This is a postprint of an article published in
Garritsen, H.S.P., Xiu-Cheng Fan, A., Lenz, D., Hannig, H., Zhong, X.Y., Geffers, R., Lindenmaier, W., Dittmar, K.E.J., Wörmann, B.
Molecular diagnostics in transfusion medicine: In capillary, on a chip, in silico, or in flight?
(2009) Transfusion Medicine and Hemotherapy, 36 (3), pp. 181-187.

Molecular diagnostics in transfusion medicine: in capillary, on a chip, in silico or in flight?

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Abstract

Serology, defined as antibody based diagnostics has been regarded as the diagnostic gold standard in transfusion medicine. Nowadays however the impact of molecular diagnostics in transfusion medicine is rapidly growing. Molecular diagnostics can improve compatibility testing (HLA), increase safety of blood products (NAT testing of infectious diseases), enabling blood group typing in difficult situations (after transfusion of blood products or prenatal non-invasive Rh-D typing). Most of the molecular testing involves the determination of the presence of single nucleotide polymorphisms (SNPs). Antigens (e.g. blood group antigens) mostly result from single nucleotide differences in critical positions. However most blood group systems can't be determined by looking at one SNP solely: to identify members of a blood group system a number of critical SNPs has to be taken into account. The platforms which are used to perform molecular diagnostics at this moment are mostly gel based, requiring time consuming multiple manual steps. To implement molecular methods in transfusion medicine in the future the development of high throughput SNP genotyping non- gel based platforms which allow a rapid, cost effective screening are essential.

Introduction

With the emerging technology of genetic diagnosis and the genetic characterization of polymorphisms a whole new area opens for clinical medicine and in particular for transfusion medicine [1-4]. Whereas molecular characterization was previously laborious and time-consuming, the introduction of automatisation reshaped the field. From the extraction of DNA or RNA to final data analysis new instruments and software enable the molecular identification of large amounts of samples within limited time. These developments have greatly facilitated the clinical utility of molecular techniques . However not only innovated molecular diagnostic techniques and software programs have shaped the applications in transfusion medicine. At least in germany, legislature prescribing the nucleic acid testing for HIV and HCV for all donated blood products has pushed the development of high throughput molecular diagnostic instruments forward. Goal of this review is to give an overview of different qualitative and quantitative molecular diagnostic options emphasizing a relative new promising option: MALDI-TOF MS genotyping.

In capillary 1

In the early nineties Carl Wittwer started to work on systems which were able to speed up DNA amplification reactions. The idea was to develop a rapid thermal cycling system by using thin-walled glass capillary test tubes (to increase the surface-to volume ratio) and hot air to increase the speed of the pcr cycling reaction. The

resulting air thermocycler Instrument matched the speed of biochemical reactions and was ten times faster than commercially available instruments. Through further developments in software, hardware and chemistries the next goal became within reach: amplification and sensitive detection of pcr products in the same instrument[5-13]. Advantages are that there is no need to open tubes or capillaries after the pcr amplification process. This reduce the risk of contamination and pipetting errors and because gel-electrophoresis as detection step is eliminated, the time for detection of pcr products is minimized. Advantages in chemistry of fluorescent dyes (e.g. SYBR-green) or use of fluorescent labeled oligonucleotides facilitated routine implementation of molecular diagnostic systems. SYBR-Green has a strong increase in fluorescence when it binds to double stranded DNA (e.g. pcr products) therefore the detection sensitivity was increased. But also the possibilities to label oligonucleotides fluorescently e.g. FRET-probes enable a sensitive and specific detection. Monitoring the amount of fluorescence during each pcr-cycle is defined as real time pcr. There is a direct relation between the starting amount of DNA (template dna) and the number of pcr cycles (ct: cycle threshold) which are needed to obtain a predefined fluorescence intensity caused by a certain number of pcr products. If you have a large quantity of template dna you need less pcr-cycles to reach this fluorescence threshold than with a small amount of template DNA. In other words: the threshold cycle is indicative for the amount of starting (template) DNA. This method allows a (semi)- quantification of starting material. Parallel to the described capillary system microtiter plate systems have been developed which have similar advantages and possibilities. Variation in fluorescence chemistry and detection filter combinations allow multiplex pcr (analysing multiple gene sequences in the same pcr-reaction). However multiplexing in real time pcr is limited to 2-3 different gene sequences.

