Int.J.Curr.Microbiol.App.Sci (2020) *9*(11): 1962-1970

EXCELLENT PUBLISHERS

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 11 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.911.233

Comparative Microbiome Analysis of Pesticide-Contaminated and Pristine Agricultural Soils of Hot Arid Rajasthan

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Keywords

Microbiome, 16S rRNA, Pesticides, Arid soil

Article Info

Accepted:
15 October 2020
Available Online:
10 November 2020

A B S T R A C T

Comparative microbial community structure analysis of agricultural soil located in hot, western Rajasthan with long term history of chemical pesticides vis-à-vis pristine soil has been attempted using the technique of PCR-RFLP. Pesticide-contaminated (24 nos.) and pristine (10 nos.) soil samples were collected from different locations of the vegetable growing agricultural fields. Metagenomic DNA was extracted each from pooled-contaminated and pooled-pristine soils and amplified with universal 16S rRNA gene primers. Amplification products were cloned in to *E. coli* DH5α cells to obtain 78 and 39 16S rDNA clones from contaminated and pristine soils respectively. The clones were subjected to RFLP analysis using three restriction enzymes (AluI, AvaII and MspI) followed by phylogenetic tree construction. The studies revealed differences in microbial community structures of both types of arid, agricultural soils. We also detected the presence of pesticide-degrading naphthalene dioxygenase gene (nahA) controlling the initial step of the degradation process in contaminated soil.

Introduction

Microorganisms play a crucial role in the degradation of organic compounds and are therefore very important for the mineralization or detoxification of toxic organic chemicals. Numerous field tests and laboratory experiments have identified many harmful organic compounds which biodegrade very slowly (Alharbi, 2018, Speight, 2017) thereby persisting in the environment for long periods. Besides physicochemical conditions, the low biodegradability could be due to low number or complete absence of m.o. present in the natural environment to metabolize the pollutants effectively (Huang *et al.,* 2018; Doolotkeldieva *et al.,* 2018). Nevertheless, microbial communities exposed to xenobiotic compounds for long periods adapt to these chemicals, and m.o. that metabolize them completely and at considerable rates have been isolated (Iqbal and Bartakke, 2014; Akbar and Sultan, 2016). Pesticide degradation usually involves more than one microorganisms and each m.o. contributes to

biodegradation reactions (Doolotkeldieva *et al.,* 2018). Therefore, characterization of individual m.o. isolated from the natural environment has its limitations for a proper assessment of different genetic events in the adaptive response of bacteria in their natural environment. A comparative characterization involving microbiomes present in contaminated as well as pristine agricultural soils is important to assess a particular bio-remediation process as well as understanding of the mechanisms in nature to deal with the persistent organic pollutants. Culture-dependent methods are not capable of thoroughly depicting the existing microbial diversity in the biosphere (Austin, 2017) since more than 99% of the potentially 10^{11}–10^{12} microbial species on Earth are unculturable and undiscovered (Bodor *et al.,* 2020; Locey and Lennon, 2016). There is an emerging need to learn more about the missing species through culture-independent approach, since these have great environmental sustainability potential for bioremediation purposes (Epstein, 2013). A comparative analysis of microbiomes of pesticide contaminated and pristine soil is also important to give us an idea as to which m.o. were enriched in soil following contamination and which ones were already present. 16S rDNA clone library preparation is suitable technique for obtaining overall information about the genetic diversity and community structure of m.o., including unculturable bacteria in an environment (Marzorati *et al.,* 2008). Several authors have successfully performed PCR-RFLP analyses to assess genetic diversity among bacterial species in the past (Rahmanifar *et al.,* 2012; Mandakovic *et al.,* 2016) in environmental samples. Desert soils are particularly problematic where leaching of pesticides is low due to scanty rainfall resulting in persistence of slowly degradable chemicals (Devi *et al.,* 2018). There is hardly any report from arid western Rajasthan that involves studying changes in the beneficial microbial diversity and composition as a result of indiscriminate usage of pesticides in agricultural soil. Therefore, the present study was undertaken to assess the genetic diversity in microbial populations of pesticide-contaminated arid agricultural soil vis-a-vis uncontaminated or pristine soil of Jodhpur district of western Rajasthan. In a parallel study, we also analyzed the presence of naphthalene degraders by amplification of nahA component of naphthalene dioxygenase gene coding for Naphthalene 1, 2-dioxygenase reductase (Ferrero *et al.,* 2002) in the contaminated soil sample.

