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Changing Panorama of Human Disease Diagnostics: Role of Molecular Diagnostic Methods

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Abstract: There has been increasing demand of molecular diagnostic tools to channel disease management, from diagnosis to treatment, particularly in the fields of infectious diseases, cancer and congenital abnormalities. A lot of information is required on the genetic level which has led to the rapid expansion of molecular techniques within clinical laboratories across globe. Advanced molecular methods have capability of detection of pathogenic isolates at genomic level so early and precise diagnosis of diseases. Various promising techniques having potential of detection at molecular level have been implemented in the laboratories since last decade such as PCR, other DNA and hybridization based approaches like PCR-RAPD, PCR-RFLP. FISH technique can provide valuable understanding of physiological and pathological processes by identifying the expression level and localization of specific miRNA within individual cells in tissue section. FISH has also been used for the diagnosis of both hematopoietic malignancies and non- hematopoietic malignancies (solid tumors). Advances in microarray technology have allowed simultaneous analysis of expression of thousands of genes. Sequencing based technologies like NGS and pyrosequencing help in exploring expression genetics and rapid discovery of SNPs. The high throughput and low sequencing costs provided by NGS systems have greatly speeded up the diagnosis of many diseases.

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I. INTRODUCTION

One of the major concerns in the present era is maintaining human health owing to a number of chronic and infectious diseases across globe. Biotechnology, an innovative field of science and technology has been found to be promising in improving the challenges associated to human health by providing robust technologies.[1] This has resulted in improved health, quality of life, and an increased life expectancy worldwide over the last century.[2]

Diagnostics play a vital role for identification of the presence and origin of disease at both the individual and the population level. Despite of availability of novel technologies for disease diagnosis, there is a deficit of diagnostic tests at low infrastructure sites in developing countries. Consequently, the differential diagnosis for diseases/infections with common symptomatology like fevers is not possible leading to difficulty in accurate treatment and monitoring the effects of intervention (preventative or therapeutic). Besides, inaccurate diagnosis also hinders the evaluation the drug resistance and recurrence of existing diseases. The diagnostic modalities/equipments presently available are mostly insufficient for achieving health needs in most developing countries. This necessitates for an enormous financial support for rapid development and delivery of diagnostic resolutions in the developing countries. [3]

Integrating Molecular Diagnostics with Microbiological Methods

Though traditional microbiologic methods are inexpensive but there are certain limitations and drawbacks associated with these methods. Long turn-around time, limited sensitivity and accuracy are associated with culture methods. To overcome these limitations molecular diagnostics have been progressively more incorporated in clinical laboratories. Molecular methods utilize the principle of DNA replication. These methods are expensive but benefits like rapid turn-around time and high sensitivity and specificity outweighs the cost.[1]

The molecular methods for the detection and characterization of microorganisms can be divided into three broad categories: 1) direct hybridization methods, 2) nucleic acid amplification, 3) methods for post-amplification analysis. The benefits such methods are evident in the clinics or the health care practitioners when they provide accurate antimicrobial therapy after the rapid and precise detection of the infecting pathogen thus reducing the probabilities of antimicrobial resistance. Other advantages include decreasing the hospital stays or the number of unnecessary admissions.[2]

Molecular Diagnostics in the Era of Precise Medicine and Treatment

The novel methods of molecular diagnostics are clearly changing the way of practicing medicine. Signal amplification and real-time nucleic acid amplification technologies provide a high sensitivity and specificity with a more rapid turnaround time than traditional methods.The parallel detection and differentiation of numerous microbial pathogens, mechanisms of antimicrobial resistance, and the development of disease-specific assays are nowmanaged by numerous methods of post-amplification analysis.[3]Besides, there is a considerable augmentation inthe identification of the genetic components of disease.[4]

During the last decade genomics, proteomics and microarray technologies have provided insights of the molecular basis of cells and tissues in health and diseases such as tumor classification and prognosis. Based on this personalized medicine came into picture in which each patient's distinctive genetic makeup is used for treatment to obtain optimal results.[5]With the invention of the high-throughput next generation sequencing (NGS) technologies, diagnostic spectrum has changed remarkably in daily routine. Though NGS implementation requires specific expertise and high cost its explicit advantages and new diagnostic possibilities make the switch to the technology unavoidable. Some of the valuable features are higher sequencing capacity, multiplexing of samples, higher diagnostic sensitivity, clonal sequencing of single molecules and workflow miniaturization.[6]

Markets [7]

The market scenario for molecular diagnostics is huge with considerable applicability on the following aspects:

1. Facilitating the routine testing across a broader range of hospitals and reference laboratories as the cost effective and simple procedure tests are required. Automation aids in developing the easy to perform and less expensive to operate tests.
2. Current trends reveal a big market size for infectious disease testing including the re- emergenceof infectious threats, multidrug-resistant TB, new strains of HIV, and swine flu(H1N1).
3. Pharmacogenomics, an emerging field might have big markets in the near future. There is significant investment in this field now-a days in expectationof cutting the long duration required for the drug discovery and regulatory approval process and thus getting market authorization sooner to bring potentially beneficial drugs to market.
4. The progressive degradation in the efficiency of traditional methods of practicing medicine favours molecular diagnostic methods strongly so as to obtain more precise molecular biomarker-assisted diagnosis and more accurate and safe molecularly guided therapeutic treatments. For the pharmaceutical industry, this will result in augmented efficacy, safety, productivity, and novel product lines. For diagnostics industry this means probability for integration and commercial opportunities for molecularly derived tests.
5. Main areas of growth market include infectious diseases, oncology, genetic testing and blood banking.

