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Bacterial Identification by 16S Ribotyping: A Review

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Abstract

PCR ribotyping is considered as a rapid method for the identification of bacteria. . In this technique, the intergenic regions from 16S-23S are amplified through PCR. The obtained PCR product is then digested using restriction endonuclease. This technique provides information about the length variability, useful in identifying the microorganisms. Ribotyping has been proved important in determining to prescribe treatment with antibiotics or not and in the recognition of *Campylobacter fetus*, a thermotolerant strain which has been found in causing bacteremia. The identification of rare and phenotypically abnormal strains is often met by multiple mistakes in clinical microbial laboratories and sometimes it is also difficult to know that the recognition of bacterial species that have been made in incorrect. Whereas ribotyping can identify rare and aberrant bacterial isolates or strains with high accuracy. The results of ribotyping in giving correct identifications fall in the range of sixty-two to ninety-two percent, depending upon the strains of bacteria and standard that is used for definition of species. All of these identifications have been helpful and important in a wide range of aspects in which the most important thing is the identification of disease causing or infectious strains and discovery of appropriate treatment against them.

Keywords: Bacteremia; Infectious strains; Restriction endonuclease; Thermo-tolerant

Introduction

One of the most suitable method for DNA fingerprinting study is ribotyping. This technique makes simpler the recognition of microbes and infection causes. With commence of RNA gene restriction pattern, ribotyping recognized for microorganism's environmental, genomic and for systematic study. Bacteria consist of operon which is a group of genes having related expression controlled by a common operon. Eukaryotes lack operons. In bacteria each ribosomal operon constitutes genes which encode different ribosomal RNA structure i.e. 23S, 5S and 16S. Within a bacterial species all these three structures are conserved. But among them 16S is highly conserved which is why it is used for the identification of bacterial phylogeny or investors and taxonomy. Also the 16 ribosomal RNA gene's function remained same over the years [1]. The rate at which 16S rRNA gene evolve over the period of time is not known and this change may not be same in different organisms of the same species either. There are

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certain regions in 16S rRNA which are termed as ‘hot spot’ which exhibit maximum mutation rate. But this cannot hinder it from being as identification marker. So we use 16S ribotyping technique in which we sequence 16S gene in particular bacteria under observation. 16S rRNA constitutes both conserved and variable region and is of 1550 bp length.

For the sequencing of 16S rRNA mostly universal primers are utilized which are complementary either to the sequence in the beginning or where the sequence ends or at 540 bp region. The variable region between these constant parts can be used for taxonomy comparison.

For most bacterial strains 16S rRNA sequence has been found and recorded in databank like Gene bank. It has record of over 20 million bacterial stains from which 90000 is of 16S rRNA sequence of different species. This concludes that 16S rRNA gene is considered as standard and universal and allows us to differentiate bacteria at the genus level. For the identification sometimes the sequencing of the whole 16S gene is necessary but for clinical purposes the sequencing and comparing the initial 500 base pairs is enough. If one want to detect any certain virulence region or comparison of stains for epidemiological studies then 16S rRNA sequencing is not enough. But if ones goal is to detect any unknown organism about which there is no information recorded then 16S rRNA sequencing is excellent [2]. According to the given instruction the sequence under observation being sequenced is compared with the data banks if the sequence is less than 97% similar then it shows that given bacterial sequence belongs to another species and the similarity is 97% above then it is classified using an alternative approach [3].

PCR ribotyping is considered as a rapid method for the identification of bacteria. For the analysis of molecular epidemiology, it uses spacer regions of bacterial rRNA from 16S-23S as a target. Every bacterium possesses several copies of genes encoding rRNA per cell. The spacer region in each rRNA operon has a varied length, therefore their amplification may yield a specific banding pattern [4].

