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# Application of Molecular Diagnostic tools in Clinical Veterinary Microbiology

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**Summary:** Nucleotide is nucleoside plus a phosphate group. Biomolecules includes all molecules such as proteins, fatty acids, it is refers to nucleic acid this days. Application of molecular technology in medicine is almost endless. Nucleic acid is the center genetic material. Two types of nucleic acids RNA & DNA. Deoxyribonucleic acid (DNA) is encoded with four interchangeable "building blocks", called "bases", Adenine, Thymine, Cytosine, and Guanine. Ribonucleic acid (RNA) has five different bases: Adenine, Guanine, Cytosine, Uracil, and more rarely Thymine. DNA synthesis can occur only in the 5' to 3' direction.

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**Key words**: Application; diagnostic tools; molecular

## 1. Introduction

Molecular biology is the science of biomolecules. Even though the term "biomolecules" includes all molecules such as proteins, fatty acids, it is refers to nucleic acid this days. **Nucleotide** is a compound consisting of a nucleoside linked to a phosphate group forming the basic structural unit of nucleic acids. **Nucleoside** is an organic compound consisting of purine or pyrimidine base linked to sugar, example Adenosine (Albano *et al*., 2007).

## 2. Application of molecular tools

The application of molecular technology in medicine is almost endless. Some of the applications of molecular methods are: Classification of organism based on genetic relatedness (genotyping), Identification and confirmation of isolate obtained from culture, early detection of pathogens in clinical specimen, Rapid detection of antibiotic resistance, Detection of mutations, Differentiation of toxigenic from non-toxigenic strains, Detection of microorganisms that lose viability during transport, impossible, dangerous and costly to culture, grow slowly or present in extremely small numbers in clinical specimen and Apart from their role in microbiology, these techniques can also be used in identifying abnormalities in human/animals, and forensic medicine.

## 3. Characteristics of Nucleic Acids

Nucleic acid is the center genetic material. Two types of nucleic acids RNA & DNA. Deoxyribonucleic acid (DNA) is encoded with four interchangeable "building blocks", called "bases", Adenine, Thymine, Cytosine, and Guanine, with Uracil rarely replacing Thymine. Ribonucleic acid (RNA) has five different bases: Adenine, Guanine, Cytosine, Uracil, and more rarely Thymine.

## 4. Characteristics of Nucleic Acids

In double stranded linear DNA, 1 end of each strand has a free 5' carbon and phosphate and 1 end has a free 3' OH group. The two strands are in the opposite orientation with respect to each other (antiparallel). Adenines always base pair with thymines (2 hydrogen bonds) and guanines always base pair with cytosines (3 hydrogen bonds).

## 5. The various molecular techniques

It requires: Plasmid profiling, molecule% G+C content, Nucleotide sequencing, and Restriction fragment length profiling (RFLP), Pulse field Gel electrophoresis (PFGE), Nucleic acid hybridization

and Amplification techniques (target amplification, signal amplification & probe amplification).

**5.1. Plasmid profiling:**

Plasmids are extrachomosomal, circular, double stranded DNA found in most bacteria. Each bacterium may contain one or several plasmids. Plasmid profile analysis involves study of **size** and **number** of plasmids. After the cells are lysed, the nucleic acids are subjected to electrophoresis. This gives the size and number of plasmids present in the cells. Since some species may contain variable number of plasmids or even unrelated bacteria may harbor similar number of plasmids, plasmid profiling may not provide useful information. **Electrophoresis** is the movement of molecules by an electric current. Nucleic acid moves from a negative to a positive pole (Albano *et al*., 2007).

**5.2. Molecule % G+C content**

DNA is a helical structure with AT and GC base pairs held by hydrogen bonds. When the solution of double strand DNA (dsDNA) is heated to near boiling temperature, the two complimentary strands separate and this is called **denaturation** or melting. The melting temperature of a particular DNA sequence is determined by its nucleotide composition. Because of three hydrogen bonds between G and C, DNA that has relatively high GC content require more energy to denature than DNA with higher AT content.

