Biotechnology in forensic science: The revolution continues

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ABSTRACT

Molecular biology has become one of the most fascinating fields of biology in recent years. Molecular methods have been applied in various fields of biology including Medical/Health Science. In this paper, light has been shed on the application of different molecular methods (DNA analysis) in the field of Forensic Medicine.

Keywords: Mol biology, DNA, PCR.

THE BEGINNING OF THE REVOLUTION

DNA analysis constitutes the most significant aspect of biotechnology related to forensic science. Since it was first introduced in the mid-1980s, DNA analysis (formerly called DNA Fingerprinting, but now increasingly referred to as DNA Typing or DNA Profiling) has revolutionised forensic science like no other technique has, especially in the area of identification of individuals. The technique was first described in 1985 by Dr. Alec Jeffreys, a geneticist in the University of Leicester. He discovered that certain regions of human DNA contained sequences that repeated over and over contiguously, and that the number of such repeats differed from individual to individual. By developing a technique to examine the length variation of these repeat sequences, Dr. Jeffreys devised the ability to fix the identity of individuals with a high degree of certainty. The technique developed by him came to be called restriction fragment length polymorphism (RFLP). This really triggered the era of forensic biotechnology, which has since moved at an amazing pace, and continues to do so, impacting virtually every area of forensic investigation of serious crimes such as homicide, rape, and assault. This rapidly developing science requires that every professional, practitioner, or student of forensics, criminalistics, and indeed biotechnology, constantly updates himself and keeps abreast of the latest developments. This review aims to present the current status of biotechnological methods in forensic investigations, and the evolving techniques of the future.

Actually RFLP technology was first described by Southern in 1975, though Jeffreys deserves credit for describing its use with hypervariable loci, and recognising its potential for forensic identification purposes.[1] Jeffreys' multilocus probes, which created a bar code-like pattern, gave rise to the term 'DNA fingerprint'.

In RFLP technology, DNA from a 'hypervariable region' or minisatellite' of nuclear material is broken up into fragments by enzymes (restriction endonucleases) that cleave the DNA strands at predetermined spots. The DNA fragments are then separated by gel electrophoresis, which spreads the fragments into bands, transferred to nylon or nitrocellulose sheets, and subjected to a 'DNA probe'. This is a single strand of DNA labeled with radioactive phosphorus-32. This latches on to the separated minisatellite fragments and – by autoradiography – the position of each band is rendered visible on an X-ray plate.[2] The end result is a series of parallel bars, similar in appearance to the 'bar code' printed on goods in a supermarket. This bar pattern from multilocus probes then became supplemented, and later replaced by simpler patterns from single-locus probes, though the underlying principles remained the same. The characteristic of single locus RFLP is that the variants detected occur at a single gene or locus. It is detected by a probe derived from that same region of the genome.

RFLP is conventionally performed by detecting relatively long core repeats, in which the repeat is greater than 15 bp in length. The number of repeats may be anything up to about 10,000. The core repeat unit for a medium-length repeat (referred to as a 'minisatellite' or VNTR, i.e., 'variable number tandem repeat' is in the range of approximately 10-100 bases in length. The large number of alleles shown by VNTR loci, and the fact that well established population genetics and statistical theory may be used to calculate the probability that two individuals selected at random will have the same VNTR type has led to the revolutionary advances achieved in recent times in conclusively identifying sources of forensic biological samples.[3]

The great range in lengths of minisatellites used for RFLP renders them the most variable of forensic loci (and, therefore, the most discriminating) because so many different alleles are possible. Unfortunately, their length also means that they are destroyed when DNA is broken up by UV light, microbes, etc., as often happens to forensic specimens.[4] Also, because it is only the original sample DNA that is detected, a relatively large amount (at least 5 to 10μg) must be obtained. This means that RFLP may not be successful in forensic samples such as a single hair, a drop of dried saliva, or a post-coital swab.

Because of this limitation, as well as other drawbacks (high expense, labour intensive), it has largely been supplanted over a period by polymerase chain reaction (PCR) based DNA analyses. The only exception is minisatellite locus D1S80 which has a repeat core only ~220 to 650 bp in length, and which can be amplified by PCR prior to electrophoresis. This variation on VNTR analysis is called 'amplified fragment length polymorphism (AmpFLP)'. New polymorphic loci were identified for this purpose, and since the small fragment sizes necessitated a stiffer electrophoretic medium than agarose, acrylamide gels were introduced. By this technology, discrete alleles are easily determined without ambiguity. But longer fragments that are likely to get degraded may not amplify well, resulting in preferential amplification of shorter fragments, which is the main problem with AmpFLP.

