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Review

Human Leukocyte Antigen alleles as an aid to STR in complex forensic DNA samples

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ABSTRACT

Human biological samples with multiple contributors remain one of the most challenging aspects of DNA typing within a forensic science context. With the increasing sensitivity of commercially available kits allowing detection of low template DNA, complex mixtures are now a standard component of forensic DNA evidence. Over the years, various methods and techniques have been developed to try to resolve the issue of mixed profiles. However, forensic DNA analysis has relied on the same markers to generate DNA profiles for the past 30 years causing considerable challenges in the deconvolution of complex mixed samples. The future of resolving complicated DNA mixtures may rely on utilising markers that have been previously applied to gene typing of non-forensic relevance. With Massively Parallel Sequencing (MPS), techniques becoming more popular and accessible even epigenetic markers have become a source of interest for forensic scientists.

The aim of this review is to consider the potential of alleles from the Human Leukocyte Antigen (HLA) complex as effective forensic markers. While Massively Parallel Sequencing of HLA is routinely used in clinical laboratories in fields such as transplantation, pharmacology or population studies, there have not been any studies testing its suitability for forensic casework samples.

1. Issues with challenging DNA samples

Advancements in DNA recovery and processing has led to the development of highly sensitive STR kits [1,2] combined with the introduction of analysis methods designed for low template DNA samples [3–6] has made obtaining DNA profiles easier, quicker and more efficient even from trace amounts of DNA material. However, at the same time, those advances have introduced new issues. Analysis of low template samples has revealed many problems with relying on STR in human identification since STRs are very prone to stochastic effects, particularly when low copy samples are involved. Stochastic effects are random fluctuations occurring during early cycles of PCR amplification that manifest as variations in the results from repeated analyses of the same sample. Amplification of the same source material may therefore result in different alleles being observed at a particular locus. Two examples of the outcome of stochastic effects are allelic drop-out and allelic drop-in. When dealing with low template DNA, the sensitivity of the analysis needs to be increased [3] by increasing the number of PCR cycles [7,8]. However, the minute amounts of source DNA combined with the increase of cycles will often lead to allele drop-out (failure to amplify a single allele) or locus drop out (loss of both alleles) [3]. An allele drop-out is particularly problematic as the follow-up analysis gives what appears to be a complete DNA profile when in fact half of the genetic information is missing. On the other hand, allele drop-in results in appearance of an extra allele, unrelated to the source profile. Interpretation of a single source DNA profile with allelic drop-ins may lead to a false conclusion that the tested sample is a mixture. Another common result of stochastic effects occurring during PCR amplification is the presence of stutter peaks in the analysed profile. The mechanism behind the generation of stutter peaks is thought to be polymerase slippage leading to slipped strand mispairing (SSM) [9]. A stutter peak may appear one repeat unit before the true allele (caused by a deletion of one repeat unit) or one unit after the true allele (insertion of a repeat unit into the new strand) [10]. The most common scenario is the appearance of a new strand which is one repeat unit shorter than the parent strand [11]. Although there have been reports of stutter peaks being two or more repeat units smaller or larger than the true allele [12].

Currently there are no methods to eliminate the issues with STR profiling of low template DNA source material. One of the proposed solutions to avoid misinterpretation of DNA profiles is simply not to analyse and profile samples with high probability of stochastic effects [13]. This 'stop testing' approach however is not free of flaws. First of all, it can significantly limit the evidence available at a crime scene,

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although the most common types of DNA evidence found at scenes of crime are usually sources with a potentially high DNA content such as blood [14], there are instances where the only available biological evidence is present in the form of a low template, trace DNA.

The other major issue with STRs is difficulty in interpretation of the results from mixed samples containing DNA from several contributors in various proportions. It can be especially tricky in cases where the major DNA contributor masks the presence of minor contributors. This is caused by the fact that the STR primers are designed for specific loci. Two or more contributors can have alleles from the same locus with different numbers of repeats. In cases with highly unbalanced mixtures, the STR markers will not be able to differentiate between the alleles from the same locus. The PCR-based analytical method generally allows for the detection of a minor DNA contributor in an unbalanced mixture only when its contribution is more than 10% of the total DNA content. However to be able to detect all the minor alleles, the minor contributor has to be at least 20% of the total DNA [15]. Low template DNA samples with many contributors are now part of many forensic investigations, still their interpretation remains challenging [16]. Despite various tools developed to help with deconvolution of mixed samples [17–22], a recent report published by Butler et al. [23] showed some worrying results. As part of interlaboratory study in 2013, the National Institute of Standards and Technology provided the same data from the profiling a DNA sample with multiple contributors to 108 laboratories across United States and Canada. The sample was accompanied by a mock crime scene scenario; the DNA evidence was recovered from a ski mask found near the scene of bank robbery. Along with the evidence profiling results, all the laboratories were sent profiles of three potential suspects (A, B, C). Out of 108 laboratories taking part in the study, 74 (69%) determined that all three suspects contributed to the DNA mixture. When in fact suspects A and B were contributors to the mixture; suspect C however was not part of the mixed profile. In a real life scenario, such a mistake in profile interpretation may result in an arrest of an innocent bystander.

