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Corrective Measures of Denaturing Gradient Gel Electrophoresis Limitations

[1,2]Essahale Adil

[1]Microbiology and Health Group, Department of Biology, Laboratory of Applied Chemistry Biology Environment, Faculty of Sciences, Moulay Ismail University, BP11201 Zitoune Meknes, Morocco
[2]Provincial Delegation of Health in El Hajeb, 1 Km Route South of Azrou AV Med V, El Hajeb, Morocco

ABSTRACT

Denaturing Gradient Gel Electrophoresis (DGGE) is a technique that attempts to monitor microbial populations, without culture dependant methods. This molecular biology approach is a fingerprinting methodology that revolutionary changes many of the traditional routines used in assessing microbial populations, PCR-DGGE is classified as part of the new discipline of molecular microbial ecology. Therefore, this study will briefly explain a number of applications that involve the technique and will also examine how DGGE works in general. In this study, various limitations of the DGGE technique and the corrective measures were briefly described that can improve the profitability, to get a real biodiversity.

Key words: DGGE, limitations, correctives measures

INTRODUCTION

Conventional method such as cloning, hybridization and culture methods are not always practical for such investigations. The inability was explained to correctly determine the genetic diversity by providing an example of cultivability methods using conventional method.

Moreover, in microbiology study; classification based on physiological or biochemical features is not always possible because an estimated percentage of 99% of all microorganisms in nature can not be isolated (Amann *et al*., 1995). For many samples, the cultivation methods are inadequate in the identification and study of bacterial communities, because these methods allow the isolation of 1 and only 10% (Amann *et al*., 1995).

The enormous difficulty to cultivate and isolate new microorganisms is directly related to growing conditions *in vitro* that are highly difficult (Table 1). Therefore, the cultural approach does not correctly identified the diversity, hence the necessity to elect a more informative methods.

Many bias limits the ability of microorganisms culture, for example; the intrinsic selectivity signified that minority populations can numerically replace the majority populations in the environmental sample but inappropriate to growing conditions, also inhibition of growth due to high concentrations of substrates (e.g., oligotrophic microorganisms), populations enriched in liquid medium may be unable to grow on solid medium, possible dilutions of the inoculum used for enrichment can influence the type of microorganisms cultivated. Another type of limitation is absence of communication between the cells in conventional culture media and finally a physiological state of cells named "Viable but not cultivable" the study of the bacteria survival indicated that some organisms lost their culturability on appropriate medium but still had signs of metabolic activity and thus viability. The state "viable but non-culturable" could be a programmed

Table 1: Determination of bacterial cultivability in comparison with cells counted (Amann *et al*., 1995)

Habitat	Cultivability (%)[a]	References
Sea water	0.001-0.1	Ferguson *et al*. (1984) and Kogure *et al*. (1979, 1980)
Fresh water	0.1-1	Staley and Konopka (1985)
Estuarine waters unpolluted	0.1-3	Ferguson *et al*. (1984)
Activated sludge	1-15	Wagner *et al*. (1993, 1994)
Sediment	0.25	Jones (1977)
Soil	0.3	Torsvik *et al*. (1990)

a: No. of colony-forming units (CFUC)

response to environmental stress (McDougald *et al*., 1998). Finally the separation of different microorganisms capable of growth under similar conditions is difficult. The principal difficulty is to replicate or at least in the laboratory to imitate the conditions of the habitat from which the samples are obtained. Recently, cultivation of microorganisms has a new perspective with a growing interest in developing alternative cultivation techniques aimed at more specialized microorganisms grow and cultivate new microorganisms (Leadbetter, 2003).

However, conventional cloning, hybridization and culture methods as mentioned above are not always practical.

For these reasons and in order to have a real biodiversity of organisms animal, plant or microbial, the use of molecular biology techniques is necessary. In addition, these methods have revolutionized the routine identification of bacteria in a sample environment and industry. These techniques are based primarily on 16S rRNA gene analysis to study bacterial diversity.

DGGE TECHNIQUE

Genetic fingerprinting techniques provide a pattern or profile of the community diversity based upon the physical separation of unique nucleic acid species (Stahl and Capman, 1994).

The methods are rapid and relatively easy to perform but more importantly, they allow the simultaneous analysis of multiple samples which makes it possible to compare the genetic diversity of microbial communities from different habitats or to study the behaviour of individual communities over time.

There are many genetic fingerprinting techniques; but in this review technique of molecular fingerprinting-based on DGGE are discussed only.