Evolution of polymerase chain reaction-based DNA testing: PCR testing began virtually simultaneous to RFLP, but its potential was not recognised in the first few years, mainly because of the poor discriminatory power of early PCR-based tests. However, because of rapid advances in recent years, PCR-based DNA testing quickly became the method of choice, and by the year 2000, RFLP was virtually eased out of the forensic domain.

The early PCR-based tests involved 'reverse dot blot technique', which depended on the development of a colour reaction from probe hybridisation on a filter membrane. Such tests analyse sequence polymorphism rather than length polymorphism. PCR-based tests quickly began to get popular owing to a number of advantages over RFLP: availability of commercial kits, rapid results, high degree of sensitivity, and applicability to degraded DNA. However, the discriminatory power of reverse dot blots was poor, and interpretation of mixed stain specimens and weak dots were often problematic. Prof Jeffreys stepped in with a method utilising PCR to take advantage of the polymorphic potential of traditional RFLP loci, which came to be called 'minisatellite variant repeat (MVR) mapping'. However, this technology never really took off, even though it continues to be used in sporadic cases.[5,6]

Fluorescent dye labeled inter simple sequence repeat-polymerase chain reaction: Non-anchored inter simple sequence repeats (ISSRs) are arbitrary multilocus markers profiled by PCR amplification with a microsatellite primer. No prior genomic information is required for their use. It is a stable technology across a wide range of PCR parameters; hence these markers have been used in DNA fingerprinting.[7] The sensitivity, speed, and informativeness of the ISSR-PCR method can be enhanced substantially by using fluorescent dye labeled nucleotides in the ISSR-PCR reaction (FISSR-PCR), followed by separation of PCR products on an automated sequencer.[8]

Short tandem repeat analysis: The need for smaller target regions resulted in the development of Short Tandem Repeats (STRs). STRs are a species of AmpFLPs in which the core repeat units are 3-7 bp in length ('microsatellites'). The usual AmpFLPs such as D1S80, contained long tandem repeats in which the core repeat units were larger than STRs.

Though STRs are shorter than VNTRs, they are more numerous, and bear approximately 8 to 10 alleles per locus. Using the 13 STR loci recommended by the FBI, the average match probability is less than one in a trillion.[9] In STR analysis, the DNA is extracted, quantitated, and then amplified by PCR. Identification of STR loci is performed by gel-based electrophoresis methods utilising fluorescent dyes, or by capillary electrophoresis using laser-induced fluorescence detection. The main advantages of STR typing include simplicity, rapidity, capability for testing very small quantities of DNA, and amenability to automation. The main disadvantage is that, because the method is extremely sensitive, it is susceptible to contamination.

The Forensic Science Service in the United Kingdom pioneered efforts to create robust and powerful multiplexed sets of STRs, and subsequently applied them to casework and databasing applications. In the US, the FBI sponsored an STR working group, which advocated the use of 13 core STR loci performed in two amplification reactions for submission into the Combined DNA Index System (CODIS) database. Manufacturers accommodated the community by developing kits, which responded to this need. Today these STR loci have replaced for the most part all other forensic DNA tests, and have become the community standard for routine identity testing in most parts of the world. The commercial STR core loci kits currently in use by forensic DNA laboratories include amelogenin, which is strictly not part of STR system, but a gender marker. Amelogenin is useful as a gender determinant because the locus is a different size in X- and Y-chromosomes. Thus, two bands on a gel or electropherogram indicate a male, while a single band indicates a female.

STR typing has been used (and continues to be used) successfully in hundreds of forensic cases from across the globe, even in samples such as bone,[10] tooth,[11] fingernail clippings,[12] postal envelopes and stamps[13], toothbrush[14], adhesive tape,[15] and even blood crusts from firearm projectiles.[16] STR typing has also been attempted with varying degrees of success in the identification of decaying corpses,[17] ancient remains,[18] and dead bodies recovered from damp environments,[19] even from under the sea.[20]

Randomly amplified polymorphic DNA: Since most DNA applications in the early years had been developed for the specific detection of human DNA, only a few VNTRs of invertebrate DNA were known. This limitation was overcome by a new technique that could be used on virtually any organism: randomly amplified polymorphic DNA (RAPD). In this method, non-specific primers are used that can amplify many regions of a sample DNA at once. The resulting PCR products are separated by electrophoresis, and a ‘band’ or ‘peak’ of a particular length can be considered a locus even though it is not known what portion of the sample DNA it represents. RAPDs can allow up to 100 or more loci in one PCR. Since the high number of amplified RAPD loci can render the sorting of informative PCR polymorphisms from non-informative ones difficult or confusing, specialised electrophoresis unit and software programme must be used.[21] In the forensic area, RAPD has special importance in the entomological investigation of decaying corpses.