Due to reproducibility, sensitivity and quantification, real-time pcr becomes an accepted method to detect viral contamination in blood products[14-19]. Real time pcr is fast but expensive and therefore the real time pcr is mostly used to quantify the viral load in studies and limited in routine applications (e.g. NAT testing HCV and HIV).

<u>In capillary 2</u>

DNA Sequencing using capillary electrophoresis succeeded the generation of automated gel based DNA sequencers in the middle of the nineties[20,21]. Fluorescently labelled DNA fragments generated in enzymatic sequencing reactions are rapidly separated by capillary electrophoresis and detected. They are mostly based on a modified sanger sequencing reaction where fluorescent fragments are at random generated in pcr reaction (cycle sequencing). In this pcr reaction only a small percentage of the nucleotides in the reaction mix are labelled with fluorescent dyes. Each nucleotide (guanine, cytosine, thymidine and adenosine) bears its own fluorescence dye which fluorescence at different wavelength of the spectrum. Furthermore these fluorescent nucleotides are chemically modified and consist of didesoxyriboses. Incorporation of the fluorescent nucleotide leads to an chain demolition (cycle sequencing). Because it is an at random process at what time and what length a fluorescence labelled nucleotide is incorporated after a fixed number of pcr cycles, statistically all nucleotide position are labelled and the pcr tube contains thousands of fragments with a different length and a specific fluorescently labelled dye at the end. The capillary electrophoresis automated DNA sequencer is used to separate these fragments (a shorted fragment will run faster as a longer fragment) and analysed. Cycle sequencing using capillary electrophoresis has become an standard method in transfusion medicine especially for high resolution HLA-typing.

The method can be modified for high throughput by simply increasing the number of sequencing capillaries which can be run in parallel.

For the analysis of predefined SNPs, DNA sequencing using cycle sequencing is not necessary and more and more replaced by other techniques. In the majority of cases these techniques are variations of a technique called minisequencing. In this technique a single stranded DNA-fragment which has the complementary sequence of the region of interest direct next to the SNP position to be tested is incorporated in the pcr-mix together with fluorescent labelled didesoxyribose nucleotides. A primer extension will take place during the pcr reaction but only until a fluorescent labelled didesoxyribose nucleotide is incorporated and then the reaction is stopped (minisequencing). The fluorescent label is indicative for the nucleotide on the SNP position. By varying the length of the single stranded DNA fragment next to SNP position it is possible to analyse different SNPs in one pcr-reaction (multiplex pcr) using capillary electrophoresis and fluorescence detection unit. An example of such an minisequencing assay is given in figure 1 where we analysed multiple mtDNA haplotype specific SNPs in a single reaction (SNaPshot Assay) [22-28].

This SNaPshot was carried out in multiplex using the SNaPshot multiplex kit (Applied Biosystems). The reaction involves the extension of a oligonucleotide probe designed to lie adjacent to the SNP of interest by one of four fluorescently labelled dideoxynucleotides complementary to the base found at the SNP site. The oligonucleotides probes within one multiplex reaction are designed to be of different lengths by addition of neutral sequence so that when they are separated by capillary electrophoresis only one product will be seen within a size range. The size range of the primer can vary between 15 to 30 nucleotides. For successful multiplexing it is recommended that one uses primers different by sets of 4 nucleotides. Theoretically four different SNPs can be run in one assay. However there is a second level of

multiplexing: you can run together an assay for an A/G polymorphism and a C/T polymorphism even if the primers are the same lengths. This theoretically doubles the number of SNPs run in one assay: 8. Extreme caution have to be taken in primer designing for multiplex assays. If one is targeting a C/T polymorphism on the forward strand, it will give you an A/G polymorphism on the reverse strand using a reverse probe. It is advisable to run an assay on it's own before multiplexing it.