Muyzer *et al*. (1993) introduced another genetic fingerprinting technique in microbial ecology that of denaturing gradient gel electrophoresis. A mixture of PCR products obtained with genomic DNA extracted from a complex assemblage of microorganisms and primers specific for a molecular marker, such as the 16S rRNA gene, is separated in a polyacrylamide gel containing a linear gradient of DNA denaturants. One of the strongest points of the techniques is that bands can be excised from the gel and subsequently sequenced to reveal the phylogenetic affiliation of the community members. Furthermore, community profiles can be hybridized with group-specific probes to detect the presence of particular bacterial populations. One of the limitations of the techniques is the separation of relatively short (ca. 500 bp) DNA fragments which limits the design of probes for hybridization analysis. Furthermore, it is not always possible to separate fragments with different sequences, because of similar melting behaviour of the fragments. In addition, the presence of double bands which might be caused by primer degeneracies or of heteroduplex molecules, might contribute to difficulties in the determination of community complexity.

CHARACTERISTIC OF THE DGGE TECHNIQUE

Reproductivity of electrophoresis: DGGE is the most commonly used environmental fingerprinting approach, yet differences in the preparation of the gradients can significantly affect profile reproducibility. Producing gradient can be difficult and to prepare gradient gels that yield reproducible DGGE profiles requires a routine. When first starting DGGE/TGGE analysis, there is also the considerable time and effort required to optimize the gradient and electrophoresis condition which can vary considerably between laboratories and different equipment (Osborn and Smith, 2005).

Reliable comparison of different samples with DGGE and SSCP typically requires the electrophoresis of all relevant samples on the same gel to overcome the problem of gel-to-gel variability (Hong *et al*., 2007).

Quality of signals and resolution: The fragment reproduced by DGGE achieving high resolution gels is influenced much more by skilled technical expertise, e.g., knowledge and understanding of the most suitable gradients and temperature ranges. Often such parameters must be determined empirically.

On particular problem with DGGE and TGGE is that some region of the gel may contain a smear of ssDNA that can obscure part of fingerprint. This is caused when some of the PCR products melt completely despite the presence of GC clamp and hence form a ssDNA fraction and maintains the integrity of partially denatured amplicon. The art of gradient adjustment requires skill and experience to generate gels in which the ssDNA fraction and the DGGE fingerprint are separated (Hong *et al*., 2007).

Prediction of band positions: The melting behaviour of T/DGGE fragments can also be predicted. This requires considerably more effort and may fail if there are ambiguities or even 1 pb errors in the sequence (Osborn and Smith, 2005).

Access to DNA sequence information: Since DGGE/TGGE separate on the basis of melting proprieties of the entire sequence, they may be used to directly separate individual amplicons prior to sequencing. In some but not all cases on T/DGGE band will represent on sequence. Hence, for complex community fingerprints one band may consist of two or more different sequences, necessitating a cloning step prior to sequencing (Osborn and Smith, 2005).

HOW DGGE WORKS

In DGGE, Polymerase Chain Reaction (PCR) generated DNA fragments of the same length but with different base-pair sequences can be separated.

This method constitutes direct extraction of the community DNA and amplification of typically 200-600 bp long of DNA fragments. These fragments are separated according to their melting point on a denaturing gradient gel (Muyzer, 2000). The DGGE technique is based on the migration of DNA fragments amplified by PCR based on their nucleotide composition in a denaturing gradient which separates the products of similar size; the method provides a direct comparison of the sequences studied (Trotsenko and Khmelenina, 2005). Molecules with different sequences may have a different melting behaviour and will, therefore, stop migrating at different positions in the gel (Fig. 1 and 2).

The analysis begins with the preparation of the gel polyacrylamide 6% (acrylamide: bis-acrylamide 37.5: 1) and a denaturing gradient ranging from 40-70% is ranked in the gel.

