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Mini Review

Emerging Trends of Next Generation Sequencing of Human Platelet Antigens in Transfusion Medicine

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ABSTRACT

Human Platelet Antigen (HPA) genotyping has played a pivotal role in platelet transfusion medicine for decades, including the characterization of immune mediated thrombocytopenias and the provision of HPA-matched platelets to these patients. Real-time PCR is currently considered the current gold standard method to detect and discriminate HPA alleles. Although readily available, cheap, and quick to perform, these methods are restricted to the detection of known Single Nucleotide Variants (SNVs) and confer disadvantages such as allele drop-out and an inability to detect novel, rare and inactivating alleles. NGS offers unique advantages that overcomes these pitfalls and more, however ongoing challenges such as cost, data storage, accurate variant calling and availability of technically trained staff means a large debate continues to exist about its widespread implementation. Despite the technical and clinical advantages of NGS over SNV-based methods, particularly in high-throughput settings such as donor, rare allele and prenatal screening programs, these challenges currently impede the implementation of NGS as a stand-alone technique for HPA typing.

Keywords: HPA; Platelet antigen; Genotyping; NGS; Next generation sequencing; Illumina

INTRODUCTION

Human Platelet Antigens (HPAs) are located on platelet-specific membranous glycoproteins and play an important role in coagulation, inflammation, and immunity [1]. Like Red Blood Cell (RBC) antigens, HPAs are immunogenic and may stimulate the production of alloantibodies if transfused into HPA-incompatible recipients, making them clinically significant in transfusion medicine [2]. 41 different HPAs spanning 7 genes have been described to date [3]. Of these, HPA-1a/1b, -2a/2b, -3a/3b, -4a/4b, HPA-5a/-5b and -15a/15b are recognized as being most frequently implicated in clinical settings and have thus been well characterized. Most HPA systems are biallelic, with high frequency alleles assigned as the 'a' allele, and low frequency alleles as the 'b' allele for that system [3,4]. HPA systems may also be assigned 'w' nomenclature (e.g., HPA-9w); in such cases, antibodies against only of the alleles have been described to date [4].

HPA alloimmunization may result from incompatible blood product transfusion or exposure to fetal HPA antigens recognized as non-self during pregnancy. Several clinical conditions have been associated with HPA antibodies, including Platelet Transfusion Refractoriness (PTR), Post Transfusion Purpura (PTP) and Fetal and Neonatal Alloimmune Thrombocytopenia (FNAIT) [5]. FNAIT is a rare but potentially serious condition characterized by varying degrees of thrombocytopenia due to maternally derived anti-HPA antibodies attacking paternally inherited HPAs in the fetus. HPA-1a is a high frequency allele in most ethnic groups (98% Caucasians, 90% Africans, 100% Asians) [4,6]. Individuals negative for HPA-1a (HPA-1bb) are able to make anti-HPA-1a antibodies and indeed, this antibody is responsible for 85% of FNAIT cases in Caucasian populations [6]. Most cases of FNAIT resolve within the first week of life, however some cases of FNAIT may progress into serious complications such as intracranial hemorrhage in utero and intrauterine fetal death [7]. The incidence of FNAIT

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is approximately 1 in 1000 pregnancies [8], however due to a lack of screening programs and international consensus on antenatal management of high-risk pregnancies, FNAIT is likely to be under-diagnosed [9].

Several Polymerase Chain Reaction (PCR) based methods have been developed to detect and discriminate between HPA alleles. These include restriction fragment length polymorphism PCR (PCR-RFLP), real-time PCR (qPCR), PCR using Sequence Specific Primers (PCR-SSP) and allele-specific oligonucleotide hybridization PCR (PCR-ASO) [10,11]. Although PCR-based methods are currently considered gold standard for HPA typing, they are restricted to the detection of Single Nucleotide Variants (SNVs) in known HPA systems, are somewhat prone to allele drop-out and are unable to identify novel, rare and inactivating mutations [10,11]. In contrast, NGS is a high-throughput, scalable and fast method of DNA sequencing that has quickly gained popularity amongst molecular scientists. The basic principle of NGS involves fragmenting purified DNA into multiple pieces, adding adapters, sequencing libraries, and reassembling the fragments to form a genomic sequence [12]. The length of the genomic sequence generated depends on the scale of the analysis. Whole Genome Sequencing (WGS), Whole Exome Sequencing (WES) and amplicon-based NGS all utilize this basic framework, but each associated with different run times, costs, complexity, and length of output data [13].