Mitochondrial DNA analysis: In comparison to nuclear DNA, mitochondrial DNA (mtDNA) has some significant advantages in forensic investigations. Firstly, it is present in high copy number, and can provide better results when nuclear DNA is scanty, e.g., analysis of hair shafts, teeth, skin, etc.[22] Secondly, mtDNA is transmitted exclusively maternally to the offspring without undergoing recombination. This clonal inheritance is of great use in identity testing because it allows direct comparison of DNA sequences of relatives with the same maternal lineage, without the ambiguities caused by meiotic shuffling and the mixing of nuclear genes.[23] In fact, when the sample sequence is compared to that of a reference person, the possibility of a maternal relationship can be assessed. One significant disadvantage of mtDNA has been that compared to nuclear DNA, the genome organisation is very compact and, therefore less polymorphic: over 90.0% of the genome is coding, introns are lacking, intergenic sequences are very small or absent, and repetitive classes of DNA are relatively uncommon. For forensic DNA testing, the most extensively studied region of mtDNA has been the non-coding DNA replication control region (‘D-loop’), located between the genes for $tRNA^{Pro}$ and $tRNA^{Phe}$, at positions 16,024 to 576. mtDNA has been used with great success in the forensic analysis of bones and historical or ancient remains.[24,25] However, amplification of mtDNA D-loop fragments with a length of 200 bp or more from ancient and even from fairly recent biological samples, can lead to erroneous results. Use of short PCR fragments for the analysis of mtDNA from shed hair, in combination with a competitive PCR assay to determine the state of degradation, should improve the reliability of forensic mtDNA analysis considerably.[26] Due to the

erroneous database collection, the validity of sequence analysis of the mtDNA-loop hypervariable regions for anthropological information about the maternal lineage has been questioned in many cases.[27] To avoid this, recommendations and guidelines have been proposed for the validity of mtDNA sequence analysis and their interpretation in the forensic context.[28]

Since heteroplasmy (same individual harboring more than one mtDNA sequence) is a potential drawback to forensic mtDNA analysis, newer methods have focused on overcoming this problem by enhancing detection capability of this phenomenon, for e.g., denaturing gradient gel electrophoresis (DGGE). Several other technologies are also now being applied to mtDNA analysis to make it more popular among the forensic community, including mass spectrometry, microchip instrumentation, and molecular beacon analysis.

Y-chromosome markers: There has been an increasing interest among forensic investigators, in Y-chromosome markers, not only for gender determination, but also for identity fixation. Y-chromosome markers are useful for discriminating male DNA from female DNA in forensic situations such as sexual assault, when a vaginal swab is submitted for DNA analysis. However, the amplification of Y-chromosomal STRs is also known to result in the formation of artefactual amplification products, mainly due to insufficient PCR specificity. This is a major drawback of the method, as both the sensitivity as well as the correct Y-STR interpretation are affected. The addition of a PCR enhancer to the reaction master-mix is claimed by some investigators to result in a significant increase of specificity of Y-STR typing.[29] Y-STRs are also useful for tracing paternal lineages, just as mtDNA is used to match maternal lineages.[30]

Alu repeats: The Alu family of short interspersed nuclear elements (SINEs) is distributed throughout the primate lineage and is the predominant SINE within the human genome.[1] The Alu family has spread throughout the genome by an RNA-mediated transposition process known as 'retroposition' and is present in the genome in extremely high copy number (in excess of 500,000 copies per haploid human genome). The majority of Alu family members are pseudogene products of a single 'master' gene. Sequence divergence in the master gene and its progeny occurs with time, resulting in subfamilies. Young Alu subfamilies are polymorphic and are present or absent on given chromosomes. The first appearance of the Alu insertion represents the beginning of the family tree, and can be used as a molecular clock to estimate the time that family or subfamily arose. Thus, unlike other forensic DNA markers, the distribution of Alu insertions, and possibly long interspersed nuclear elements (LINEs) and other SINEs loci, permit tracing of population ancestral heritages.[31] Information about the likely ethnicity of the sources of the sample is one piece of information that investigators may use when pursuing leads based on the genetic analysis of crime scene evidence.

GOING INTO THE FUTURE

Single nucleotide polymorphisms: Single nucleotide polymorphisms (SNPs) represent the ultimate in the trend toward smaller DNA fragments. Recent advances in SNP research have raised the possibility that these markers could replace the forensically established STRs. SNPs are more numerous than other polymorphisms, and occur in coding and non-coding regions throughout the genome. They are single base-pair changes in the DNA sequence, which can be detected by sequencing, RFLP-PCR or single-strand conformational polymorphism (SSCP) techniques. A set of SNPs decoding identification of an individual demands only a short stretch of DNA (<100 bp) for analysis. This is of great advantage over the conventional methods in genotyping highly degraded forensic and archaic samples. The presence of 1.8 million SNPs in the human genome makes it even more attractive for forensic investigations.[32] The most important attribute of SNPs is their suitability to new automated instrumentation platforms, especially mass spectrometry and microchip instrumentation, as well as in-solution techniques such as molecular beacon and fluorescence polarisation.

