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# Molecular Techniques Used In the Detection of Fungi

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# التقنيات الجزيئية المستخدمة في الكشف عن الفطريات وتأثيراتها والكائنات الدقيقة الاخرى

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## ABSTRACT

Diagnostic microbiology has been transformed by molecular biological technologies for the characterization and identification of microorganisms, which are currently routinely used in specimen processing. The methods of Polymerase Chain Reaction (PCR) have paved the path for this new era through enabling for the quick detection of microorganisms which have been formerly impossible or difficult to detect using standard microbiological methods. Along with detecting fastidious microorganisms, pathogens of public health relevance may currently be detected more quickly using molecular techniques. Molecular approaches have presently evolved beyond identification for detecting antifungal resistance genes as well as providing public health information like genotyping strain characterization. The costs of molecular methods are reducing due to the introduction of multiplex PCR, real-time PCR, and advances in efficiency via automation, and the role of the molecular techniques will continue to grow.

Key words:
fungi, PCR, sequencing, techniques



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**الخلاصة**

تم تطوير علم الأحياء المجهرية بواسطة التقنيات البيولوجية الجزيئية لتوصيف وتحديد الانماط الوراثية لتلك الكائنات المجهرية، والتي تُستخدم حاليًا بشكل روتيني في تشخيص العينات .مهدت طريقة تفاعل البلمرة المتسلسل (PCR) الطريق من خلال الاكتشاف السريع للكائنات الحية الدقيقة التي كان من المستحيل اكتشافها سابقًا أو يصعب اكتشافها باستخدام الأساليب الميكروبيولوجية الكلاسيكية . إلى جانب اكتشاف الكائنات الحية المجهرية ، يمكن حاليًا اكتشاف مسببات الأمراض ذات الصلة بالصحة الانسان بسرعة أكبر باستخدام التقنيات الجزيئية .تطورت الأساليب الجزيئية حاليًا إلى ما هو أبعد من التحديد للكشف عن جينات مقاومة مضادات الفطريات بالإضافة إلى توفير معلومات مهم مثل التنميط الجيني لتوصيف سلالات مرضية مختلفة ، كما ان تكاليف الطرق الجزيئية اخذت بالانخفاض بسبب إدخال تفاعل البلمرة المتسلسل وسيستمر دور التقنيات الجزيئية في التطور بشكل مستمرمن اجل الوصول الى مراحل متقدمة في وراثة الاحياء المجهرية.

**الكلمات المفتاحية**

النمذجة التكيفية، التغذية الراجعة ذات الصلة، استرجاع الوثائق، الخوارزمية الجينية.

## INTRODUCTION

A fungi is a wide collection of eukaryotic organisms that belong to their own Kingdom: Fungi. Molds, yeasts, smuts, rusts, morels, truffles, and mushrooms are among the many organisms found in fungi [1]. At least 69,000 species were identified, although there are likely to be more than 1.5 million fungi in existence. Only about 5-10% of such fungi can be cultured artificially [2].

With about 1.5 million species, fungi have the most eukaryotic variety on the earth and are one of principal de-composers in the eco-system [3] . Fungi can be foes as well as friends ، Due to their medical, economic, and commercial uses, various fungus species are helpful to vegetal, human, environmental, and animal health. A lot of them are also dangerous due to their capability to function as pathogens and result in diseases [1]. The cornerstone regarding the plant pathology discipline is considered as the identification of fungi that cause plant diseases [4].

Molecular technology advances our understanding regarding the population structures and biology of fungus pathogens, gives precise and quick solutions to fungi epidemiological problems, and aids the decisions of disease management [5]. Because of the significance of understanding fungi and how crucial they are in producing holistic effects on human well-being. The first relies on phenotypic characteristics, whereas the second, which depends on genotypic characteristics, provides highly specific, quick, effective, and perhaps more precise results. Contrary to traditional procedures, organism isolation does not necessitate culturing [6,7].

