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POLYMERASE CHAIN REACTION IN LABORATORY DIAGNOSIS

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ANNOTATION
Modern medicine successfully uses the achievements of the natural sciences, intensively applies new technologies for the diagnosis and treatment of diseases. Recently, new methods based on the use of molecular genetic technologies have been added to the traditional microbiological and immunological methods for the laboratory diagnosis of infectious diseases.

Keywords: polymerase chain reaction, laboratory diagnostics, microbiology, disease.

Polymerase chain reaction (PCR) is an artificial process of repeated copying (amplification) of a specific DNA sequence, carried out in vitro. DNA copying during PCR is carried out by a special enzyme - DNA polymerase, as in the cells of living organisms. DNA polymerase, moving along a single DNA strand (matrix), synthesizes its complementary DNA sequence. It is important that DNA polymerase cannot start the synthesis of a DNA chain "from scratch", it needs a short "seed" chain of RNA or DNA, to which it can begin to add nucleotides.
The basic principle of PCR is that the polymerization reaction (the synthesis of a DNA polymer chain from monomeric nucleotide units) is initiated by specific primers (short fragments of "seed" DNA) in each of the many repeating cycles. The specificity of PCR is determined by the ability of primers to "recognize" a strictly defined DNA region and bind to it according to the principle of molecular complementarity.

In a conventional PCR reaction, a pair of primers are used that “limit” the amplified region on both sides by binding to opposite strands of the DNA template. To multiply the number of copies of the original DNA, a cyclic reaction is needed. As a rule, each of the sequentially repeated PCR cycles consists of three stages:

1. Enaturation, or "melting" of DNA, when double-stranded DNA under the influence of high temperature goes into a single-stranded state;
2. Binding (annealing) of primers with template DNA;
3. Elongation, or chain elongation.

The change of stages of each cycle is carried out by changing the temperature of the reaction mixture. At first, primers can only bind to a certain sequence of the original DNA, but in subsequent cycles they bind to copies of this sequence synthesized in previous cycles. In this case, the amount of the main PCR product (a copy of the DNA sequence limited by primers) theoretically doubles in each cycle, that is, it grows exponentially with the number of cycles.

Improvements in PCR technology Initially, conventional DNA polymerases were used to carry out PCR, which were subjected to temperature inactivation in each cycle at the stage of DNA denaturation. The polymerase had to be repeatedly added to the reaction mixture, which was rather laborious and did not allow automating the process [1,3,5].

The reaction uses thermostable DNA polymerases that withstand high temperatures at all stages of the PCR cycle for several tens of cycles. The number of commercially available thermostable DNA polymerases, which differ in some of their properties, is quite large. The most commonly used is Taq- polymerase, originally isolated from the thermophilic microorganism Thermus aquaticus. Other polymerases are more commonly used for specific PCR applications. Modern commercial preparations of thermostable polymerases provide, as a rule, stable reproducible activity, which allows the use of PCR technology in standard laboratory practice. For the implementation of PCR, devices (thermal cyclers) are mainly used that change the temperature automatically based on a given program.

Thus, a standard PCR can be carried out in 1-3 hours. Many devices allow programming of special complicated temperature profiles necessary for specific modifications of the PCR process. In parallel with the improvement of PCR technology, methods for analyzing reaction products also developed. Gel electrophoresis followed by staining with a DNA-specific dye, such as ethidium bromide, is traditionally used in many laboratories to detect amplified DNA and determine its size.

The use of hybridization with internal DNA probes makes it possible in some cases to significantly increase the sensitivity and specificity of detection of PCR products. Due to the absence of the need to prepare and conduct electrophoretic separation, the possibility of automation for the analysis of a large number of samples, and the use of a non-radioactive detection format, this method is becoming more common. In some cases, the use of special fluorescent "markers" makes it possible to control the conduct of amplification or the detection of PCR end products directly in the reaction tube [4, 6].

The Use of PCR in Medical Microbiology

Among the many different areas of clinical diagnostics, medical microbiology occupies perhaps the leading position in terms of the number and variety of applications using PCR technology. The introduction of this method into practice, along with serological diagnostics, has significantly expanded the possibilities of modern clinical microbiology, which is still based on methods for isolating and cultivating microorganisms on artificial nutrient media or in cell culture.

The use of PCR for direct diagnosis and identification of pathogens of infectious diseases. In cases where the use of culture methods is problematic or associated with insufficient diagnostic performance, the possibility of replacing biological amplification (i.e., growth on artificial media) with enzymatic doubling of nucleic acids in vitro using PCR seems to be particularly attractive. There are various approaches to the use of PCR for the diagnosis of infectious agents. The most common variant of PCR (specific PCR) involves the use of primers that are complementary to a specific DNA sequence characteristic of a strictly defined type of microorganism. For example, PCR amplification of a specific region of the gene encoding the major outer membrane protein (MOMP) of Chlamydia trachomatis, combined with non-radioactive hybridization to detect reaction products, can detect single copies of chlamydial DNA in the studied samples.