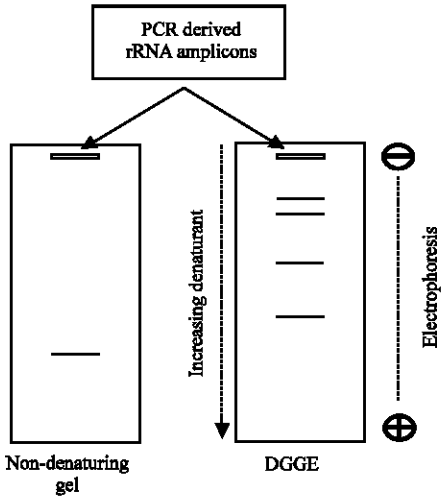

Fig. 1: Schema illustration of Density Gradient Gel Electrophoresis (DGGE) of a PCR derived rRNA amplicon from four different bacteria. Gel electrophoresis in a non-denaturing gel results in a single band, whereas electrophoresis through a gel containing increasing concentrations of a denaturant results in the separation of the four different products, based on their sequence dependent melting patterns (O'Sullivan, 2000)

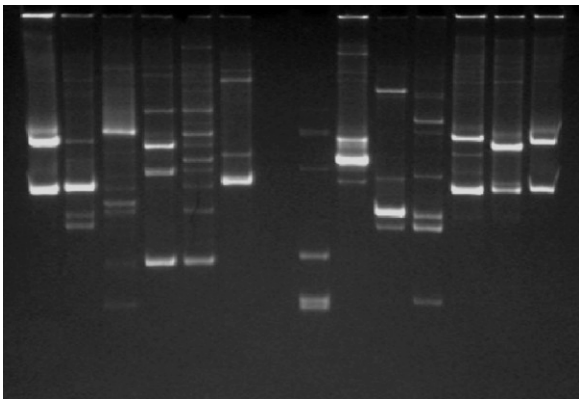

Fig. 2: Example of a DGGE gel (Forno *et al.*, 2008)

APPLICATIONS OF PCR-DGGE

The use of the denaturing gradient gel electrophoresis technique is useful for the rapid screening of parasite rDNA for sequence variation without the need for exhaustive cloning or DNA sequencing. The resolution of this variation by DGGE provides a diagnostic fingerprint for a species (Gasser *et al.*, 1996).

Application technique-relative: The use of environmental 16s rDNA, revealed that bacterial communities in natural environments are much more diverse than was previously found with traditional culture methods.

Application of microbial community fingerprinting: DGGE is generally used in microbial community fingerprinting (Essahale *et al.*, 2010). Consequently; the predominant fingerprint signals may also represent the unknown bacteria. Therefore, fingerprinting is especially useful to monitor the uncultured majority within environmental bacterial communities (Hong *et al.*, 2007). However, DGGE is a resolution to very small habitats problem, because it is adapted to milliliter or microliter volumes, setting practical limits to nucleic acid extraction volumes and hence the size of the sample to be treated. Nevertheless the observation that microbial communities may be common across significant distances has been supported by the studies in other areas of the world (Hossain and Sugiyama, 2011).

Recent reports using DGGE, indicating that heteroplasmy in the control region is more common than was previously believed a finding that is of potential importance to evolutionary studies and forensic applications that are based on mtDNA variation (Tully *et al.*, 2000).

Combination of cloning with DGGE: This approach represents a way to high-quality sequence data from sequence identified in fingerprinting are applied in parallel from the beginning of an experiment, they can overcome the limitation of each method. Additionally, it's sometimes difficult to excise fingerprint bands is not necessary. Comparison of amplicons from the cloned inserts on the same DGGE gel as the environmental fingerprint can be used to link clone and fingerprint sequences. Thus cloned that might represent dominant sequences in the community profile can be really identified.

Screening isolated strains next to DGGE: Screening isolated strains may be based on quite different 'DNA template' this template is, in effect, the gene pool represented in the respective culture collection. It is well known that cultivation methods are highly selective, varying with the media and culture conditions used and that they introduce a considerable bias in the representation of the bacterial community.

Southern blot hybridization and DGGE: This approach has been performed successfully to identify small DNA region. Borresen *et al.* (1988) developed a method for the efficient Southern transfer of genomic DNA fragments from the denaturing gradient gels in order to be able to analyze larger regions in several loci for variation. The gels were made using polyacrylamide containing 2% Low-Geling Temperature agarose (LGT). The polyacrylamide gel (PAG) was crosslinked with a reversible crosslinker and after electrophoresis the crosslinks were cleaved, the structure of the gel being maintained by the agarose. After this treatment of the denaturing gels, more than 90% of the DNA fragments could be transferred to nylon membranes by alkaline transfer while electroblotting transferred only 10% of the DNA. Hybridization with gene-specific probes was then performed (Borresen *et al.*, 1988).

