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The Quantitative Assessment of Bacterial Species from Soil Samples through Real Time PCR

Anjali Tomar[1], Deepak Bharati[2], Samrah Rehan[3], Rohit Kumar Vishwakarma[1] and Manisha Shukla[1*]

[1]Department of Biotechnology, Pandit S.N. Shukla University, Shahdol, Madhya Pradesh, India
[2]Centre for Molecular Biology Research (CMBR) Biotech. Pvt Ltd. Bhopal, Madhya Pradesh, India
[3]Department of Biochemistry, Ganesh Shankar Vidyarthi Memorial Medical College, Kanpur, Uttar Pradesh, India

**Corresponding author*

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A B S T R A C T

This study assessed microbial populations in soil samples from the Bhopal, Capital, Madhya Pradesh, India using traditional dilution plating, supplemented with qPCR for molecular insights. The CFU counts ranged from 6.3 to 8.2 $\log_{10}$ CFU/gm, with sample 7 having the highest and sample 4 the lowest bacterial load, highlighting variability due to soil properties and environmental conditions. qPCR analysis showed an inverse relationship between CFU counts and CT values, confirming higher DNA concentrations with higher viable bacterial counts. This dual approach of combining CFU and qPCR methods offers a comprehensive understanding of microbial presence, consistent with prior research findings. High bacterial counts, as seen in sample 7, suggest healthy soil with robust nutrient cycling and organic matter decomposition, while lower counts in sample 4 may indicate less favorable conditions for microbial growth. Future studies should incorporate advanced molecular techniques, like high-throughput sequencing, to fully capture soil microbial diversity.

Introduction

Among soil organisms, bacteria and fungi play a crucial role in breaking down organic materials, releasing chemical nutrients and promoting plant growth. Bacteria are the most prevalent microorganisms in soil, with populations varying across different soil types and conditions. According to Whitman *et al.,* (1998) bacterial counts in various soils range from 4×106 to 2×109 per gram of dry soil. Soils naturally cover the earth's surface and act as an interface between solids (geological and defunct biological components), liquids (water), and gases (air in soil pores) (Whitman *et al.,* 1998). Each soil is a unique product of its underlying geological material, glacial and geomorphological history, biotic activity, and land-use history. Soils support a diverse array of bacteria, archaea, fungi, insects, annelids, and other invertebrates, in addition to plants and algae. Soils provide essential nutrients to species both above and below ground and are crucial for filtering and buffering freshwater habitats.

Consequently, soils are integral to human cultures. They are indispensable for the production of food, construction materials, and other resources, significantly influencing the ecosystem services we rely on (Dominati *et al.,* 2010). Soil microbes—bacteria, archaea, and fungi significantly contribute to these ecosystem functions. Their diverse metabolic activities drive or influence the cycling of key elements such as carbon (C), nitrogen (N), and phosphorus (P), affecting soil ecosystem structure and function and their capacity to benefit humans. Bacteria and archaea, the world's smallest autonomous single-celled organisms, typically range in diameter from 0.5 to 1.0 µm. These microorganisms come in common forms like cocci, rods, and spirals, with some bacteria, such as Actinomycetales, branching into filaments. Bacteria and archaea lack a membrane-bound nucleus, with their DNA freely located in the cytoplasm. Their genome typically comprises a single circular molecule of double-stranded DNA but may also include plasmids, smaller DNA fragments. Genome size varies with the organism's lifestyle and complexity, typically ranging from 4 to 6 million nucleotides and containing genetic material for 3000 to 4000 genes.

Their cells are surrounded by a phospholipid cell membrane and a cell wall composed of proteins, carbohydrates, and lipids, varying by organism. Many microorganisms move using flagella (whip-like cell extensions) and produce fine filaments called pili, which connect cells to each other or the soil surface. Some microbes use specific pili for conjugation, a process of adhering to other microbes and transferring DNA. Microbes usually reproduce asexually by binary fission, with some dividing as quickly as every 12-20 minutes, while others take longer. They are classified as autotrophs or heterotrophs. Autotrophs synthesize carbohydrates, fats, and proteins using energy from sunlight or inorganic compounds, while heterotrophs rely on organic carbon compounds for carbon and energy. While archaea and bacteria are morphologically similar, molecular phylogenetic methods based on 16S ribosomal RNA sequences classify life into three domains, with Archaea more closely related to Eukarya than Bacteria (Woese *et al.,* 1990). Soils harbor abundant microbial diversity, with bacteria most prevalent and archaea tenfold less abundant. A gram of soil is estimated to contain 2000 to 18000 bacterial species (Ritz *et al.,* 2003).

