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Advances in Biochemistry and Biotechnology

Review Article

Iles R and Mahmoud N. Adv Biochem Biotechnol 3: 157.
DOI: 10.29011/2574-7258.000057

Future Laboratory Medicine: Rapid, Efficient and Affordable Screening for Haemoglobinopathies by MALDI-ToF Mass Spectrometry

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Citation: Iles R, Mahmoud N (2018) Future Laboratory Medicine: Rapid, Efficient and Affordable Screening for Haemoglobinopathies by MALDI-ToF Mass Spectrometry. Adv Biochem Biotechnol 3: 157. DOI: 10.29011/2574-7258.000057

Received Date: 31 January, 2018; **Accepted Date:** 16 February, 2018; **Published Date:** 26 February, 2018

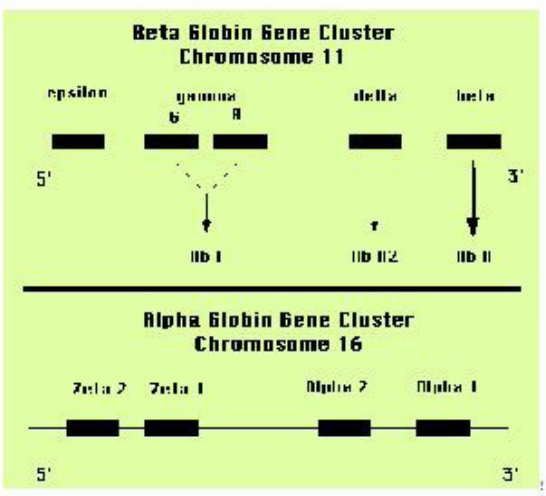
Abstract

Latest advances in clinical proteomics now mean that, from a pin-prick of blood, definitive diagnosis of sickle cell disease and carrier is diagnosed by direct MALDI-ToF mass spectrometry. In addition, characteristic spectra of alpha and beta-thalassemia are revealed. The combination of easy sampling and analytical efficiency makes population screening an economic reality for the most common genetics disease to affect humanity.

Introduction

Haemoglobinopathies are the largest group of inherited human monogenic disorders worldwide and are due to mutations in one or more of the genes encoding the globin proteins that form the $\alpha_2\beta_2$ hetero-tetrameric complex that binds oxygen in red blood cells - Haemoglobin (Hb). The resulting structural alteration in the patients Hb can be clinically mild, even occult, to being the cause of severe chronic morbidity and even neonatal death.

Haemoglobin itself should be a hetero-tetrameric complex containing two alpha gene globin protein products associated with two beta gene globin proteins. The genetics and protein biochemistry of the constituent globin proteins are complex: human β-globin locus is composed of five genes located on a short region of chromosome 11, responsible for the creation of the beta parts of Haemoglobin. This locus contains not only the gene for the classically expressed beta (β) globin gene but also delta (δ), gamma-A (Aγ), gamma-G (Gγ), and epsilon globin (Ɛ). The human α-globin gene cluster is found on chromosome 16 and contain expressed globin genes Alpha 1 (α1) and Alpha 2 (α2) and Zeta (ξ) (see Figure 1); but a new α-globin gene has recently been identified termed Hb Mu (μ). As human life moves from early fetal to late pregnancy, birth and infant/adult life the alpha and beta gene pairing change to match the circumstance of in utero and extra utero life. For example, the very early embryo produces Hb combinations $\xi_2\varepsilon_2$ (Gower); $\alpha_2\varepsilon_2$ (Gower II) and $\xi_2\gamma_2$ (Portland) by the red blood stem cells formed in the Yolk sac during the first trimester of pregnancy. This is rapidly superseded by α2γ2 (HbF), produced by the red blood stem cells of the fetal liver during the second and third trimester. After birth this changes to predominantly $\alpha_2\beta_2$ (HbA), some $\alpha_2\delta_2$ (HbA2) and trace amounts of HbF (see Figure 1).



Embryonic hemoglobins	Fetal hemoglobin	Adult hemoglobins
Gower I- zeta$_2$epsilon$_2$ Gower II- alpha$_2$epsilon$_2$ Portland- zeta$_2$gamma$_2$	Hemoglobin F- alpha$_2$gamma$_2$	Hemoglobin A- alpha$_2$beta$_2$ Hemoglobin A2- alpha$_2$delta$_2$

Figure 1: Diagrammatic representation of the major Functional

genes coding for beta-globins on Chromosome 11 and alpha-globins Chromosome 16 (pseudogenes and newly discovered alpha-globin - mu, are not shown on this diagrammatic map for clarity).

Not surprisingly, the various haemoglobinopathies identified were named as characteristics of the hetero-tetrameric hemoglobin complex rather than the feature of an isolated globin proteins. In Sickle cell disease the haemoglobin Hb S variant is where globin gene β has a specific amino acid change mutation β6Glu-Val at position 6 of the β globin, termed $^{S}\beta$; and the hetero-tetrameric Hb is $\alpha_2\ ^{S}\beta_2$ Thus, Sickle cell trait is a complex of normal β and $^{S}\beta$ within the red cells constituent haemoglobin molecules as a result of hetrozygosity and is often unnoticed.