DGGE with protein-coding genes: Screening of environmental samples for protein-coding genes promises to yield specific functional data (Wawer and Muyzer, 1995). A major drawback for functional gene community profiling is the often limited number of reference sequences available and therefore functional gene fingerprinting would often require extensive sequencing efforts to gain a suitable database for reliable primer design. For example a denaturing gradient gel electrophoresis method was developed to assess the diversity of *dsr*B (dissimilatory sulfite reductase β-subunit) genes in sulphate reducing communities (Geets *et al.*, 2006).

APPLICATION ON THE DOMAIN OF STUDY

PCR-DGGE in medical: DGGE was applied in clinical microbiology, this technique allowed the identification of over 65 *Mycoplasma* species of human origins in less than one day (McAuliffe *et al.*, 2005). *Mycoplasmas* cause various diseases associated (pneumonia, arthritis, conjunctivitis, infertility and abortion), this bacteria require many weeks to culture and other serological tests to be identified. This application of PCR-DGGE could potentially allow considerable savings of time, life and treatment costs (McAuliffe *et al.*, 2005).

Another important achievement was that DGGE has been established to have a real potential in screening large number of patients for rapid and reliable identification of deleterious changes in both breast cancer genes BRCA1 and BRCA2 (Diez *et al.*, 2003). Hence, PCR-DGGE allowed the detection of numerous mutations and revealed the existence of unclassified variants that were not reported before (Diez *et al.*, 2003). This method was also able to demonstrate the infection by bacteria suspected to be responsible for stomach ulcers (Schabereiter-Gurtner *et al.*, 2002). In addition, Al-Soud *et al.* (2003) demonstrated that 16S rDNA genotyping in combination with DGGE fingerprinting are appropriate molecular methods for the investigation of severe bacterial infections which might not be detected by conventional cultivation. The application of the PCR-DGGE technique can also identified endodontic microbes; this research has the potential to shed light on several aspects of the different types of endodontic infection as well as on the effects of treatment procedures with regard to infection control (Siqueira *et al.*, 2005).

PCR-DGGE in environmental microbiology and food safety: PCR-DGGE is also a useful tool in studying complex microbial communities such as the gastrointestinal tract of food producing animals. These animals can carry in their gastrointestinal tract disease-causing organisms throughout the production chain to the retail market and from the retail market to the consumer's dinner table (NRC., 1999). It is, therefore very important to elucidate the exact microbial populations of food producing animals' gastrointestinal tract. DGGE is recommended as well as a better control of the shedding of deadly bacteria strains into manure which is used as fertilizer for produce such as fruits and vegetables (NRC., 1999).

The need for rapid and accurate methods for screening of total microbial populations in complex ecosystems is more evident than ever. PCR-DGGE has proved to be a powerful tool in assessing total gut microbial populations and was also used to detect previously unknown bacteria species (McAuliffe *et al.*, 2005; Walter *et al.*, 2000; Al-Soud *et al.*, 2003). Understanding the relationship between the host and the disease-causing organisms will certainly assist us in defining efficient pathogens control measures. This is of paramount importance in food safety and food processing where quality control and assurance programs necessitate proficient methods to discontinue the transmission cycle of life-threatening microbes.

PCR-DGGE in biotechnology: Denaturing Gradient Gel Electrophoresis (DGGE), were also applied in the intrinsic bioremediation. In several environmental microorganisms were detected and identified susceptible to play a major role in biotechnological (Table 2) . These microorganisms are identified by DGGE bacteria which degrade the pollutants and will be an alternative to classic methods (Ferris *et al.*, 1996).

LIMITATION OF DGGE

The most important limitations of DGGE are technical for example in the closely related organisms, the relationship between nucleotide sequence, phylogenetic affiliation and the

Table 2: Application of DGGE technical from different organisms and the gene fingerprinting used

Organisms	Gene	Environment	Application	References
Fenneropenaeus chinensis	16S rRNA	Intestine of Chinese shrimp	Diversity	Liu *et al*. (2011)
Bacteria	16S rRNA	Bovine ruminal epithelial	Ecology and function of bacteria	Li *et al*. (2012)
Bacteria	16s rRNA	Aaquatic	Ecology and environmental relationship	Essahale *et al*. (2010)
Pyrenophora species	16s rRNA	Plants tissues	Bring about economic and food safety concerns	Mavragani *et al*. (2011)
Bacteria	16s rRNA	Sediments	Biodegradation of PAHs	HuiJie *et al*. (2011)
Nematode	18S rRNA	Soil	Biodiversity taxonomie	Foucher *et al*. (2004)
Microflora	16S rRNA	Intestinal	Comparison of DNA extraction	Ariefdjohan *et al*. (2010)

melting point is not well established and the retardation of the fragment in the gel matrix may not properly indicate phylogenetic relatedness at high resolution, like the species level (Kisand and Wikner, 2003).