3.1 Nucleic Acid Based Techniques

Molecular diagnostic tools increase the application of gene therapy or biologic response modifiers. It provides tools for disease prognosis and therapy response and allows detection of minimal residual disease. Over the years, a variety of molecular biology techniques have been utilized in clinical diagnostic laboratories for the analysis of patient samples. [8]

3.1.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was first discovered by Kary B Mullis in 1984 which basically revolutionized molecular diagnostics. PCR provided the ability to produce billions of copies of a target DNA region, facilitating rapid analysis and direct mutation identification.[9]The principle of PCR is based on utilization of natural DNA replication mechanisms and results in the *in-vitro* production of large amount of a desired sequence of DNA from a mixture of heterogeneous sequences. The amplification potential of PCR is quite high, it can generate billions of copies of desired DNA sequence ranging from 50 to several thousand base pairs.[10] The biochemical reaction of the synthesis of specific DNA fragments is catalysed by a thermostable *TaqDNA Polymerase*, which takes part in the replication of the cellular genetic material.[11]The advantages of PCR include:

- Diagnosis of many genetic diseases and the detection of bacterial, viral and fungal pathogenic organisms. [11]
- Study of gene expression by cloning of a particular DNA fragment which has applicability in forensic medicine. [11]

The sequential steps to carry out the PCR are given in Figure 1.

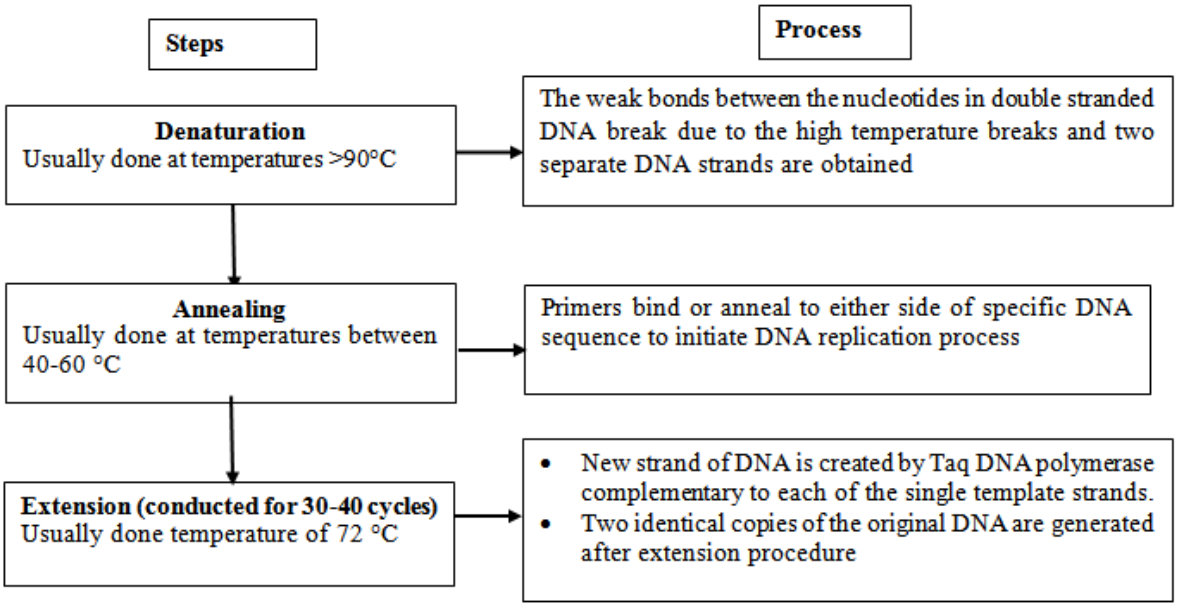

Figure 1: Steps in Polymerase Chain Reaction[10]

A careful application of PCR procedure is required for diagnostics to prevent contamination of the samples because the remarkable sensitivity of the technique can easily lead to false-positive results. Results of the published multicentre studies indicate that though positive samples are detected reliablybut that false positives werealso frequently obtained, indicating the ongoing presence of contamination problems. A number of systems have been developed to overcome contamination problem. One of the systems, the dUTP-UNG system (d-uracil triphosphate and uracil-N-glycosylase), utilizes an enzymatic reaction which allows specific degradation of PCR products from previous amplification cycle (in which dUTP has been inserted) without degrading native nucleic acid templates. However, this does not inhibit contamination of the sample with extraneous virus. Another innovation is new generation robotic workstations in which PCR reactions may be set up with only a single tube open at any one time. This significantly reduces the risk of contamination. The contamination by patient's DNA template is also important to avoid potential 'negative' results. These precautions allows the PCR to become a convincing option for the diagnostician. [10]

3.1.2Real-time Quantitative PCR (qPCR)

Traditional PCR methods for diagnosis of bacterial and viral are now being accompanied and sometimes replaced with qPCR assays. Even though traditional PCR can qualitatively detect nucleic acid sequences or genes, qPCR enables a more sensitive and quantitative analysis of a sample. It allows identification of the cycles during which near-logarithmic PCR product generation occurs. Contrary to conventional PCR methods, qPCR is rapid, highly sensitive and specific, with a closed-tube format thereby reducing risk of cross-contamination and without post amplification analysis.A rapid diagnosis in less than 2 hours during disease outbreaks is now possible with the portable real-time PCR machines and assays.[10]

The detection of real-time PCR products is done by insertion of fluorescent reporter molecule in each cycle which yields increased fluorescence as the number of cycles increase and amplified DNA is accumulated. The qPCR fluorescence chemistries have been classified into two main groups; 1) double-stranded DNA intercalating molecule(SYBR Green I) 2) fluorophore-labeled oligonucleotides.[12] The amplification is carried out in special thermal cyclers with fluorescence detection modules to monitor fluorescence signals. The fluorescence is measured to estimate the amount of amplicon produced after each amplification cycle and the emitted fluorescence is directly proportional to the total amount of amplicon. The main advantage of qPCR over conventional PCR is that it can be used for detection of presence or absence of sequence(qualitative) or to identify copy number(quantitative). Moreover, qPCR does not require gel electrophoresis, resulting in reduced TAT and increased efficiency. [13]

The rate by which the generation of fluorescent signal occurs in qPCR can be defined by:

Rf=Ri(1+E)n

where,

Rf = the final fluorescence signal of the reporter dye[14]

Ri =the reporter dye's initial fluorescent signal [14]