Following the SNaPshot reaction, the mixture is digested with shrimp alkaline phosphatase to prevent any further primer extension by eliminating unincorporated ddNTPs. The fluorescently extended probes are then separated on a ABI 3130 capillary electrophoresis system in the presence of a fluorescently labelled size standard. Using this approach and 10 SNPs indicative for caucasian frequent mtdna haplotypes as published by Wiesbauer[29], we analysed 800 platelet apheresis donors. Again as in real time pcr separation and analysis is in one instrument although the amplification and purification of the pcr product is separate but can be automated to a large extend.

A new generation of powerful non-gel based DNA sequencers are introduced at this moment. It will however take some years before the capillary DNA sequencers generation is replaced by them.

On a chip

Since micro-arrays were first described 13 years ago [30], they have evolved into a standard experimental technology that has had a profound impact in molecular biology. Especially micro-arrays with high numbers of features are still relative expensive. Global transcription patterns as well as nucleotide polymorphisms can now be readily evaluated using DNA micro-arrays. Micro-arrays exploit preferential binding of DNA or RNA to their complementary single-stranded sequences. A micro-

array chip consists of thousands of single-stranded DNA molecule attached in fixed positions onto a solid silicate surface. Usually the micro-array chip is incubated with a biological extract (mRNA or cDNA) that is labelled with a fluorescent dye. Thus a single micro-array experiment produces thousands of datapoints, each of which is a measure of the quantity of fluorescent label, bound at a feature on the micro-array chip. These fluorescent intensities are determinated using a fluorescence reader from which the concentration of mRNA/cDNA molecules are inferred. Tens of thousands of features can be fixed to a single micro-array chip, thus providing an opportunity to quantify thousands of gene transcripts, indeed the entire human transcriptome in a single experiment. Knowing the presence or absence of transcripts for all the genes in the genome at a particular moment and their changes relative to some reference state, can be extremely valuable information, and these detailed transcriptomic portraits provide hitherto unimaginable insights into the regulation of biological processes in individual cells, normal tissue or tumors. The number of applications of micro-arrays has increased dramatically.

In addition to assessing mRNA abundancy, micro-arrays have also been applied to the quantification of DNA-copy number, DNA sequence variations and protein-binding sites in a genome. Technological advancement ensures that higher numbers of features can be spotted on micro-arrays. The applications of chip arrays in transfusion medicine are limited. There was an approach to perform high resolution HLA-Typing using micro-array technology. This approach was not successful due to technical problems with the micro-arrays. One of the main problems is that almost all SNPs relevant for high resolution HLA typing are more or less concentrated on a relative short stretch of chromosome 6 instead of being dispersed over the complete genome. Furthermore due to intense HLA genotyping novel SNPs are detected every

month leading to continuous redesigning of the micro-array. Moreover HLA-ambiguities caused analysing problems in silico.

A current project is the bloodgen project supported by the EU[31-33]. In a subproject a number of research groups have joint to implement immunohematological relevant SNPs onto a DNA micro-array. Because the number of datapoints are significant lower than needed in HLA high resolution analysis and the relevant SNPs are dispersed more equally over the complete genome, this project has been successful. We applied micro-array technology and in silico analysis to investigate potential differences in global transcription patterns comparing cultivation and maturation of human genetically modified and non-modified mature dendritic cells. Micro-arrays allow us to monitor gene expression profiles of thousands of genes in a single experiment. An example is given in figure 2 where we concentrated on transcriptional changes in immuno-relevant genes between genetically modified and non-modified mature dendritic cells.

in silico

The terminology ‚in silico' refers to the processing and modelling of huge amounts of physical data of biological origin by computers. Computer chips are mostly made of the element silicium, therefore: in silico. This process of computational analysis of biological information gained more and more importance through the availability of high throughput molecular diagnostic methods like automated sequencing and most of all through the development and availability of micro-arrays.

Scientists are encouraged to submit their raw data of these technologies in public data repositories. These repositories contain a rapidly expanding collection of gene expression data of numerous cell types, in numerous experimental conditions in several species, which form an excellent resource for in silico evaluation.