In order to understand the process of PCR based ribotyping, a brief discussion of PCR has to be given. In molecular biology PCR is advance scientific technique. From PCR we can generate millions of particular DNA sequence copies by single amplified piece of DNA. Development of PCR by Kary Mullis who is an American biochemist, won Japan Prize and Nobel Prize in 1993 and in 1984, respectively for his great invention i.e. PCR. Due to its broad variety of applications, quick simple and inexpensive PCR becomes an essential method in biological and medical research labs. PCR technique involves three major steps first is denaturation of DNA after denaturation, annealing is the second step and in the end extension of DNA [5]. Mounting the number of diseases with the passage of time PCR becomes a useful tool for investigation and diagnosing of disease. We can also detect the genes of virus and bacteria by using qualitative PCR technique this is not only for human genes. In forensic laboratories PCR playing a crucial role because only minute amount of DNA is required to perform a PCR. Take a look on other function of PCR which is the recognition of genes that are involved in the growth of cancerous cells. In development of PCR techniques that give benefit in Molecular cloning. Due to quick easy method PCR producing unlimited copies of DNA this become a major development in science and deserve a well-worn titles like ‘breakthrough’ and ‘revolutionary’. PCR sets a new level of accuracy and reliability of examine minute amount of DNA even damaged genetic material. Recombinant DNA technology, Southern blotting, DNA cloning and DNA sequencing can be used by PCR in research laboratories [6]. Classical PCR method can detect pathogens and give importance in medical application. Viruses have RNA instead of DNA. In this case before performing PCR viral genome have to be transcribe first and can use Real Time PCR (RT-PCR; qPCR). Detecting the pathogen outer to the body which is necessary sometimes. Microorganism DNA can be detect from any sample from body fluids, water and foods from the method of PCR. Furthermore by detecting DNA quantitative PCR can give additional information that how much DNA present in there and whether the specific DNA segment are present or not in the sample. On the basis on molecular finding the disease can be diagnosed by the Quantitative PCR application rather than using physiological symptoms. Quantitative PCR gaining increase importance in constructing the diagnosing of the viral diseases [7].

Kostman and Gurtle are the scientists who combined the two techniques and developed PCR ribotyping in 1990’s to overcome the

need for epidemiological discrimination among infectious microorganisms [8]. PCR ribotyping is applicable because it does not require probes, instead it uses primers complementary to 16S and 23S rRNA. The amplified PCR products possess length heterogeneity. The computational analysis of length variability of PCR amplified products of bacteria revealed that PCR ribotyping is more reliable for recognizing bacteria to specie level instead of identifying them at strain level [9]. Many bacterial species exhibit variability in sequences among different copies of intergenic regions. Intergenic regions in some cases encode one or two transfer RNA's generating sequence variability [10].

PCR ribotyping is sometimes followed by endonuclease digestion for subtyping. In this technique, the intergenic regions from 16S-23S are amplified through PCR. The obtained PCR product is then digested using restriction endonuclease. This technique provides information about the length variability, useful in identifying the microorganisms [9]. The long PCR ribotyping that includes the amplification of 16S-23S-5S followed by restriction digestion has more advantages over PCR ribotyping amplifying only 16S region [11].

The process by which 16S rRNA PCR based ribotyping is done is explained as first of all bacterial strain whose identification is required is selected and isolated from its source. Then it is grown under optimized conditions and in the culture containing the components required for its growth. Then genome of the bacteria is separated by adding RNAase. The DNA obtained is diluted. The aliquot obtained from the reaction mixture is mixed with the buffer. To some amount of mixture restriction endonucleases are added which cuts the DNA at specific points i.e. one cut is made in 23S rRNA gene and another in 16S rRNA gene [12]. The fragments of DNA are separated on the basis of their sizes through gel electrophoresis. Controls are run alongside to compare the size required to separate. Probes are designed in the initial stage of analysis which can be designed from the sequence of ribosomal operon. PCR is carried out in which probes or primer designed in the beginning are used to amplify the whole operon i.e. 16S-5S-23S for confirmation of size the PCR product is then run along agarose gel and cut directly while present in the gel. The product embedded in agarose gel is solubilized by boiling. Probe (radiolabeled) is then generated by using ribosomal template (20-50 mg) with random primers and DNA polymerase 1. Next is hybridization in which membrane transferred having digested DNA is bonded with prior made prob. This hybridized product is given audio radiographic film exposure. This is followed by analysis of bands of ribotyping RFLP either manually or by using any software like fingerprint analysis. This software is used for band normalization, identification and complementing position of bands across strains. The end step is the comparison of our band with that of the standard i.e. number of bands shared between the two [1].