At the same time, PCR is significantly superior in diagnostic efficiency to cultivation and methods of direct detection of chlamydial antigen (microimmunofluorescence and enzyme immunoassay), traditionally used to detect C. trachomatis.

There is also the possibility of using several pairs of species-specific primers at once in one reaction tube for simultaneous amplification of DNA of various pathogens. This modification is called multiplex PCR.

Multiple PCR can be used to identify the etiological role of various microorganisms that cause certain types of diseases. For example, options for using multiple PCR for the simultaneous detection of two (C. trachomatis and N. gonorrhoeae in diseases of

the urogenital tract) or even four pathogens (H. influenzae, S. pneumoniae, M. catarrhalis and A. otitidis in chronic suppurative otitis) are described.

An alternative approach to PCR diagnostics is associated with the use of universal primers that allow amplification of gene fragments present in all microorganisms of a certain taxonomic group. The number of species that can be identified using this method can be limited both by the framework of small systematic groups (genus, family) and large taxa at the level of order, class, type. In the latter case, the target for PCR is most often ribosomal genes (16S and 23S rRNA), which have a similar structure in various prokaryotic microorganisms.

The use of primers complementary to the conserved regions of these genes makes it possible to amplify the DNA of most bacterial species. The resulting PCR ribosomal gene fragments can then be analyzed using various laboratory methods to identify the bacteria to which they belong. The most accurate method of "molecular" identification is to determine the complete nucleotide sequence (sequencing) of amplified DNA and compare it with the corresponding sequences of known species.

Despite the availability of automated systems that use the described identification principle, in practice, less laborious and expensive methods are usually used, which nevertheless allow certain differences in the sequence of DNA fragments to be reliably detected.

The most common methods are based on the analysis of the location in DNA of cleavage sites by restriction enzymes - the RFLP method (RFLP) - restriction fragment length polymorphism, or on the determination of the electrophoretic mobility of DNA in single-stranded form (SSCP method - single-stranded conformational polymorphism) [2,7].

PCR using universal primers can be used both to identify microorganisms isolated in pure culture and to directly diagnose a wide range of pathogens directly in clinical samples. It should be noted, however, that the sensitivity of "broad spectrum" PCR is generally lower than that of "species-specific" test systems.

In addition, PCR with universal primers is usually not used to study samples that may contain a large number of different microorganisms, due to the difficulty of analyzing reaction products obtained by amplifying DNA of different species. The use of PCR to detect drug resistance in microorganisms.

Recently, PCR has been increasingly used to study various properties of pathogenic microorganisms, in particular, to identify the resistance of certain types of pathogens to certain drugs. As a rule, the use of PCR to determine the sensitivity of microorganisms is appropriate only in cases where traditional phenotypic methods are not applicable or are not effective enough. For example, the determination of the

sensitivity of Mycobacterium tuberculosis to anti-tuberculosis drugs using culture methods usually takes 4 to 8 weeks. In addition, the results of phenotypic tests in such cases may be distorted due to a decrease in the activity of antimicrobials during long-term cultivation of microorganisms.

The study of the molecular mechanisms of drug resistance in M. tuberculosis and some other pathogens has made it possible to develop PCR-based methods for the rapid detection of genetic markers of resistance. For such an analysis, DNA or RNA of the pathogen isolated in pure culture is usually used. However, in some cases, there is the possibility of direct PCR analysis for antibiotic resistance without pre-cultivation of the pathogen. The studied sample of clinical material is used as a source of target DNA for PCR, and the copied PCR product is analyzed to detect mutations associated with antibiotic resistance. For example, a method has been developed that allows using PCR to detect in patients suffering from tuberculous meningitis the resistance of the pathogen to rifampicin.

There are, however, natural limitations to the use of genetic methods for assessing the drug resistance of microorganisms:

- data on specific genetic mechanisms of resistance may not be available;
- resistance to certain drugs is often associated with different mechanisms and mutations in different genes that independently affect the phenotype.

In addition, the lack of international standards and recommendations for the use of PCR to determine sensitivity to antimicrobial drugs is an additional factor limiting the possibility of a wide application of this approach in practical diagnostics.

Advantages and Disadvantages of PCR The many advantages of using PCR over traditional microbiological methods have already been discussed. Such properties of PCR as speed and high productivity (that is, the possibility of parallel analysis of a standard procedures for isolation of microbial DNA from clinical material or pure culture and subsequent PCR amplification usually require no more than a few hours to complete number of samples) are the indisputable advantages of this method. Standard procedures for isolation of microbial DNA from clinical material or pure culture and subsequent PCR amplification usually require no more than a few hours to complete.

PCR products can be identified using simple methods (gel electrophoresis, hybridization) in about the same time. The advantage of PCR in speed compared to culture methods is especially noticeable in the study of slow-growing microorganisms. However, even in the case of fast-growing cultures, the speed of PCR can be beneficial. For example, isolation, identification and determination of drug resistance in strains of methicillin-resistant Staphylococcus aureus (MRSA) using traditional

microbiological methods requires at least 3-5 days, while PCR analysis can detect MRSA in less than a day.