E =amplification efficiency [14]

n = the number of cycles[14]

E can be calculated from a standard curve using the following formula:

Exponential Amplification=10(−1/slope) [14]
E=(10(−1/slope)−1)×100[14]
where,
Slope = the standard curve's regression line.
A 100% efficient PCR reaction means it will double every cycle and produce a 10-fold increase in amplification product every 3.32 cycles (it will have a slope of −3.32).
Mathematically, it is[14]
log210=3.3219
or
23.3219=10

Box 1 Advantages of qPCR[15,16]

- Viral Quantification
- Gene Expression Analysis
- Microarray Verification
- Efficacy of Drug Treatment
- DNA Damage Measurement
- Quality Control and Assay Validation
- Pathogen detection
- Genotyping
- Detection as small as a two-fold change((i.e. 10 Vs. 20 copies)

Interpretation of plot
The results of qPCR results are depicted by an amplification plot. The X-axis of the plot represents number of PCR cycles while Y-axis of the plot represents fluorescence. The amplification curve consists of three phases:

1. **An initial phase:** Fluorescence emitted is lowerthan the detection level of the thermocycler. [17]
2. **A second phase:**There is an exponential increase in the fluorescence. In this phase a threshold value can be ascertained which indicates the area of exponential increase. Graphically, this value is represented by a horizontal line. [17]
3. **A third phase (plateau):** The reaction ends and the fluorescence reach a plateau. [17]

The threshold cycle Ct (intersection point between the amplification curve and the threshold line) indicates that the fluorescence has reached the threshold value. [17]

3.1.3 Diagnosis by restriction fragment length polymorphisms and related DNA-based approaches

3.1.3.1 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) method primarily detect variation at the homologous DNA sequence level of different biological samples like blood, tissues etc. The principle behind RFLP is the comparative analysis of restriction enzyme cleavage profiles following the existence ofa polymorphism in a DNA sequence related to other sequence. It acts as a molecular marker because of its specificity to a single clone/restriction enzyme combination. The DNA of individuals to becomparedis subjected to restriction digestion with specific enzymes called restriction endoucleases. In the next step the resulting fragments are separatedaccording to molecular size using agarose gel electrophoresis. After separation of fragments by agarose gel electrophoresis, a unique blotting pattern to a specific genotype at a specific locus is obtained by hybridization of sequence specific probe(Southern blotting).Throughthis approach two individuals which display different restriction profiles are detected.[18,19]

Genetic Basis for Restriction Fragment Length Polymorphisms (RFLP)
A variation in thenumber of tandem repeats of a short DNAsegment accounts for the genetic basis of RFLP. These variations termed as "variable number of tandemrepeats" (VNTR) forms the basis of DNA

fingerprinting.So, if one personhas 14 repeatsof a DNA segment on one chromosomeand eight repeats on the other, another person may have differentVNTR patterns which are detected by RFLP.VNTRs can be very shortrepeats of a simple DNA sequencescalled as $(CA)_n$ blocks (for example:(deoxy cytidine-deoxy adenine)$_n$ (deoxyguanine-deoxy thymine)$_n$.In view ofthat the human genome consists of 50,000 to 100,000 $(CA)_n$ blocks, each nin the range of 15 to 30, the probability of a single $(CA)_n$ block is every 30 to 60kilobase segment of the human genome.These blocks might play role in gene regulationor recombination.[20]

Types of Restriction Endonucleases(RE)

Restriction endonucleases are one of the important tools in molecular biology, which cut both strands of DNA in a site-specific manner.According to structure, recognition site, cleavage site, cofactor(s), and activator(s), REs are classified into four classes as I, II, III, and IVand subclasses (IIe, IIf, IIs, etc.)(Table1).There can be multiple subunits and holoenzyme assemblies to obtain the required restriction, methylase, andspecificity domains.[21, 22]

Restriction Endonuclease Type	**Examples**	**Cleavage site**
I	*Eco*KI, *Eco*AI,*Eco*BI	Distant and variable from recognition site ***Eco*KI:** AAC(N6)GTGC(N>400)↓ TTG(N6)CACG(N>400)↑
Orthodox II	*Eco*RI, *Bam*HI, *Hind*III	Distinct, within recognition site, and might result in a 3' overhang, 5' overhang, or blunt end ***Eco*RI:** G↓A A T T C C T T A A↑G
IIe [3]	*Nae*I, *Nar*I, *Bsp*MI	• Cleave in defined manner within the recognition site or a short distance • Needs activator DNA for carrying out cleavage ***Nae*I:** GCC↓GGC CGG↑CCG
IIf	*Sfi* I, *NgoM* IV, *Cfr* 10 I	Distinct, within recognition site, may result in a 3' or 5' overhang ***NgoM* IV:** G↓C C G G C C G G C C↑G
IIg (formerly Type IV)	*Eco57I, Bce83I, Hae*IV	• Cleavage in a defined manner a short distance away from recognition site • Might not cut tocompletion ***Eco*57I:** CTGAAG(N) 16↓ GACTTC(N) 14↑
IIm	*Dpn*I	• Cleave within the recognition site to form a blunt end • Methylation of recognition site is required
IIs	*Fok*I, Alw26I, BbvI	• Cleave in defined manner • At least one cleavage site outside of the recognition site • Blunt ends are rare ***Fok*I:** GGATG(N) 9↓ CCTAC(N) 13↑
IIt	*Bpu*10 I, *Bsl* I	Distinct,within recognition site or a short distance away, results in a 3' overhang ***Bsl* I:** C C N N N N N↓N N G G G G N N↑N N N N N C C

III	*Eco*P15I, *Eco*PI, *Hin*fIII	• Cleavage roughly 25 bases away from recognition site • Might not cut to completion ***Eco*P15I:** CAGCAG(N) 25–26↓ GTCGTC(N) 25–26↑
IV(formerly Type IIb)	*Bcg*I, *Bsp*24I, *Bae*I	• Cleaves short distance away in a defined symmetric manner on both sides • Leaves 3' overhangs ***Bcg*I:** ↓10(N)CGA(N)6TCG(N)12↓ ↑12(N)GCT(N)6ACG(N)10↑