The Bacterial Identification by 16S rRNA Sequencing or Ribotyping

Ribotyping is a process that is used for the identification and characterization of different organisms belonging to different species, for example 23S ribotyping for fungi and 16S ribotyping for bacteria. But mostly this technique is used for bacterial identification among all other life domains. Using 16S rRNA sequencing a large number of bacterial species and genera has been classified and named again, many bacteria whose cultivation is nearly impossible have been classified, the determination of phylogenetic relationships has been done and most of all, novel species of bacteria have been discovered and classified. 16S rRNA sequence among many other gene sequences, is used for phylogenetic analysis of bacteria because of some important reasons like its function has remained same over, it is present in a large number of bacteria and its gene is large enough, consisting of 1500 base pairs, for bioinformatics analysis [13].

Recognition of Rare and Phenotypically Aberrant Bacteria

The bacterial strains that generate only a small possibility of identification through commercially used systems, the isolates that

do not fall in any acknowledged biochemical profiles and taxa that are not or hardly associated with any infectious disease of humans, get recognized by 16S rRNA sequencing and also provides the species and genus of such strains and isolates. The combined results of studies on ribotyping provide that 16S rRNA sequencing mostly gives genus identification i.e. more than ninety percent and in case of species the percentage is comparatively low i.e. sixty-five to eighty percent, with some isolates being unidentified after performing test i.e. one to fourteen percent. Some problems that hinder the recognition of genus and species include the identification of a new taxa, the presence of same and/or similar 16S rRNA gene sequences in different species, very small number of sequences present in nucleotide databases and multiple genomovars allotted to single complex or species giving rise to nomenclature issues [14]. In many cases, the identification of hardly encountered aetiological agents of infectious bacteria can only be done by using 16S rRNA sequencing and not only provides correct recognition and suitable treatment, but also gives a better awareness and knowledge about pathogenicity and epidemiology of these strains, for example *Streptococcus iniae* invasive infections, which had been reported in North America only, was recognized and reported in Asia also [15].

The character's expression which show high variability and the absence or presence of genes that are non-housekeeping can easily affect phenotypic identification but 16S rRNA sequencing gives absolute isolates' recognition having atypical or unique phenotypic properties. Thus, ribotyping has been proved important in determining to prescribe treatment with antibiotics or not and in the recognition of *Campylobacter fetus*, a thermotolerant strain which has been found in causing bacteremia. The identification of rare and phenotypically abnormal strains is often met by multiple mistakes in clinical microbial laboratories and sometimes it is also difficult to know that the recognition of bacterial species that have been made in incorrect. Whereas ribotyping can identify rare and aberrant bacterial isolates or strains with high accuracy [16].

Recognition of Slow Growing Bacteria

16S rRNA sequencing has an edge to other identification techniques and that is the reduction of time to recognize slow growing strains like *mycobacteria* which usually takes six to eight weeks for growth in media for phenotypic identification techniques. Even in case of fast growing *mycobacterium*, a long time is required for some biochemical reactions to complete for example 28 days. Microbiological laboratories always do not have full equipment for some techniques for example gas chromatography for analysis of fatty acid content of whole cell. Thus, ribotyping has been proved to be efficient and easily done method to identify different species of *mycobacteria* obtained from clinical samples leading towards fast diagnosis and appropriate treatment [17].

