World Journal of Current Medical and Pharmaceutical Research
Content available at www.wjcmpr.com ISSN: 2582-0222

REVIEW: GENE OF *16S RRNA* IS THE GOLD STANDARD TO DIAGNOSE BACTERIA

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Article History
Received on: 15-06-2023
Revised on: 04-07-2023
Accepted on: 26-08-2023

Abstract

The success of the biochemical tests depends on the purity of the culture, and this requires special preparations for obtaining, transporting and purifying of sample, use of selective media, duration of incubation, temperatures and others and all this takes time and effort.It may require sending slow-growing pathogens to laboratories that provide the requirements for their growth, but they may be far from the source of their collection, and thus it takes a long time to diagnose pathogen. Also, biochemical tests reflect the activity of the gene responsible for the production of a particular substance, and this gene may be present, but it does not work, and therefore the result may be a misdiagnosis. Gene of *16S rRNA* is found in all bacteria, and it contains stable regions through which bacteria can be identified, and other variable regions through which bacterial species can be distinguished. As well as, diagnosis of *16S rRNA* gene does not require the requirements that are required by biochemical tests for the diagnosis of bacteria, and mutations that occur in this gene do not affect its basic function, which is diagnosis, so *16S rRNA* gene is the gold standard for bacterial diagnosis. In principle, it is possible to rely on phenotypic characteristics to diagnose bacteria, but confirmatory diagnosis and registration of new species is done through *16S rRNA* gene using polymerase chain reaction.

Keywords: Biochemical tests, Isolation of bacteria, Identification of bacteria, *16S rRNA* gene, Polymerase chain reaction,Agarose gel electrophoresis, Sequence of gene.

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DOI: https://doi.org/10.37022/wjcmpr.v5i4.283

Introduction

The treatment of pathogens depends mainly on speed and accuracy in diagnosing those causes (Maurer *et al.* 2017) especially with the rapid emergence of pathogens that are resistant to multiple drugs (Burillo and Bouza 2014).

Routine and advanced morphological and biochemical tests can be used to diagnose bacteria tentatively and not deterministically where these tests are not very accurate (Jesumirhewe et al. 2016), as confirmatory diagnosis is achieved using *16S rRNA* gene. However, the methods of morphological and biochemical diagnosis require more time (Srinivasan et al. 2015) and effort than that required by the conventional PCR diagnosis, where the work is in stages and the results give many possibilities, which requires other steps and other materials that are expensive and may be difficult to obtain, in addition to the need to provide other environments that cannot be provided in routine laboratories to distinguish between bacteria.

Some routinemorphological and biochemical tests require a long time to be implemented, which leads to exacerbation of the disease and death of the patient, and pathogens may be transmitted from the patient to other people (Rizal et al. 2020),but there are advanced diagnostic and confirmatory biochemical tests such as analytical profile index (API) (Abd al-Razzaq and Hamim 2019; Beden and Mousa 2022; Al-Hilali and Al-Mozan, 2023) and VITEK®2 system (Abd al-Rida et al. 2019; Beden and Mousa, 2022; Dhahir, 2023) save time, effort and cost.

However, the phenotypes and enzymatic activities of each microbe are affected by the conditions that the organism goes through, so they are not stable (Petti et al. 2005), thus theconventional and advanced biochemical tests may fail to diagnose bacteria (Ayeni et al. 2015) because they give a result of the activity of the gene (useful for measuring the virulence of bacteria) and not to detect the presence of the gene itself (Clarridge 2004) , therefore, it does not give an accurate result on the diagnosis of the identity of bacteria (Gee et al 2004) .

The diagnostic gene of bacteria in general, which, by sequencing it, can determine the genus and species of bacteria if the primers used are universal primers, or without sequencing them if the primers are specific primers (Clarridge 2004).The related gene to the diagnosis of bacteria is 16S rRNA gene (Al-Yousif 2022) which is considered confirmed test (Al-Mozan, and Al-Amara 2020) , as it is amplified using the PCR

assay, then the sequence of its sequences is detected, and then the bacterial species is reached using the relevant software.