The relatively high rate of mutation of STRs adds another limitation to their reliability in human identification [24]. Those mutations may cause major difficulties in kinship analysis, as they can lead to the combined kinship index (CKI) or combined paternity index (CPI) not reaching the required threshold. In consequence, additional STR markers have to be applied. This involves additional costs, workload and time spent on the analysis, without any guarantee that the additional markers will prove to be sufficient.

2. Current methods of sample deconvolution

With current methodologies, mixture deconvolution using STR markers alone is not as reliable as would be desirable. Therefore the investigation focus has shifted into exploring other markers as a potential aid to STR in complex mixtures.

An example of such is deletion/insertion polymorphism. Deletion/Insertion Polymorphism (DIP) is part of the human DNA length polymorphism group based on the insertion or deletion of one or more nucleotides, also known as indels [25]. The indels are responsible for approximately 16 to 25% of human DNA variation [26]. With the majority of sequences being shorter than 100 bp [27], DIP seems like a suitable tool for analysis of degraded DNA samples.

DIP's potential to aid forensic casework in relation to resolving the issue of mixed samples was first mentioned in 2011 [28]. The innovative method linked the deletion/insertion polymorphism with standard short tandem repeats (DIP-STR), utilising the polymorphic properties of both markers and therefore resulting in higher discriminatory power than STR markers alone. Despite the fact that on their own, the biallelic DIPs are associated with a low power of discrimination [15]. In order for DIPs and STRs to form a marker, the two loci cannot be independent from each other, have to be less than 500 bp apart and close enough together to make recombination events highly unlikely [29].

The DIP-STR markers are not only highly polymorphic and located throughout the whole genome [15], but are also very easy to genotype as the typing method is the same as it is for standard STR profiling [30]. The markers have been successfully applied to resolve extremely unbalanced DNA mixtures with two contributors, producing a high resolution profile from a donor with only 0.1% of contribution to the analysed DNA mixture [28]. Targeting DNA of the minor contributor for the alleles missing in the major donor's profile is possible due to variations in DIP sequence that allow for designing two allele specific primers, one for the deletion (S-DIP) and one for the insertion (L-DIP primer) [28]. To test the utility and feasibility of the marker, Castella et al. [15] applied the system to an unbalanced DNA mixture from a homicide case. The collected evidence pointed to three potential suspects; a man and his two sons, each from a different mother. The tested samples included a biological stain recovered from the victim's body and reference DNA samples from the three suspects. The initial analysis was done with the standard autosomal kit and the generated results pointed out to just one female STR profile with no traces of a minor male DNA contributor. Only after applying Y chromosome specific markers, the researchers were able to obtain one Y chromosome profile. However with all 3 suspects being part of the same paternal line, their Y chromosomes were practically identical hence the profiling results had no discriminatory power to distinguish between the individuals. Only after DIP-STR typing, Castella et al. detected a haplotype matching only one of the three suspects.

A few sets of the DIP-STR markers have been developed and tested for a variety of samples including 'touch' DNA [30,31]. These DIP-STRs markers are suitable for typing from degraded and cell-free DNA and produce fewer PCR artefacts, including stutter peaks. Thanks to the high degree of polymorphism for each DIP, the combined markers have greater chances of identifying the alleles unique to the minor contributor in an unbalanced mixture [30] and consequently help to determine the number of contributors in a mixed sample. The application of DIP-STR in real forensic cases shows clear advantage of the combined markers over standard STR and Y chromosome typing when it comes to unbalanced DNA mixtures. Where STR markers failed to detect any contribution of a minor donor in the mixture and Y-STR marker was not able to differentiate between the related male suspects, the DIP-STR marker set has emerged as a promising tool for challenging samples. With the ability to utilise already established methods and analytical techniques, the combination of DIP and STR markers can be easily implemented in forensic laboratories without any extra costs involved.