In principle single bands of interest can be excised from gel, reamplified and subsequently analyzed by sequencing. But certain problems have to be considered.

DNA extraction, amplification and phylogenetic errors: The band must be excised accurately to avoid contamination by adjacent bands. It is important to note that, not all of the PCR products will be separated during DGGE into a defined fingerprint. In addition all fingerprints will be 'sandwiched' by faint smears of differently separated PCR products. Another constraint of the amplification is the recovered amplicon which will only represent a few hundred nucleotides of the target sequence, since only amplicons of <500 bp in size give good results on DGGE, this can be generated prevents the more accurate phylogenetic analysis that could be undertaken with a full-length sequence.

The errors in DNA extraction or in PCR give the erroneous nucleotides (Kisand and Wikner, 2003; Speksnijder *et al*., 2001). Petersen and Dahllof (2005) were used the internal standards throughout the DNA extraction and PCR-DGGE such variability causes that decreased reproducibility among replicate samples as well as compromise comparisons between samples, since experimental errors cannot be differentiated from actual changes in the community abundance and structure.

GC clamp disadvantage: The GC clamp on a primer is an essential requirement for successful DGGE (and TGGE) analysis, GC-clamped primers consisting of an extended primer together with a GC-rich at the 5' end of the primer can be readily purchased from commercial suppliers. It is very likely that the GC clamp may hamper primer annealing during PCR and reduce amplification efficiency. Hence, it is advisable to add GC clamps to standard primers that have already been shown to have high PCR efficiency (Muyzer, 2000).

The GC-clamp portion of primers for DGGE amplicon preparation provides a key component in resolving fragments of similar size but different sequence (Rettedal *et al*., 2010) collaborators hypothesized that repeat syntheses of identical 40-base GC-clamp primers lead to different DGGE profiles. Three repeat syntheses of the same GC-clamp primer and two different GC-clamp primers directed at the V3-5 region of the 16S rRNA gene were compared. Genomic DNA of two separate soil bacterial communities and three bacterial species was amplified and resolved by DGGE. The DGGE profiles obtained with repeat-synthesized primers differed among each other as much as

with alternate primers, for both soil DNA and pure single species. The GC-clamp portion of members of amplicon pools varied among each other, deviating from the design sequence and was the likely cause for multiple bands derived from a single 16S rRNA gene sequence.

Heterogeneous genes: The presence of multiple heterogeneous gene copies in many strains influences the interpretation of microbial ecology sequences.

Kang *et al.* (2010) suggested that these remarks must be taken when interpreting. In their study; among the total strains, 639 strains (82%) possess multiple 16S rRNA gene copies, 415 strains (53%) whose multiple copies are heterogeneous in sequences as revealed by alignment, 236 strains (30%) whose multiple copies show different restrict patterns by CSP6I+HinfI, MspI+RsaI or HhaI as analyzed *in silico*. Polymorphisms of the multiple copies in certain strains were characterized by G+C% and phylogenetic distances based on the sequences of V3 region which are linked to DGGE patters (Kang *et al.*, 2010).

Ueda *et al.* (1999) concluded that certain helices of the 16s rRNA may be mutation tolerant and that misincorporation during DNA replication and horizontal gene transfer could be the causes. This heterogeneity within a single strain has the potential to mislead interpretation of fingerprinting. DGGE is, in fact, a very valuable tool for studying this phenomenon (Nubel *et al.*, 1996).

Co-migration: DGGE of complex polymicrobial communities may be limited by co-migration of different sequences (Gafan and Spratt, 2005). The band excision and sequencing of DGGE bands might not provide unequivocal identifications as a result of the co-migration of DNA fragments from different taxa to the same positions within DGGE gels (Gafan and Spratt, 2005).

Gel constraints: DGGE gel may be one of the major constraints of the technique because; Gels of complex communities may look smeared due to the large number of bands, in the other hand band position does not provide reproducible taxonomic information, also results are difficult to reproduce between gels and laboratories.

CORRECTIVE MEASURES AND RECOMMENDATIONS

We note that it is very important to take into account the disadvantage of the technique and establish corrective action in order to improve the usefulness of technology; in this case it is recommended the use of.