Haemoglobin C (HbC) is a structural variant of normal haemoglobin (HbA) caused by an amino acid substitution at position 6 of the β-globin chain (β6Glu-Lys). Globally, it is one of the most prevalent abnormal haemoglobin mutations alongside haemoglobin S, which occurs at the same position (HbS; β6Glu-Val), and haemoglobin E (HbE, β26Glu-Lys). In HbC heterozygote individuals (AC), this trait is asymptomatic. Homozygosity (CC) causes clinically mild haemolytic anaemia, due to the reduced solubility of the red blood cells, which can lead to crystal formation. HbC is mainly of clinical significance when inherited in combination with HbS (sickle-haemoglobin C disease).

Thalassemias arise because of a mutation that prevents the expression of either the β-globin gene (β-thalassemia) or the alpha-globin genes (α-thalassemia). As a consequence, unusual hetero- and homo- tetrameric Hb pairings arise.

In the β-thalassemia there is reduced synthesis of β globin (β^{+}-thalassemia) or the absence of synthesis of β globin (β^{0}-thalassaemia). Clinically mild forms of β- thalassaemia are called thalassemia intermedia. There is also a δβ-thalassaemia which arises from gene fusion and the production of both β and δ chains is diminished. The imbalance in globin chain synthesis with more α-globin than beta-chains leads to precipitation of α-globin in the red cell, which leads to premature destruction of the cell in the bone marrow or the peripheral blood. Excessive α-globin can be very damaging to the cell; generating Reactive Oxygen Species (ROS) that destroy cellular proteins, lipids and nucleic acids in the red cell precursors. In addition, tetrameric-α haemoglobin is structurally unstable, with a tendency to denature upon oxidation, filling the cytoplasm and cell membrane with precipitated α-globin chains, free heme, porphyrins and iron, which further propagate ROS production. Erythroid cells have proteolytic pathways to degrade excess free α-globin, but these pathways can be overwhelmed.

In the α-thalassemias there is the loss of expression of α globin genes; but since humans have four gene copies (two on each parental chromosome see Figure 1) there is a greater capacity for tolerance. However this also means an equally greater capacity for carriers and as consequence when this has arisen a higher incidence of α-thalassemia within defined populations. Thus, this condition occurs very frequently in Asia; from India to China, including Southeast Asia, and also occurs in the Middle East and Africa.

Within the Arabic nations haemoglobinopathies are the most common genetic diseases; with reported carrier rates of 1-11 % for β-thalassemia, 1-58 % for α-thalassemia and 0.3-30 % for sickle cell trait. In India, the most common haemoglobinopathies are sickle cell trait, Hb D and Hb E. It was estimated that, in the year 2000, with a population of 1000 million and a birth rate of 25 per thousand, there would be about 45 million carriers and about 15,000 infants born in India each year with haemoglobinopathies.

The Logistics of National Screening

Preventive genetic services based on population screening are now an integral part of public health and specifically maternal and child health programmes, in many countries. However, the effectiveness of such programmes is not only a component of the efficiency of the test, in terms of sensitivity and specificity, but also compliance. The importance of compliance can-not be underestimated and is more important than the difference between a 75% and a 100% detection rate for a test in achieving a reduction of the incidence and economic burden of any disease in a given society (1).

Compliance to testing is a combination of cost of test and the ease to which a sample can be given; it should not be invasive, time consuming or arduous. Mass screening and genetic counseling for sickle cell disease and other haemoglobinopathy have been carried out widely, but they are not as effective as antenatal screening programmes. This is due not to a difference in test efficiency but in compliance: All babies born in Hospitals can be sampled by as little as a Heel prick of blood as part of their routine care. When dealing with adult populations the question of cost, time and the invasive nature of a screening test becomes more pressing [2].

Although newborn screening for sickle cell and thalassemia are effective at detecting suffers early, they have a very limited capacity to impact on disease prevalence within a society. In the case of haemoglobinopathies it is much better to establish carrier status of couples prior to child rearing. In so doing the risk of a child suffering such a condition can be prevented and, by measures such as pre-implantation genetics, effectively circumvented.

In Sickle cell disease carrier and thalassemia trait detection we see a clear distinction between an “at high risk test” and a definitive diagnostic test. Carriers of haemoglobinopathy are, in the vast majority of cases, asymptomatic. Mild anemia, if shown to be non-iron deficient in origin, is a strong indicator of a haemoglobinopathy carrier. However, definitive diagnostics of an individual’s carrier status is a combination of more laborious tests and effective sampling for genetic analysis. This is neither straight forward or inexpensive. Thus, screening for carriers of sickle cell

and thalassaemias is a combination of mass screening for mild anemia and a more costly & involved, analysis of those positive for the risk marker.