An important property of PCR is its high specificity, determined primarily by the uniqueness of the genetic material of each type of microorganism. Therefore, when using primers that are complementary to a certain "species-specific" DNA sequence, and observing the optimal reaction temperature, only DNA of the desired species undergoes multiple copying even in the presence of a large amount of other, "ballast" DNA, for example, human DNA or other types of microorganisms. When using PCR to identify a specific pathogen, specificity is an undoubted advantage of this method.

On the other hand, one should always keep in mind the "narrow focus" of PCR, in contrast to cultural methods, which make it possible to detect the growth of various types of microorganisms when seeded on primary accumulation media.

In addition to high efficiency and specificity, PCR is characterized by extremely high sensitivity. Theoretically, only one copy of the desired DNA (RNA) sequence in the test material is sufficient to carry out the reaction.

If a DNA fragment represented by a large number of copies in the pathogen genome is used as a target for PCR (for example, 16S rRNA genes in many bacterial species), then PCR can detect less than one microorganism in the sample (that is, a DNA fragment from a lysed microbial cell).

Therefore, a sensitivity of the order of 0.5- 1 microorganism per sample is quite realistic for PCR, which allows it to be used even in cases where serological and bacteriological studies do not give a positive result due to an extremely low microbial titer (for example, when monitoring the infectious safety of donated blood and organs, diagnosis of chronic and latent infections).

At the same time, the extreme sensitivity of PCR requires new approaches to the clinical interpretation of the results obtained in the laboratory. In particular, the detection of saprophytic and opportunistic microorganisms in clinical samples may not mean the presence of a pathological process and therefore cannot be automatically interpreted as a diagnosis, especially against the background of a favorable clinical picture in a patient. For the same reason, PCR should be used with caution when analyzing samples with a characteristic polymicrobial community (feces and material obtained from the upper respiratory tract and genitals).

The extreme sensitivity of the DNA enzymatic amplification reaction is also the Achilles' heel of PCR technology. This paradox is also known as the problem of PCR sensitivity to contamination (contamination) with foreign DNA molecules that can serve as a target for the primer set used. Even single molecules of contaminating DNA

can be repeatedly copied during PCR, leading to the formation of the target DNA product, and, consequently, to a false positive result.

Due to the risk of false positive results in laboratories that routinely use PCR for diagnostic purposes, stringent guidelines and special approaches are required to reduce the risk of PCR contamination.

One of the most significant requirements for diagnostic PCR laboratories is the need to divide the laboratory into "pre-PCR rooms", where samples are processed and reaction mixtures are prepared, and "post-PCR rooms", where reaction products are analyzed.

Separate storage of reagents and materials used at different stages of PCR, maximum use of disposable plastic materials at the stage prior to amplification, and regular treatment of premises with ultraviolet (FF) radiation that damages contaminating DNA sequences are also mandatory.

Additional measures to protect PCR from contamination may include the use of laminar boxes for working with samples and preparing PCR mixtures, special biochemical (uracil glycosylase) and physicochemical (FF radiation + isopsoralen) methods for inactivating PCR products. It is especially important to assess the reliability of PCR data and the absence of false positive results by regularly testing negative controls (containing no target DNA) in parallel with clinical samples.

In addition to the risk of false positive results, there is an inverse problem associated with a decrease in the sensitivity of PCR, which results in false negative results. In practice, the question of the ratio of theoretical (that is, the maximum possible) and real sensitivity of PCR is not always resolved unambiguously. The sensitivity of PCR can be reduced due to many reasons, the most important of which is the inhibition of the reaction by the components of biological samples.

PCR inhibitors may be present in blood samples (hemoglobin), sputum, urine, biopsy material.

Various substances, such as commonly used anticoagulants (especially heparin) or components of blood culture media, can also inhibit the DNA amplification reaction. PCR inhibition can usually be detected by artificially adding the target DNA to the test sample and then amplifying it. A negative result obtained with a known positive control may indicate the presence of inhibitors, which require the use of sample dilution or special sample preparation methods to eliminate them. A serious problem is also the choice of specific methods for preparing clinical samples for research using PCR. Currently, there are no universal approaches to the isolation of DNA of different types of microorganisms from various sources. Rapid sample preparation methods that allow for automation sometimes fail to achieve the required level of sensitivity.

On the other hand, multi-step techniques to purify and concentrate microbial DNA for PCR analysis are laborious and may increase the risk of sample contamination [1,2,6].

Finally, PCR is expensive. Its implementation requires complex laboratory equipment, including not only a thermal cycler and a device for DNA electrophoresis, but also separate centrifuges, refrigerators, dispensers and other equipment. PCR costs include the high cost of reagents and consumables. Therefore, only in the case of detection and study of difficult-to-cultivate pathogens, PCR can be comparable in cost to traditional microbiological methods.

CONCLUSION

Already, PCR is an indispensable tool in the diagnosis and investigation of many pathogens of infectious diseases, and the number of laboratory tests using PCR continues to grow rapidly. Further development and introduction of this method into the practice of clinical diagnostic laboratories may be associated with the improvement and standardization of the technology itself, especially the stages of sample preparation and analysis of reaction products.

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