In 1980s, it became possible to accurately diagnose bacteria by comparing the stable parts of the genetic regions, *16S rRNA* gene is considered one of the genes that serve this purpose in a large way, as it is important in the diagnosis and classification of bacterial species (Clarridge 2004).

The stability found in 16S rRNA gene(Clarridge 2004; Tran*et al* 2017)is evidence of its importance in cell function, as mutations that affect other genes such as those responsible for the production of enzymes can affect this gene, but in a way that does not lead to the abolition of its basic function (Clarridge 2004).

Gene of *16S rRNA* is amplified by polymerase chain reaction assay (PCR)(Amutha and Kokila 2014) which is practical method by which millions of amplified copies are obtained for one copy of a specific piece of amplification represented by three stages denaturation, annealing, and extension (Joshi and Deshpande 2011).

polymerase chain reaction method has a major role in early detection of many viral, bacterial and cancerous diseases (Joshi and Deshpande 2011; Marx 2016) and It is the basis on which the cloning process and other methods depend (Ghannam and Varacallo 2023). The importance of PCR is evident in forensic evidence, because it requires only a small part of the sample at the crime scene to take it upon itself to amplify it and reveal the truth (Joshi and Deshpande 2011).This method does not require much time(Anahtar *et al* 2016) or much money and can be saved in routine laboratories (Joshi and Deshpande 2011).

Data source

Many researches refer to the stages of bacterial diagnosis by traditional morphological and biochemical methods, and confirm that it takes a long time and requires materials, equipment, and experienced workers. As the diagnosis of bacteria based on morphologicaland biochemical methods should go through the following steps:

First isisolation of bacteria, where the aim of the isolation and purification process is to obtain a pure culture to avoid errors in diagnosis. Culturing of a sample of soil, water, or something else on the surface of a general medium, such as a nutrient agar, leads to the emergence of multiple colonies of different shapes, sizes, and colors.Pure cultures are prepared by picking up a specific colony with an inoculating needle and transferring it to a solid culture medium. The process of preparing pure cultures is carried out in two ways (Qazangi 2017):

A: Streak plate method which is the simplest method used in the purification of microorganisms, especially bacteria (Irwan et al 2016; Qazangi 2017;Putria et al 2020), and it is done as follows: I- A solid culture medium such as nutrient agar is prepared, sterilized, cooled, then poured into sterile Petri dishes and left until complete solidification. II- A portion of a distinct colony from the mixed culture is transferred by a sterile loop and streaked onto the surface of the prepared solid medium(Qazangi 2017).

B: Pour plate method which is the second method for obtaining isolated single colonies (Hunt et al 2017;Qazangi 2017). Where a smear from one of the colonies is mixed by loop with a solid culture medium prepared in test tubes after cooling it to 50ºC, shortly before solidification, then the mixture of bacteria and the medium is poured into sterile petri dishes and incubated at 37ºC for 24 hours, and colonies are grown on the medium appear singular and identical or very similar. A series of dilutions can be made in the culture medium before pouring it into petri dishes, in order to reduce the number of colonies that grow on the medium.

Second isidentification of bacteria which includes: A-Morphological test: The colonies are growing on the solid culture medium differ in size, shape and color if they belong to different bacterial genera or species.The colony can be distinguished with the naked eye,as the colony consists of a large number of cells. However, These cells are characterized by their high transparency, as they are not clear when seen under the microscope, also these cells cannot be distinguished from the glass slide, therefore, they must be stained.

The main goal of staining is to make the cells distinctly visible under the microscope.The staining also helps for distinguishing the external forms of the bacteria and some of its parts such as spores and capsules.Dyes can be divided on the basis of the purpose of the dyeing process into simple staining, differential stains, negative stains, and special stains. Differential stains are the most common in laboratories, as these dyes are useful for distinguishing bacteria into two or more groups depending on the color of the acquired dye.