Table 1: Classification of Restriction Endonucleases[21, 22]

Disadvantages of RFLP

RFLP detection method can lead to false results due to gain or loss of restriction fragments probably due to intraspecies mutations at restrictionsites. As the detection is based on only a few informative DNA sequence positions, numerous restriction enzymes are usually required to obtain a correct interpretation. Consequently, due to use of several restriction enzymes a highly complex RFLP pattern is generated which is difficult for interpretation. Furthermore, RFLP detection requires ample amount of high quality DNAand thus not agreeable for automation and standardization. [23]

3.1.3.2 Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP)

PCR- RFLP is a well- known technique for the detection of both intraspecies and interspecies variation. It is also called as cleaved amplified polymorphic sequence (CAPS).[24]

Genetic variations are broadly classified into three groups:

i. **Single nucleotide polymorphisms (SNPs):**Minor genetic variations. [24]
ii. **Multi-nucleotide polymorphisms (MNPs):**Multiple, consecutive nucleotide variations of a single common length. These type of polymorphisms can include two variable nucleotides (double nucleotide polymorphisms (DNPs)) or three variable nucleotides (triple nucleotide polymorphisms (TNPs)). [24]
iii. **Microindels:**Variations like deletions, duplications and combinations leading to the gain or loss of 1-50 nucleotides. [24]

The detection of SNPs and microindels is of diagnostic importance as these are associated in monogenic as well as complex diseases and can alter individual drug responses. For example, Occurrence of SNPs and microindels in genes encoding CYP2D6 (important drug metabolizing enzymes) results in the loss of enzyme activity and slowing in the metabolism of a number of important drugs. Therefore, it is necessary to develop techniques for genotyping of SNPs, including PCR-RFLP. [24]

The basic principle lies in the fact that SNPs, MNPs and microindels are usually associated with the formation or abolition of a restriction enzyme recognition site. Allele identification is based on the presence or absence of the restriction enzyme recognition site resulting in the formation of restriction fragments of different sizes which are resolved by gel electrophoresis.[24]

Box 2 Potential Advantages and Disadvantages of PCR-RFLP [24]

Advantages

- Economical (Cost-Effective)
- Easy to design
- Analysis of SNPs and miroindels is possible
- Expensive instruments are not required
- Miniaturisable

Disadvantages

- Formation or loss of a restriction enzyme recognition site is required for this analysis
- High price of some restriction endonucleases
- If more than one nucleotide variation in a restriction enzyme recognition site, exact genotyping can't be achieved
- Long turn-around- time
- Not apposite for high-throughput analysis

Variants of PCR-RFLP

Several variants of PCR-RFLP have emergedin the last decade (Table 2). The technique of the electrophoretic separation has also been replaced by a gel-free method in addition to techniques for DNA fingerprinting.[24]

PCR-RFLP Variant	Utilization
• PCR combined with restriction fragment melting temperature (PCR-RFMT) analysis • Also known as melting curve analysis of SNPs (McSNP)	Genotyping of SNPs and microindels by utilization of differences in the heat dissociation profiles of restriction enzyme-treated fragments
Amplified fragment lengthpolymorphism (AFLP)	• A limited set of primers is used to amplify subsets of fragments of genomic DNA. • Used for genetic profiling of microbial communities
Terminal restriction fragmentlength polymorphism (T-RFLP)	• Restriction digestion of amplified fragments(obtained by using end-labelled primers) • Used for genetic analysis of microbial communities and differentiation of animal species

Table 2 Variants of PCR-RFLP [24]

3.1.3.3 Amplified Fragment Length Polymorphisms (AFLP)

The basic principle of AFLP technique is the selective PCR amplification of restriction fragments from a total digest of genomic DNA.The restriction digestion is carried by two enzymes, one with an average cutting frequency (like *Eco*RI) and a second one with a higher cutting frequency (like *Mse*I. The sticky ends both 5´ and 3´ ends of DNA fragments produced by restriction digestion are then ligated with double-stranded oligonucleotide adapters (10-30 bp long).[23]The ligated DNA fragments are subjected to two successive PCR amplifications with adapter-specific primers under highly stringent conditions. One selective nucleotide amplifies 1 of 4 of the ligated fragments, while three selective nucleotides in both primers amplify 1 of 4,096 of the fragments.[24]

The potential uses of AFLP include genomic profiling of bacteria, phylogenetic analysis, quantitative trait locus mapping, and population genetics .[25]

3.1.3.4 Random amplified polymorphic DNA (RAPD)

RAPD markers are DNA fragments obtained by PCR amplification of random segments of genomic DNA with single primer (9-10 nucleotides long) of arbitrary nucleotide sequence. These primers anneal with enough affinity to different genomic regions at low annealing temperatures. Contrary to traditional PCR analysis, no knowledge of DNA sequence of target organism is required for RAPD. A specific banding pattern resulting from the different genomic location of primer-binding sites is used for profiling of the species. [23, 26]

Chromosomal rearrangements like insertions/ deletions are primary reasons of RAPD polymorphisms. Therefore, after amplification the presence and absence of bands in the RAPD profile are indicative of the products of different length from the same alleles in a heterozygote. This is called as RAPD fingerprinting. [26]

DNA-based Approach	DNA hybridization	RFLP	AFLP	RAPD	Conventional PCR	qPCR	Sequencing	Microarray
Criterion								
Quantity of achieved information	Low	Moderate	High	Moderate	Moderate	High	High	High
Requirements of previous information	No	Yes	No	No	Yes	Yes	Yes	Yes
Aptness of the detection of mixtures	No	Yes	Variable	Variable	Yes	Yes	Yes	Yes
Inter-laboratory repeatability	Moderate	Moderate	Moderate	Poor	Good	Good	Good	Good
Cost of equipments and reagents	Moderate	Moderate	Moderate	Moderate	Moderate	High	High	High
Throughput capacity	Low	Moderate	Moderate	Moderate	Moderate	High	High	High
User-friendly	Easy	Easy	Moderate	Moderate	Easy	Moderate	Moderate	Difficult