Other potentially useful markers can be found in single nucleotide polymorphism (SNP) substitutions. A SNP is defined as a single nucleotide variation between individuals. Such a variation occurs at a specific point in the DNA sequence. These polymorphisms are thought to be responsible for 90% of all of human sequence variants [32]. The nucleotide variants are very common in the human genome, occurring on average once in every 100 to 300 nucleotides [33]. Due to their abundance, SNPs are widely used in disease related gene mapping [34].

There are several characteristics of SNPs that make them useful markers for forensic analysis. First of all, when amplified, their PCR products are less than 100 base pairs in length, which means that they are the perfect size markers for analysing highly degraded DNA samples. For comparison, the standard STR amplifications generate amplicons that are about 300–400 bp long [35]. Another advantage of SNPs over STR markers is their low mutation rate, making them valuable markers for paternity tests as well as other kinship analyses [36]. Unlike STR typing, profiling of SNPs does not produce any stutter peaks [37] thus allowing for easier result interpretation. Additionally, some of the tetra-allelic SNPs are close in their discrimination level to simple STRs (CSF1PO and TPOX) and can be easily amplified from much shorter sequences [38].

Unfortunately due to their binary nature, the majority of SNPs are unable to detect more than one contributor in a mixed sample [39].

However, as shown by Westen et al. in their study from 2009, applying the tri-allelic SNPs to a 2 donor sample can reveal the presence of a second donor with a ratio up to 1:8 [40]. The authors suggest that the second donor presence is detected by the appearance of a third allele on one locus, unexpected peak height ratios and/or peaks above the detection threshold. Still, their experiment was conducted in a controlled lab environment with a known number of contributors. When analysing a crime scene sample with an unknown number of donors, it would not be possible to determine the contribution of more than two individuals. Similar to the tri-allelic SNPs, the tetra-allelic SNPs system only allows for the detection of two-contributor mixtures [38].

Fortunately, as in case of DIP markers, the forensic potential of SNPs can be increased by linking them with STRs. In 2013 L. Wang et al. introduced a new method for simultaneous analysis of short tandem repeats together with single nucleotides called SNP-STRs [41]. The combined markers along with allele specific primers allow for detection of a minor contributor in a mixed sample and have a higher sensitivity than the standard STR markers alone. Additionally, SNP-STR typing does not require any additional genotyping techniques and can be analysed using methods routine for STR profiling. In their follow-up study, L. Wang et al. suggested that typing of SNP-STR is more efficient than applying the DIP-STR combination when dealing with unbalanced mixtures [42].

Furthermore, SNP based markers known microhaplotypes have been recently emerging as potential tool in mixture interpretation [43,44]. Micorohalpotypes (microhaps) consist of two or more SNPs located within less than 200 base pairs from each other [45]. Those closely linked SNPs can have multiple allelic variations, however due to the short distance between SNPs, the chances of recombination are very low [46]. Heterozygosity of microhaps can be influenced by various factors such as rare cross over events, accumulation of allelic variants at different locations within the region of interest throughout time and random genetic selections and drifts [46]. Similarly to SNPs, microhaps contain fewer alleles in comparison to STR. Therefore, in order to reach comparable level of information, a high number of microhap panels are required. Such panels are being developed and tested against unbalanced mixtures [43,44] with promising results when combined with Massively Parallel Sequencing (MPS), a technique capable of sequencing whole human genome. Additionally, microhaplotypes have proven to be the highest performing markers in ancestry analysis when compared against SNPs and indels [47]. Their application and benefits to forensic science has recently been extensively reviewed in other publications [48,49] and therefore will not be considered further in this review.

3. Human leukocyte antigen

The Human Leukocyte Antigen (HLA) system known also as the Human Major Histocompatibility Complex (MHC) consists of more than 20,000 identified alleles [50] located on chromosome 6p21. This complex has been found to be the most polymorphic gene system discovered in the human genome [51]. Apart from being highly polymorphic, the system is also characterised by a high linkage disequilibrium and high density of genes [52]. The role of the HLA system is to help the body's immune system distinguish between its own proteins and the foreign proteins made by viruses and bacteria.