I-Differential stains, Gram's stain, acid fast stain, and Giemsa stain are examples on these stains. In this stains, more than one dye is used during stages of dyeing, as a result, bacterial cells acquire the color of one of these dyes, not all of theused dyes. It is worth mentioning that Gram's stain is the most widely used stain in laboratories. **A- Gram's stain ,** this stain is named after its discoverer, a Danish physician Christian Gram, in 1883. It is made of crystal violate, iodine solution, ethanol, and safranin stain. It is performed as follows:1- Bacterial smear is prepared and fixed by heat, and several drops of crystal violet is added to it and left for one minute.2- The excess dye is washed off with tap water or using a wash bottle.3- Drops of iodine solution are added to the bacterial smear and left for one minute.4- The excess dye is washed with water as well.5- A few drops of ethyl alcohol are added and left for 30 seconds, then washed with water.6- Drops of safranin dye are added to the smear and left for 30 seconds, then washed with water.7- The slide is dried and examined with a micro lens, then with an oil lens.If bacteria acquired the color of the first dye (crystal violet), it appears with purple color and it is considered positive for gram stain. But if it acquires the color of the second dye(safranin), it appears with red color, and it is negative for gram stain(Qazangi 2017).

Some types of bacteria retained the dye crystal violet and appeared under the microscope withpurpule color, while others retained the safranin dye and appeared under the microscope with red color due to the discrepancy in the chemical composition of the cell wall between these two types of bacteria. The cell wall of gram-negative bacteria contains a high percentage of fatty substances that are removed by adding alcohol, which leads to the exudation of the crystal-iodine complex from the cell to the outside, so the cell is ready to receive another dye (safranin) (Silhavy *et al*. 2010 and Qazangi 2017).

It should be mentioned that cell wall of Gram negative bacterium is thinner than Gram positive bacterium (Mai-Prochnow *et at* 2016).

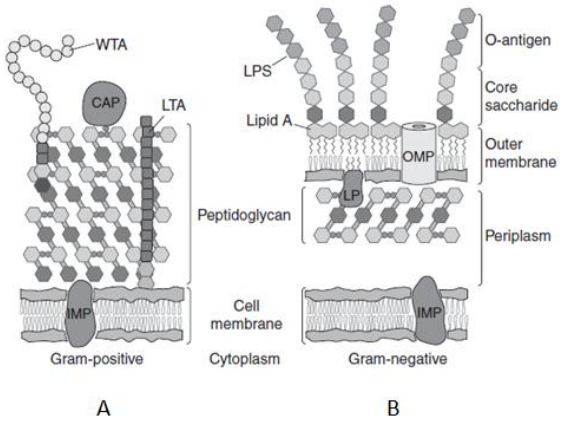
Where this method was discovered by scientistChristian Gram. In 1884 Christian Gram found a method for dividing all existing bacteria into two groups on the basis of their cell envelope by staining them. This stain was called Gram stain (Silhavy et al. 2010). The bacteria that retain with this dye are known as Gram-positive and are represented by *Staphylococcusaureus*bacteria (Silhavy et al. 2010; Romaniuk and Cegelski 2015; Devlynne et al 2018; Elma et al.2020) as one example on it. As for the bacteria that do not retain this dye, they are known as Gram-negative bacteria andEscherichia coli is represent one of the examples on these bacteria(Silhavy et al. 2010; Rasheed*et al*. 2014; Jang *et al*. 2017; Jeong *et al*. 2023).

There are three layers in the envelope of Gram-negative bacteria which are outer membrane (OM), the peptidoglycan cell wall, and the cytoplasmic or inner membrane (IM).The outer membrane is characteristic of Gram-negative bacteria, as it is not present in Gram-positive bacteria. It is a bi-lipid layer, devoid of phospholipids, but contains Lipopolysaccharide (LPS)which is considered an endotoxin that associated with Gram-negative bacteria and It is composed of glucosamine disaccharide and six or seven chains of acyl, these chains on two parts which are a polysaccharide core, and O antigen which is an extended chain polysaccharide.

The outer membrane contains proteins like outer membrane proteins (OMPs), some of them are wrapped in a cylindrical shape so that they form pores that contribute to the passive diffusion of some small molecules such as amino acids, and others have another functions.