Table 3 Qualities of different DNA-based approaches[23]

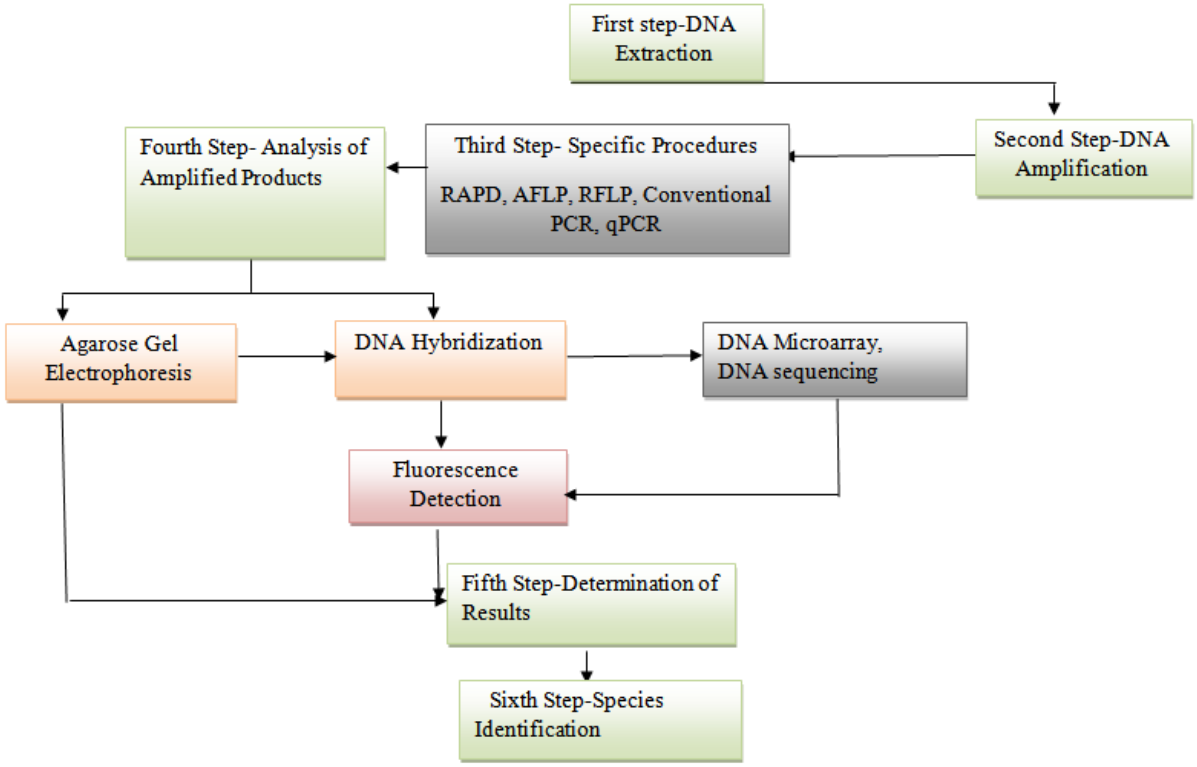

Fig 2: Procedural Steps in DNA-based Approaches[23]

3.1.4 In-situ Hybridization(ISH) and Fluorescence In-situ Hybridization (FISH)

3.1.4.1 In-situ Hybridization

The basis of in-situ hybridisation (ISH) is the complementary binding of labeled DNA or RNA probes with target nucleic acid sequences in intact chromosomes, cells or tissue sections. Unlike other molecular biology techniques, ISH allows localisation and visualisation of target nucleic acid sequences within the cells.[27] Once the annealing of the labeled probe to the target complementary sequences in fixed cells or tissue is completed, the hybridized probe is visualized by various isotopic and non-isotopic (fluorescent and non-fluorescent) approaches.[28] Isotopic probes (^{3}H, ^{32}P, ^{35}S, ^{125}I) generally have high sensitivity than non-isotopic ones but associated with some limitations like less stability, longer processing times and stringent disposal methods. Amongst non-isotopic probes biotin and digoxigenin are the reporters of choice.[27, 29] Different types of probes used for ISH include double-stranded DNA probes, riboprobes (single stranded antisense RNA probes), singlestranded DNA probes generated by polymerase chain reaction (PCR), synthetic oligodeoxynucleotide probes, and oligoriboprobes. [28, 29] Choosing a probe for ISH procedure is critical step and parameters like sensitivity and specificity, production facilities, easy penetration of probe into the tissue, stability of hybrids and reproducibility of the method should be kept in mind while selecting the probe of interest. Generally, the ideal size of the probe used in ISH technique is 50–300 bases.[28]

Application of ISH typically involves:

1. Detection of various infective agents in tissue sections(like cytomegalovirus(CMV), herpes papilloma virus(HPV), HIV, John Cunningham(JC) virus, B19 parvovirus, herpes simplex virus(HSV)- 1, Epstein–Barr virus (EBV), hepatitis B virus(HBV), hepatitis delta virus, *Chlamydia trachomatis*, *Salmonella* and *Mycoplasma*) [27]
2. Localisation of sites of infection [27]
3. Explanation of mechanisms of virus transmission [27]
4. Determination of the link between viral agents and cancer [27]
5. Sex typing [27]
6. Detection of structural and numerical chromosomal changes in tumours [27]

