Review Article

Nucleic Acid Extraction from Clinical Specimens for PCR Applications

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Abstract: Polymerase chain reaction (PCR) has been widely used due to its high specificity, sensitivity, and rapid turn-around time. However, the major limitation of PCR based diagnosis is inhibition of the reaction caused by a variety of components within clinical specimens. The demand for PCR diagnosis in medical microbiology has highlighted the need for efficient methods of nucleic acid extraction. Although there has been progress in the simplification and purification of microbial nucleic acids, many researchers' procedures are still inconvenient for routine use with any specimen and a universal method has not been devised yet. In this review, some of the basic principles for extraction procedures that have been used to date and the present situation of nucleic acid extraction are summarized.

Key Words: PCR, Clinical Specimens, Inhibitors, DNA extraction

Klinik Örneklerden PZR için Nükleik Asit Ekstraksiyonu

Özet: Polymer zincir reaksiyonu (PZR), yüksek oranda özgüllüğü, hassasiyeti ve sonuçların çabuk alınmasından dolayı yaygın bir şekilde kullanılmaktadır. Fakat, PZR temelli teşhisin en büyük engeli klinik örneklerdeki çeşitli bileşenlerden kaynaklanan reaksiyonun inhibisyonudur. Tıbbi mikrobiyolojideki PZR teşhisi için rağbet, etkili nükleik asit extraksiyonu ihtiyacı artırmıştır. Mikrobiyal nükleik asit saflastırılması ve kolaylığı açısından bir çok ilerleme yapılmış olmasına rağmen, çoğu metod hala bir çok örnek için günlük kullanıma uygun olmayıp genel bir metod geliştirilmemiştir. Bu çalışmada bugüne kadar kullanılan ekstraksiyon prosedürlerinin bazı temel prensibleri ve nükleik asit ekstraksiyonlarının mevcut durumu kısaca özetlenmiştir.

Anahtar Sözcükler: PCR, Klinik Örnekler, İnhibitörler, DNA ekstraksiyonu

Introduction

The conventional microbiological culture method for the detection of specific microbial infectious agents requires the use of diverse growth media and conditions, followed by identification of the pathogens by their multiple physiological characteristics through a series of biochemical tests. The culture-based methods of identification of microbial pathogens may take several days to weeks depending on the nature of the infectious agent (1,2). Although conventional approaches to identification are still valid and useful, rapid methods and rapid reporting truly became an inevitable goal for clinical microbiology laboratories. Rapid diagnostics can greatly impact patient care, direct the use of appropriate antimicrobial therapies, and reduce hospitalization and overall medical costs. With this goal, many molecular biological developments have emerged and one of them with great promise for the detection of microbial pathogens is DNA-based molecular diagnosis. Most of these methods unfortunately require highly specific genetic information for a special pathogen, which might not be always available.

The development of the Polymerase Chain Reaction (PCR) method and its application to the diagnosis of microbial pathogens has become a cornerstone for diagnosis in clinical laboratories across the world. Nowadays PCR is being increasingly used for direct detection of microorganisms, detecting genes coding for virulence factors and determining the presence of genes responsible for anti-microbial resistance etc.

However, the major limitation of PCR based diagnosis is inhibition of the reaction caused by a variety of components within the clinical specimens. Several extraction methods have been developed and used by investigators from clinical samples prior to performing PCR to avoid inhibition, because of the varied specimens such as blood, urine, sputum, and CSF. Therefore, this short review discusses the preparation of clinical samples, mainly blood and CSF, prior to PCR application.

Rapid Identification of Pathogenic Microorganisms

Rapid methods have not only decreased the turn-around time for patient results, but have also increased the clinical relevance of the information provided by the laboratory.

One of the rapid methods for identification of a microorganism is to use gas chromatography, which can detect specific bacterial products directly in clinical specimens. Brooks and associates have detected various organism-specific products in extracts of several biological fluids (3-5).

Immunological methods have also found a place in the diagnosis of certain infections as one of the rapid methods. Although those methods have been used for detection of antigens in body fluids, Countercurrent Immuno-Electrophoresis (CIE), which uses an electric current to speed up the migration of the antigen and antibody, was the only one to be accepted in clinical practice. However, even this method has been largely supplanted by the more rapid latex particle agglutination. As the Radio Immuno-Assay (RIA) has no impact on routine applications, testing for markers for Hepatitis B infection is still done by RIA, but has mostly been now replaced by the Enzyme Immune Assay Method (EIA) (6).

Until late 1988, most of the molecular based clinical diagnostic methods were limited to Southern Blot analysis of DNA and Northern Blot analysis of RNA. Both methods are limited by the large number amount of DNA or RNA required and at least one to several thousand cells had to be present in the sample to show a detectable amount of signal.

Drawbacks of the methods

The culture-based methods are very laborious and take time. Moreover, some microorganisms require culturing in animals, which takes a considerable amount of time and effort.