As for the second layer of the envelope, it is the peptidoglycan, which consists of many units of disaccharide N-acetyl glucosamine-N-actyl muramic acid, Which are linked together by side chains of pentapeptides. The peptidoglycan gives bacteria the ability to maintain their distinctive shape. The peptidoglycan layer is very thin. The peptidoglycan is followed by the so-called periplasm.Periplasm contains proteins and is more viscous than cytoplasm. Periplasm is the source of the material that makes up the envelope. The third layer is The Inner membrane (IM) that consists of two layers of phospholipid. The function of the inner membrane is to transport the materials that are required for the biosynthesis of the envelope. As for Gram-positive bacteria, their envelope does not contain the outer membrane, as the envelope of Gram-positive bacteria begins with many layers of peptidoglycan much more than those layers found in Gram-negative bacteria. The links that bind the peptidoclaycan layers to each other differ from those that bind the peptidoclaycan layers found in Gram-negative bacteria. The peptidoglycan layers are interspersed with long polymers called teichoic acids which are composed of glycerol phosphate, glucosyl phosphate, or repeats of ribitol phosphate.

Wall techoic acids covalently linked to peptidoglycans (WTA) and lipoteichoic acids associated with membrane lipids (LTA) are considered types belong to these polymers. The large percentage formed by these polymers in the mass of the Gram-positive cell wall reflects their structural and functional importance.Many Gram-positive bacteria possess branched stem proteins in the peptidoglycan layer that act as binding sites for covalently linked proteins that play a role in bacterial resistance to beta-lactams shape1(Silhavy et al. 2010) .



Shape 1: A- Envelope of Gram positive bacteria, B- Envelope of Gram negative bacteria (Silhavy et al. 2010)

B- Biochemical tests, the most common tests that should be performed:

1- Catalase test

This assay aims to detect the ability of bacteria to produce catalase (Qazangi 2017; Hartline 2023), which decomposes hydrogen peroxide into oxygen and water (Qazangi 2017;Kaushal *et al* 2018). It is done by addingdrops of (3%) hydrogen peroxide to a colony of fresh bacterial culture that is placed on clean slide, and the appearance of bubbles is evidence of a positive test (Qazangi 2017; Al-Mozan 2021; Muktaf, 2022).

2- Oxidase test

This test aims fordetection of cytochrome oxidase enzyme that is responsible for transferring electrons from cytochrome to oxygen as a final electron acceptor in the respiratory chain (Wikström et al. 2018). A drop of oxidase reagent is mixed with a colony from fresh bacterial culture by a sterile loop on filter paper where result is being positive when the color changes to purple(Qazangi 2017).

The above two tests are general tests, whether for Gram-positive or Gram-negative bacteria, to distinguish between the species of each group.

However, there are tests specific to distinguish between gram positive bacteria(AL-Joda and Jasim 2021), while otheras IMViC tests are specific to distinguish between gram negative bacteria especially family of enterobacteriaceae (Hemraj et al. 2013) .

Tests of IMViC derived from the first letters of the tests that aim to diagnose members of enterobacteriaceae family and differentiate between them, these tests are indole test, methyle red test, Voges-Proskauer test, and citrate test.

A: Indole test, this assay aims to test the ability of bacteria to degrade the amino acid tryptophan into pyruvic acid, ammonia, and indole (Aminollah and Supriyanto, 2016; PHE, 2018). The latter (indole) is detected chemically by the Kovacs reagent, which has pale yellow color, as the aldehyde present in the reagent combines with indole to form a complex with red color that collects on the surface of the medium.The importance of this test lies in the fact that there is a limited group of bacteria

that have the ability to analyze tryptophan and form indole, which paves the way for their diagnosis easily through this test.This test can be done in peptone, tryptone, or other media, where the test is performed as follows:

1-The prepared peptone broth in test tubes is inoculated with the bacteria that should be tested and incubated in 37ºC for a period of not less than 24 hours.

2- About 0.3 ml of Kovacs reagent is added to each 5ml of cultured peptone broth, mixing of them is done by moving the tube well,. **3-** It has been left aside for some time, where As the positive result is the appearance of a pink or red ring, which it is indicating to the presence of indole (Goswami *et al*. 2015 and Qazangi 2017).