3.1.4.2 Fluorescence In-situ Hybridization

FISH is a macromolecule recognition technology in which specific fluorophore coupled nucleotides called as probes are used to hybridize on to the complementary DNA sequences in targeted cells and tissues. The visualization is then done through a fluorescence microscope or an imaging system. The three potential benefits of FISH technology include: 1) high sensitivity and specificity in target DNA or RNA recognition, 2) direct applicability to both metaphase chromosomes and interphase nuclei, 3) single cell level visualization of hybridization signals. Due to an increased resolution from Giemsa bands to the gene level, detection of structural chromosomal abnormalities can be done rapidly by this technique.[30] It is a faster and less-time consuming compared to traditional cytogenetic metaphase karyotype analysis as the detection ue involves use of either fresh water or paraffin-embedded interphase nuclei without the requisite for culturing.[31] Due to high sensitivity and specificity it acts as a potent tool for genetic diagnosis of microdeletion/microduplication syndromes, common aneuploidies and subtelomeric rearrangements. Now-a-days it is one of the robust technologies in detecting genetic anomalies (somatic recurrent losses, gains, and translocations) related to cancer wherein a set of gene-specific probes is used for hematologic and solid tumors. Besides, detection of infectious microbes and parasites like malaria in human blood cells are also the applications of FISH technology. [30]

Genetic diagnostic applications	**Constitutional chromosomal and genomic abnormalities** • Fast screening of familiar aneuploidies • Recognition of microdeletion/microduplication syndromes • Identification of subtelomeric rearrangements • Study of supernumerary marker and ring chromosomes
	Somatic recurrent chromosomal abnormalities • Scrutinizing disease progression and clonal evolution • Finding translocations, deletions, duplications/amplifications • Measurement of sex-mismatch bone marrow transplantation
	Infectious diseases • Identification of malaria by 16s rRNA
Research applications	**Analysis complex chromosomal rearrangements** • Mapping breakpoints and genomic orientation • Studying 3D chromosomal structures

	• Characterizing complex rearrangements
	Characterizing nuclear genomic structures • Spatiotemporal organization of centromeres/telomeres • Chromatin interaction during cell cycle • *in situ* chromosome haplotyping
	Profiling RNA transcription and localization • Quantitfication of multiplex mRNAs in single cells • Subcellular localization of mRNAs and non-coding RNAs

Table 4 Genetic diagnosis and research applications of FISH technology [30]

3.1.4.3 Detection of MicroRNAs (miRNAs) by ISH

MicroRNAs (miRNAs) constitute a class of evolutionarily conserved small non-coding RNAs that regulates the post-transcriptional gene expression.[32]In the recent times, association between initiation and progression of the disease and miRNAs are becoming increasingly apparent. These miRNAs have been reported to play biological role in homeostasis so the tissue localization of miRNAs is essential. ISH technique can provide valuable understandingof physiological and pathological processes by identifying the expression level and localization of specific miRNA within individual cells in tissue sections. [33]The base pairing specificity and stability of miRNA-mRNA complex has been robustly augmented by the application of locked nucleic acid (LNA) probes in miRNA-ISH. These LNA probes are most accepted, because these efficiently increases the specificity in target detection. [32]

LNA is an oligonucleotide containing at least one LNA monomer i.e., one 2'-O,4'-C-methylene β-dribofuranosyl nucleotide. LNA probes comprise a class of bi-cyclic, high-affinity RNA analogues in which the furanose ring of LNA monomers is conformationally locked in an RNA-mimicking C3'-endo (N-type) conformation. The unique physicochemical properties of LNA include good aqueous solubility and a full compatibility with standard coupling chemistry and synthesis programs of commercial DNA synthesizers. The principle of miRNA ISH is depicted in Figure 3. [33]

Applications of miRNA-ISH

The miRNA-ISH detection provides a dependable assessment of the physiologic function of miRNA at three-dimensional location and the single-cell level. For example, using FISH detection systems, identification of the two tumor specific miRNAs, miR-205 and miR-375 was done in two skin tumors namely basal cell carcinoma (BCC) and merkel cell carcinoma (MCC) respectively. [32]

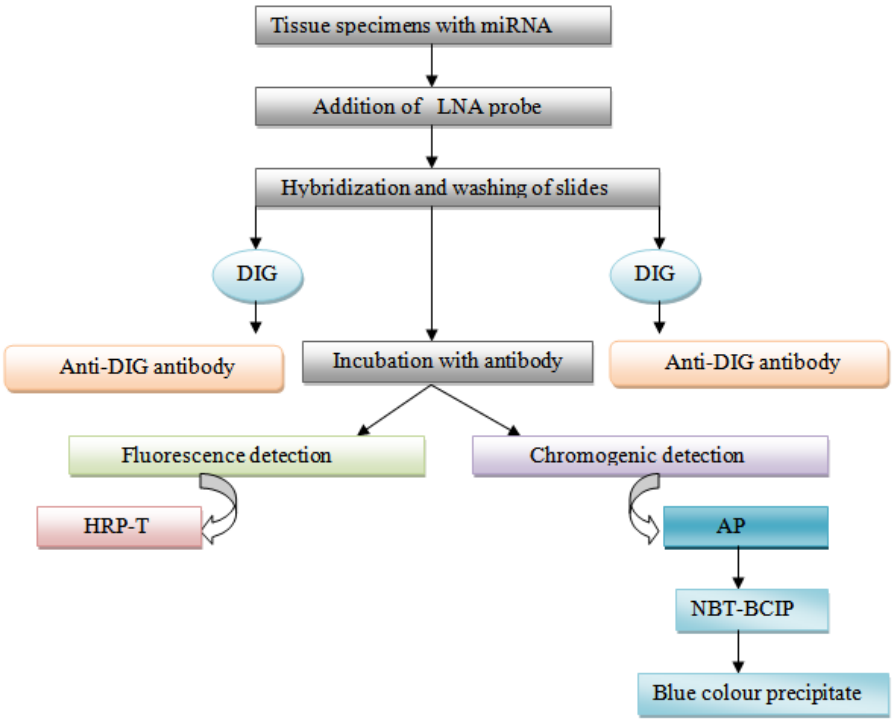

Fig 3: Procedural steps in miRNA in-situ hybridization [32, 33]

FISH in Oncology Diagnostics

Treatment for cancer is one of the biggest challenges in modern medicine. Increasing evidence reports association of diverse genomic aberrations with both hematologic and solid tumors. Extensive research in this area for defining the role of genomic fluctuation and disease pathogenesis shows that number of genomic abnormalities such as gain, loss or rearrangement of chromosomal fragments and gene mutations are the primary factors in the pathogenesis of various types of malignancies. [34]