A protocol internal standards in molecular analysis of diversity (ISMAD): ISMAD should be taken into consideration when interpreting the diversity in a sample based on a DGGE. That is simple to use, inexpensive, rapid to perform and it does not require additional samples to be processed (Petersen and Dahllof, 2005). Banding patterns from DGGE analysis may therefore be misinterpreted in terms of the species richness in natural bacterial communities, when using commonly applied universal primers (Petersen and Dahllof, 2005).

Co-migration solution: The principle of DGGEGE is based on resolving the co-migrating taxonomic units (CTUs) into individual OTUs. It would therefore be possible, albeit laborious, to excise every DGGE band from a single lane and then attempt to resolve each individual DGGE band into its constituent CTUs by DGGEGE. By aligning each DGGEGE profile accordingly which

generate a fingerprint that truly represented the microbiota. Gafan and Spratt (2005) have used Denaturing Gradient Gel Electrophoresis Gel Expansion (DGGEGE) that would overcome the limitations imposed on DGGE by co-migration. Demonstrated that co-migration of different sequences to the same electrophoretic position takes place in DGGE of dental plaque microbial communities. Separation was reproducibly shown to occur through a 50.6-51.7% gradient of denaturants.

GC-clamp primer yield: We suggest procuring an oligonucleotide batch large enough to conduct an entire project. This should help to avoid any DGGE profile variations due to performance differences between repeat syntheses of GC-clamp oligonucleotide primers (Rettedal *et al*., 2010).

DGGE apparatus: We recommend the use of one and the same DGGE apparatus throughout an experiment, the monitoring of microbial community structures requires multiple gel-to-gel analysis. Ascher *et al*. (2010) established an attempt to investigate the hypothetical impact of the DGGE system due to different technical characteristics. They show the effect of changing the DGGE equipments on the effectiveness of the technique.

CONCLUSION

DGGE provides new opportunities for better knowledge of the living world and very suitable technique for the identification of novel or unknown organisms.

We reported in this review the advantage of the denaturing gradient gel electrophoresis technique, some limitations of this technique were shown and we tried to resolve them with the necessary recommendation.

Nevertheless DGGE remains among the best methods of molecular biology.

REFERENCES

Al-Soud, W.A., M. Bennedsen, S.L.W. On, I.S. Ouis and P. Vandamme *et al*., 2003. Assessment of PCR-DGGE for the identification of diverse *Helicobacter* species and application to faecal samples from zoo animals to determine *Helicobacter* prevalence. J. Med. Microbiol., 52: 765-771.

Amann, R.I., W. Ludwig and K.H. Schleifer, 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev., 59: 143-169.

Ariefdjohan, M.W., D.A. Savaiano and C.H. Nakatsu, 2010. Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. Nutr. J., Vol. 9. 10.1186/1475-2891-9-23

Ascher, J., M.T. Ceccherini, A. Chronakova, J. Jirout and F. Borgogni *et al*., 2010. Evaluation of the denaturing gradient gel electrophoresis-apparatus as a parameter influencing soil microbial community fingerprinting. World J. Microbiol. Biotechnol., 26: 1721-1726.

Borresen, A.L., E. Hovig and A. Brogger, 1988. Detection of base mutations in genomic DNA using Denaturing Gradient Gel Electrophoresis (DGGE) followed by transfer and hybridization with gene-specific probes. Mutation Res./Fundam. Mol. Mech. Mutagen., 202: 77-83.

Diez, O., A. Osorio, M. Duran, J.I. Martinez-Ferrandis and M.D.L. Hoya *et al*., 2003. Analysis of *BRCA1* and *BRCA2* genes in Spanish breast/ovarian cancer patients: A high proportion of mutations unique to Spain and evidence of founder effects. Human Mutation, 22: 301-312.

Essahale, A., M. Malki, I. Marin and M. Moumni, 2010. Bacterial diversity in Fez tanneries and Morocco's Binlamdoune River, using 16S RNA gene based fingerprinting. J. Environ. Sci., 22: 1944-1953.

Ferguson, R.L., E.N. Buckley and A.V. Palumbo, 1984. Response of marine bacterioplankton to differential filtration and confinement. Applied Environ. Microbiol., 47: 49-55.

Ferris, M.J., G. Muyzer and D.M. Ward, 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-Defined populations inhabiting a hot spring microbial mat community. Applied Environ. Microbiol., 62: 340-346.