In the case of bacteremia, the blood cultures' sensitivity may be limited because patients may not be bacteremic at the time that the blood is drawn for culture (7). Furthermore, a substantial number of cases of bacteremia in children may be missed by the performance of a single blood culture (8) because of low density and the fastidious nature of the pathogen. Additionally, some organisms are cell dependent such as *Coxiella burnetii*, *Bartonella* spp. and *Chlamydia* spp. and detection of fungi is still difficult even with improved automated blood culture methods and materials (9). In the case of Methicillin Resistant *Staphylococcus aureus* (MRSA), which is one of the most common causes of nosocomial infection, standard bacterial identification susceptibility testing frequently require as long as 72 h to report results, and there may be difficulty in rapidly and accurately identifying Methicillin Resistant Coagulase Negative (MRCoNSA) (10). The diagnosis of *Listeria meningitis*, meningoencephalitis, or septicemia is based on the culture of cerebrospinal fluid (CSF) and blood. The direct examination of Gram stained clinical specimen is generally of little value, because *L. monocytogenes* is often present in some samples, particularly in CSF (11). Another complication is that many patients are pretreated with antibiotics before their blood is cultured. The culture method may be negative if the patients have received antibiotics before blood was taken. It is possible to enlarge the list of such obstructions.

Although RIA has great sensitivity and specificity the problems associated with radioactive waste disposal and the instability of certain radionuclides is limiting the techniques. The amount of antigen are generally present during the acute phase of the illness; thus the sensitivity of EIA may be compromised by trapping of antigen in the specimen and the affinity of antigens for antibodies produced.

Alternate methods with poly or monoclonal antibody can show cross-reactivity with other related pathogens. Antigen molecules with only a single epitope are rarely encountered; rather, hundreds or even thousands of potential antigenic determinants may exist on a cell surface. Cross-reactivity occurs because of shared antigenic determinants or because of mutations resulting in the evolution of epitopes. Therefore the interpretation of positive results may be difficult and require expert judgment.

RIA is principally similar to ELISA, but the antigens in ELISA are bound to the antibodies that are conjugated with enzymes. Although RIA is more sensitive than ELISA, ELISA is preferred due to the problems caused by the radioactive isotopes used in RIA.

Nucleic Acid Probe Technology

A nucleic acid probe is a sequence of single stranded nucleic acid that can hybridize specifically with its complementary strand via nucleic acid base pairing. The use of nucleic acid hybridization probes is common as an alternative means for rapidly identifying infectious microorganisms (12-15). This is particularly useful for screening large numbers of specimens. However, nucleic acid hybridization suffers from several disadvantages. Traditionally, nucleic acid hybridization has relied on radioactive labels for detection. Although non-radioactive labels have been developed (16,17) and used successively (18-20), the sensitivity of the assays requires large number of organisms for detection. Thus, the currently used hybridization assays are generally for culture confirmation rather that direct detection and identification.

In spite of this, the detection of organism-specific DNA sequences by nucleic acid hybridization offers the possibility of detection pathogenic microorganisms without the tedious process of prior isolation of a pure culture. The development of a standardized, highly sensitive and specific, nonradioactive detection system in which organism-specific gene sequences are used would facilitate rapid diagnosis.

Saiki and co-workers (21,22) have described a system, PCR, for amplifying the concentration of specific nucleic acid sequences. This powerful technique can produce millions of copies of a selected DNA target in only a few hours. The technique can be used to detect very small amounts of specific nucleic acid material in clinical specimens where bacterial, viral or fungal agents are thought to play a causative role. The fundamental basis of the technique is that each infectious disease agent possesses a unique "sequence" in its DNA or RNA composition by which it can be identified.

Principles of PCR

PCR is based on 3 simple steps required for any DNA synthesis reaction: **Denaturation** of the template into single strands; **Annealing** of primers to each original strand for new strand synthesis; and **Extension** of the new DNA strands from the primers. These reactions are carried out with any DNA polymerase and result in the synthesis of defined portion of the original DNA sequence. Heat-stable DNA polymerase from a thermophilic bacterium, *Thermus aquaticus* (Taq), enable one to synthesize new DNA strands repeatedly. The thermal cycler is able to elevate, hold, and cool the temperature of the vials in a manner that allows initial DNA denaturation and then repeated cycles of DNA synthesis and denaturation to occur. The initial denaturation step "melts" the ds-DNA at 95 to 100 °C. Single stranded, oligonucleotid "primers" that sided to the DNA sequence of interest are allowed to anneal to the denatured DNA strands during a cooling step. Extension of the primers by DNA synthesis is done by thermostable Taq polymerase.

Detection of the PCR end-products may be accomplished by electrophoretically separating the components of the final amplified sample in agarose or acrylamyde gels, followed by staining of the gel. The separation can then be visualized on a shortwave ultraviolet radiation transilluminator, comparing the separated bands with those of a standard in parallel.