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The application of molecular biology methods for microorganism follow-up and identification depends on the genome properties of the organism to be characterized or detected. Yet, various factors continue to obstruct their use in microbiology laboratory, including slow growth, difficulty in isolation, high test costs, and inadequate sensitivity of the detection for identifying certain species of the bacteria found in complicated samples, to name a few [8].

This research aims to give a focused overview of the field of microorganism detection using molecular techniques

## 2. Selection of target genes

Various target geneswere identified as effective methods for identifying bacteria, and fungi ،Because of their higher genetic diversity and our incapability to discover common genetic link, broad-range gene targets for viruses have not been identified [5] . The gene targets chosen should have a functional constant, acting as molecular clocks for microbial evolution (phylogeny) [8]. A conserved segment which is shared by all the bacteria (or fungus) and is flanked via variable or highly variable sections must be present in the gene. The "universality" regarding gene target in which PCR and DNA sequencing primers anneal is due to conserved regions. Variable or highly variable parts yield distinct nucleotide base sequences or fragments throughout cycle sequencing, which play the role of "signatures" for diverse species. For determining relatedness, the sequence is put to comparison with reference sequences that have been deposited in private or public sequence libraries. The allowable extent of the variation between the two sequences for species or genus classification varies based upon target gene and micro-organism, and is a point of contention [9,10].

### 2.1 Fungi

The biochemical tests and expertise in detecting fungal reproductive structures' morphology microscopically and macroscopically are required for traditional identification of fungi, just as they are for bacteria. The sequencing of the genes doesn't need a viable organism or sporulation (i.e. molds), allowing for faster diagnosis. The gene targets for the medically valuable fungi and yeast aren't equally well defined as these for the bacteria. The internal transcribed spacer regions ITS-1 and ITS-2, which represent variable regions that are positioned between conserved genes encoding for 5.8S, 28S and18S rRNA, appear to be the best targets (figure 2). Yeasts

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including Cryptococcus, Candida, Trichosporon [11,12], and Aspergillus species; dematiaceous molds; zygomycetes; and other fungus that have medical relevance [13,14] have all been identified using ITS region. Alternative gene targets, like D2 and D1 domains of 28S rRNA subunit [15], elongation factor a (Fusarium species), and b-tabulin, are frequently required for identifying particular genera (Phaeoacremonium species).

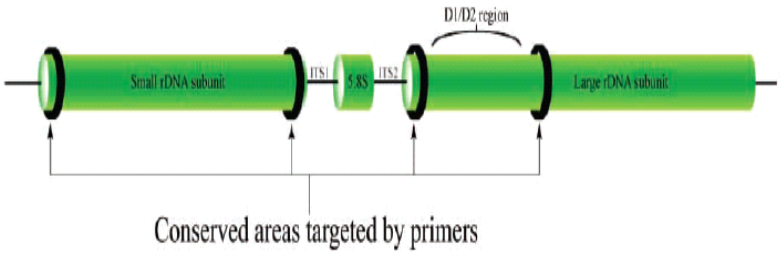


**Figure 1.** Between genes for large and small rDNA sub-units, there are fungal ITS regions. Outside of the ITS2 and ITS1 variable regions, circles denote conserved areas serving as gene targets for the amplification of the PCR and sequencing of the DNA in the fungus[12].

## 2.2 DNA barcoding

DNA barcoding can be defined as one of the molecular diagnostic tools for identifying the species regarding all domains of eukaryotic life using a small DNA segment. In all target lineages, barcode regions can be detected. It has enough DNA sequence variation to differentiate between species[15]. Standardized sequences regarding 500–800 base pairs are employed in DNA barcoding for identifying species, with markers that are valid for a broad variety of taxonomic groups [16]. For microbiological organisms like bacteria, fungi, and algae, such PCR-based approach isn't just useful for recognizing cultured species, yet it may also be utilized to identify species from natural environment [15]. The number of representatives in database determines whether or not a species may be identified with the use of a barcode. Fungi could be detected in life-cycle stages that aren't suited for morphological identification using DNA barcoding [10]. Barcoding's effectiveness is based on the notion that genetic differences within a species are significantly fewer compared to those across species. DNA barcoding could be used for identifying and discovering fungi that aren't suitable for the morphological identification and discovery Furthermore, effective markers are highly useful in this approach for identifying the poorly understood diversity of the fungal species in natural environments [17]. Figure 2 depicts the DNA barcoding process in its entirety.