Materials and Methods

Soil source and characteristics

Pesticide contaminated soil cores weighing approximately 10 gm were obtained from 0-15 cm. depth from 24 different sites covering three villages namely Tinvri, Balarwa and Indroka of hot, arid western Rajasthan, India (Latitude, longitude; 24-300N, 70-76.20E). The 24 soil samples were from 12 fields, 2 samples / field, and 4 fields / village, each village situated within a radius of 25 km from the other. The selected fields were mostly growing chilli, groundnut and mustard in different combinations. The pesticides commonly used were carbofuran, thiram, dinocap, carbendazim, dimethoate for chilli; chlorpyriphos, imidacloprid for groundnut and quinalphos, monocrotofos for mustard to name a few. For comparison, pristine soil samples were also collected from ten different locations from agricultural fields with no history of pesticide usage from the same agro-climatic zone. For ensuring maximum microbial diversity, both the contaminated and the pristine soil samples were pooled separately and mixed thoroughly before further analysis. Major soil characteristics of sampling sites were loamy, sandy soil with sand 87.2%, silt 5.2% and clay 7.1%. The pH

of the soil was in the range of 8.1 to 8.5 with Ece 0.088 ds/m-1. The soil samples were kept in the cold room in sterile sample bags at 4ºC until used.

Metagenomic soil DNA extraction

The DNA extraction method was a modified version of the direct lysis method [Saano *et al.,* 1995] which was briefly as follows- To 1g of soil sample in a 15 ml polypropylene tube was added 2.5 ml of the following buffer- 120 mM Na_2HPO_4 (pH 8.0), 1% sodium dodecyl sulphate (SDS), 100 µg ml^{-I} proteinase K+, mixed well and incubated for 1h at 37°C with occasional shaking. 450 µl of 5M sodium chloride was then added followed by vortexing and addition of 375 µl of 10% cetyl trimethyl-ammonium bromide in 0.7M NaCl and incubation at 65°C for 20 min. Chloroform was added in equal volume followed by vortexing. The mixture was centrifuged for 15 min. at 9000xg at 4°C. The water phase was collected into a fresh tube and an equal volume of absolute alcohol was added, mixed and incubated for 1h at -20°C followed by 70% ethanol wash. It was then centrifuged for 5 min and the DNA pellet was dried. The amount of DNA extracted was estimated by electrophoresis of 2µl aliquot on a 0.8% agarose gel.

PCR amplification and cloning

PCR was performed to amplify bacterial 16S rRNA and naphthalene dioxygenase (nahA) gene sequences using metagenomic soil DNA. Universal rDNA primers- Eub1(5'AG AG TTT GAT CCT GG CTCA 3') and Eub2 (5'GCTCGTTGCGGGACTTATCC 3') from *Eubacteria* and naphthalene dioxygenase gene primers (NA-1-1/F 5'GATGTTCGCG CTCGGA3' and NA-1-1/R 5' AGCTGCT GACGTGTG 3') from *Pseudomonas putida* were used for amplification purpose. All PCR amplifications were performed with Taq DNA polymerase (Bangalore Genei) in PERKIN ELMER GeneAmp PCR System using following steps; an initial denaturation for 5 min. at 94°C, 35 amplification cycles of denaturation (30s at 94°C), annealing (30s at 55°C) and elongation (30s at 72°C) and a final extension step of 7 min. at 72°C. The PCR products were purified by running on low melting point agarose (1%), followed by elution of bands and phenol-chloroform-alcohol purification of the DNA. Plasmid clones were generated by blunt end ligation of the PCR product with PUC 19 at the Sma I site after end filling with klenow fragment of DNA polymerase I. The ligation products were transformed into *Escherichia coli* DH5α competent cells and the selection of the transformants containing the inserts was done on Luria agar plates containing ampicillin (100 µg/ml^{-1}) in presence of 50 µI x-gal (stock- 20 mg/ml in dimethyl formamide) for blue-white color selection. White colonies selected were inoculated into luria broth containing ampicillin. The cultures were grown overnight and plasmid minipreps were done. The plasmid preps showing higher molecular weight on the electrophoresis gel as compared to the vector were further confirmed as recombinant clones by restriction enzyme digestion with Hind III and Eco R1 which cut the vector PUC 19 on either side of Sma I site to release the insert.