The amplified target DNA or RNA sequences can also be detected more specifically by hybridization of the amplified DNA to a synthetic, labeled probe that is complementary to all or part of the amplified DNA sequence (23,24).

Several modifications of the technique have been described for different applications. By **multiplex PCR**, multiple primer pairs for different target molecules are included in the same amplification mixture. Such a preparation can theoretically be used to simultaneously amplify target sequences for several pathogenic microorganisms in a single reaction vial (25). **Nested PCR** is the other modified technique, in which several rounds of amplification are performed with one set of primers, and the product of this amplification is subsequently amplified using another set of primers that lie within the internal sequence amplified by the first primer set. Although nested PCR is extremely sensitive, it does have some disadvantages, including the need to transfer reaction products synthesized with the first set of primers to another reaction vial for subsequent amplification using the "nested" set of primers, thus risking aerosolization of the amplified DNA. Beside these, **single-tube nested PCR** strategies (26-29) are a rather promising approach, which do not involves the opening of

the reaction tubes after first PCR, and consequently a major source of aerosol contamination prior to second PCR is eliminated and sensitivity is enhanced (30,31). In **Hot Start PCR**, one of the reagents required for PCR is kept out of the reaction mixture until the temperature of the reaction tube reaches 50 °C. This approach could be explained by the *Taq* polymerase having important activity at room temperature. Because the target DNA may contain single stranded regions formed during the extraction process, primers could anneal non-specifically at surrounding temperature during the PCR set-up phase (32). Non-specifically annealed primers are then extended by *Taq* polymerase; therefore these unwanted products will compete with the specific target during the following cycles. Using a wax barrier that physically separates the various reaction components at room temperature is also possible for Hot Start PCR. Subsequently, at a certain temperature the wax melts down and allows reagents to mix with each other. Thus single molecule sensitivity in a complex mixture is possible without nesting and radioactive probing (32) with Hot Start PCR.

Additionally, it might be possible to mention other modified PCR techniques such as Asymetric PCR, RT-PCR, Inverse PCR, Immuno-PCR, in-Situ PCR, and Anchored PCR used for various scientific purposes. Among these, **Real-time PCR** has an important place in the scientific community. It is extremely useful for studying microbial agents of infectious diseases (33-35) and can be regarded as a method of choice due to its rapidity, sensitivity and reproducibility, because the risk of carry-over contamination is minimized. Although the conventional PCR has had a big impact with very good results in research, researchers have had difficulties due to the post-PCR steps for amplicon evaluation such as agarose gel electrophoresis. But real-time PCR allows the scientist to actually view the increase in the amount of DNA as it is amplified and post-PCR manipulation of the amplicon is not required, since the fluorescent signals are directly measured as they pass out of the reaction vessel, because most of the real-time PCR components involve hybridization of oligoprobes to a complementary sequence of the amplicon strands. In another words, the detection is being carried out by the labeling of primers, oligoprobes or amplicons with molecules of capable of fluorescing (36). Several reporter systems have also been discussed in detail (37-39).

Despite the extreme power of the PCR, in terms of its sensitivity, the main disadvantage is due to contamination, which leads to false positive results (40). A number of guidelines are available for minimizing the risk of contamination, such as the use of negative control, meticulous care and the separation of pre- and post-work areas. To destroy the contaminants various procedures have been formulated including restriction enzyme treatment prior to amplification, Dnase I treatment (41), exonuclease treatment (42), and incorporation of dUTP and treatment with uracil-N-glycosylase (UNG) prior to PCR (43). Principally, Uracil DNA glycosylases are DNA repair enzymes that function by excising from DNA uracil residues from either misincorporation of dUMP residues by a DNA polymerase or deamination of cytosine (44). The UNG method is very efficient, being able to render up to 20,000 molecules of carry-over contaminant unamplifiable by an assay sensitive enough to amplify a single target molecule (45). Carry-over contaminant from a previous PCR amplified product containing dUTP, but not the specific native target nucleic acid, becomes susceptible to degradation by the enzyme Uracil N-glycosylase. Therefore, if the PCR reaction mixture is treated with UNG prior to amplification, the carry-over contaminant will not reamplify (43).

Beyond these modified PCR techniques, the most important thing in identification of infectious diseases is the limitation of PCR arising from using clinical samples.

Sample-originated Limitation of PCR

The PCR sample to be used may be a DNA (ss or ds) or RNA (poly A-RNA, viral RNA, tRNA or rRNA). The amount of starting material required can be as little as a single molecule. Theoretically up to 10^5 DNA target molecules are the best for initial testing (24).

Although the PCR is the most sensitive of the existing rapid methods to detect microbial pathogens in clinical specimens, the application PCR to clinical specimens has many potential pitfalls due to the susceptibility of PCR to inhibitors, contamination and experimental conditions.