The HLA complex can be categorised in three main groups; Class I, Class II and Class III. Class I consists of the three main subgroups: HLA-A, HLA-B and HLA-C with 4200, 5091 and 3854 identified alleles per group, respectively [50]. The other three subgroups, HLA-E, HLA-F, HLA-G contain less than 100 described alleles each [50]. The proteins produced by the genes from Class I are transmembrane glycoproteins (antigens) located on the surface of every nucleated cell. These glycoproteins bind to the peptides that are derived from the protein breakdown system of the cell. If the immune system recognises those peptides as foreign (i.e., sourced from a bacteria or virus), it triggers the immune response and the infected cells are targeted for destruction. Out of all three classes, Class I is the most polymorphic [53] with HLA-B genes showing the highest polymorphism [52], consisting of over 5000 identified alleles [50]. The majority of the HLA alleles identified so far belong to Class I (13,324) [50,54]. Class II molecules consist of DP, DQ, DR, DM and DO gene groups. These gene groups create the following allele groups: HLA-DPA1 & HLA-DPB1, HLA-DPA2 & HLA-DPB2, HLA-DQA1 & HLA-DQB1, HLA-DMA & HLA-DMB, HLA-DOA &HLA-DOB, HLA-DRA, and HLA-DRB1 to DRB9. The majority of alleles in class II belong to HLA-DRB1 (2165 alleles) and DQB1 (1196 alleles) [50]. The Class II proteins are expressed on B-lymphocytes and monocytes where their role is similar to that of Class I. The Class III group does not encode any HLA molecules [55] but it contains many genes involved in the inflammatory responses [56].

Genes of the HLA system are closely linked together and as a result, the whole complex is inherited as a haplotype. However, there are possibilities for random recombination within the haplotype. In the majority of the cases, the distance between genes on the chromosome determines the chances of such an event. As documented by Martin et al. [57,58] recombination rates within HLA class II region (between DRB1 and DPBI/DPB2) were 0.74% and 0.94% for class III region (between HLA-B and DRB1 loci). Surprisingly the recombination rate for class I, defined by the authors as the region extending from HLA-A to HLA-B (1.4 Mb), was only 0.21%. The determined value was much lower than expected considering the distance between both genes. Based on the observed results, the authors suggest that some haplotypes may be more prone to recombination than others and that the reported recombination rates indicate a non-random pattern of recombination. Since their study, instances where recombination within HLA haplotype has occurred within the same family have been documented [59,60]. Generally, the inheritance is ruled by Mendelian law, with one HLA haplotype coming from each parent. Two children from the same parents have 50% chance of sharing one haplotype (haploidentical), 25% chance of having no haplotypes in common and 25% chance of being genotypically identical [55]. In case of unrelated individuals, the possibilities of random combinations of HLA antigens on a haplotype are immense.

Variation and polymorphism of the HLA complex has played a crucial role in studying population migration [61] as well as being used to establish ethnic genetic database and linkage analysis [62]. The HLA proteins are responsible for mediating immune responses and therefore also play a very important role in organ transplantation [53,54] as they are unique for every unrelated individual [63]. HLA testing, also known as tissue typing, is primarily used to determine the compatibility between an organ donor and organ recipient by identifying the antigens on their blood cells [53].

Historically, HLA typing was done by serological testing, where antibodies were used to distinguish between different variants of antigens. Each antigen had its own specific antibody and the HLA serotyping allowed determination of the degree of match between the donor and recipient serotypes. The higher the number of the identical antigens between the potential donor and recipient, the more likely it is that the transplant will be successful. This method however is limited by the number of antibodies available for the specific HLA antigens.

Standard HLA serotyping has recently been replaced by superior molecular testing techniques, allowing for more alleles to be identified. HLA typing is currently done by a combination of PCR amplification and the analysis of the genomic DNA [54]. There are three main types of DNA-based typing [64]; low resolution, characterised by being able to define a gene subtype by allocating first two digits for the gene alleles (Fig. 1.), high resolution which can be defined as a set of alleles coding for the same protein but with different DNA sequences, and finally the allelic resolution, sensitive enough to determine single alleles in the coding sequence. The level of required resolution may depend on the type of transplant.