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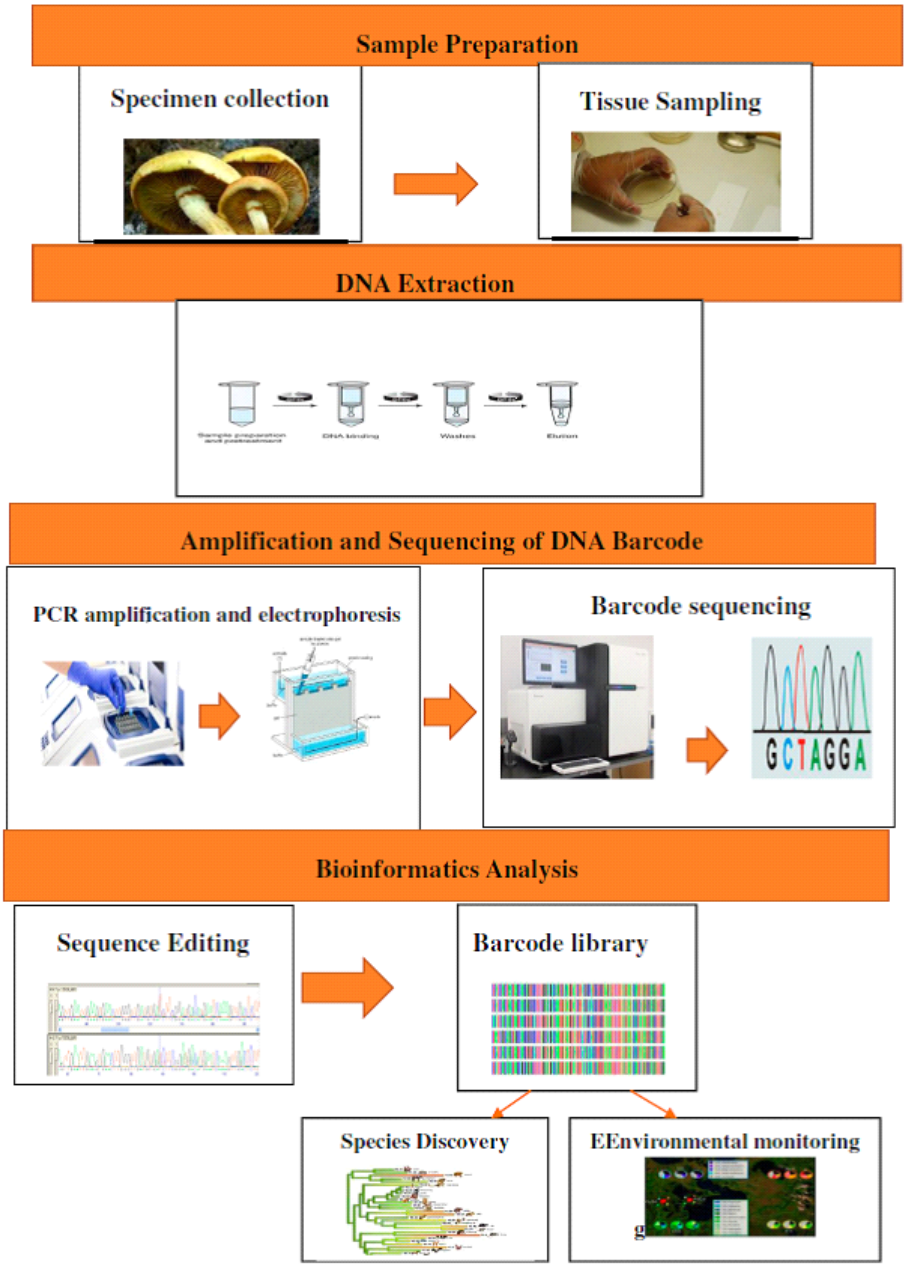


**Figure2. Overview of DNA barcoding method** [9].

## 3. Molecular Techniques

Recently, with the introduction of new molecular-based approaches, considerable advances were made in diagnosing clinically relevant bacteria. Amongst which, the detection of the ribosomal RNAs using DNA probe and the amplification of nucleic acids from the clinical samples stand out [15] . Those approaches have improved the diagnostic specificity and sensitivity of other detection methods, such as culture, and had allowed a simultaneous identification of multiple microbial agents from same sample in certain situations [18] .