Micro-arrays produce transcriptional data and indirect measure of expression (translational data). Processing of these data in biologically meaningful units is not a trivial task. Complex processing, normalisation and statistical analysis are required to obtain even the most basic information from these data. Numerous bioinformatics statistical and machine learning methods have been applied to micro-array data. The goal of micro-array data preprocessing is to transform detected fluorescent signal values into biological meaningful measurements. Unfortunally, the relationship between the fluorescence intensity and the abundance of a given RNA/DNA molecule is not straightforward.

In flight

Recently, MALDI-TOF MS was introduced for molecular diagnostics. At this moment, it offers a solution for high-throughput qualitative and quantitative analysis of up to 36-40 multiple SNPs in a single reaction.

MALDI-TOF MS stands for Matrix-Assisted Laser Desorption/Ionisation –Time of Flight Mass Spectrometry. The process consists of two parts:

1) Laser induced desorption/onisation of analyte/matrix molecules (MALDI)
2) Separation and analysis of different biomolecules based on intrinsic physical properties (TOF)

1) Laser induced desorption/ionisation of analyte /matrix molecules

In preparation for MALDI-TOF MS SNP analysis, oligonucleotides can be generated using a minisequencing reaction without the need to label any nucleotides. PCR is performed either in multi- or uniplex format, followed by a themosequenase sequencing reaction using an oligonucleotide bound directly

adjacent to each SNP and stop mixes containing cocktails of ddNTPs. Adding an anion exchange resin removes adduct-forming ions. 15 nanoliter of the extension cocktail is spotted onto a silica-based chip array containing 384 matrix pads using a nanoliter plotting robot. Choosing the right matrix is essential for the success of the reaction. At this moment 3-hydroxy picolinic acid (3-HPA) is in use as a matrix for DNA/RNA analysis. Although the ratio matrix: analyte is approximately 1:1000, the matrix is essential to support the laserlight induced desorption and ionisation of the analyte (DNA). The silica chip array is placed into the mass spectrometer and a laser (usually N2-laser with 337nm wavelength) fires 3-5 nanosecond pulses of approximately 100µJ to each matrix pad to initiated the desorption/ionisation process of the DNA analyte.

2) Separation and analysis of different biomolecules based on intrinsic physical properties (TOF).

By applying an electrical charge the intact ionised analytes are accelerated in a vacuum detector chamber and are attracted to a detector. The time taken by each particle to fly to the detector (‘Time of Flight’) is characteristic for each analyte. This time is inversely related to the velocity, which in turn is proportional to the mass to charge ratio (m/z) of a flying particle (e.g. DNA-fragment). The time of flight is a specific characteristic of each DNA fragment since the mass/charge ratio is an intrinsic property of the DNA and depends only on base composition. In the case of SNP analysis the mass differences in the nucleotide in the polymorphic position is enough to discriminate an A, T, G or C (e.g. desoxyadenosine: 313 Dalton, desoxythymidine: 304 Dalton, desoxyguanosine: 329 Dalton and desoxycytosine: 289 Dalton). These small differences in molecular mass result in different peak positions. This situation allow us to

discriminate DNA fragment which differ in only one base (SNPs) and by specific design of the size of the DNA fragments to test multiple SNPs in one reaction.

Development of powerful software enables the automated analysis of generated MALDI-TOF MS spectra, with the possibility to assign these spectra to different genotypes.

In our group, MALDI-TOF MS has been used successfully for genotyping single fetal nucleotide polymorphisms in maternal blood for non-invasive prenatal diagnosis of genetic diseases [34-36]. We applied this technique also for the investigation of relevant SNPs for platelet antigens (HPA1-5, HPA15) and relevant mtDNA polymorphisms in the hypervariable regions (HV I, II and III)[37,38] in a multiplex assay. An example is shown in figure 3.

Discussion

The different molecular approaches presented in this paper show the rapid transition from gel-based systems to alternative systems. As to the applications which are going to be widely used, we feel that genotyping assays (SNP analysis) and DNA-sequencing (e.g. HLA) are most important for transfusion medicine.

Real-time pcr systems offer a good sensitivity and accuracy of gene expression quantification however are only limited capable in performing multiplex- PCR reactions. Also their ability to handle a large number of samples is limited.