Forno, E., A.B. Onderdonk, J. McCracken, A.A. Litonjua and D. Laskey *et al.*, 2008. Diversity of the gut microbiota and eczema in early life. Clin. Mol. Allergy, Vol. 6

Foucher, A.L.J.L., T. Bongers, L.R. Noble and M.J. Wilson, 2004. Assessment of nematode biodiversity using DGGE of 18S rDNA following extraction of nematodes from soil. Soil Biol. Biochem., 36: 2027-2032.

Gafan, G.P. and D.A. Spratt, 2005. Denaturing Gradient Gel Electrophoresis Gel Expansion (DGGEGE)-An attempt to resolve the limitations of co-migration in the DGGE of complex polymicrobial communities. FEMS Microbiol. Lett., 253: 303-307.

Gasser, R., P. Nansen and P. Guldberg, 1996. Fingerprinting sequence variation in ribosomal DNA of parasites by DGGE. Mol. Cell. Probes, 10: 99-105.

Geets, J., B. Borremans, L. Diels, D. Springael, J. Vangronsveld, D. van der Lelie and K. Vanbroekhoven, 2006. *DsrB* gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. J. Microbiol. Methods, 66: 194-205.

Hong, H., A. Pruden and K.F. Reardon, 2007. Comparison of CE-SSCP and DGGE for monitoring a complex microbial community remediating mine drainage. J. Microbiol. Meth., 69: 52-64.

Hossain, Z. and S.I. Sugiyama, 2011. Geographical structure of soil microbial communities in northern Japan: Effects of distance, land use type and soil properties. Eur. J. Soil Biol., 47: 88-94.

HuiJie, L., Y. CaiYun, T. Yun, L. GuangHui and Z. TianLing, 2011. Using population dynamics analysis by DGGE to design the bacterial consortium isolated from mangrove sediments for biodegradation of PAHs. Int. Biodeterior. Biodegrad., 65: 269-275.

Jones, J.G., 1977. The effect of environmental factors on estimated viable and total populations of planktonic bacteria in lakes and experimental enclosures. Freshwater Biol., 7: 67-91.

Kang, Y.J., J. Cheng, L.J. Mei, J. Hu, Z. Piao and S.X. Yin, 2010. Multiple copies of 16S rRNA gene affect the restriction patterns and DGGE profile revealed by analysis of genome database. Microbiology, 79: 655-662.

Kisand, V. and J. Wikner, 2003. Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. J. Microbiol. Methods, 54: 183-191.

Kogure, K., U. Shinoda and N. Taga, 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol., 25: 415-420.

Kogure, K., U. Simidu and N. Taga, 1980. Distribution of viable marine bacteria in neritic seawater around Japan. Can. J. Microbiol., 26: 318-323.

Leadbetter, J.R., 2003. Cultivation of recalcitrant microbes: Cells are alive, well and revealing their secrets in the 21st century laboratory. Curr. Opin. Microbial., 6: 274-281.

Li, M., M. Zhou, E. Adamowicz, J.A. Basarab and L.L. Guan, 2012. Characterization of bovine ruminal epithelial bacterial communities using 16S rRNA sequencing, PCR-DGGE and qRT-PCR analysis. Vet. Microbiol., 155: 72-80.

Liu, H., L. Wang, M. Liu, B. Wang, K. Jiang, S. Ma and Q. Li, 2011. The intestinal microbial diversity in Chinese shrimp (*Fenneropenaeus chinensis*) as determined by PCR-DGGE and clone library analyses. Aquaculture, 317: 32-36.

Mavragani, D., C. Hamel and V. Vujanovic, 2011. Species-specific PCR-DGGE markers to distinguish *Pyrenophora* species associated to cereal seeds. Fungal Biol., 115: 169-175.

McAuliffe, L., R.J. Ellis, J.R. Lawes, R.D. Ayling and R.A.J. Nicholas, 2005. 16S rDNA PCR and denaturing gradient gel electrophoresis: A single generic test for detecting and differentiating *Mycoplasma* species. J. Med. Microbiol., 54: 731-739.

McDougald, D., S.A. Rice, D. Weichart and S. Kjelleberg, 1998. Nonculturability: Adaptation or debilitation? FEMS Microbiol. Ecol., 25: 1-9.

Muyzer, G., 2000. Genetic fingerprinting of microbial communities-present status and future perspectives. Proceedings of the 8th International Symposium on Microbial Ecology, August 9-14, 1998, Halifax, Canada, pp: 503-512.