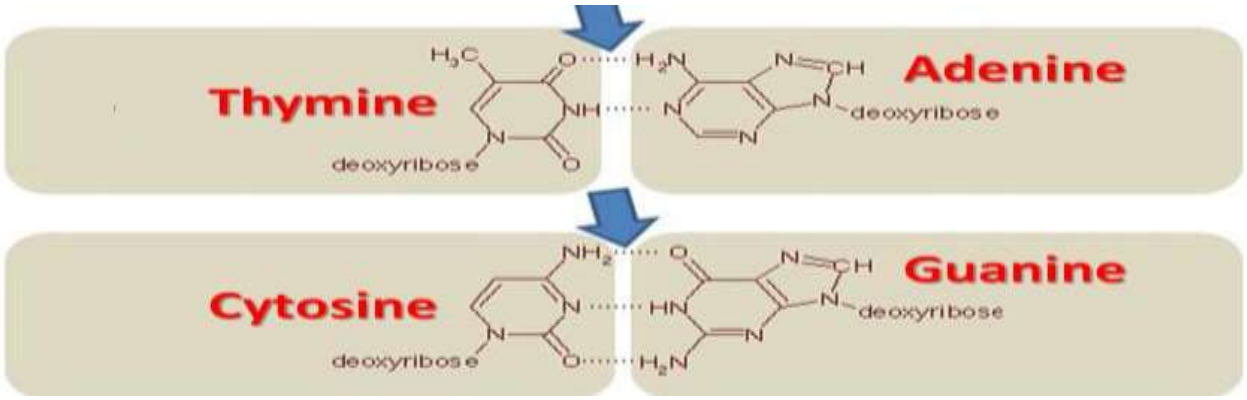


Figure: 1, Nucleotide bonds showing AT and GC pairs. Arrows point to the hydrogen bonds.

At a wavelength of 260 nm DNA absorbs light and the melting process can be monitored by continuous measurement of optical absorbance at this wavelength. As the temperature is raised, the complementary strands disassociate resulting in increase in absorbance until the two strands are completely separated. A curve is drawn noting the time on x axis and temperature with absorbance on y axis. The midpoint of the curve represents the temperature at which half of the base pairs have separated. This temperature Tm is the function of mol% G+C content of DNA (Albano *et al*., 2007).

The bases composition of DNA from bacteria range from 25-75 moles percent guanine + cytosine (mol% G+C). If the mol% G+C of two organisms differ widely then it is more likely that the two are unrelated. If the mol% G+C values of two isolates are identical, then it is likely that the two are similar or related. It is also possible that the two isolates are unrelated but coincidentally have similar G+C ratio. This method was in use earlier to study phylogenetic relationship amongst bacteria. Due to ambiguity of the interpretation and the availability of more specific techniques (such as rRNA homology), this is no longer used in classification.

**5.3. Nucleotide sequencing**

This method involves the determination of nucleotide sequence in the given DNA molecule. There are two popular methods for sequencing DNA: **Chemical Cleavage Method** and **Chain Terminator Method**. Both these methods have now been automated and the sequence can be read using a computer. Since it is time consuming process, it does not much role in diagnostic microbiology. This technique can be used to study the structure of gene, detect mutations, and compare genetic relatedness and to design oligonucleotide primers (Al-Harbi, and Uddin, 2004).

**5.4. Restriction fragment length polymorphism (RFLP)**

Polymorphism (or variability) in nucleotide sequence is present in all organism including microbes. RFLP technique relies on the base pair changes in restriction sites, which arise due to mutations. Restriction sites are strands of DNA that are specifically recognized and cleaved by restriction endonucleases. **Restriction endonuclease** an enzyme produced chiefly by certain bacteria that has the property of cleaving DNA molecules at or near a specific sequence of bases. Some enzymes cleave segment away from restriction sites and some within the sites (Al-Harbi, and Uddin, 2004).

Restriction site sequences range from 4-12 bases in length. When cleaved by the specific endonuclease enzyme, the average length of the fragment obtained is determined in part by the base pair recognized by the enzyme. In general restriction enzymes recognize 4, 6 or 8 base sequence. Recognition of 4 base pair (bp) sequence yields fragments with average length of 250 bp, 6 bp yields fragment of 4000 bp, the enzyme that recognizes 8 bp sequences generates fragments of approximately 64000 bp in length and thus, the enzyme that recognizes 4 bp sequences produces more short fragments.

Once the desired target DNA (bacterial chromosome, plasmid or of any origin) is cut using known or randomly selected restriction enzyme, the resultant fragments are separated by electrophoresis on agarose or polyacrylamide gel. Upon separation on gel, the fragments can be visualized as bands after staining with ethidium bromide (which binds to dsDNA) and viewed under uv light. Depending on the numbers of fragment and their sizes either discrete or overlapping bands are seen. These bands can be transferred to nylon membranes for hybridization. This technique is very useful as an epidemiological typing tool as it can be used to type isolates. The DNA of two or more isolates is subjected to digestion by the same restriction endonuclease enzyme, the fragments are separated by electrophoresis and the bands are compared. This process is also known as DNA fingerprinting and makes it very useful tool in forensic medicine (Al-Harbi, and Uddin, 2004).