The soil ecosystem is complex, with diverse microbial habitats influenced by parent material, landform, organisms, and climate. Over time, these factors interact, creating distinct soil horizons. Processes such as humus formation, mineral weathering, and organic material decomposition affect the soil profile. Soil microbes aid in these processes. Typical soil horizons include L, F+H, A, E, and B, with the highest microbial activity in the organic-rich A horizon. Molecular identification of bacterial species in soil is efficient and time-saving. This involves directly extracting ribosomal RNA (rRNA) genes from soil cells, followed by using rRNA-specific primers and polymerase chain reaction (PCR) to amplify the genes from total community DNA.

Materials and Methods

Collection of bacteria from soil samples

Total 10 soil samples were collected from different sources of the Bhopal, district of Madhya Pradesh. One gram of soil sample was dissolved in 9 ml of sterile distilled water and serial dilutions were performed from 10^{-1} to 10^{-6}. The 10^{-3} to 10^{-6} serial dilution were then relocated to the nutrient agar plates using the spread plate method.

1g of soil from each sample was dissolved in 10ml of autoclaved double distilled water. The samples were then serial diluted up to10^{-6}. The suspensions of each sample were spread on LB media plates under aseptic condition (Robertson and Egger, 2010). All the inoculated plates were allowed to absorb the inoculums at 37° temperature for 24 hrs. Plates were inverted and incubated as optimum conditions. Morphological appearances of all different colonies were examined which included size, shape, colour, elevation and transparency. Then, all the plates were analyzed for Colony Counting (Atlas Ronald, 1984). Plates with 30 to 300 colony forming units i.e., CFUs/plate were used to calculate CFUs/ml.

DNA Extraction

Isolated bacterial cultures were grown overnight in nutrient broth at 37°C. A volume of 1-2 ml of the bacterial culture was centrifuged at 10,000 rpm for 5 minutes to pellet the cells. Genomic DNA was extracted using a commercial DNA extraction kit, following the manufacture DNA Extraction users' instructions. The extraction process typically includes cell lysis, binding of DNA to a silica column, washing, and elution of pure DNA. The quality and concentration of the extracted DNA were assessed using a NanoDrop

spectrophotometer. DNA purity was evaluated by measuring the absorbance ratio at A260/A280 nm.

Real-Time PCR analysis

Real-Time PCR amplifies specific DNA sequences, allowing for the detection and quantification of bacterial DNA in the samples. Primers specific for the 16S rRNA gene, a conserved region in bacterial genomes, were selected from previous studies.

These primers ensure the amplification of bacterial DNA across a wide range of species. Real-Time PCR reactions were set up in a total volume of 25 µl, consisting of: 12.5 µl of 2x SYBR Green Master Mix, which contains DNA polymerase, dNTPs, MgCl2, and SYBR Green dye for fluorescence detection. The PCR reactions were performed in a thermocycler with the following cycling conditions:

A melting curve analysis was conducted after the amplification cycles to confirm the specificity of the PCR product.

To determine the bacterial load in the soil samples by quantifying the amount of target DNA using standard curves. Serial dilutions of a known concentration of bacterial DNA were prepared to generate a standard curve. The dilutions typically range from 10^1 to 10^7 copies per reaction. Each standard dilution was subjected to Real-Time PCR under the same conditions as the samples. The Ct (cycle threshold) values obtained from the standards were used to plot a standard curve, relating Ct values to the known DNA concentrations. The Ct values of the soil samples were compared to the standard curve to determine the bacterial load. The bacterial concentration in the soil samples was calculated and expressed as the number of bacterial cells per milliliter (cells/ml).

Data Interpretation

The results obtained from Real-Time PCR were then compared with CFU counts to analyse bacterial load and diversity in soil samples.

Results and Discussion

In present study, we have assessed microbial population (Bacterial species) in soil samples, collected from different fields of Bhopal district and were analyzed using traditional dilution plating method for the quantitative study of microflora (Atlas Ronald, 1984).

CFU analysis in soil samples

The viable bacterial counts in the soil samples were determined by the Colony-Forming Unit (CFU) method on nutrient agar plates. The results of the viable counts for the different soil samples are presented in Table 1. The CFU counts, expressed as log10 cfu/gm of soil, indicate the microbial load present in each soil sample.

The viable bacterial counts in the soil samples ranged from 6.3 to 8.2 $\log_{10}$ cfu/gm. The highest bacterial count was observed in sample 7 (8.2 $\log_{10}$ cfu/gm), indicating a significantly higher microbial load compared to the other samples. The lowest count was found in sample 4 (6.3 $\log_{10}$ cfu/gm), suggesting a relatively lower bacterial density. The variation in bacterial counts among the soil samples can be attributed to several factors, including differences in soil composition, moisture content, organic matter, and the presence of nutrients.