HPA typing by NGS provides information on nucleotides outside of the discriminating base for individual HPA systems. By providing the sequence of entire platelet glycoproteins containing HPA systems, NGS has the potential to identify novel variants, rare alleles, silencing mutations, weak phenotypes and naturally occurring chimerism [10,12]. Furthermore, the ability to multiplex and sequence hundreds of patient samples for platelet, leucocyte, neutrophil, and/or red blood cell antigens simultaneously confers a massive high throughput capability unparalleled by SNV-based typing methods [10,11,13]. The potential of NGS to replace current gold-standard methods as a stand-alone HPA typing platform is promising, however several technical and practical challenges continue to be major impediments to its implementation for routine use. This mini-review aims to provide a focused analysis of the advantages and challenges of NGS for HPA typing for routine clinical investigations and donor/prenatal screening programs.

LITERATURE REVIEW

HPA Genotyping accuracy: NGS vs. SNP-based approaches

Single nucleotide variants in regions outside of antigen determining positions of HPA alleles may affect primer and probe binding in PCR-based typing methods, potentially causing allele drop-out and false negative results. We previously published a systematic review and meta-analysis assessing the concordance between NGS and SNV-based genotyping methods for common HPA systems (HPA-1, HPA-2, HPA-3, HPA-4, HPA-5 and HPA-15) [14]. The pooled proportion agreement for the overall concordance of the 6 included studies was shown to be 0.998, 95%CI [0.995, 0.999], P<0.001. In this study, 2 out of 1587 genotyping events performed by NGS and SNV-based comparator were discrepant. These discrepancies were due to allele dropout in the qPCR analyses; NGS was shown to be more accurate when genotyping HPAs. For example, in two separate patients, NGS predicted heterozygosity (ab) for HPA-1 and HPA-15 respectively, whilst qPCR predicted homozygosity (aa) for these systems [10,15]. In these cases, silent SNVs within the primer and probe binding regions in the TaqMan qPCR assays resulted in allele dropout and failure to detect the HPA-1b/HPA-15b alleles [10,15].

The issue of allele drop-out is not unique to SNV-based genotyping methods such as qPCR. Indeed, amplicon-based NGS too utilizes a set of forward and reverse primers to capture, amplify and sequence genes or systems of interest [16]. SNV-based typing methods, however, utilize an additional probe for each HPA system over the discriminating base region, increasing the likelihood of a variant occurring in these regions and the risk of amplification failure. NGS can be further optimized to capture multiple HPA systems (over one or more HPA-containing glycoprotein genes) in a single amplicon, further reducing the need for additional primers and reducing the occurrence of allele dropout [10,15]. Biotinylated oligonucleotide probes may also be employed with NGS analyses to reduce the occurrence of allele dropout. These probes are longer than conventional PCR probes and can therefor tolerate a small number of mismatches within the binding site without affecting probe hybridization [17].

Role of NGS in the detection of rare and novel HPA variants

Real-time PCR, along with other SNV-based typing methods, are only able to detect the presence or absence of a single base at a particular position in a sample of DNA. Whilst the most common HPAs are defined by SNVs at known positions, sequences outside of the discriminating base may impact the predicted HPA phenotype. For instance, the phenomenon of silencing HPA mutations causing discrepancies between phenotype and genotype is well documented, and in such cases only NGS would be able to correctly predict the HPA phenotype where SNV-based typing would not [18,19]. HPA-1a phenotype/genotype discrepancies due to rare integrin β3 alleles (ITGB3, GPIIIa and CD61) have also been reported on numerous occasions [19-21]. In these cases, rare HPA alleles may introduce missense mutations that modify the HPA epitope [20] and would only be detectable by sequencing; HPA genotyping by SNV-based approaches would be inadequate. Furthermore, most, if not all rare and low-frequency HPA alleles are generally not tested for by SNV-based typing methods. SNV-based genotyping typically assesses the common HPA genes HPA-1, HPA-2, HPA-3, HPA-4, HPA-5 and HPA-15. To extend analyses beyond these 6 systems unique sets of primers and discriminating probes would be required for each HPA system. Testing for all systems would be inefficient, costly, and unlikely to have a significant clinical impact.

NGS not only has the potential to identify rare HPA alleles by sequencing entire sets of platelet glycoprotein genes, but also has the potential to identify novel HPA systems. Importantly, classification of new HPAs requires the existence or identification of an alloantibody to the newly proposed HPA system. Without such information, laboratories may waste time, money and resources attempting to characterize insignificant variants. In some instances, such investigations may be warranted. For example, Davey et al. report a novel ITGB3 gene variant preset in a father and baby but not the mother during the investigation of an unresolved case of FNAIT using NGS [15]. Undoubtedly, the use of NGS for HPA genotyping has the potential to identify rare and low frequency HPAs, contribute to the classification of novel HPA systems and aid in the resolution of unresolved FNAIT, PTP and PTR cases Table 1.