For example, haem and other substances found in blood are known to inhibit Taq polymerase (46). To date, blood, mucus, urine, CSF (Cerebrospinal fluid), sperm and fecal material, e.g., bile salts, bilirubins, and polysaccharides (47), are some of the body fluids/products that have been shown to inhibit Taq polymerase (48). The nature of these inhibitors is poorly

understood, but they consist of both intrinsic and extrinsic factors. Intrinsic factors include intracellular substances such as the porphyrin ring of haem, which is thought to bind to the Taq polymerase (46). Extrinsic factors that may inhibit PCR amplification include anticoagulants such as heparin (49) and chelating agents such as EDTA (Ethylenediaminetetraacetate), which are used to prevent degradation of target DNA. Similarly, the use of EDTA in samples to prevent degradation of DNA must be limited since Mg^{2+} is required by the Taq polymerase (50). Traces of phenol and SDS (Sodium Dodecyl Sulfate) can also inhibit the Taq polymerase (51). This makes a PCR-based diagnosis directly from clinical material difficult. Since a variety of clinical specimens, such as blood, urine, csf and others, vary in regard to the nature of the content, some extraction procedure for each specific specimen before PCR applications are conducted is essential.

Some Extraction Methods

The isolation of DNA is an essential step before performing the PCR. Although there are numerous methods for doing this, the particular procedure must be tailored to the organism from which the DNA is to be obtained, as the structure and composition of organisms vary. The common feature of the extraction methods is that the cell or virus is first disrupted, and then DNA is separated from the other components such as proteins, lipids and carbohydrates. Most protocols also include an Rnase digestion to degrade RNA.

The simplest method of releasing nucleic acid entails only a crude lysis. There are a variety of methods for the release of nucleic acid from microorganisms, such as boiling in distilled water or PCR buffer (52), detergents with or without heat (52), sodium hydroxide with heat (53), freeze-thaw (54), SDS-proteinase K (55), percloric acid (52), enzymes (56), sonications (54), and heat (57).

Microbial cells can be lysed by repeated freezing and thawing (5 to 20 °C) of the sample. Rapid freezing is performed in a dry-ice methanol/ethanol bath, and thawing is performed in a 50 °C water bath (25,58).

Following freeze-thaw cycles, the sample is heated to 80 °C over 10 min and cooled to room temperature; PCR reagents are added, and PCR amplification is performed. Most Gram-negative microorganisms are sensitive to repeated freeze-thaw lysis, and this method can be applied for their preparation prior to PCR amplification.

The cells can be frozen as described above, followed by boiling in a water bath or in a thermal cycler (Freeze-Boiling) 3 to 5 times. The PCR reagents are added to the sample, and PCR amplification is performed (59). Most Gram-negative and some Gram-positive cells such as *Staphylococcus* spp. can be detected by this approach.

Alternatively DNA suitable for PCR amplification can be produced from specimens by simple boiling in distilled water. The major advantage of this technique is its speed, taking only a matter of minutes. Prolonged boiling of the sample reduces the yield of released DNA, and so the optimum boiling time must be applied. DNA extracted by this method can be stored at 4 °C or frozen.

Samples can be incubated at 60 °C for 10 min followed by boiling for 10 min in a water bath or in a thermal cycler in the presence of 50 to 100 ml of Chelex-100. Chelex-100 is known to stabilize the genomic DNA in boiling water by maintaining the ionic strength of the sample. Following boiling, the sample is centrifuged in a table-top microcentrifuge at 10,000 g for 3 min, and 5 to 10 ml of the supernatant can be used for PCR amplification. This approach is more effective than the freeze-boil method for releasing nucleic acids by lysis of the cells, for most of Gram negative, Gram positive, and even some cysts such as *Giardia* spp. (60).

The chelex agent is active on a variety of organisms even without the boiling process, such as *Mycobacterium* spp. (61), *Plasmodium* spp. (62), *Candida* spp. (63), and *Borrelia* spp. (57).

The method, boiling with detergents, is based on the treatment with SDS and proteinase K. When the sample is mixed with the TE buffer, after centrifugation it is resuspended in TE buffer. After SDS and proteinase K incubation at 55 °C for 30 min, boiling takes place for 5 min (64).

In the sonication procedure enough ultrasonic energy is transmitted through the walls of the microcentrifuge tubes to effectively disrupt the microorganisms. This can be used in almost any laboratory setting and a number of specimens can be treated simultaneously (54).

After releasing DNA with any method, it is essential that any substances that may cause inhibition of the PCR amplification are successfully removed using a reliable, reproducible and sensitive purification procedure.

To these releasing DNA applications, the most known conventional method phenol-chloroform extraction of proteins, precipitation of DNA with ethanol, isopropanol and spermine or butanol concentration of DNA might be combined. Alternatively to these methods solid phase carriers (Silica matrix, glass particles), ion exchange resins, or magnetic particles (65), column application strategies have been developed as a new protocol for purification of DNA.

GuSCN-diatoms method (or Silica or Glass particles), which are among the most effective protein denaturants, is based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidium thiocyanate together with the nucleic acid-binding properties of silica particles or diatoms in the presence of this agent. By using size-fractionated silica particles, nucleic acids can be purified in less than 1 h (66).