Another important application is the ribotyping. The 16s rRNA (~1500 bp) is the smaller subunit of the bacterial ribosome is said to be most conserved sequence. It has a constant sequence that is common to most microorganisms and a variable sequence that is unique to a specific genus or species. Such sequences can be subjected to RFLP to determine relatedness with other organisms and can be confirmed by following with southern blotting. This technique has now superseded mol% G+C ratio for phylogenetic classification (Al-Harbi, and Uddin, 2004).

**5.5. Pulsed Field Gel Electrophoresis (PFGE)**

PFGE is a technique used for the separation of large DNA molecules by applying to a gel matrix an electric field that periodically changes direction (Principle). It is a technique that is similar to RFLP. If a bacterial chromosome is fragmented by an endonuclease that cleaves frequently, it may result in generation of large number of fragments. These fragments may not produce discrete bands but may form unresolved overlapping bands. This problem can be overcome by using restriction enzyme that cuts the DNA infrequently, producing large but few fragments.

Separation of these fragments is done by passing current that is reversed regularly in polarity. In conventional gels, the current is applied in a single direction (from top to bottom). But in PFGE, the direction of the current is altered at a regular interval. PFGE separates DNAs from a few kb to over 10 MB pairs. Thus larger DNA fragments can be separated into few well resolved bands. The application of PFGE is same as those of RFLP, with enhances resolution of fragments that differ by few bases.

**5.5.1. Application of PFGE**

PFGE has proved to be an efficient method for genome size estimation. In PFGE DNA fragments obtained by using endonucleases produce a descript pattern of bands useful for the fingerprinting and physical mapping of the chromosome. The PFGE is useful to establish the degree of relatedness among different strains of the same species. Parts of PFGE system are: Gel box, High voltage power supply, Switch unit and Computer system (Bairagi, *et al*., 2002).

**5.6. Nucleic Acid Hybridization**

The two strands of a DNA molecule can be separated by exposing the DNA to high temperature, low salt or various chemicals. The process of denaturation or melting can be reversed by lowering the temperature, raising the salt concentration or removing the denaturation agent. The separated strands reassociate into double helix (duplex) and the process is known as renaturation or annealing. Since the hybridization requires sequence homology, a positive hybridization reaction between two nucleic acid strands each derived from different source indicate genetic relatedness between the two organisms. Hybridization assays require that one nucleic acid strand is from the known organism while the other is derived from the organism to be identified or detected.

If DNA from isolate obtained from a clinical specimen is mixed with a probe (labeled DNA) and denatured, the strands separate. Following reversal of the conditions, the probe strand would anneal with the isolate's strand if there is homology between the two. This reaction is called **hybridization**. The results of such experiments are expressed as percent hybridization/ percent similarity/ percent relatedness or D value (Bairagi, *et al*., 2002).

DNA is composed of two purine nucleoside bases, adenine (A) and guanine (G), and two pyrimidine nucleoside bases, thymine (T) and cytosine (C). Double-stranded DNA is formed through hydrogen bonds that can occur only between the complementary bases A and T or G and C. (Top) perfectly reassociate DNA base sequence in which all nucleosides are paired by hydrogen bonds. (Middle) Perfectly the paired DNA base sequence in the center with unpaired, single-strand ends on each strand. (Bottom) None of the bases in the sequence (left to right) GCTACGTCAGT on the top strand are complementary to the sequence TACGATGCAGT in the bottom strand (Bairagi, *et al*., 2002).

Requirements for hybridization experiment include: target nucleic acid (DNA/RNA), restriction endonuclease enzyme, labeled probes, polyacrylamide gel/ agarose electrophoresis apparatus and nylon/nitrocellulose membrane and stringent conditions and Steps involved in hybridization reactions are: Production and labeling of single stranded probes, Preparation of single stranded target nucleic acid, Mixture of target and probe to allow annealing and Detection of hybridization reaction (Bairagi, *et al*., 2002).

**Probes** are short nucleic acids with known nucleotide sequences designed to hybridize with the target nucleic acid. Probes are labeled to enable their detection after hybridization. To synthesize a probe against a target sequence, the nucleotide sequence of the target must be known. Probes are prepared against target sequences that are unique to a given organism or a group of organism or a virus to prevent non-specific binding. Probes are prepared using one of these methods: Cloning on vectors such as plasmids, λ phages, Chemical synthesis of oligonucleotide probes (~ 20 nucleotides) or PCR amplification of known sequence.