The current typing technologies include sequence-specific

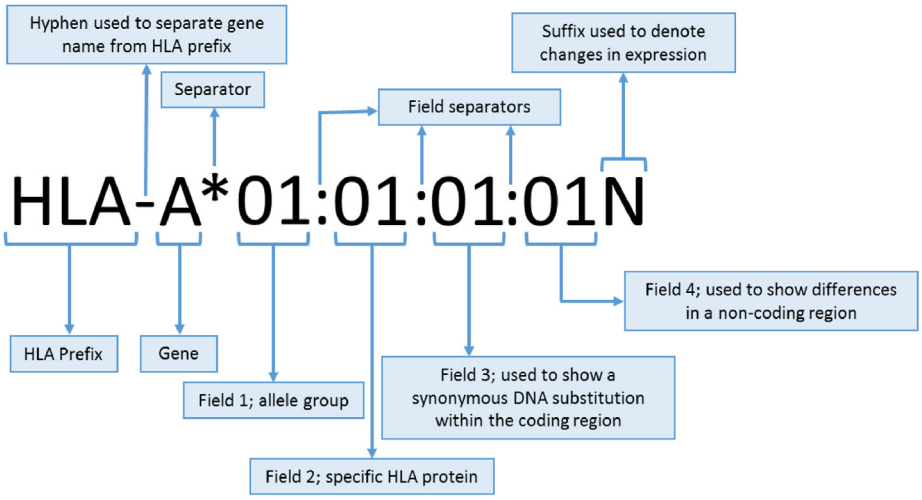

Fig. 1. New HLA nomenclature system adopted in 2010 (S.G.E. Marsh, HLA Informatics Group, http://hla.alleles.org/nomenclature/naming.html).

oligonucleotide probes, sequence-based typing (SBT), PCR restriction fragment length polymorphism (PCR-RFLP), and sequence specific primer amplification and micro-array techniques [62]. Of these, the micro-array techniques have proven to be the most efficient for detecting all the allele variants [62]. The micro-array method has made it possible to analyse over 1000 HLA alleles on a single microarray slide [62] while using a small sample volume [65]. As of late, HLA alleles are being sequenced with high throughput MPS [61,66–68]

4. Application of the human leukocyte antigen complex in the field of forensic science

The potential of the HLA system for forensic DNA typing has not yet been fully evaluated as the majority of the conducted studies focus on medical applications such as tissue transplant and links between HLA alleles and disease states [69]. Based on previous applications it is safe to assume that the most important characteristic of the HLA complex, which could be utilised in forensic casework, is the high genetic polymorphism within the antigen peptide-binding region. Exons 2 and 3 within HLA class I and exon 2 within class II are the sequences that encode the peptide antigen binding site of the HLA proteins [50]. Due to the high degree of polymorphism, these sections are sequenced in majority of non-forensic related applications [70], and are also the main regions of interest in forensic studies.

Of the studies focusing on the potential utilisation of HLA sequences in forensic science the majority [62,71–78] predate the development of MPS technology. Interestingly, the first ever PCR-based DNA kit was developed for detecting a variation at class II HLA gene DQA1 and released in 1990 [79]. The kit was successfully evaluated by forensic laboratories for its suitability for common forensic samples [77,80]. The DQA1 based PCR tested positively against a variety of forensic specimens such as blood, hair and tissue [74], bloodstains exposed to various contaminants [76] and semen-containing materials [78]. HLA-DQA1 typing was also successful when dealing with mixed fluid samples (sperm and vaginal cells) from rape cases [81] and allowed for positive identification of severely burnt cadavers [75]. An example of a more recent study is an investigation conducted in 2006 by researchers from Shanghai [62]. The aim of their research was to evaluate the forensic potential of HLA-DRB1, the subgroup within Class II with the highest number of alleles (2146) [50]. The process of HLA based DNA typing is similar to standard DNA typing, in that the extraction of DNA and amplification by PCR is identical to the corresponding steps in STR based analysis, with the exception of the primer sets used. The follow-up micro-array hybridization allowed for a rapid and large-scale analysis. Even though the HLA systems are present in various animal species, the HLA typing proved to be human specific. None of the DNA samples extracted from the animal tissue (blood and muscle) produced PCR amplicons capable of hybridisation to the array. Sensitivity of the system was tested by using different DNA dilutions, ranging from 200 ng to as low as 10 ng. In their *'Evaluation for use in forensic identification'* section, the authors claim that the template DNA concentration of 10–15 ng was enough for the HLA-DBR1 typing. In the abstract however, they state that 15 ng is the minimal amount required for a successful genotyping. This suggests that any DNA concentration below 15 ng would fail to produce satisfactory results. The analysed dilutions reported are suitable for standard DNA typing but further sensitivity tests would be required to determine whether the method could be suitable for detection of low template DNA, ranging from 10 pg to as low as 0.25 pg. The method was also not applied to any samples with more than one contributor, making it impossible to predict whether it could be suitable for complex DNA mixture analysis. Nonetheless, the method proved suitable for typing from variety of materials. Apart from human blood used as the main source of DNA in the project, the other analysed sources included semen samples, cigarette butts and hair roots. All of which produced good HLA-DBR1 typing results.