Cytogenetic aberrations might be undetectable in traditional karyotyping methods. Diagnosis by FISH has emerged as an indispensable tool for leukemia and cancers. FISH-based tests such as multicolor karyotyping, comparative genomic hybridization (CGH) and array CGH are largely used now-a-days in clinical applications as they enable resolution of complex karyotypic aberrations and whole global scanning of genomic imbalances. Different types of FISH probe systems are given in Table 5.[35]

Types of FISH probe systems for cancer diagnosis	**Unique applications**
Centromeric probes	Expression of trisomy, monosomy and ploidy level abnormalities
Chromosome painting probes	• Developed to detect the whole chromosome of interest • Beneficial in decoding cytogenetic aberrations complicated to resolve by morphologic detection
Locus specific probes: Two types	Gene fusion, gene deletion or duplication
a. Dual colour translocation probes(D-FISH): Large DNA probes encompassing upstream and downstream of the translocation breakpoint of both fusion partners, enabling one signal each for the wild type alleles and two fusion signals*one for the fusion gene and the other for the reciprocal product) in a positive metaphase or cell	
b. Dual color break-apart probes: **i. Identify gene rearrangements:** as one fusion signal and two split signals. **ii. Identify gene deletions:** as signal fusion and the loss of the other fusion signal, constant with preservation of one allele and deletion of the other. **iii. Identify gene amplification or duplication:** of the corresponding chromosome band that includes the wild-type gene. The copy number of the fusion signal will be increased (> 2) in these cases.	

Table 4 Different type of FISH probe systems in clinical laboratories [35]

Advances in FISH in Oncology

FISH has become gradually more important in clinical diagnosis due to its simplicity and reliability in detecting key biomarkers in various tumors. Moreover, a high precision in prognostic has been obtained by combination of cytogenetic and molecular profiling by this technique. It has been used for the diagnosis of both hematopoietic malignancies and non- hematopoietic malignancies (solid tumors). [34, 35]

Hematopoietic malignancies

Leukemia

The frequently occurring chromosomal rearrangements in acute myeloid leukemia (AML) are t(8;21)(q22;q22), t(15;17)(q22;q12) and inv(16)(p13.1q22) or t(16;16)(p13.1q22). A more extensive risk assessment system was developed by combining the new molecular information with cytogenetic findings: For example: The genes which are associated with clinical outcome: t(8;21)(q22;q22)/RUNX1-RUNX1T1(Runt-related transcription factor1);t(15;17)(q22;q12) /PML- RARa; inv (16)(p13.1q22) or t(16;16)(p13.1;q22)/Core-binding factor, beta subunit- Myosin, heavy chain 11, smooth muscle (CBFB-MYH1) and mutation in both the CCAAT/Enhancer binding protein alpha (CEBPA) and nucleophosmin member 1 (NPM1) genes. A diagnostic hallmark of CML is the universal presence of breakpoint cluster region protein / abelson murine leukemia viral oncogene homolog 1 (BCR/ABL1) rearrangement in chronic myeloid leukemia (CML). [34, 35]

Multiple myeloma

One of the well known heterogeneous malignancies is multiple myeloma (MM) that affects plasma cells. The pathology of MM, include accumulation of malignant plasma cells in the bone marrow thus inhibiting the normal plasma cells to help fight infection. The malignancy is clinically presented as monoclonal plasma cells that penetrate the bone marrow, presence of monoclonal immunoglobulin in the blood and/or urine, and huge osteolytic bone lesions. The genetic aberrations found in MM are categorized into two types based on the chromosome ploidy status: **1)** the hyperdiploid karyotype (these are generally trisomies of many chromosomes,

such as 3, 5, 7, 11, 15, 19 and 21**); 2)** the hypodiploid karyotype (generally associated with a translocation of immunoglobulin heavy chain (IGH) locus at 14q32). [34, 35]

Non-hematopoietic malignancies (solid tumors)

Lung cancer

Anaplastic lymphoma kinase (ALK) rearrangements are commonly associated with pulmonary adenocarcinomas in non-small cell lung cancer (NSCLC). Diagnosis by FISH plays a critical role in directing précised ALK-targeted therapy. ALK FISH testing is generally advised for patients with wild-type EGFR/KRAS non-squamous NSCLC because of mutually exclusive ALK rearrangements with EGFR/KRAS mutations. [34, 35]

One more receptor tyrosine kinase rearrangement located on the chromosome 6p22 is ROS1 with the prevalence of only 2-3% of the NSCLC adenocarcinomas. ROS1 gene rearrangement is another predictive FISH biomarker that can be applied to individually tailored management of lung cancer. [34, 35]

Prostate cancer

Androgen-regulated transmembrane protease, serine 2 (TMPRSS2) and E26 transformation specific (ETS) family members (ERG, RTV1, and ETV4) have been reported in 50% of the prostate cases. The rearrangement pattern engages the fusion of TMPRSS2 to the oncogene ETS-related gene (ERG), resulting in the abnormal activation of ERG. In recent times the strategy includes the identification of either TMPRSS2 or ERG rearrangements by a four-color FISH assay. [34, 35]

Breast Cancer

Melanoma

Melanoma is a heterogeneous group of skin malignancies caused by melanin-producing (melanocytes) with acquired genetic aberrations. FISH can be used for precise diagnosis. Four probes targeting 6p25 (RREB1), 6q23 (MYB), 11q13 (CCND1), and centromere 6 (CEP6) are used for identification of melanoma. [34, 35]

3.1.5 DNA microarray technology

DNA microarray technology is an emerging diagnostic tool owing to rapid advances in the bioinformatics, computational biology, robotics and fabrication of high quality glass slides. A microarray is a group of microscopic spots arranged in an array on grid- like format and attached to a solid surface or membrane. In DNA microarray each individual spot depicts single stranded DNA fragment known as probes. The basic principle of DNA microarray technology is same as southern blotting and comprise of hybridization of probes with specific target (nucleic acid sequence) which is labeled with a fluorescent dye. One advantage of is that it allows the simultaneous detection and expression of several genes on a single chip. Two important types of DNA microarrays are oligonucleotide microarray and spotted microarray. [36]