Restriction fragments analysis or ribotyping

Five µl aliquot of each plasmid DNA having cloned 16S rRNA gene were digested with three different restriction enzymes namely AluI, AvaII, and MspI (New England Biolabs,) according to the manufacturer's instructions in a 25 µl system. In case of nahA gene clones, instead of AvaII, Sau3AI was used, the other two enzymes were the same. The enzymes were inactivated by heating the reaction mixture at 65°C for 15 min. The digestion products were analyzed on horizontal agarose (Gibco BRL) gel (1.5 %

w/v) electrophoresis with TBE buffer (1x) containing ethidium bromide (0.5 mg/ml). Electrophoresis was carried out at 80 V for 3 hr in 11x14 cm gel. The gels were viewed and photographed under UV illumination. Clones with similar pattern of digestion and intensity generated from a particular restriction enzyme were put in to the same ribotype group.

Phylogenetic analysis

Genetic relationship between two amplified 16S rRNA (ribotypes) and nahA genes was evaluated by determining the presence or absence of a particular DNA restriction pattern for a given enzyme. Dice similarity coefficient based on proportion of shared restriction patterns was calculated, distance matrix determined and distance values were displayed as a dendrogram by using the un-weighted pair group method with arithmetic mean (UPGMA) using NT Sys-Pc package (Exter Corp., USA). Cluster analysis was done using Neighbour-joining option of the same package.

Results and Discussion

Metagenomic soil DNA extraction

The sample soil was clay loam in texture and alkaline in nature (pH 8.0). The typical feature of alkaline soils or sediments is the presence of low level of microbial biomass (Verma and Satyanarayana 2011). But the DNA obtained by the method used in the present study (pooling of soil samples from all 24 locations) was sufficient as well as suitable for PCR amplification and also ensured diversity of rDNA clones obtained. Use of metagenomic molecular-based approach not only circumvented the limitation of culture-based approach but also revealed vast diversity of microbes from soil sample. Similar results were also reported by Daniel (2005).

PCR amplification and cloning

The primer set used in the present study targets and amplifies the 16S rRNA gene present in majority of the environmental bacterial groups (Huws *et al.,* 2007). The extracted metagenomic DNA from soil samples was amplified with the 16S ribosomal primers and a 16S rDNA clone library with 117 clones in *E. coli* DH5α cells was constructed from pesticide contaminated and non-contaminated soils. Authenticity of the recombinant clones was confirmed by subjecting the clones to restriction digestion with HindIII and EcoRI enzymes to release 1.1 Kb inserts from the vector. The nahA gene amplification produced bands of 393 bp size and total 7 clones were obtained.

RFLP analysis or ribotyping

Ribotyping is the identification and classification of bacteria based on polymorphisms in universal and highly conserved ribosomal RNA molecules or their genes (Bouchet *et al.,* 2008). The 78 rDNA clones from the pesticide-contaminated and 39 from the non-contaminated soil were cut with restriction endonucleases namely AluI, AvaII and MspI having internal sites in the 16S rRNA gene. PCR-RFLP method has been used previously by several workers to distinguish and identify bacteria to species and strain level (Kashyap *et al.,* 2014; Mandakovic *et al.,* 2016). Three restriction endonucleases gave different numbers of distinct restriction patterns - AluI (15), MspI (18) and AvaII (12) in both types of soils combined together (Table 1).