Routine Recognition

Many studies have been carried out to make a comparison between ribotyping and commercial or conventional techniques for the recognition of routine isolates and/or different groups of bacteria which are medically important. Generally, ribotyping gives high percentage of accurate identifications of bacterial species than conventional techniques. The results of ribotyping in giving correct identifications fall in the range of sixty-two to ninety-two percent, depending upon the strains of bacteria and standard that is used for definition of species [18]. However, there are some cases when ribotyping is not able to efficiently give identification of bacterial species by not being able to recognize some blind spots in their genera. In this case identifications have to be carried out at alternative spots. For example there are some bacterial groups whose recognition and classification can be done by using gene GroEL instead of 16S rRNA and in example of such bacteria, species of *Staphylococci*

and *Burkholderia* are involved [19]. As conventional and/or commercial techniques are available to identify commonly confronted bacteria, ribotyping is useful and important in recognition of routine isolates in the sense of identifying bacterial species that are difficult to recognize with commercial phenotypic techniques. Gram negative anaerobic bacterial strains are some examples whose identification by conventional techniques is not really valid but after 16S rRNA sequencing many anaerobic bacterial strains which were ignored and/or unrecognized were identified and found to be involved in bacteremia. For example *S. dysgalactiae* subspecies *equisimilis* has been found to be the causing agent of β-haemolytic *Streptococcus* strain *G. bacteraemia*. Also *S. anginosus* has been found to be the causative agent of infectious endocarditis. Moreover, ribotyping has also been proved efficient in recognizing unidentified bacteria and then suggesting appropriate antibiotics for the treatment and also duration of treatment. For example *Enterococcus cecorum* has been found to be the causative agent of empyema thoracic by ribotyping whose causative agent was unknown even by using conventional phenotypic techniques and this strain was found to be vulnerable to cefotaxime and ceftriaxone due to its atypical phylogenetic position, allowing the proper treatment of the said disease. Also ribotyping is efficient in discriminating actinomycetes from anaerobic non-actinomyces Gram-positive bacteria [20].

Discovery of Novel Bacterial Species

When bacterial identification has been carried out some novel strains of bacteria are also encountered by having notable different phenotypic characteristics and sequence of 16S rRNA gene from closely related known species. In case of novel species, a single technique or sequence is not enough to define them thus a polyphonic approach is taken and 16S rRNA sequence, DNA-DNA hybridization and some other housekeeping gene sequences are used. For example some novel species were found which were isolated from human specimens and some examples include one (1%) anaerobic Gram-negative coccus, four (4%) anaerobic Gram-positive cocci, twenty-four percent aerobic Gram-negative rods, ten percent anaerobic Gram-positive rods, four percent aerobic Gram-positive cocci, thirty percent aerobic Gram-positive rods, two percent spirochetes, twenty-two percent anaerobic Gram-negative rods and three percent aerobic Gram-negative cocci, *Clostridium hathewayi, S. sinensis, Laribacter, Borrelia spielmanii and hongkongensis* [20].

Discussion and Conclusion

The 16S rRNA gene is conserved in all the bacterial strains and play major role in bacterial identification and taxonomy comparison. It is also used to establish ancestral relations between bacterial species. 16S rRNA sequencing involves isolation of bacteria, extraction of bacterial DNA, amplification of 16S rRNA gene via PCR, sequencing a region of this gene and comparison with the sequence in Gene bank. 16S ribotyping is used for the identification and characterization of different bacterial genus and species in which the recognition rate of genus is high than that of species. To date the 16S ribotyping has been able to identify different bacterial strains having aberrant phenotype due to which they have been remained unidentified, slow growing bacteria, the disease causing bacteria who have been wrongly identified by conventional methods and also some novel strains. All of these identifications have been helpful and important in a wide range of aspects in which the most important thing is the identification of disease causing or infectious strains and discovery of appropriate treatment against them.

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