Probes can vary in length, they can be short oligonucleotides (20-40 nucleotides) or cDNA probe of 1500-3000 bp in length. Oligonucleotide probes are convenient because they can be synthesized in large quantities artificially and their short length allows for highly specific discrimination of single nucleotide changes in hybridization reactions. However, shorter probes have some limitations too. Shorter the probe the more likely it is find a closely similar sequences within target DNA. This may result in background cross hybridization and false positive hybridization results. CDNA probes are thus more specific than oligonucleotide probes. The probes are labeled to facilitate their detection following hybridization to their target sequence. Once the probe is ready it must be labeled with a signal generating moiety. Labeling can be done using radioactive or non-radioactive labels. Once hybridized, the labeled probes can be detected by scintillation counter or on X-ray autoradiography. Even though radioactive isotope labels provide maximum sensitivity, their disadvantages include higher expense, difficulty in handling, health hazard, short shelf life and disposal issues (Bairagi, *et al*., 2002).

The non-radioactive labels include biotin, digoxygenin and acridinium ester. Other methods include use of fluorescein conjugated avid in or fluorescein conjugated antibody to biotin molecule. Hybridization is observed for fluorescence using UV light. The various methods of detection of labeled probes are: radiometric (radioactive isotope labeled probes), enzymatic (biotin or digoxygenin labled probes), fluorometric (fluorescein tagged avidin or

antibody) or chemiluminescence (acridinium ester labeled probe). The source of target nucleic acid can be microorganism from the clinical specimen or from the culture. The nucleic acid from the organism is extracted chemically or enzymatically. The nucleic acid is treated to stabilize as well as preserve structural integrity and then denatured (if DNA) to derive single strands (Saha *et al*., 2006).

**5.7. Amplification techniques**

The sensitivity of a DNA based method can be enhanced by manipulating any of the three reagents (also known as the three amplification techniques). These are: a) Target amplification, b) Probe amplification and C) Signal amplification.

**5.7.1) Target amplification**

Target amplification requires that the DNA to be tested should be amplified, i.e., the number of copies of the DNA is increased. To understand this we must first review the activity of the enzyme, DNA polymerase that is used to amplify the DNA.

DNA polymerase cannot initiate synthesis on its own. It needs a primer to prime or start the reaction. The primer is a single stranded piece of DNA that is complementary to a unique region of the sequence to be amplified. **DNA synthesis can occur only in the 5' to 3' direction**. Remember that DNA replication is semiconservative (Saha *et al*., 2006).

**6. The PCR reaction**

Polymerase chain reaction (PCR) – used to amplify something found in such small amounts that without PCR it would be undetectable. Uses two primers, one that binds to one strand of a double-stranded DNA molecule, and the other which binds to the other strand of the DNA molecule. All four nucleotides and a thermostable DNA polymerase. The primers must be unique to the DNA being amplified and they flank the region of the DNA to be amplified.

The PCR reaction has three basic steps. **Denature** – when you denature DNA, you separate it into single strands (SS). In the PCR reaction, this is accomplished by heating at $94^0$ C for 15 seconds to 1 minute. The SS DNA generated will serve as templates for DNA synthesis. **Anneal** – to anneal is to come together through complementary base-pairing (hybridization). During this stage in the PCR reaction the primers base-pair with their complementary sequences on the SS template DNA generated in the denaturation step of the reaction (Saha *et al*., 2006).

The primer concentration is in excess of the template concentration. The excess primer concentration ensures that the chances of the primers base-pairing with their complementary sequences on the template DNA are higher than that of the complementary SS DNA templates base-pairing back together. The annealing temperature used should ensure that annealing will occur only with DNA sequences that are completely complementary. The annealing temperature depends upon the lengths and sequences of the primers. The longer the primers and the more Gs and Cs in the sequence, the higher the annealing temperature. The annealing time is usually 15 seconds to 1 minute.

**Extension** – during this stage of the PCR reaction, the DNA polymerase will use dNTPs to synthesize DNA complementary to the template DNA. To do this DNA polymerase extends the primers that annealed in the annealing step of the reaction. The temperature used is $72^0$ C since this is the optimum reaction temperature for the thermostable polymerase that is used in PCR. The extension time is usually 15 seconds to 1 minute. The combination of denaturation, annealing, and extension constitute 1 cycle in a PCR reaction. Most PCR reaction use 25 to 30 of these cycles to amplify the target DNA up to a million times the starting concentration (Saha *et al*., 2006).

Common problems in PCR are **false negatives** due to presence of PCR inhibitors, poor nucleic acid isolation and poor amplification efficiency, and **false positives** due to contaminations. Common sources of error are: **False positive** reactions are caused by contamination with a new or previously amplified DNA. Non-specific primer hybridization. Binding of primers to area other than desired region resulting in false positive results. Primer dimer formation. This condition can arise if the two primers used are complimentary to each other and end up hybridizing with each other instead of hybridizing with the target. This may lead to little or no amplification of target sequence.

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