B: Methyl red test, this test aims to detect the ability of bacteria to produce a fixed amount of organic acids, as a result of fully ferment glucose, and the test is done as follows:

1- A colony of fresh bacterial culture is cultured in a tube contains methyl red Voges- Proskaur broth(where this broth is used for IMViC test(Aryal 2018a)) and incubated at 35ºC for 48 hours.2- A few drops (8-10 drops) of methyl red reagent is added to the cultured tube (Qazangi 2017), the positive result of this test is the appearance of a bright red color (Igbeneghu and Lamikanra, 2014; Qazangi, 2017).

C: Voges-Proskauer test, this test targets bacterial species that have the ability to partially analyze glucose and form an intermediate compound (Acetyl Methyl Carbinol).Acetyl Methyl Carbinol is detected by Voges-Proskauer reagent (Qazangi 2017), where this reagent consists of two solutions, the first contains alpha-naphthol and the second contains potassium hydroxide (Aryal 2018b) where these solutions are important to detect aceton (Aminollah and Supriyanto 2016).Voges-Proskauer test is performed as follows:

1- A colony of fresh bacterial culture is cultured in a tube contains methyl red Voges- Proskauer broth and incubated at 35 ºC for 48 hours.

2-About 1-1.5ml of the first solution and 0.5-1ml of the second solution are added to the cultured tube and shake well for a minute to dissolve the largest possible amount of oxygen in the medium which is necessary to oxidize the acetone and show the desired color.The tube is left for at least one hour for development of color into red (Qazangi 2017).

As mentioned above, MRVP test is used to test the ability of bacteria to ferment glucose (Shanmugaraj et al. 2021).

D: Citrate test, citrate test aims to detect the ability of bacteria to consume citrate or citric acid as a single source of carbon. There are culture media have been designed for this purpose and are prepared by specialized companies, such as Simmons citrate medium, which contains sodium citrate, as source of carbon and energy, and inorganic compounds, as source of the rest of the nutritional elements needed by bacteria, in addition to bromothymolblue which acts as color guide.Citrate test can be done as follows:

1-The medium is prepared, distributed in tubes, sterilized, and left slanted until solidified.

2- A colony of fresh bacterial culture is cultured in the tube that contains Simmons citrate medium and incubated at 35ºC for 48 hours. The incubation period can be extended to 7 days, because some bacterial species slowly consume citrate, and it is worth prolonging the incubation period.The positive result is a change in the color of the medium from green to blue (Qazangi 2017).

However, morphological and biochemical methods are not as accurate as molecular methods (Kshikhundo and Itumhelo 2016).

16S rRNA geneand its preference over other diagnostic methods

Gene of 16S rRNA is characterized by its unique ability to identify bacterial species(Srinivasan et al 2015;Dickson et al 2020; Fernández-Barat et al 2020) within a sample, especially with regard to taxonomic and phylogenetic studies (Dickson et al 2020; Fernández-Barat et al 2020), especially that diagnosis of bacteria and the period of its presence in the environment is essential in studies that aim to reveal microbial comunities(Lindner and Renard 2013) where16S rRNA gene is very efficient for this role . This gene contains conserved regions interspersed with the nine variable regions(Dickson et al 2020;Fernández-Barat et al 2020),through the variable regions, it is possible to reach the bacterial species, not just the bacteria (Rizal *et al*.2020). This gene has 1500 base pairs in long (Dickson et al 2020**).**

There are universal primers that integrate with the constant regions of the gene that are similar among all bacteria, through which the microorganism can be diagnosed as a bacterium, as these primers can reveal any bacteria, even if they are not intended in the diagnosis(Verma *et al* 1999)

There are other regions in this gene called hot spots (which are different for all bacterial species) on which the effect of mutations appears(Shepherd *et al* 2022). For example, it is possible that antimicrobial agents affect hot spots in this gene, leading to a change in its phenotypic characteristics related to the organism's resistance to antimicrobial agents without affecting its diagnostic use for bacterial species.