The identification of nucleic acids of the micro-organisms using probe via the hybridization has been the first step in developing approaches depending on molecular biology methods. The genetic probe can be defined as one of the nucleic acid molecules which could detect complementary sequence of the DNA. Natural DNA oligonucleotide probes are made through the cloning of the fragments of the DNA into proper plasmid vectors and isolating cloned DNA, or by direct synthesis using the combinatory chemistry. Under the right conditions, probes could be labeled with substances which create colored reactions [19].

The approaches for performing and interpreting DNA hybridization are quite simple. Amplification methods depending on PCR detection of DNA and transcription-mediated specific rRNA amplification are already established and commercially available. Those methods deliver faster findings with more specificity and sensitivity compared to traditional methods. Based on the sample type, such approaches identify 15 to 20% more infectious agents compared to traditional methods, and 25% - 70% more than the immuno-fluorescence or enzyme immuno-analysis (EIA) [20].

The development of the probes for detecting markers of virulence, such as the ones that have been directed to genes expressing toxins, enables for the identification of organisms carrying such genes in the clinical samples with no need to cultivate them. Probes for *E. coli* enterotoxins, Clostridium difficile toxins, and *Vibrio cholerae* toxin which could be directly applied to fecal samples, are examples of the latter [21].

### 3.1 polymerase chain reaction

PCR is defined as the most commonly utilized target nucleic acid amplification approach. Using this approach, one nucleic acid copy is multiplied to over 107 throughout quite a short duration of time. A thermo-stable DNA polymerase and 2 specific oligo-nucleotide primers are utilized for the production of several copies of certain regions of the nucleic acid throughout 25-50 repetitive cycles [22]. PCR had gained certain attention for the detection of presence of small numbers of the virus or bacteria particles in the environmental and clinical specimens. The commercial kits that employ the PCR technology in detecting *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Mycobacterium tuberculosis* were advanced. Even though the PCR represents the most commonly utilized approach, other approaches of amplification for the DNA and RNA molecules have been found. After the occurrence of amplification reaction, the simplest approach for the identification of the product by the size is electrophoresis and migration on agarose gel [23].

The PCR can be described as *in vitro* approach of DNA syntheses by which certain DNA portion undergoes the amplification through being delimited with a pair of the flanking primers. Copying has been exponentially accomplished via repeated cycles of various periods of incubation and degrees of temperature in presence of thermo-stable DNA polymerase enzyme. Thereby, millions of wanted DNA sequence copies may be obtained in about two hours. Molecular biology technique which has high specificity, speed, sensitivity, and versatility for the detection of smallest

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specific DNA amounts, promoting its easy identification as well as avoiding utilization of the radioisotopes [24].

### 3.2 Real-time amplification

The majority of the traditional PCR-based tests need several operations and PCR products have been detected in separate system of gel electrophoresis. In comparison to the traditional methods of the PCR, the real-time PCR is faster and the risks of contamination are decreased. It can detect the amplified target through fluorescently labeled probes as hybrids have been created (in other words, detecting amplicon in the real time). The produced signal is associated with the amount of the amplicon that is present at every cycle's end and it is increased with the increase in the amount of the specific amplicons [25]. The commercially available systems for the nucleic acid extraction, in combination with the rapid thermal cyclers and instrumentations (for example TaqMan© and Light Cycler) able to detect and differentiate several amplicons, make the real-time PCR one of the viable and attractive propositions for routine diagnostics lab. The real-time assays were highly beneficial to study the microbial agents of the infectious diseases. The biggest impact until now was in the virology area, in which the real-time assays were utilized for the rapid detection of many different viruses in the human samples and for the quantitative monitoring of the viral loads and responses to the anti-viral treatments. Advantages for the patient may be observed as well in the bacteriology, in which the rapid detection of the antibiotic resistance genes and/or bacterial pathogens may be helpful in ensuring the suitable utilization of the antibiotic treatments, minimize hospital stay duration and reduce the potentials for the emergence of the resistant bacterial strains. The latest advancements in the real-time PCR had suggested a future where rapid quantifications, identification and typing of different microbial targets in single multiplexing reactions will turn to be a usual process [26].