The outcome of this study allowed the definition of a few further characteristics that could aid forensic DNA typing. First of all, the distribution of alleles makes the HLA-DBR1 system a useful tool in DNA typing. The analysed population data suggested that allele frequency did not seem to be in Hardy–Weinberg equilibrium. Significant differences were observed not only between people of different races but also within different ethnic groups of people belonging to the same race. Secondly, all the alleles specific to the HLA-DBR1 group can be detected using only one single PCR amplification. This is possible due to the fact that all the array oligonucleotide probes are between the two target primers. This feature makes it a valuable marker in cases involving disaster victim identification, when the only source of available DNA can be found in the form of highly degraded samples. Furthermore, due to its polymorphism and large-scale output with micro-array testing, HLA typing can be used in conjunction with standard STR typing, giving it a higher discriminatory power.

In another study from 2006 the sequence specific primer and probe typing of HLA was validated in forensic testing [71]. The study used a commercially available PCR-SPP (sequence specific primer and probe) HLA typing kit, capable of typing Class I and II HLA alleles from small amounts of DNA (10 ng) at low resolution by allocating the first two digits in the allele nomenclature system described above. Even though the highly polymorphic HLA loci can be very useful in forensic science, it has proven difficult to simultaneously profile alleles from Class I and Class II when dealing with low template DNA samples such as formalin-

fixed paraffin-embedded (FFPE) tissues, nail, hair pieces and other trace DNA evidence encountered during a forensic examination [71]. The source material used in the study consisted of DNA samples obtained from 1 μl of blood deposited on a piece of cotton cloth and stored from 1 week to 3 months, buccal cells and samples from real forensic cases in the form of blood stains that had been stored at room temperature for 18 years.

The results showed that full HLA typing was possible for DNA obtained from blood covered cloth at each stage of decomposition. The HLA-SPP typing system proved to be very sensitive and did not require isolation of the DNA prior to the PCR amplification. This is significant since by reducing the number of steps involved in DNA typing, the chances of sample contamination with any carryover reagent during the process of extraction are significantly reduced.

In a study conducted in 2003 [72], HLA typing with the PCR-SSP method was used to identify bodies using DNA obtained from aortic tissue. The aortas were collected from eight bodies, one of them was burnt, one was mummified and the remaining six were found in water. The attempt to identify the bodies was made by typing alleles from HLA-A, HLA-B, HLA-DR and HLA-DQ. The DNA was isolated from 1 mg of fixed tissue or from 10 to 20 mg of aortic tissue and the yield of DNA obtained ranged from 0.04 to 3.84 μg. The amounts were sufficient to type for multiple HLA loci and the obtained results were consistent with the alleles typed from the bones of the corresponding bodies. Additionally, extracting DNA from the aortic tissue proved to be easier than extracting DNA from teeth and bones, establishing the aortic tissue as a valuable forensic material. Unlike the two previous cases, this study attempted HLA typing from amounts of DNA much lower than 10 ng. The alleles from HLA-DR locus were successfully typed for every single case, even for the DNA content as low as 0.04 μg and 0.06 μg.

5. Potential of massively parallel sequencing in forensic analysis of the human leukocyte antigen complex

Before the introduction of MPS, high-resolution HLA typing was challenging due to the large number of alleles and patterns of polymorphism [82]. The standard SBT method was unable to determine phasing of sequences, often requiring application of complimentary methods [83]. Phasing of HLA sequences can be defined as determining haplotypes from alleles and mutations. Unlike the previous methods, MPS allows for thorough sequencing at a molecular level. MPS techniques are capable of detecting a single nucleotide variation. This feature is particularly important for sequencing of HLA genes, due to their high degree of polymorphism which is often dependent on point mutations.

As previously mentioned, everyone inherits a maternal and paternal set of the HLA alleles. Those alleles are co-dominantly expressed in a cell; therefore, it may be difficult using serotyping or low resolution allele determination to interpret the HLA typing data from both alleles together as genotypes with two separate alleles. The results that are consistent with more than one genotype are referred to as 'ambiguous data' [84]. This ambiguity is a consequence of the complexity of the HLA system and is considered to be a major challenge when typing and interpreting the HLA data using the standard sequence-based methods such as Sanger sequencing [84]. Resolving those issues would often prove complicated, expensive and time consuming [82].With the addition of MPS, typing of HLA without ambiguities became a possibility [85,86] since MPS enables detecting polymorphisms in unknown alleles alongside the already known alleles, novel alleles are still being discovered [83].