Soil sample 7, which exhibited the highest bacterial count, might have higher organic matter content or more favorable conditions for bacterial growth compared to the other samples. This variability is consistent with findings from previous studies, which have reported that soil bacterial populations are influenced by environmental factors and soil properties (Torsvik and Øvreås, 2002; Fierer and Jackson, 2006). The bacterial counts observed in this study are in line with those reported in similar studies.

For instance, Torsvik *et al.,* (1990) reported bacterial counts ranging from 10^6 to 10^9 cfu/gm in various soil types, highlighting the high diversity and abundance of soil bacteria (Torsvik *et al.,* 1990). Similarly, Aislabie *et al.,* (2013) found that agricultural soils typically contain bacterial counts in the range of 10^6 to 10^8 cfu/gm (Aislabie *et al.,* 2013). The counts in our study fall within this range, indicating a normal microbial load for soil samples.

High bacterial counts, such as those observed in sample 7, are generally indicative of healthy soil with active microbial communities. These bacteria play crucial roles in nutrient cycling, organic matter decomposition, and soil fertility (Coleman *et al.,* 2017). Conversely, lower bacterial counts, such as those in sample 4, may suggest poorer soil health or less favorable conditions for

microbial activity. Maintaining a diverse and abundant microbial population is essential for soil health and agricultural productivity (Van Elsas *et al.,* 2006). While CFU counting provides valuable information on viable bacterial populations, it only accounts for culturable bacteria.

Many soil bacteria are not readily culturable under standard laboratory conditions, and thus, the actual bacterial diversity and abundance may be underestimated (Amann *et al.,* 1995). Future studies should incorporate molecular techniques, such as high-throughput sequencing, to gain a more comprehensive understanding of soil microbial diversity (Daniel, 2005).

The results from the CFU counts highlight the variability in bacterial load among different soil samples, with sample 7 exhibiting the highest bacterial count. These findings are consistent with previous studies and underscore the importance of soil properties and environmental factors in shaping microbial communities. For a more holistic view of soil microbial diversity, combining culture-based methods with molecular techniques is recommended.

Comparison between CFU Count and DNA Concentration (CT Values, qPCR)

The standard plot for DNA-based quantification of all bacterial species is a valuable tool in qPCR experiments. It assists researchers in determining the DNA concentration in their samples and assessing the efficiency and accuracy of their qPCR assay. Properly constructed standard plots are critical for achieving reliable and reproducible quantitative PCR results.

The results presented in Table 2 show the CT values obtained from known DNA concentrations, which were used to construct a standard calibration curve Figure 2. The CT values decrease as the DNA concentration increases, indicating an inverse relationship. This calibration curve is essential for quantifying DNA concentrations in unknown samples based on their CT values.

The results presented in Table 3 show the comparison between colony-forming unit (CFU) counts and DNA concentrations determined by qPCR (represented by CT values) for various soil samples. The viable counts, expressed as $\log_{10}$ cfu/gm of soil samples, provide an estimate of the number of viable bacteria present in each sample. Concurrently, the CT values and corresponding DNA concentrations (in ng/µl) provide a molecular quantification of bacterial presence.

The results indicate a general trend where higher CFU counts correspond to lower CT values, reflecting higher DNA concentrations, as seen in samples 4 and 7. This inverse relationship is consistent with expectations, where a greater number of viable bacterial cells leads to higher DNA yields, resulting in lower CT values during qPCR analysis.

Correlation between CFU and DNA Concentration

Sample 1

A CFU count of 6.4 $\log_{10}$ /gm corresponds to a CT value of 23.33, with a DNA concentration of 59.13043 ng/µl. This indicates a moderate bacterial load.

Sample 4

This sample shows a lower CT value of 22.22, corresponding to a higher DNA concentration of 107.3913 ng/µl, despite having a slightly lower CFU count (6.3 $\log_{10}$ cfu/gm). This discrepancy could be due to variations in DNA extraction efficiency or differences in bacterial cell lysis.

Sample 7

The highest CFU count of 8.2 log10 cfu/gm correlates with the lowest CT value of 21.32 and the highest DNA concentration of 146.5217 ng/µl, confirming the expected trend. Variation in CFU and CT Values: Both samples (Sample 5 and 6) exhibit the same CFU count (6.4 $\log_{10}$ cfu/gm) but slightly different CT values (23.81 and 23.73, respectively) and DNA concentrations (38.26087 ng/µl and 41.73913 ng/µl). These minor differences could arise from slight inconsistencies in sample processing or DNA extraction.

The consistency in the inverse relationship between CFU counts and CT values (and thus DNA concentrations) highlights the reliability of combining traditional microbial counting techniques with molecular quantification methods for assessing bacterial load. Such dual approaches provide a comprehensive understanding of microbial presence and can be particularly valuable in ecological and environmental studies.