Table 1: Advantages and challenges of NGS for HPA genotyping.

Advantages	Disadvantages
Less prone to allele dropout due to variants in primer binding positions	High upfront instrument costs and ongoing expenses related to sequencing, maintenance and validation.
Not limited to known HPAs	Data storage may be an issue
Not limited to the most commonly tested for HPAs (HPA-1 to HPA-5 and HPA-15)	Overwhelming amounts of information generated; issues with discriminating silent SNVs with clinically significant variants (accurate variant calling)
Able to detect rare and inactivating alleles	Highly skilled, technically trained staff required for performing sequencing and interpreting results
Potential to discover novel HPA systems	
Aid in the resolution of unresolved cases of FNAIT, PTP and PTR	
Potential for massive multiplexing, high-throughput donor screening and identification of rare donors for HPA alloimmunized patients requiring platelet products, improved inventory management	
Potential to establish prenatal screening programs and reduce the risks of severe complications of FNAIT	
More cost-effective in high-throughput settings	

Multiplexing and high-throughput screening programs: Clinical utility of mass HPA typing

Unlike real-time PCR and other SNV-based typing methods, NGS offers the unique advantage of being able to pool hundreds of samples into a single experiment without drastically increasing the cost or run time [22]. Known as multiplexing, unique 5' and 3' barcodes can be added to individual patient samples *via* a PCR step. Samples can then be combined, fragmented, sequenced and reidentified using the assigned barcodes. Multiplexing for HPA typing has been used in a number of studies to date. Vorholt et al. demonstrate an effective amplicon-based NGS sequencing protocol with pools as high as 760 samples shown to be effective for typing HPA-1 to HPA-5, HPA-15 and more [10]. Beyond HPA typing alone, NGS also offers the unique advantage of being able to sequence an entire set of HPAs, Human Leucocyte Antigens (HLAs), Human Neutrophil Antigens (HNAs) and RBC antigens in a single experiment. Orzinska et al. also developed an amplicon-based NGS approach whereby 48 samples were multiplexed and typed for HPA-1 to HPA-5, HPA-15, RHCE, RHD, FY, JK and MNS [13]. HPA typing may be viable to implement for laboratories with a pre-existing NGS workflow. High-volume, high-throughput settings where samples are more effectively batched (multiplexed) in such laboratories improves testing economy and reduces the cost per sample compared to small-batch typing [23,24]. Large-scale blood donor typing for HPAs is not yet widely accepted, however such screening programs may be useful in identifying rare donors, improving inventory management of apheresis platelets and aiding the provision of antigen-negative platelet products to highly immunized patients [11,25].

In the absence of screening, FNAIT is usually only diagnosed postnatally when platelet counts of newborns are observed to be abnormally low. In severe cases, initial diagnosis of neonates may coincide with symptomatic hemorrhage such as severe bleeding and Intracranial Hemorrhage (ICH) in utero [26]. Due to this delay in diagnosis, especially in first pregnancies, a prenatal NGS screening program for low frequency maternal HPA alleles (e.g. HPA-1bb) may be beneficial in identifying pregnancies at risk of FNAIT. Serological screening programs for HPA antibodies may also be of use, however such programs would be limited to known HPA systems and likely only feasible for common antibodies implicated in FNAIT, such as anti-HPA-1a [27]. Furthermore, antibody screening would be incomplete as low frequency HPAs are often underrepresented on platelet panels used in HPA-typing assays. Thus, an advantage of NGS over both serological and SNV-based prenatal screening programs is to assess the entire maternal HPA haplotype to identify novel and rare HPAs. Currently, prenatal HPA genotyping screening is not routinely performed or internationally accepted due to a lack of consensus on appropriate antenatal care and use of prophylactic treatments for pregnancies at risk of FNAIT [28]. Nonetheless, if an appropriate management system is established and screening is cost effective, early screening of HPA-1bb and other rare HPA alleles may help to identify at-risk pregnancies and reduce the risk of ICH and other severe complications of FNAIT [28]. Finally, a NGS-based approach for prenatal HPA screening may also serve to increase understandings of HPA alloimmunization in pregnancy and provide greater insight into the factors that contribute to pregnancies being "at-risk" of FNAIT and developing serious complications.

Impediments of NGS for HPA typing

Despite the vast number of advantages of HPA typing by NGS, several real challenges continue to prevent the widespread implementation of this technology in clinical and reference laboratories. High upfront instrument costs, data storage and reliable variant calling are all major obstacles of adopting NGS as a stand-alone technique for HPA typing.