Muyzer, G., E.C. de Waal and A.G. Uitterlinden, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied Environ. Microbiol., 59: 695-700.

NRC., 1999. The Use of Drugs in Food Animals: Benefits and Risks. National Academy Press, Washington, DC., ISBN: 9780309175777, Pages: 243.

Nubel, U., B. Engelen, A. Felske, J. Snaidr and A. Wieshuber *et al*., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J. Bacteriol., 178: 5636-5643.

O'Sullivan, D.J., 2000. Methods for analysis of the intestinal microflora. Curr. Issues Intestinal Microbiol., 1: 39-50.

Osborn, A.M. and C.J. Smith, 2005. Molecular Microbial Ecology. Garland Science, USA., ISBN: 9781859962831, Pages: 381.

Petersen, D.G. and I. Dahllof, 2005. Improvements for comparative analysis of changes in diversity of microbial communities using internal standards in PCR-DGGE. FEMS Microbiol. Ecol., 53: 339-348.

Rettedal, E.A., S. Clay and V.S. Brozel, 2010. GC-clamp primer batches yield 16S rRNA gene amplicon pools with variable GC clamps, affecting denaturing gradient gel electrophoresis profiles. FEMS Microbial. Let., 312: 55-62.

Schabereiter-Gurtner, C., S. Maca, S. Kaminsky, S. Rolleke, W. Lubitz and T. Barisani-Asenbauer, 2002. Investigation of an anaerobic microbial community associated with a corneal ulcer by denaturing gradient gel electrophoresis and 16S rDNA sequence analysis. Diagn. Microbiol. Infect. Dis., 43: 193-199.

Siqueira, J.F., I.N. Rocas and A.S. Rosado, 2005. Application of Denaturing Gradient Gel Electrophoresis (DGGE) to the analysis of endodontic infections. J. Endodontics, 31: 775-782.

Speksnijder, A.G., G.A. Kowalchuk, S. de Jong, E. Kline, J.R. Stephen and H.J. Laanbroek, 2001. Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. Applied Environ. Microbiol., 67: 469-472.

Stahl, D.A. and W.C. Capman, 1994. Applications of Molecular Genetics to the Study of Microbial Communities. In: Microbial Mats: Structure, Development and Environmental Significance, Stal, L.J. and P. Caumette (Eds.). Vol. 35, Springer, New York, ISBN: 9783540579755, pp: 193-206.

Staley, J.T. and A. Konopka, 1985. Measurement of in situ activities of non-photosynthetic microorganisms in aquatic and terrestrial habitats. Ann. Rev. Microbiol., 39: 321-346.

Torsvik, V., J. Goksoyr and F.L. Daae, 1990. High diversity in DNA of soil bacteria. Applied Environ. Microbiol., 56: 782-787.

Trotsenko, Y.A. and V.N. Khmelenina, 2005. Aerobic methanotrophic bacteria of cold ecosystems. FEMS Microbiol. Ecol., 53: 15-26.

Tully, L.A., T.J. Parsons, R.J. Steighner, M.M. Holland, M.A. Marino and V.L. Prenger, 2000. A sensitive denaturing gradient-gel electrophoresis assay reveals a high frequency of heteroplasmy in hypervariable region 1 of the human mtDNA control region. Am. J. Human Genet., 67: 432-443.

Ueda, K., T. Seki, T. Kudo, T. Yoshida and M. Kataoka, 1999. Two distinct mechanisms cause heterogeneity of 16S rRNA. J. Bacteriol., 181: 78-82.

Wagner, M., R. Amann, H. Lemmer and K.H. Schleifer, 1993. Probing activated sludge with proteobacteria-specific oligonucleotides: Inadequacy of culture-dependent methods for describing microbial community structure. Applied Environ. Microbiol., 59: 1520-1525.

Wagner, M., R. Erhart, W. Manz, R. Amann, H. Lemmer, D. Wedi and K.H. Schleifer, 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for *in situ* monitoring in activated sludge. Applied Environ. Microbiol., 60: 792-800.

Walter, J., G.W. Tannock, A. Tilsala-Timisjarvi, S. Rodtong, D.M. Loach, K. Munro and T. Alatossava, 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Applied Environ. Microbiol., 66: 297-303.

Wawer, C. and G. Muyzer, 1995. Genetic diversity of *Desulfovibrio* spp. in environmental samples analyzed by denaturing gradient gel electrophoresis of [NiFe] hydrogenase gene fragments. Applied Environ. Microbiol., 61: 2203-2210.