Extended gene arrays which offer the possibility of monitoring the complete transcriptome will only be of limited value in transfusion medicine either to aid in the development and quality control of new cellular blood products as demonstrated in

figure 2, or for use of new materials and manufacturing procedures. Their value will be more in answering of experimental clinical questions. Costs of the gene arrays and instrumentation needed is also a disadvantage.

Gene arrays focussing on a predefined limited number of SNPs and therefore producing a limited number of datapoints will certainly have their place in transfusion medicine diagnostics. One of their disadvantage is that once produced they are relative static. New informative SNPs can only be added to the new version of the assay. On the cost side they certainly will be compatible.

Sanger sequencing combined with capillary electrophoresis will certainly be the standard for the next years as to DNA-sequencing. The number of samples needed to be sequenced are limited in transfusion medicine and DNA sequencing is mainly focussed in tissue typing for stem cell transplantation. As shown in this paper capillary electrophoresis can be combined with minisequencing reactions using fluorescent labeled nucleotides. At this moment theroretically 8 different SNPs can be analysed in one multiplex PCR-reaction. The system is fully automatable .

MALDI-TOF MS is a new technology for molecular diagnostics. It was first introduced as tool of choice in proteomics applications. In 1995 Tang et al [39]demonstrated its usefulness in DNA analysis. Three years later methods for RNA applications using MALDI-TOF MS were published[40]. Although the whole process from sample preparation to automated analysis takes 8-9 hours at this moment we feel we're still at the beginning of the clinical application of this technique. In contrast to the above mentioned techniques for SNP genotyping, MALDI-TOF MS allows the required sample throughput and it doesn't rely on indirect detection reactions, involving specific nucleic acid labelling or hybridization steps. By measuring molecular mass, a direct physical property of the analyte, MALDI-TOF MS is not susceptive to background problems resulting from hybridization based detection reactions.

The minimal amount of sample (15 nanoliter) underscores the potential for cost reduction. On the other hand it requires purified samples free of ions and other impurities, increasing technical time and sample processing costs. High cost efficiency at medium levels of multiplexing, high sample throughput und possibility to incorporate a high level of laboratory automation will make this a serious competitor in molecular diagnostics. Due to the high instrument costs at this moment MALDI-TOF MS will probably only be available at selected facilities for the near future.

Whatever approach is taken we feel that genotyping using non-gel based technologies will have a bigger impact in the transfusion medicine diagnostics spectrum however it will not replace serology completely in the near future

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Tables and Figures

Fig. 1

MT-DNA haplotype determination using capillary electrophoresis and a multiplex primer extension reaction.

A SnaPshot assay was developed which incorporated multiple MtDNA-SNPs to identify common European mtHaplotypes (in this case MtDNA-Haplotype H) The informative SNPs for Haplotype H are 7028 C,9055 G, 10398 A, 12308 G, and 13368 G which can be identified unambiguously on the electropherogram.

Fig 2

cDNA arrays data for comparing gene expressions from human genetically modified and non-modified dendritic cells (DC's). Mature DC's from three different donors (LK39, LK49, LK53) or modified culture conditions (RF) were compared with genetically modified mature DC's using different adenoviral vectors (AdVegfp, AdVM3TG,AdVTyreG, AdVM3m1eG). Data for selected immunorelevant are shown here. They

compare gene expression of immature DC on day 4 of cultivation to DC gene expression of mature DC on day 7. Overexpression is labelled green, downregulation is colored red. Cluster analysis shows that the gene expression pattern of the genes shown is independent from the genetic modification.

Fig. 3

Mass spectra of multiplex PCR Maldi-tof data (Iplex) from one plateletapherese donor (donor 89) to identify relevant platelet antigens (HPA-1 to 5 and HPA-15) are shown. The peaks corresponding to the HPA-Alleles are indicated with colors (turkish blue for HPA-1, blue for HPA-2, green for HPA-3, red for HPA-4, gray for HPA-5 and pink for HPA-15). HPA genotype of this donor: HPA 1a/b, 2a/a, 3a/b, 4a/a, 5/a, 15a/b