So far it has been proven that MPS allows for a successful typing of DNA with concentrations of 2 ng/μL [87] and since there is no need to test very low concentrations of DNA for clinical samples, further tests to determine suitability of the HLA typing for low template DNA are required. An important point to take into consideration when running a PCR plate is making sure that the samples with similar typing are positioned distant from each other. Increasing the distance between the potentially HLA-matched samples will reduce the chances of contamination [87]. However, it is only important in the first step of library preparation. The follow up PCR reaction involves tagging each sample with a unique molecular barcode, allowing the sequencer and software system to separate and isolate the data generated from each sample [88].

There are certainly some disadvantages of the MPS method. First and foremost, there are high costs involved in implementing MPS technology in a crime laboratory. The main costs involve an MPS instrument and kits that are much more expensive than the standard STR profiling kits. The average price for a reference sample is about £45 while a casework sample will cost around £65 however these costs are continuing to fall as general MPS use is increasing. In comparison the average price for standard STR analysis is about £14 to £19 [89]. Secondly, there are potentially longer turnaround times. With massive parallel sequencing, it can take 24 or more hours to obtain the relevant profiling information. In comparison, getting 20 CODIS markers can be achieved in 6 to 8 h [90]. Furthermore, an MPS analysis generates a vast amount of data. Each run produces approximately 18 gigabytes of data [87]. This in turn creates a need for specialist software to enable extraction of the desired information. The most cost-effective approach to MPS analysis could be triage of samples. The simple, straightforward samples with plenty of source material could still be analysed with standard STR techniques, while the complex, mixture and low concentration samples would be sequenced with high-throughput methods.

The potential disadvantages involved in the MPS technology should not stop researchers from exploring and utilising its full potential. No technique is free of faults and even if in the long run MPS proves to be unsuitable for routine use, there are always the standard methods to fall back on. However, as many recent publications confirm; massive parallel sequencing has certainly demonstrated its value as an emerging technology in many fields of science. Markers such as STRs, mtDNA and YSTR have already significantly increased their forensic value due to MPS [91]. While MPS of HLA is being routinely utilised in clinical laboratories in fields such as transplantation [92], pharmacology [93] or population studies [61], there have not been any studies testing its suitability for typical forensic samples. However a recent study by Wu et al. [66] showed promising results of applying HLA-based typing to kinship analysis. In the study HLA haplotypes of 24 individuals from the same family spanning six generations were determined. The HLA genes used in the study were part of a standard Illumina TruSight HLA typing kit (Illumina, Inc., San Diego, CA) and included HLA-A, -B, -C, -DRB1, -DRB3, -DRB4, -DRB5, -DPA1, -DPB1, -DQA1 and -DQB1. Alongside HLA typing, the samples provided by the family were analysed with 23 STR loci, 20 of which were CODIS core loci [94] and a sex marker. The resolution of HLA typing was conducted at field 4 level (see nomenclature above) which resulted in a total of 88 unique alleles in 24 tested samples 8 of which were not previously recorded in the IMGT/HLA database [50]. This is not a rare occurrence during an MPS analysis, as previously stated the technology allows for detection of novel polymorphisms as well as known at the same time [83]. The immense polymorphism of the HLA complex was further confirmed by a total of 21 different haplotypes found in 24 closely related individuals 3 of which were created due to random recombination, as described in the first section of this review and detailed by Martin et al. [57,58]. This generation of haplotypes by recombination events adds additional discriminatory power to kinship analysis and human identification. STR analysis revealed only two mutations, which required additional confirmation by MPS. Furthermore, the calculated likelihood ratio (LR) showed higher overall performance of HLA alleles when compared with STR markers in relationship testing. This novel study shows how HLA alleles may aid in complicated kinship and paternity analyses. Wu et al. [66] have provided an important starting point for reintroducing HLA genes back into forensic analysis. Paternity and kinship testing however are not the most challenging aspect of forensic identification, therefore