A single nucleotide polymorphism (SNP) multiplex has been developed recently to analyse highly degraded and low copy number (LCN) DNA template, i.e. <100 pg, for scenarios including mass disaster identification.[33] The multiplex consists of 20 autosomal non-coding loci plus amelogenin for sex determination, amplified in a single tube PCR reaction and visualised on a capillary electrophoresis system. As the multiplex is intended for use with samples too degraded for conventional profiling, a computer program has been specifically developed to aid interpretation. The discrimination power of the system is estimated at 1 in 4.5 million, using a White Caucasian population database. Reproducibility studies are claimed to have showed concordance between SNP profiles for different sample types, such as blood, saliva, semen and hairs, for the same individual, both within and between different DNA extracts.

However, some other recent reports demonstrate that a battery based exclusively on SNPs, matching the informative power of current STR kits, if applied to routine paternity investigation, would be statistically inadequate.[34] The current consensus is that the introduction of an SNP-based strategy, as a substitute to the now classical STR approach can pose statistical problems that must be carefully evaluated.

EMERGING INSTRUMENTATION

Microarrays: One of the most powerful new technologies to emerge from the age of genome sequencing comes from the tiny microarray slide, carrying the capacity to comparatively scan genome-wide patterns of gene expression for any organism with a sequenced genome[35], or expression profiling to measure allele-specific expression.[36] First developed in research laboratories examining model organisms (yeast, mustard), microarrays are now being used worldwide to study everything from cancer biology and drug development to the evolutionary biology of microbes and functional studies at molecular level.[37] One highly anticipated application that has tremendous potential in forensic science has been the rapid determination of genotype using oligonucleotides arrays. Individuals in any population display differences in phenotype (traits), and currently it is very difficult to identify the specific genetic makeup (or genotype) that determines any given phenotype. Ultimately scientists need to follow the segregation of each gene as it passed from one generation to the next, and establish a correlation between traits and the alleles of every gene. Traditional strategies for genotype determination have been laborious and limited, scanning hundreds or a few thousand genetics markers to crudely examine the genotype of each individual at relatively low resolution. The markers being used in these newer genotyping strategies are at the level of single nucleotide polymorphisms (SNPs), which occur at high frequency in the genome, about every 1,000 base pairs.[38] If all SNPs for each individual in a pedigree could be determined, researchers could follow genetic information at high resolution as it is passed from generation to generation. But determining over a million SNPs for each sample is a daunting task. To offer high-throughput determination of SNPs, oligonucleotide microarrays have been developed for the rapid and accurate analysis of genotype.[39] Perlegen Sciences (Mountain View, CA) and Affymetrix have collaborated to develop microarrays for the detection of SNPs in humans. Introduced in 2001, the first generation of these tests (GeneChip® HuSNP) examines 1,500 SNPs for each DNA sample. Using a manufacturing process that has been adapted from the semiconductor industry, Perlegen Sciences is now developing a protocol that will utilize tens of millions of probes on a glass wafer to characterise 1.5 million SNPs for each individual sample.

Mass spectrometry (MS): Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has been explored widely for DNA sequencing.[40] It can perform genetic analysis within seconds. With robotic sample preparation and sample loading, literally thousands of analyses can be accomplished in a single day. Compared with gel electrophoresis-based sequencing systems, MS produces a very high resolution of DNA-sequencing fragments, fast separation on microsecond time scales, and completely eliminates the compressions associated with gel electrophoresis. The high resolution of MS allows accurate mutation and heterozygote detection. MALDI-TOF MS is especially suitable for SNP analysis.[41] Future improvements in detector sensitivity for large DNA fragments in MS instrumentation will further improve MS for DNA sequencing.

Today DNA analysis in various forms is extensively applied in various types of criminal investigations all over the world. As techniques for manipulating and analysing DNA become increasingly sophisticated, forensic DNA testing will keep improving. Currently, using a wide array of techniques, DNA can be used almost conclusively for identifying individuals. Also, depending on the quantity of sample available and the extent of its degradation, various techniques can be applied to fix a person at a crime scene. An additional advantage of DNA testing is the ability to reinvestigate previous cases (‘cold cases’), as well as review cases, which were decided primarily by older, less conclusive tests. In the latter, DNA techniques can be used to reanalyse material that may provide previously convicted individuals an opportunity for acquittal. It is clear that DNA technology is here to stay and will continue to help forensic investigations, though it will keep advancing at a rapid pace, necessitating constant updating of relevant information on the part of investigative agencies, forensic professionals, and biotechnologists.

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