DISCUSSION

The raw sequencing data generated by NGS is overwhelmingly large. The average file size of an entire genome is 120GB, 6GB for exomes and 1GB for panel tests per patient [29]. Although the cost of cloud storage is decreasing, organizations must still appreciate the costs and logistics of storing patient data for x amount of years. When considering the volumes of data generated from a national donor or prenatal HPA screening program with accruing costs per year of storage, the volumes of data generated may quickly become difficult to manage in high-throughput settings.

Since the advent of Sanger sequencing and the technological advancements that have followed, the cost of NGS has drastically declined in the last 40 years [30,31]. Despite this, the high upfront and ongoing costs of NGS remains one of the biggest obstacles to its use, especially in poorly funded labs and resource poor countries. The typical cost of NGS sequencing varies, and lack of transparency makes accurate cost reporting challenging. Table 2 illustrates the instrument and sequencing costs of some popular NGS platforms.

In contrast, the cost of qPCR is significantly less than NGS; a typical qPCR thermal cycler will range from $15K to $90K depending on the instrument [32]. Although qPCR is cheaper for low-throughput HPA typing studies, NGS is likely to be more cost-effective in high-throughput settings such as donor and prenatal screening. The sequencing cost per sample is reduced in NGS experiments where multiplexing is optimized, and in laboratories already performing HLA/HNA and/or blood group typing this may be even more so true.

The rapid rise in global popularity of NGS as a genotyping platform has meant that this is one of the least standardized analytical platforms used today. Some commonly reported quality indicators of NGS include % targets at Q30, cluster densities; mean depth of coverage and filter pass rate, however reporting of these parameters is far from consistent amongst published studies. Much debate exists around minimum NGS quality indicators and a current consensus on acceptable depth of coverage for SNV detection is not agreed upon [33-35]. As a consequence of the rapid developments in sequencing technologies and the expanding field of personalized medicine, standardized protocols, data output formats and quality management considerations are scarce. This presents a major challenge for implementation of NGS in clinical laboratories. Accurate variant calling and distinguishing true SNVs from noise continues to present another challenge to the use of NGS, establishing minimum criteria would aid in overcoming this impediment. It would thus be of great interest to incentivize development of international standardization and minimum quality criteria for sequencing studies.

Table 2: Instrument and sequencing costs for popular NGS instruments. Table adapted from [14,35].

	Illumina MiSeq System	Illumina NextSeq 550 System	Illumina NextSeq 1000 System	Illumina NextSeq 2000 System	Illumina NovaSeq 6000 System	Thermo Fisher Scientific Ion PGM Dx System
Instrument price	$99,000	$275,000/	$210,000	$335,000	$985,000	$120,000
Cost per run	$325–$1,705	$1,205–$5,065	$1,420– $3,540	$1,420– $6,000	$2,100–$14,400	~$2,250 on Ion 318 D × chip for 16 samples
Cost per sample[a]	Variable	Variable	Variable	Variable	Variable	Variable
Maximum no. of libraries amplified in single amplification event	384 samples (>384 samples with custom barcodes)	384 samples (>384 samples with custom barcodes)	384 samples	384 samples	384 samples (>384 samples with custom barcodes)	16 samples (>16 samples with custom barcodes)
Output per run	up to 15 Gb	up to 120 Gb	up to 120 Gb	up to 330 Gb	up to 3,000 Gb	up to 1Gb; 200 bp workflow on Ion 318 D × chip
Read length	2 × 300 bp	2 × 150 bp	2 × 150 bp	2 × 150 bp	2 × 250 bp	Up to 200 bp
Total time from library construction to variant callingb	4–56 hours	11–29 hours	13–29 hours	13–48 hours	13–44 hours	<2 days

Note: [a]Cost per sample depends on the number of samples pooled per run. bDepends on read length; sequencing only.

CONCLUSION

NGS is a rapidly developing technology gaining popularity in many clinical settings, including transfusion and transplant medicine. When compared to SNV-based typing methods, NGS presents a number of unique advantages for HPA typing. NGS may be more accurate than SNV-based typing methods due to reduced occurrence of allele drop-out and false negative results. Furthermore, the ability to detect rare HPA alleles, silent mutations and novel HPA systems may provide opportunities to improve patient care whilst increasing our understandings of platelet antigens and their immunogenic properties. The ability to multiplex several hundred patient samples into a single experiment makes NGS capable of performing ultra-high-throughput analyses, creating potential for rare donor and prenatal screening programs. Despite these advantages, NGS for HPA typing is met with many challenges, including high upfront instrument costs, ongoing expenses associated with performing sequencing, validation and maintenance, data management and storage, standardization of protocols and accurate variant calling. Currently, these impediments mean that NGS as a stand-alone technique for typing HPAs is unlikely to be efficient. Future studies on the use of NGS for HPA genotyping with large sample sizes and cost-benefit analyses will aid in assessing if implementation of this technology is viable.

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