Phenol-Chloroform extraction of DNA

The fundamental aim of the phenol extraction is to deproteinize an aqueous solution containing DNA. Chloroform, when used in conjunction with phenol, improves the efficiency of the extraction by its ability to denature proteins. Its high density makes separation of the 2 phases easier and it also removes any lipids from the sample. The extraction with phenol and chloroform can either be performed sequentially or as a single extraction step with an equal volume of phenol-chloroform-isoamylalcohol. The DNA in the aqueous phase can subsequently be recovered by ethanol precipitation. For DNA isolation from solution, salt concentration does not significantly influence the efficiency of the extraction. However, if the salt in the aqueous phase is below 50 mM, oligomers, in particular, may be lost to the phenol phase.

In many cases the ability to obtain undegraded nucleic acids using phenol extraction depends on methods for inhibiting nucleases. The presence of EDTA is sufficient to inhibit any contaminant nucleases. Heparin is another common ribonuclease inhibitor that cannot be removed by extracting with phenol.

Ethanol precipitation is probably the most versatile method of concentrating nucleic acids and is commonly used to recover DNA or to retrieve phenol-extracted DNA products.

The precipitate is formed by leaving a mixture of the sample, salt, and ethanol at low temperature (-20 °C or lower). The precipitated salt of the nucleic acid is then sedimented by centrifugation, the ethanol supernatant removed, and the nucleic acid pellet resuspended in an appropriate buffer. The choice of salt used for the precipitation is determined both by the nature of the sample and by the intended use of the nucleic acid. Samples containing phosphate or 10 mM EDTA should not be subjected to ethanol precipitation as these materials also precipitate along with the nucleic acid; preliminary dialysis of such samples is essential before ethanol precipitation.

Isopropanol-induced precipitation of DNA minimizes the total volume of the precipitating sample. The precipitation is carried out by adding equal volumes of isopropanol(2-propanol) to the sample, and treating the mixture in the same ways as for ethanol precipitation. The drawbacks of this method are that 2-propanol cannot be easily lyophilized due to its relatively low volatility, and also that salts present in the original sample tend to coprecipitate with the DNA. Because of these problems it is common practice to carry out an ethanol precipitation of the sample immediately following a 2-propanol precipitation.

The precipitation of DNA by the polyvalent cation spermine is useful for the recovery of DNA from the dilute solution (0.1-100 µg/ml). Due to the selective nature of the precipitation, the procedure yields DNA of relatively high purity. Indeed, DNA can be precipitated from solutions containing proteins. Nucleoside triphosphates are not precipitated by spermine, thus providing a method of recovering dNTP-free DNA; 2 sequential precipitations are recommended.

The efficiency of spermine precipitation is sensitive to the ionic strength of the sample, and the amount of spermine to be added is determined by the salt content of the sample.

DNA can be recovered from dilute solutions by extracting several times with butanol to concentrate the sample prior to ethanol precipitation. An equal volume of 2-butanol is mixed vigorously with the sample followed by phase separation on a bench-top microcentrifuge. The upper organic layer is carefully removed with the aid of a micropipettor. The volume of the aqueous, DNA-containing lower phase will be reduced as water partitions into the butanol phase, thus increasing the DNA concentration. Butanol extraction is carried out until the

desired final volume of sample is attained. Because this procedure also concentrates the salts present in the sample, a final ethanol precipitation is carried out and the pelleted DNA resuspended in the desired buffer (67).

Commercial Nucleic acid extraction tools

The need for a nucleic acid extraction step in the diagnostic microbiology from pathogenic agents in the patient sample has been previously emphasized. Many sample preparation tools are commercially available from many suppliers that are designed for nucleic acid extraction without complicated and laborious work. Puregene DNA isolation kit (PG) (Gentra Systems, Inc., Minneapolis, MN, USA) Generation Capture Column kit (GCC) (Gentra Systems, Inc.), MasterPure DNA purification kit (MP) (Epicentre Technologies, Madison, WI, USA), IsoQuick nucleic acid extraction kit (IQ) (MicroProbe Corp., Bothell, WA, USA), QIAamp Blood Kit (QIA) (Qiagen, Inc., Valencia, CA, USA) and NucliSens isolation kit (NS) (Organon Teknika Corp., Durham, NC, USA) are some of them. Most commonly used are the silica-based gel membrane or glass fiber filter columns or magnetized particles, which specifically bind DNA, while contaminating substances or PCR inhibitors are effectively removed. These tools were also tested and compared in terms of sensitivity, specificity, ease of automation and overall efficiency (65,68-70).