Table.1 Real-Time PCR Cycling Conditions

PCR Steps	Temperature	Duration	Number of Cycles
Initial denaturation	95°C	3 minutes	1
Denaturation	95°C	10 seconds	40
Annealing	54°C	10 seconds	
Extension	72°C	15 seconds	
Final Extension	72°C	5 minutes	1

Table.2 Viable Bacterial Counts in Soil Samples

Soil Sample No.	NA (Nutrient Agar) Viable count (log10cfu/gm of soil samples 103) (CFU)
1	6.4
2	6.8
3	6.6
4	6.3
5	6.4
6	6.4
7	8.2
8	6.4

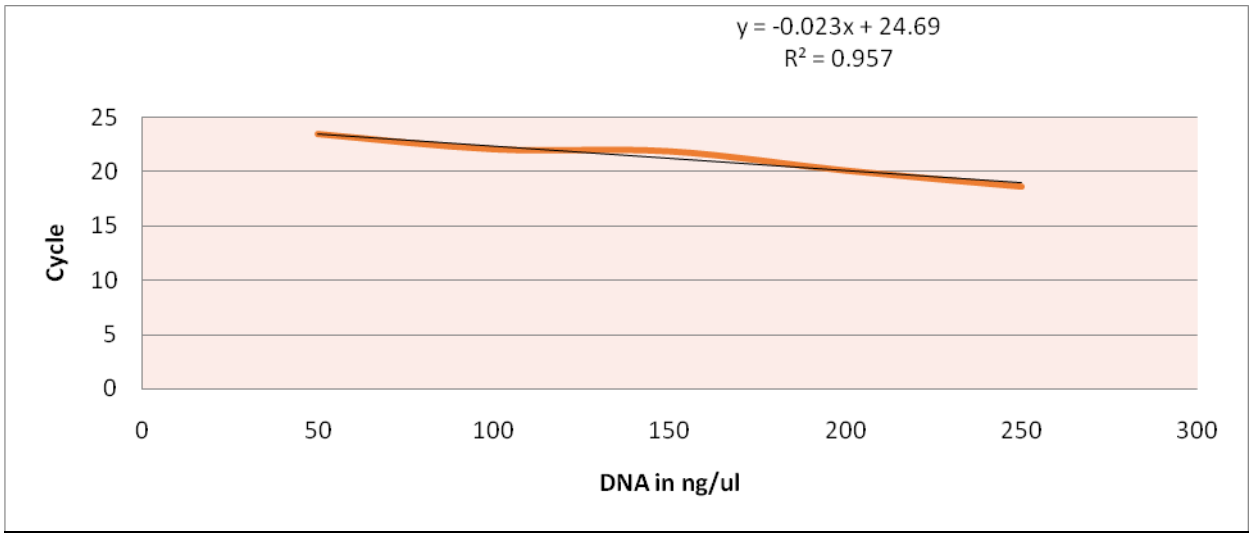
Table.3 CT values of known bacterial DNA sample for standard plot.

S. No.	Concentration (ng/ul)	CT value
1	50	23.45
2	100	22.07
3	150	21.88
4	200	20.13
5	250	18.67

Table.4 Present table showing comparison between CFU count and the DNA concentration (CT values -qPCR) in different collected soil samples

Soil Sample No.	NA (Nutrient Agar) Viable count (log10cfu/gm of soil samples 103) (CFU)	CT value (qPCR)	Concentration in (ng/µl)
1	6.4	23.33	59.13043
2	6.8	23.14	67.3913
3	6.6	23.05	71.30435
4	6.3	22.22	107.3913
5	6.4	23.81	38.26087
6	6.4	23.73	41.73913
7	8.2	21.32	146.5217
8	6.4	23.47	53.04348

Figure.1 Calibration curve of known bacterial DNA sample for standard plot.



The standard plot constructed from known DNA concentrations (not shown here) allows for precise quantification of bacterial DNA in unknown samples, facilitating accurate assessments of microbial populations. Proper calibration and validation of qPCR assays are crucial for obtaining reliable and reproducible results, as demonstrated by the data presented. Integration of CFU counts with qPCR-based DNA quantification provides a robust framework for evaluating bacterial loads in environmental samples. This combined approach enhances the accuracy of microbial assessments and supports a deeper understanding of bacterial dynamics in different environments.

In conclusion, our study assessed microbial populations in soil samples from Bhopal district using traditional dilution plating and supplemented with qPCR for molecular insights. The CFU counts ranged from 6.3 to 8.2 log10 cfu/gm, with sample 7 having the highest and sample 4 the lowest bacterial load, highlighting variability due to soil properties and environmental conditions.

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Author Contributions

Anjali Tomar: Investigation, formal analysis, writing—original draft. Deepak Bharati: Validation, methodology, writing—reviewing. Samrah Rehan:—Formal analysis, writing—review and editing. Rohit Kumar Vishwakarma: Investigation, writing—reviewing. Manisha Shukla: Resources, investigation writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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