in order to fully introduce HLA as an aid to difficult forensic samples, additional testing requirements must be taken into consideration. First of all, the already existing HLA testing kits are not suitable for analysis of a typical crime scene sample with low DNA concentration. For instance, the TruSight HLA protocol recommends a DNA input of 400 ng and because the kit relies on the long-range PCR, the DNA should be intact with at least 50% of input DNA being greater than 10 kb. While such requirements can be easily fulfilled in clinical settings, they are not applicable to the majority of crime scene samples. In comparison, the minimum requirement for most common STR profiling systems is 0.1–0.5 ng [95]. The forensic application of HLA will certainly require implementation of a new approach with primers targeting shorter sequences. Instead of targeting the majority or even the whole gene as it is currently being done for clinical kits, it would be preferable to design primers targeting only Exons 2 and 3 which as mentioned before are the regions of highest polymorphism. The PCR products produced from these exons would range from 250 to 450 bp depending on the HLA allele and class which is an ideal size for creating MPS sequencing libraries without any need to further size select or process the PCR product. The only drawback of shorter target sequences would be the partially reduced chances of discovering novel HLA alleles. As with any other marker, a low amount of starting material can cause issues with analysis. However, unlike STRs, MPS of HLA would not be susceptible to stutter and would not as prone to stochastic effects since the read depth of the sequence data would clearly indicate any problems. The issues of low template or highly degraded DNA sample could still result in a lower level of resolution due to allele dropout, giving the sequenced alleles a lower power of discrimination.

The use of HLA to resolve forensic cases may seem questionable due to the coding nature of the markers. Wu et al. [66] comments on the potential of revealing external visible characteristics due to correlation of some HLA alleles with diseases as an aid to criminal investigation [66]. It is hard to imagine that this feature will be applied to forensic investigation in the near future due to the ethical considerations. One of the reasons why STRs are suitable identification markers is their location in the non-coding part of the human genome and as such are not capable of revealing any sensitive data about an individual. In view of the ethical considerations, the use of DNA for forensic purposes in some countries is currently restricted by law to neutral, non-coding markers only. This legislation however does not appear to be a barrier to the development of markers located in the coding part of the genome. An example of such are Forensic DNA Phenotyping (FDP) markers, based by default on non-neutral markers. Additionally, FDP markers include SNPs, many of which are located in coding parts of DNA such as SNPs associated with eye colour which are located within HERC2 gene group [96]. In fact, there is a whole European Consortium. VISAGE (Visible Attributes through Genomics) dedicated to development of new tools for FDP. Taking all of this into consideration, investigating HLA as potential forensic markers should not be viewed as controversial so long as the markers are used as a reference or comparison tool instead of being used to report any unsolicited findings, just as in the case of any other identification marker located in the coding part of the genome. Undoubtedly, introduction of any novel techniques and markers that may rely on analysis of the coding part of human genome will require a new set of standards governing the analysis, interpretation and use of the forensically relevant data.

6. Conclusions

Despite their limitations, STRs remain the most reliable and widespread markers used in forensic identification. Nonetheless, in cases where the conventional techniques fail to produce satisfactory results, there is a demand for alternative or supplementary markers to aid the analysis. There are already a notable number of markers that have been studied and applied alongside STRs, yet none of them has been studied as thoroughly nor have they acquired a status similar to common short tandem repeats. However, with the recent development of sequencing techniques, the focus should be shifting to the application of alternative and often more discriminative and polymorphic markers. To date, the scientific literature does not mention application of MPS technology in HLA typing with regard to forensic casework. All previous studies testing HLA alleles in forensic science were conducted before massive parallel sequencing became available which has limited the investigation of the full potential of the HLA markers in a forensic context. Based on the literature published until now, there is undoubtedly a gap in forensic research. Taking into account all of the past and successful applications of both HLA and MPS in forensic science, it is safe to assume, that when combined, they could create a very powerful method for future implementations in forensic analysis. At the same time, introduction of HLA alleles into the field of forensic identification should not be seen as a replacement but rather as an aid to already existing and widely used markers. With such high polymorphism and discriminatory power, addition of even a single HLA marker to any standard STR-based analysis can significantly increase chances of positive identification

Novelty statement

The alleles from human leukocyte antigen complex have a great potential to aid human identification in challenging forensic cases. With over 20,000 identified alleles, their polymorphism makes them unique and powerful markers that have a chance to revolutionise deconvolution of DNA samples with multiple contributors. While massively parallel sequencing is routinely applied to improve discriminatory power of standard and commonly used markers, not enough research is being dedicated to investigation of novel markers. The purpose of this review is to consider re-introducing HLA markers to the world of forensic science. We believe that with the power of high-throughput techniques, those markers have capability to become a valuable tool in DNA analysis and should be evaluated in forensic context.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scijus.2019.09.003.

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