Recently, along these extraction kits, several new automated-robotic systems were also designed with intelligent features, which are commercially available, such as MagNa Pure compact system (Compact; Roche Diagnostic Corp., Indianapolis, IN, USA), NucliSens miniMAG extraction instrument (miniMAG; bioMerieux, Inc., Durham, NC, USA), and BioRobot AZ1 system (EZ1; QIAGEN Inc., Valencia, CA, USA). Mostly their technology is based on using magnetic particles, mainly consisting of iron oxide particles (Fe_3O_4)(71), in combination with glass or silica. With the instrument, the nucleic acid can be efficiently isolated from a wide range of sample types including plasma, serum, whole blood, csf, stool and respiratory samples and sample volume. It is still necessary to compare the data from different instruments for limitation of the systems that might have been chosen by users for a variety of reasons.

Clinical samples

Blood

Blood is widely utilized for the diagnosis of various infective microorganisms. If the clinical specimen is blood, there are still a number of choices that should be considered e.g., plasma, serum, and whole blood. Plasma or serum is preferred over whole blood or purified leukocytes when the target microorganism is predominantly extracellular, e.g., *Hepatitis B virus* (HBV) or *Hepatitis C virus* (72,73). The anticoagulant used for plasma and the method of storage may affect the ability of the assay to detect the presence of target sequences (74), and as little as 1% whole blood can inhibit a typical PCR, and so it has been suggested that the inhibition is due to the binding of haeme or porphyrin to *Taq* DNA polymerase (46). Considerable effort has been devoted by many investigators to develop a method for rapid isolation and purification of target DNA from blood samples.

Treponema pallidum has been detected from whole blood by lysis with SDS-proteinase K followed by adsorption to a silica matrix (75). Originally in this method the DNA extraction is carried out using 50 ml of the lysate adsorbed to diatoms in the presence of guanidium thiocyanate (GuSCN) (66). The yield of DNA by use of diatoms is higher (66) than that by use of the combination of phenol extraction and alcohol precipitation (76,77). Extraction of larger volumes of samples yields an increase in the sensitivity needed for a positive PCR (75).

Using proteinase K digestion followed by phenol-extraction and ethanol precipitation for detection of *Francisella tularensis* in blood and extracted the total nucleic acid with phenol-chloroform-isoamyl alcohol and precipitated with ethanol is sensitive for PCR assay, producing successful results to detect a single bacterium in a 5 ml aliquot blood sample (78). On the other hand, phenol extraction method practiced on *Streptococcus pneumonia* produced a decreased diagnostic sensitivity when DNA was extracted from whole blood (79).

A simple DNA extraction method (80) was adopted so that PCR could be performed for yeast cell extracts without DNA precipitation (63). With this method yeast DNA is extracted from blood as to lysis. Centrifugation reduces the concentration of blood components that are inhibitory to PCR. After spheroblasts are performed the

DNA is released by adding Chelex 100 resin to the spheroblast suspension and the mixture is heated (63). The addition of Chelex 100 reduces the sample preparation time and further reduces the inhibitory effects of blood components on PCR (81).

Another Chelex method has also been described by Iralu et al. (61) for the identification of *Mycobacterium avium* directly from a blood specimen. The sample mixture is incubated for 30 min at 56 °C to remove PCR inhibitors and is heated for 30 min at 95 °C to complete mycobacterial lysis. The heated mixture is centrifuged to pellet the Chelex-100 resin, and the supernatant is retained for the PCR. When this extraction was applied prior to PCR, the sensitivity was 80%, and the specificity 86%, although the existence of inhibitors and probably the primers used were unable to recognize certain serovars of *MAC(Mycobacterium avium complex)*. However, it has shown that this method detects amplicons from 1 to 10 bacilli (61).

An alternative extraction method has been described using sera rather than whole blood for the diagnosis of *Trypanozoma cruzi* infection (Chagas' disease) by PCR. Samples from both acutely and chronically infected patients yielded positive results by this method. No significant difference was observed when either whole blood or serum samples were used (82). This might represent a considerable advantage due to the easier handling and transportation of serum as compared with whole-blood samples

Amplifiable *Plasmodium vivax* target was isolated from whole blood by processing blood spotted onto Whatman 3M chromatography paper (83). The filter paper samples are cut out and placed in a microcentrifuge tube with a 5% (wt/vol) Chelex-100 solution. Plasmodium vivax DNA is released from filter paper samples by vortexing and boiling for 10 min. The samples are centrifuged at 12,000 g for 1.5 min and the supernatant is removed to a new microcentrifuge tube and centrifuged again at 12,000 g for 1.5 min. The supernatant is removed and stored at 4 °C until use (83).

Barker et al. (84) has also reported a promising method for treating blood samples permitting direct detection of *Plasmodium falciparum* parasites for PCR amplification. Blood samples are first lysed, and then dimed onto filter paper. This paper is added directly to the PCR mixture for amplification. This method permits detection of less than 10 parasites in a 20 ml sample, and minimizes the effects of PCR inhibitors generally found in blood (84).

The filter paper approach offers many advantages, including ease of collection and transportation, stability of samples, economy of sample volume, little bio-hazard risk, and centralization and batching of specimens for processing and analysis.