The problems on the basis of which the diagnosis of 16S rRNA gene was preferred over the diagnosis with other tests are: in some organisms, there is no perfect match between the phenotype and the genotype (Clarridge 2004) because there is more than one phenotype for the same genotype(Clarridge 2004; Ceyssens et al 2011) and there are also similar genetic patterns, but the phenotype is different (Clarridge 2004)which may occur because of the so-called phenotypic plasticity (Ernande and Dieckmann 2004; Murren et al 2015; Gomez-Mestre and Jovani 2013; Hendry2015); 16S rRNA gene is accurate(Lakshmi et al 2020) and immediate for identifying the pathogen of serious infections; 16S rRNA gene is the only method for diagnosing bacteria that are difficult to culture (Clarridge 2004) where 16S rRNA gene does not require bacterial culture(Clarridge 2004 ; Aguilera-Arreola et al 2016; Sune et al. 2020) according to what is called metagenomics (Rizal et al. 2020); some bacteria are more expensive to diagnose by other methods than by *16S rRNA* gene, and in some cases, bacteria are many, numerous, and diverse, and it is difficult to isolate and purify them in order to diagnose them correctly (Clarridge, 2004).

As well as, some bacteria such as Rickettsia spp. and Coxiella burnetii require intracellular transplantation such as embryonic eggs and some of them, such as Mycobacterium leprae and species of Borrelia, require injection into animals (Rizal et al. 2020) and this requires special conditions and

capabilities that may be difficult to achieve for the success of the process in routine laboratories (Wilson et al. 2018 and Chiu and Miller 2019), but, for amplification of 16S rRNA gene, only DNA is required to diagnose this bacterium without culturing it (Rizal *et al.* 2020).However polymerase chain reaction is the method required to amplify this gene with an affordable price and in a short time.

Polymerase chain reaction

It is the process that depends on the change in temperature to obtain a large amount of DNA from a specific small part of the DNA (Joshi and Deshpande 2011). American scientist Kary Mullis won the Nobel Prize and the Japan Prize in 1993 as a result of his development of the PCR(Joshi and Deshpande 2011; Karnath 2019; Heckmann and Paradisi 2020; Negahdaripour and Pharm2020; Venegas and Donoso 2022)., the basic principle of PCR test was described by researcher Gobind Khorana in 1971, but progress was limited at that time to matters related to that method.

Polymerase chain reaction has become an indispensable technology in forensic medicine and scientific centers. It is widespread because this technique is fast, not very expensive, and does not require obtaining an excellent sample (Joshi and Deshpande 2011), it is successful even if the sample is of poor quality (Joshi and Deshpande 2011; Favrot 2015). The basic principle of this process is to use primers to amplify a specific sequence of DNA(Cahyadi *et al* 2017).

Name of this method (chain reaction) indicates that one copy gave two copies, then three, then four, and so on.Where, in every step of PCR, the number of copies of DNA increases, and there are enzymes that are responsible for the increase process called polymerases, where these enzymes bind the blocks of the DNA to form the long DNA molecule, and in order to enzyme can perform its work, the following must be available:**1-** The basic building blocks which are four nucleotides cytosine (C), adenine (A), guanine (G), and thymine (T).**2-** Primer, which is a short sequence of nucleotides, as the primer binds the nucleotides through the polymerase, where the polymerase enzyme brings the appropriate nucleotide based on the nucleotide present in the old DNA strand.3- The old DNA strand serves as a template for building the new strand of DNA.