### 3.3 PCR-RFLP

Consists of the PCR for amplifying a gene or segments of that gene, combined with consequent PCR product digestion utilizing one or multiple enzymes of restriction. The restriction products' electrophoretic analysis has revealed gene polymorphisms or of fragments of gene (RFLP) and evidenced genetic variations amongst the isolates. This technology has the ability to rapidly reveal the polymorphisms of the sequence; it's highly reproducible and technologically simple. In addition to that, it compared well with other approaches, for example: Temperature Gradient Gel Electrophoresis (TGGE), Denaturing Gradient Gel Electrophoresis (DGGE), or the Cut Fragments Length Polymorphism (CFLP) and single strand conformation polymorphism (SSCP), also revealing the polymorphisms of the sequence amongst the strains without the need for the determination of the entire sequence [27]. For the mycobacteria, the PCR-RLFP was commonly utilized, in particular, for studying insertion element IS-6110, where Pvull enzyme has been utilized for the generation of the fragments of restriction from the genomic DNA [28].

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### 3.4 *RAPD finger-printing*

RAPD method is PCR-based approach of discrimination, where short random primers anneal to a number of the random target sequences, which lead to diagnostic value patterns. In the RAPD analysis, target sequence (or sequences) that need being amplified is unknown and primer with a random sequence (10bp sequence or 10-base pair sequence that has been generated randomly by the computer) has been designed then synthesized. After the synthesis of those sequences, they have been utilized in the PCR reactions with the low-stringency conditions of annealing, leading to amplification of random-size fragments of the DNA. This approach is being studied currently for identifying LAB, which includes the probiotic strains. Due to the fact that RAPD patterns' reproducibility is poor occasionally; this approach should be carried out under thoroughly regulated conditions. A variety of the groups had adopted using RAPD for the identification and characterization of the LAB strains from a variety of the sources, in other words,, human, milk and food samples [29,30].

### 3.5 Multiplex PCR Assays

Generally, using multiplex PCR assays, identifying over two pathogens, toxin genes, or other targets, could result in the reduction of costs as well as time for obtaining the results. For instance, through charging a little more than for the regular single PCR assays, our lab presents assay detecting several enterotoxigenic E. coli (ETEC) virulence factors utilizing multiplex gel-based PCR, which has high specificity and sensitivity [31]. Another gel based multiplex PCR assay that has been designed for simultaneous detection of the *L. intracellularis, B. hyodysenteriae, and B. pilosicoli* had exceptional to nearly ideal agreement with nested PCR assay for the Brachyspira, as well as a moderate agreement with immuno-fluorescence tests for the Lawsonia [32]. In the present day, the m-PCR may as well be beneficial for defining structures of specific microbial communities and for the evaluation of the community dynamics, like throughout the fermentation or as a response to the environmental changes. Casey & Bosworth. [31] had described rapid m-PCR approach that allows for simultaneously detecting 6 widely encountered water-borne pathogens in one tube.

### 3.6 DNA sequencing

Determining the exact nucleotide order within the DNA molecule is commonly referred to as the DNA sequencing. Approximately 30 years ago in 1977, Maxam–Gilbert and Sanger have accomplished an innovation, which has revolutionized the biological science world through the sequencing of 5386-base bacteriophage uX-174. From 1977 until now, the DNA sequencing had come across considerable advancements concerning the techniques and tools of sequencing. The DNA sequencing of the modern era deal with the sequencing of the next generation and several of the other advancements are available to practitioners, academics and researchers at a highly reasonable cost with the maximal precision. Biological data-bases are flooded with massive flows of the sequences that come out from a variety of the organisms worldwide. In the present day, scientists and researchers from different areas are using those data for various applications, which

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include food security through the developments of better livestock, crops and crop yields, improved diagnostics, therapies and prognostics for a wide range of the complicated illnesses [33].