Malloy et al. (85) were able to detect *Borrelia burgdorferi* from whole blood by simply centrifuging and boiling the sample without any DNA extraction, and so the inhibitory effects of constituents such as hemoglobin are removed by a simple washing step. Through these kinds of example, the trend is to find a universal method that will work on various types of microorganism. In terms of the need for this, Golbang et al. (86) describe an extraction method based on guanidium thiocyanate use, effective for extracting bacterial and fungal DNA from blood products.

When the target microorganism is extracellular such as Hepatitis B virus (HBV) or HCV, HBV DNA, HCV RNA and HIV RNA have been prepared from serum or plasma specimens (72,73). By heating sera prior to amplification to inactivate inhibitors of PCR, Frickhofen and Young (87) were able to detect the presence of 10^4 copies of viral targets. Such an approach may not be applicable to samples containing lower target copy numbers or RNA targets. However, patients with low titers of circulating virus would be missed by this method. Many of the current protocols incorporate extraction of plasma sample with organics. Extraction with phenol, phenol-chloroform-isoamyl alcohol, applied to the isolation of *HCV* target (74,88) *Trypanosoma cruzi* targets (82) from serum and plasma. In general, GuSCN applications (66,86) or cesium chloride gradient procedures may be too difficult or hazardous for routine use (89).

Binding of nucleic acid to solid-phase extractants can circumvent the removal of proteins and inhibitors with organic solvents. Hepatitis E (HEV) RNA (90) HIV RNA and proviral DNA of human T-lymphotropic virus (91) have been extracted from plasma by using commercially available glass powder or silica suspensions. While commercial kits are being developed and becoming used widely for DNA extraction, most researchers are incorporating the kits into their works to get more efficient results (92).

CSF

CSF is considered germ-free and detection of microbes in CSF provides considerable information about the possible infection. Therefore, the detection, identification and quantification of microorganisms in CSF are important diagnostic parameters for meningitidis and other central nervous systems infections (93,94). Bacterial meningitidis can be caused by a number of different bacteria, including *Haemophilus influenzae, Neisseria meningitidis, Streptococcus pneumoniae, S. agalavtiae, Listeria monocytogenes*, *Enteric bacteria*, and *Mycobacterium tuberculosis*.

CSF has been used as a specimen for preparing templates for the detection of *Herpes simplex virus (HSV), Enteroviruses, CMV* and *T. pallidum*.

Since clinical specimens have PCR inhibitors, DNA extraction is necessary to avoid such effects. Dennet et al. (95) showed that the inhibitors are detected frequently in CSF and boiling of CSF is not sufficient for removal of inhibitors, which affect the detection of microbes by PCR (96).

The classical DNA extraction protocol is based on purification with organic solvents like phenol-chloroform, followed by a precipitation step (97,98). Additional modifications, such as boiling (99), have also been applied to phenol-chloroform DNA extraction for *T. pallidium* (76) and, alternatively, *Borrelia burgdorferi* has been detected by heat-thaw method with Triton X-100 (57).

Nucleic acids can bind to silica or glass particles in the presence of chaotropic agents such as GuSCN, GuHCl or NaClO$_4$. In this regard, a recent development has introduced a new protocol for purification of DNA by using solid-state carriers, which selectively adsorb nucleic acids (66). With this method, it was possible to detect about 100 treponemes in 1 ml of CSF (100).

However, most investigators' methods are too laborious and impractical for routine use in the clinical laboratory, and the direct application method has been thought of as an alternative to extraction methods. Ni et al. (101) studied the CSF with *Neisseria meningitidis* for PCR directly by lysis. The samples are incubated for 5 min at 95 °C to lyse cells and to minimize subsequent biohazard; then 3 ml of CSF is used directly in a standard 50 µl PCR protocol. They obtained 91% sensitivity and specificity of PCR for diagnosis of meningococcal meningitidis, without causing inhibition of the test,

provided that there is less than 5 µl of CSF (101). *Toxoplasma gondii* was also detected directly by thawing CSF on ice, and heating to lyse the cells, for PCR amplification assay (102). Exposure of CSF to various environmental conditions, such as room temperature versus 4 °C for up to 96 h and freeze-thawing up to 3 times, does not affect the ability of a highly sensitive PCR assay to detect bacterial DNA in CSF samples (103).

Commercial extraction kits are also practiced on CSF samples too (68).

Other samples

Fecal specimens remain the most difficult and least studied with respect to the development of extraction methods that would allow DNA diagnosis. Feces sample are processed for the detection of various enteric microbial pathogens such as *Vibrio cholerae*, *Clostridium difficile*, *Salmonella* spp., *Shigella* spp., *Escherichia coli, Giardia* spp., and *Entamoeba histolytica*. Although some workers have used phenol-chloroform techniques, inhibitory substances are often coextracted with DNA and only dilution seems to help (104,105).