When the aforementioned components are available, the polymerase enzyme can form millions of copies that are completely identical to the original template DNA strand Sequencing of PCR product can be done later (Joshi and Deshpande 2011). Polymerase chain reaction method depends on the stability of the polymerase enzyme at a high temperature during the replication process (Joshi and Deshpande 2011; Ishino and Ishino 2014). The first of the three PCR steps is denaturation, which occurs at a temperature of 90-97 degrees Celsius, where the two strands of DNA separate at this temperature. The second step, annealing, takes place at temperatures 50-60, where the primers are attached to the template DNA strand to start the extension. As for the third step, extension, it takes place at a temperature of 72 degrees Celsius , where the addition of nucleotides occurs to the end of the primer attached to the template DNA to form a new complementary strand to the template strand. After about 25-30 cycles, a DNA samplethat contains many copies identical to the original DNA sample. is obtained. It is worth noting that DNA polymerase I has failed to link the nucleotides to the end of the primers for the purpose of completing the elongation step. Where the reason is that this enzyme is not stable to the high temperatures required to perform the replication process. Therefore, Taq DNA polymerase isolated from *Thermus aquaticus* bacterium was used in PCR method. *Thermus aquaticus* has the ability to live in volcanoes at a temperature of over 110degrees celsius, thus, Taq DNA polymerase enzyme can withstand therequired temperatures for the replication process (Joshi and Deshpande 2011). In order to ensure a successful workflow, contamination must be avoided(Joshi and Deshpande 2011; Yuan 2016) when preparing the mixture, adjusting the volumes of the mixture, and ensuring that the conditions of the programthat are entered into the device are correct. Taking into account that the cycles do not exceed 35 cycles, as they affect the correct result (Joshi and Deshpande 2011). It can be confirmed whether or not amplification has occurred by taking PCR product and subjecting it to the electrophoresis process(Desjardins and Conklin 2011;Joshi and Deshpande 2011; Mardis and McCombie 2017; Wittmeier and Humme 2022).

Agarose gel electrophoresis

Electrophoresis is the method by which nucleic acids and proteins are separated using an electric current, which is the basic method by which nucleic acids are analyzed and purified in preparation for future studies. When an electric field is applied on the charged particles with a certain charge in an aqueous solution, these particles migrate to the side with the opposite charge, and this is the principle on which electrophoresis is based. The extent of thecharges strength of particles, in addition to the applied electric field, is reflected in the migration speed, and thus the migration speed of the particles differs(Westermeier and Freiburg 2005; Tantray et al 2023). Electrophoresis process is performed in media that achieves maintaining the speed to the appropriate extent, it should be mentioned that a gel medium is the best example of such media, as it works as a sieve or as a grille, and there are two types of this medium:agarose and polyacrylamide gels. Size of particles is also one of the factors that determine the speed of their migration (Westermeier and Freiburg 2005). Agarose has many properties, so it is the most widely used in routine laboratories (Li and Arakawa 2019; Tomioka et al 2022). It is a polysaccharide extracted from red algae(Graham et al 2019; Jiang *et al* 2023). The agarose dissolves in boiling water, when the dissolved agarose is cooled,(Westermeier and Freiburg 2005) a moldof a gelatinous substance permeated with pores is formed (Lira et al 2016), size of these pores varies depending on the agarose concentration, so the pore sizes are small if the agarose concentration is large and vice versa correct. When preparing agarose, either ethidium bromide or SYBR green must be added to it, because without these fluorescent dyes, it is not possible to see the bands of DNA, where using one of these dyes(Westermeier and Freiburg 2005) , it is possible to see the DNA bands using ultraviolet light(Haines et al 2015). It must be pointed out that SYBR green is safer than ethidium bromide dye in terms of toxicity or mutagenicity (Westermeier and Freiburg 2005).

To agarose gel electrophoresis is carried out, the following materials and tools must be available:

1- Chamber of electrophoresis and power supply.

2- Trays made of plastic, which transmits ultraviolet rays.

3- Combs to form wells in the gel.

4- Buffer of electrophoresis as Tris-borate-EDTA (TBE).

5- Fluorescent dyes as ethidium bromide.

6- Box of ultraviolet light.

The preparation is done as follow:

1- The required weight of the agarose, such as 1 gm, is mixed with the appropriate volume of buffer solution (TPA), which was 100 ml in a flask.

2- Melting of mixture is done by boiling using a microwave, the process may take only seconds, provided that the result is a clear solution like water.

3- The solution should be allowed to cool to 60°C, and when it reaches that temperature, ethidium bromide should be added with moving the flask to homogenize the solution.

4- The ends of tray should be sealed with caps, and the comb must be placed at its right place.

5-The cooled solution is poured into the tray and allowed to solidify.