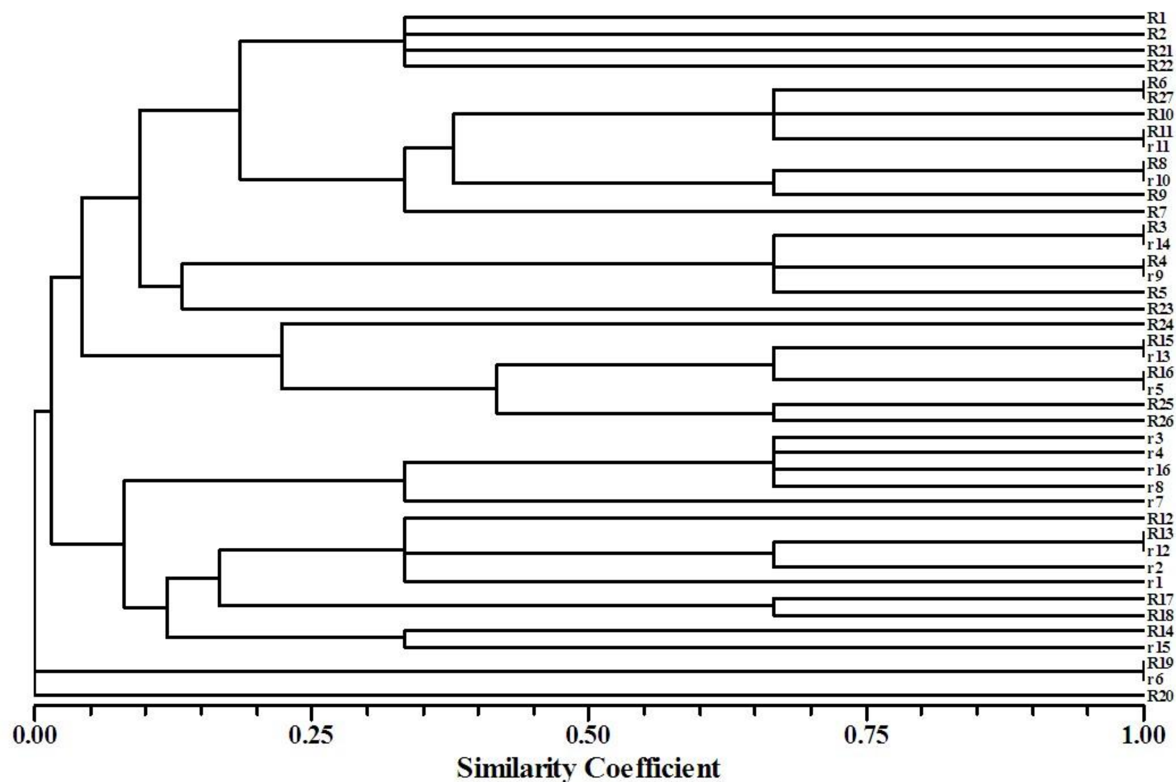
The restriction patterns obtained were then compared to generate 27 different combinations representing 27 16S rDNA genotypes (or ribotypes) from 78 rDNA clones obtained from the contaminated soil and 16 ribotypes from the uncontaminated soil (Table 1).

Table.1 RFLP patterns (ribotypes) obtained by restriction analysis of cloned 16S rRNA gene from two types of soil

Contaminated soil				**Pristine soil**			
Ribotypes	**Restriction fragments patterns**			**Ribotypes**	**Restriction fragments patterns**		
	AluI	MspI	AvaII		AluI	MspI	AvaII
R-1(4)*	A1	M1	Av1	r-1(2)	A6	M17	Av4
R-2(2)	A2	M2	Av1	r-2(4)	A6	M8	Av11
R-3(3)	A7	M3	Av1	r-3(3)	A14	M18	Av12
R-4(2)	A7	M3	Av6	r-4(2)	A3	M18	Av12
R-5(2)	A7	M3	Av4	r-5(2)	A3	M17	Av11
R-6(2)	A4	M4	Av1	r-6(3)	A6	M18	Av12
R-7(3)	A4	M5	Av4	r-7(2)	A14	M17	Av12
R-8(4)	A4	M13	Av2	r-8(3)	A15	M18	Av12
R-9(2)	A4	M4	Av2	r-9(3)	A7	M3	Av6
R-10(2)	A4	M11	Av1	r-10(2)	A4	M13	Av2
R-11(1)	A4	M10	Av1	r-11(1)	A4	M10	Av1
R-12(12)	A6	M7	Av2	r-12(3)	A6	M14	Av11
R-13(2)	A6	M14	Av11	r-13(1)	A5	M12	Av4
R-14(1)	A3	M6	Av3	r-14(2)	A7	M3	Av1
R-15(2)	A5	M12	Av4	r-15(3)	A5	M12	Av5
R-16(1)	A5	M12	Av5	r-16(3)	A12	M15	Av7
R-17(3)	A9	M8	Av9				
R-18(2)	A9	M8	Av11				
R-19(3)	A12	M15	Av7				
R-20(4)	A10	M16	Av8				
R-21(2)	A11	M9	Av1				
R-22(2)	A13	M8	Av1				
R-23(2)	A13	M10	Av6				
R-24(7)	A8	M12	Av10				
R-25(3)	A5	M14	Av1				
R-26(2)	A5	M14	Av4				
R-27(3)	A4	M4	Av1				

