-  | AB         | 18 | $2 \times 10^6$                      | 0.99827       |
| 32-  | OB         | 14 | $3 \times 10^6$                      | 0.99855       |
| 33-  | AS         | 19 | 4563                                 | 0.99754       |
| 34-  | OS         | 18 | 7850                                 | 0.99549       |
| 35-  | OS         | 25 | 3450                                 | 0.98724       |
| 36-  | OB         | 19 | $3 \times 10^7$                      | 0.98780       |
| 37-  | OS         | 30 | 1290                                 | 0.98753       |
| 38-  | OB         | 17 | $2 \times 10^6$                      | 0.99507       |
| 39-  | OS         | 27 | 4500                                 | 0.98678       |
In conclusion, these results were in agreement with data from other studies in relation of *Pseudomonas aeruginosa* biodegradable which is responsible for the representation and consumption of many polluting hydrocarbons (Le Gall *et al.*, 2013), detection of *Pseudomonas aeruginosa* by direct DNA extraction from soil and using Rt-PCR for bacterial count were more sensitive to prevent false negative results, and rapid and specific test (less than 4 hours) using Rt-PCR by targeting gyrB gene for *P. aeruginosa* detection in contaminated and non-contaminated soil samples, combined with correctly adjusted samples processing methods, as well as Soil DNA extraction were made which is applied within the sensitivity required for methods.

The gyrB gene of *P. aeruginosa* which produce a type II topoisomerase, is considered important for the detection and identification of bacterial isolates as well as the accuracy and sensitivity of collecting and processing samples (Lan J, *et al.* 2008), furthermore, DNA soil extraction was used to achieve the desired sensitivity of the method. We also conclude that molecular techniques for *P. aeruginosa* identification is more accurate, sensitive and require more rapid time than standard biochemical testing without high costs (Saiman, L., *et al.*, 2003).

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