6- The end caps are removed and the comb must also be removed carefully so as not to damage the wells.

7-The tray with its soldified gel shold be paced in chamber of electrophoresis and covered with 1X buffer of electrophoresis .

8- Each sample of the PCR product is loaded into its own well in the gel.

9- The electrophoresis chamber should be covered and DNA samples should be placed on the negative electrode side of the electrophoresis chamber.

10- The power supply must be connected to the electrophoresis chamber for the electrophoresis process to begin.

11- After the end of electrophoresis, (Reddy and Raju 2012) the gel is examined under ultraviolet light to see the bands (Reddy and Raju 2012;Badreh *et al* 2022).

Importance of 16S rRNA gene sequence technique

Sequence technique is the method that is carried out by a sequencing machine based on biochemical methods to determine the sequence of nitrogenous bases in the piece of DNA are required to be analyzed (Heather and Chain 2015; Kchouk et al. 2017).The discovery of the helical structure of DNA by Watson and Crick(Tan and McCoy 2020) and the knowledge of the four bases (A, T, C, G) that make up DNA and how they are arranged (Watson and Crick 1953;Kchouk et al. 2017) made it easy to know the genetic structure of all living organisms. Especially that the genetic makeup became available through the discovery of the so-called DNA sequence (Tourneau et al, 2015; Kchouk et al. 2017; (Lei et al.,2022; Bashar et al.,2022; Qasim et al.,2022; Al-Hchaimi et al.,2022; Mohammed and Qasim, 2022; Lafta et al.,2023).

The problem of not correctly diagnosing the unknown microorganism or the microorganism that was subjected to some conditions that led to suppressing some of its approved activities in phenotypic and biochemical diagnosis was solved by the emergence of genetic sequencing methods for 16S rRNA gene (Petti et al. 2005). The genetic sequencing method for 16S rRNA gene is the most accurate and reliable method (Chen et al 2014) as well as the popular method (Bailen et al 2020), as it avoids errors that may occur in other tests, such as variation in temperatures, concentrations, isolate size and other working conditions, in addition to that errors that occur in sequencing can be addressed, as well as most of the errors do not have a significant impact on the result (Petti et al. 2005). The sequence of 16S rRNA gene allows comparison between all types of bacteria, being general, by means of which species and subspecies of bacteria can be reached, where this gene is usedto surveyof communities of bacteria (Edgar 2018; Nagai et al 2023). Gen Bank contains a large number of sequences genes (Strasser 2008; Porter and Hajibabaei 2018;Leraya et al 2019) that can be used as a source for comparison (Benson et al 2013), and the number of sequences for the *16S rRNA* gene is about 90,000 sequences that can be compared to reach the diagnosis of bacteria, where the use of *16S rRNA* gene to judge or pinpoint the exact bacterial species depends on the (Clarridge 2004) exact sequence of that gene and the relevant sequences available in the databases. (Clarridge 2004;Das et al 2014). Sequencing is done for the gene amplification product, and before sequencing analysis, some important manipulations must be performed, which include filtering low-quality reads to obtain high-quality, accurate reads in order to reach an accurate diagnosis (Rizal *et al*. 2020). Contamination should also be avoided when amplifying during sequences(Davis et al 2018), as it leads to misdiagnosis. The sealed laboratories, which operate in a one-way workflow system, reduce the problem of contamination and thus ensure the correct diagnosis(Gu *et al*. 2019).

Conclusion

Morphological and biochemical tests can be used in the initial diagnosis of bacteria in order to confirm them later with a molecular diagnosis that includes the diagnosis of *16S rRNA* gene, as relying only on morphological and biochemical methods in the diagnosis may mislead the truth. The main function of *16S rRNA* gene is to identify the bacteria in general because it contains the stable regions found in all bacteria, and to identify the species of bacteria in particular because it contains the variable regions specific to each bacterial species. The diagnostic function performed by 16S rRNA gene is not canceled when mutations occur in that gene. Therefore, *16S rRNA* gene is the gold standard for the diagnosis of bacteria. Polymerase chain reaction method is fast, not very expensive, and does not require obtaining an excellent sample.

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