Some important applications of DNA microarrays are genotyping and determination of disease-relevant genes, mutation analysis, screening of single nucleotide polymorphisms (SNPs), identification of chromosome aberrations, and determination of posttranslational modification.[37]

Some of the common diseases for the diagnosis of which microarray technology has been utilized are avian influenza (H5N1), foot and mouth disease (FMD), viral hemorrhagic fever (Marburg virus), severe acute respiratory syndrome (SARS) virus etc. [36]

An improvised version of Flu chip have been developed by University of Florida and Centre of Disease Control US. It requires matching sequences only from a single slowly mutating gene of the influenza virus than other 2 genes in the old version (hemagglutinin (H1) and neuraminidase (N1) genes). This chip requires only 15 sequences from a single gene to reliably identify avian influenza. [36]

3.1.6 Next generation sequencing (NGS)

The clinical diagnostics took a dramatic change about a decade ago with the introduction of the next-generation sequencing (NGS) in genomic laboratories. It has become a widely used diagnostic platform as an alternative approach to the direct Sanger sequencing after improvements in accuracy, robustness and handling.[38] The high throughput and low sequencing costs provided by NGS systems have greatly speeded up the diagnosis of many diseases. The Several techniques are utilized in NGS including: (1) Micro-chip based electrophoretic sequencing, (2) Sequencing by hybridization, (3) Real-time sequencing and (4) Cyclic-array sequencing. [39]

A unique feature of NGS sequencing is that it allows WGS of numerous pathogens in single sequence run, either from bacterial isolates of different patients, or from multiple isolates present in patient material from one patient (metagenomics). One great advantage of NGS over Sanger sequencing is that a single procedure can be used for all pathogenic isolates for both identification and typing applications.[40]

A number of NGS platforms are available now-a-days. Some of them are Roche 454 System, AB (Sequencing by Oligo Ligation Detection (SOLiD) System, Illumina GA/HiSeq System, Compact PGM Sequencers. [41]

In the recent times, the NGS has been widely used in cancer diagnostics, specifically since gene mutations have been found to play crucial role in cancers. The NGS technology allows the identification of gene mutations, characterization of tumour type, diagnosis of tumour progression by biomarker prediction. NGS technology integrates genomics, transcriptomics or proteomics for early diagnosis and targeted therapy. Novel anticancer drug development by NGS technology relies on multiplexing of samples and high diagnostic sensitivity for genetic and epigenetic biomarkers.

There are three methods by which NGS can be utilized in cancer therapy:

1. Diagnosis of tumour type caused by mutations leading to genetic alternations
2. Prediction of targeted gene therapy against specific tumour type
3. Detection of mutations that cause resistance to targeted therapy

3.1.6.1 Pyrosequencing

The basic principle of pyrosequencing involves sequencing-by-synthesis. This method utilizes the luminometric detection of pyrophosphate released as a result of primer-directed DNA polymerase nucleotide incorporation. It employs a kinetically well-balanced of four enzymes (DNA polymerase, ATP surfurylase, firefly luciferase and apyrase) for precise detection of nucleic acid sequences during the synthesis.[42, 43]

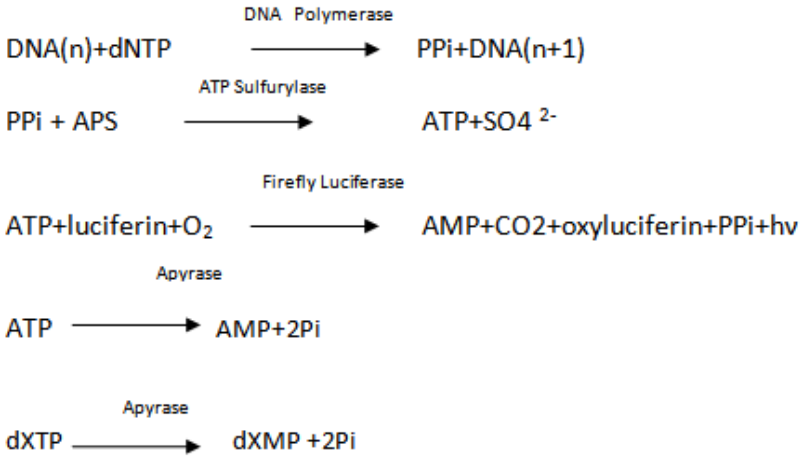

Fig.4: Pyrosequencing reaction cascade

ATP: Adenosine tri phosphate; AMP: adenosine mono phosphate; APS: adenosine 5´ phosphosulfate; dNTP: deoxyribonucleotide triphosphate; Pi: phosphate; $SO4^{2-}$: Sulphate ion

Applications of pyrosequencing include SNP genotyping, bacterial, fungal and viral typing, determining of difficult secondary structures, mutation analysis, DNA methylation analysis, and whole genome sequencing.[42, 43]

II. CONCLUSIONS

An early and rapid diagnosis of diseases is one of the key factors which influence the appropriate and targeted management of many chronic ailments. Numerous techniques have been used since long times including conventional methods like culture techniques however, there are limitations of being slow and time consuming procedures. To overcome these limitations focus has been shifted to molecular tools for rapid and accurate diagnosis and thus targeted therapy to patients. There has been a dramatic shift in clinical diagnosis by implementation of methods like PCR, PCR-RAPD, PCR-RFLP, qPCR, FISH and technologies like microarray, NGS and pyrosequencing in the clinical laboratories worldwide. These advanced technologies act as forefront of medical innovation and provides a platform in transforming cancer care. Now-a-days valuable insights can be obtained at every stage of cancer — prevention, detection, diagnosis, treatment and successful management. These robust technologies have the potential to fundamentally change clinical practice, and are especially important for personalized medicine in the modern era.

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