*Numbers in parentheses are the numbers of each ribotypes

Fig.1 Dendrogram (UPGMA) of genetic relationships among 16S rRNA ribotypes identified by PCR-RFLP analysis. The ribotypes (R1-27 & r1-16) are defined in Tables 1



The numbers in parentheses show the numbers of rDNA clones sharing a particular ribotype. Ribotype R-12 and R-24 appeared more frequently (12 and 7 times respectively) as compared to others indicating their relative abundance in the soil. Partial sequence (350 bp) of one representative clone each from the R-12 and R-24 revealed *Sphingomonas sps*. and *Bacteroides uniformis* respectively as the closest matches. *Sphingomonas sps* has been found to degrade carbofuran and carbofuran-7-phenol by hydrolysis at the Furanyl Ring (Seon *et al.,* 2004). Seven nahA gene clones were digested with restriction enzymes AluI, MspI and Sau3AI. The RFLP pattern obtained by restriction enzymes indicated all 7 nahA clones to be identical. Amplification of nahA gene from contaminated soil pointed towards the presence of naphthalene degraders in the analysed soil sample.

Genotypic relationships among ribotypes

To estimate the genotypic relationships, a matrix of pair-wise genetic distances was produced for all the ribotypes obtained from both types of soils. The distance matrix was then used to construct a dendrogram based on neighbour-joining algorithm (Fig. 1). The ribotypes represented by 'R' were from the soil of contaminated sites and those from the uncontaminated sites were represented by 'r'. As evident from Fig. 1, the ribotypes r-1, r-2, r-3, r-4, r-7, r-8 and r-16 formed separate clusters and thus seem to be unique to the pesticide-free soil, indicating disappearance

of certain microbes in the soil post contamination by toxic pesticides. Large number of ribotypes (R1, R2, R5, R6, R7, R9, R10, R12, R17, R18, R20, R21, R22, R23, R24, R25, R26 and R27) were either present singly or clustering among themselves in the dendrogram and so, appeared to be concentrated in the contaminated soil samples. Other workers have also reported that presence of some pesticides favour specific type of microbial population in the soil environment (Chen and Edwards, 2001; Nasreen *et al.,* 2015). On the other hand, certain ribotypes in pairwise combinations from both the soils were found to be clustered together with high bootstrap values (R-3 & r-14; R-4 & r-9; R-8 & r-10; R11 & r11; R13 & r12; R14 & r15; R15 & r13; R16 & r5; R19 & r6) indicating their concurrent presence in both types of soils.

To determine the possible whole range of microbial diversity, various strategies were applied in the present study which included isolation of bulk DNA by direct lysis of bacteria within their natural habitat to recover more representative fraction of the genetic diversity, selection of oligo-nucleotide primer pair to cover broad range of bacteria, and use of Restriction Fragment Length Polymorphism, one of the most useful molecular technique for diversity analysis in terms of cost effectiveness and reliability (Gayathri Devi and Ramya, 2015). Comparative microbiome analysis in the present study clearly indicated that there was alteration in microbial diversity and composition in the contaminated soil environment as a result of long term indiscriminate use of pesticides. Such alteration in the beneficial microbial community can be unfavorable to plant growth and development either by reducing nutrient availability or by increasing disease incidence (Meena, 2020). The selected ribotypes and degrader types in the present study represented the most abundant and active species. With the help of the genetic relationships and the distance values estimated in the present study, the ribotypes and the degrader types can be used as habitat-specific probes for further studies depending upon the assessment or the characterization work to be undertaken.

Acknowledgments

Authors are thankful to Director CAZRI for providing facilities during the period of this study.

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How to cite this article:

Anjly Pancholy and Singh, S.K. 2020. Comparative Microbiome Analysis of Pesticide-Contaminated and Pristine Agricultural Soils of Hot Arid Rajasthan. *Int.J.Curr.Microbiol.App.Sci.* 9(11): 1962-1970. doi: https://doi.org/10.20546/ijcmas.2020.911.233