A simple extraction method was described by Saulnier and Andremont (106) with a sensitivity of 10,000 bacteria/g feces. Brian et al. (104) described 2 techniques of DNA extraction from feces sample for PCR analysis. In one of these, stool is suspended in phosphate-buffered saline and some centrifugation steps are applied. The second technique is a heat-lysis method. Lund et al. (107) detected enterotoxigenic *E. coli* with immunomagnetic separation of targets and McCausland et al. (90) was able to retrieve the target sequences using glass powder. The relative efficiency and effectiveness of the extraction methods used and commercially available kits were tested for Gram positive and Gram negative species (108) and for *E. coli* (47) from feces for successful and valid PCR studies.

Purification of amplifiable nucleic acid is complicated by the formation of insoluble amorphous salts, which precipitate during urine sample storage. Some bacteria lyse during the storage of urine, so that nucleic acids will be lost in the supernatant in any procedure that depends on an initial centrifugation step to concentrate the microorganisms.

Although urine specimens have been extracted by a variety of methods that have yielded amplifiable nucleic acid, a method that routinely eliminates inhibitory substances has yet to be published. PCR has been

performed directly on urine for *Cytomegalovirus* (*CMV*), but the presence of inhibitors usually requires clarification steps often failing to remove inhibitors, which can even partition with nucleic acid during phenol-chloroform extraction.

A phenol-chloroform DNA extraction method has been designed for the *Mycobacterium bovis* from urine samples (109). *Borrelia burgdorferi* target was isolated from urine by centrifugation and boiling in Chelex-100 resin (57) and *Leptospira interrogans* DNA, giving positive amplification, was prepared from bovine urine by repeated centrifugations and washing (110). Small scale purification of nucleic acid from urine directly is also possible for a rapid and simple method with a size fractionated silica particles or diatoms and a GuSCN (Guanidinium thiocyanate) containing lysis buffer (66).

Sputum samples (111), endocervical swabs (112), nasal washes (113) etc. have been widely tested for a suitable extraction procedure as well.

Conclusion

Automation of the PCR amplification of nucleic acids and the selection of specific targets and primers makes the PCR procedure a routine tool, but the success of the technique still depends primarily how the sample is processed. Therefore, an easy but effective sample processing method for the target nucleic acids is an essential component of PCR. An inefficient DNA extraction method will greatly affect the sensitivity or even the success of the whole amplification of the target.

Many DNA extraction methods have been adopted by different investigators for various organisms. Phenol-chloroform (-isoamylalcahol) extraction is the one used widely (76,79,82,97,98,104,109,114-118).

Despite its wide use, phenol extraction methods can be hazardous, especially phenol burns, which can be very serious (67). These need special and prompt care, and make specimen preparation time-consuming and cumbersome (79). The method is also laborious, making it impractical for routine use in most clinical laboratories (54). Residual phenol may also inhibit amplification of the extracted DNA (119).

The chelex agent proved to be successful for sample preparation on a variety of organisms even without the boiling process. Applying the chelex for blood samples greatly reduced the concentration of these blood components inhibitory to PCR, making the method convenient for blood samples (63).

GuSCN has been shown to be a powerful agent in the purification and detection of both DNA and RNA because of its potential to lyse cells combined with its potential to inactivate nucleases (89,120-124).

Beside these promising results, the important point still remaining for optimum sample preparation is the avoidance of toxic solutions. In general, standard GuSCN procedures may be too difficult or hazardous for routine use (89). On the basis of these findings none of these methods appear likely to decrease the need for optimum sample preparation methods.

In summary, an optimal sample processing method should concentrate the DNA in the required volume to be processed, especially that derived from the target organism, and remove the amplification inhibitors commonly present in biological fluids. Since some microbes have rigid cell walls, which may resist an ordinary digestion protocol for extraction, the protocol might need an additional consideration for sample preparation. To be applicable for routine diagnostic use, the process should be suitable for use with an array of clinical specimens, simple or preferably at least semiautomatic, reproducible and safe for staff handling the specimens by avoiding toxic solutions. The absence of a requirement for specialized equipment such as freezers, and centrifuges is also important for optimum sample preparation.

With regard to a universal sample preparation method, although the kits are efficiently used for many applications, some kits are found to be unsuitable for diagnostic purposes for PCR (70). According to the announcements, automated sample preparation systems based on magnetic bead separation such as NucliSENS easyMag, MagnaPure and Agowa-mag seem to be highly efficient for various molecular diagnostic techniques with eliminating laborious steps, and enabling easy and fast handling. Despite the great convenience of the automated systems, the yield differs according to type of the sample and superiority is inevitable between the systems (125).

In this review, it was intended to highlight the importance of the nucleic acid extraction from clinical specimens for PCR identification of infectious organisms and to give a brief review about some of the works those

accomplished to date. Most data published on nucleic acid extraction for this purpose were published by researchers from the US and European countries. When considering its commercial aspect, Turkish researchers' attention should be directed towards the development of a universal sample preparation method.

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