### 3.7 Nested PCR

The nested PCR is utilized for the purpose of achieving a high sensitivity and specificity level. For instance, the sensitivity of the nested PCR is 1,000 times higher compared to the single PCR for the fungal identifications [34].

In this approach, 2 successive rounds take place, where one primer pair is utilized for the amplification of a large DNA region, after that, this amplified DNA sequence plays the role of target for 2nd round with the use of 2 internal primers.

In this PCR type, a high contamination risk is measured due to the fact that 2 amplification cycles need to be carried out in separated tubes. For the purpose of overcoming those shortcomings, lab have to follow a set of the strict precautions for using the separate equipment and place for every one of the PCR cycles [35]. Orchid disease that results from the Phytophthora spp. has been detected by using the nested PCR, 2 *Phytophthora species, which are: P. parasitica and P. palmivora* have been detected with the use of the nested PCR that cause disease [36]. *P. Cactorum* has been detected particularly in the diseased plants of the strawberry with the use of the nested PCR [37].

## 4. Future Directions of Molecular Technology

The PCR that is combined with the sequencing became one of the most powerful tools for identifying pathogens that have been unknown previously and epidemiological investigations of the new and emerging infectious illnesses. The molecular approaches were helpful in revealing the fact that more than 30 bacterial species have the ability of forming uncultivable forms under the unfavorable environment conditions. The molecular technology went beyond simply identifying the causative organisms for the infectious illnesses and either now or in near future is going to be essential in studying pathogen evolutions, maintaining infective cycles in the Nature, the research of the mechanisms and causes of the new pathogens, the susceptibility mechanisms of various host groups and developments of DNA and RNA banking of the genes encoding the pathogenic factors, Which are going to be accomplished through the use of new molecular approaches like the micro-array, micro-chips, in situ PCR and automation of the molecular processes [38].

The micro-array and gene chip assays, which have been discussed first in 1991, have benefits of the miniaturization and automated constructions utilizing the industrial robots in combination with the sensitivity as well as the rapid reading of large amount of the detailed genetic insights. Actually, as many as 106 different probes may be linked with certain sites on micro-array platform-68 of either glass slide or nylon membrane. The micro-arrays have the ability of the simultaneous identification of numerous pathogens for certain illnesses, like the infective diarrhea, meningitis or pneumonia, in addition to genetic markers of the virulence and antibiotic susceptibilities. One of the first applications of that innovation has been in HIV field, where an array has been utilized for the detection of the protease gene resistance [39].

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The nucleic acid extraction and purification in addition to manual loading of isolated nucleic acids and master mixtures into vessels of PCR reactions are still the most labor-intensive molecular technology parts. None-the-less, a new technology was advanced for the purpose of performing those tasks as automated systems for extraction and purification and pipetting robots. One of the first systems of the automated extractions that have been developed was COBAS AmpliPrep from Roche [40].

## 5. Conclusions

The molecular tests that are currently available, which include the PCR as well as the genetic sequencing approaches, made it feasible to identify the non culturable pathogens of the bacterial. The PCR-based approaches of typing are possibly beneficial for the molecular typing of the bacteria DNA that have been directly obtained from the diagnostic samples. With the increase of the affordability and simplicity of the procedures, massive DNA sequencing could eventually be one of the routine assays for identifying and characterizing the non culturable pathogens of the bacteria. One of the critical issues and major challenges is validating technologies for the purpose of establishing specificity and sensitivity of the innovative approaches towards the clinical and pathological specimens of the known causation, in addition to identifications of the new ‘‘gold standards’’ that are based upon the molecular diagnoses instead of the isolation of the infectious agents.

## Conflict of interests.

There are non-conflicts of interest.

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