Thus, a cheap high throughput general anemia test followed by more expensive diagnostics appears to be a sound strategy. However, in practice the efficiency of the "high risk for Haemoglobinopathy" test of, anemia alone is regarded by many as insufficiently sensitive and specific. For example, the recommended clinical practice guideline by the Genetics Committee of the Society of Obstetricians and Gynaecologists of Canada (SOGC) and the Prenatal Diagnosis Committee of the Canadian College of Medical Geneticists (CCMG) is: "Screening should consist of a complete blood count, as well as hemoglobin electrophoresis or hemoglobin high performance liquid chromatography. This investigation should include quantitation of HbA2 and HbF. In addition, if there is microcytosis (mean cellular volume < 80 fL) and/or hypochromia (mean cellular hemoglobin < 27 pg) in the presence of a normal hemoglobin electrophoresis or high performance liquid chromatography the patient should be investigated with a brilliant cresyl blue stained blood smear to identify H bodies. A serum ferritin (to exclude iron deficiency anemia) should be performed simultaneously". [3]

This is not unusual in national guidelines, but illustrates the point that the all-important primary screening test can become more complex, due to poor detection efficiency; but the subsequent increase in cost and arduous nature of the testing, makes the uptake/compliance rate more difficult to achieve. Despite these short comings, Iran, Saudi Arabia, UAE, Qatar and other MENA and Asian counties have instigated trial screening programmes as the impact of these disorders are so significant to the population of these nations.

Latest Technology

The ideal solution is a simple pin-prick blood sample that can be directly identify abnormal globin proteins at low cost. This is now possible using recently developed matrix assisted laser desorption - time of flight - mass spectrometry (MALDI ToF MS) techniques for analysis of complex bio fluids with little or no processing [4].

MALDI-ToF MS is revolutionizing clinical microbiology as small cultures of bacteria or fungi are lysed directly onto MALDI-ToF mass spectrometry target plates and identified via characteristic spectra of proteins detected. This not only reduces identification of infecting species from weeks to minutes; but also, the cost in reagents, staffing and dedicated laboratory space to about a 10^{th} of that required in conventional clinical microbiology [5-7]. MALDI was developed for the ionization of relatively large polypeptides and proteins but its application has widened to incorporate glycoproteins, oligonucleotides and complex carbohydrates. A great advantage of MALDI MS is that the process of soft-ionization causes little or no fragmentation of analytes, allowing the molecular ions of large molecules to be identified, even within mixtures. Once ionized, the complex molecules are accelerated towards a detector along a flight tube: The time it takes from acceleration voltage switch on, to signal registering at the detector, is proportional to the mass to charge ratio. Hence time of flight or ToF mass analysis. Unlike most other ionization technics MALDI ionization is 1,2 or 3 charges and so interpretation of signal data from a detector is relatively simple and does not require complex processing, deconvolution of multiple 10s or 100's of ionized forms of the same molecule [8].

MALDI TOF MS analysis is sensitive and very rapid as once the sample has been mixed with a 'matrix' on a MALDI target, a spectrum can be generated within seconds. Hence, the majority of protein and oligonucleotide analysis in the context of high throughput proteomics and genomics is carried out by MALDI TOF mass spectrometry (see Figure 2).

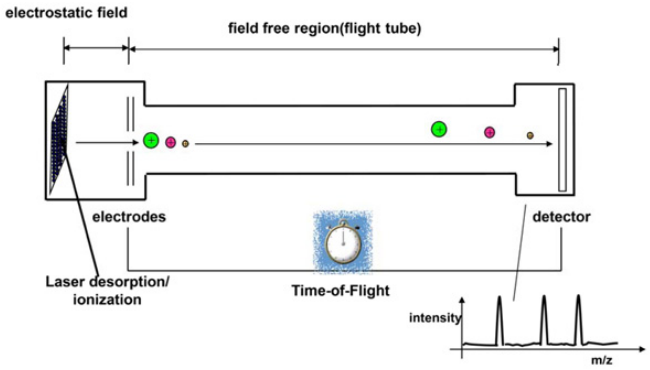


Figure 2: Diagrammatic illustration of the basic principles and components of a MALDI ToF mass spectrometer: A laser is fired at the sample mixed with matrix. The matrix absorbs the laser energy forming a reactive plume and soft ionizes the molecules in the mixture. When a high voltage is applied, the charged molecules are accelerated into the field free flight tube. The time it takes for the molecules to hit the detector is proportional to the molecule size and charge (m/z ratio).

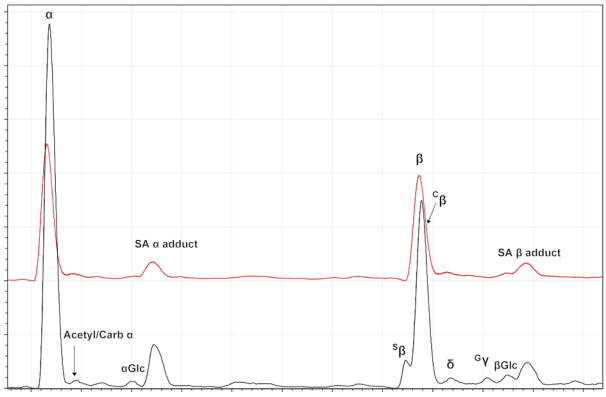
Logistics of a MALDI-ToF Mass Spectrometry Screening Program for Haemoglobinopathy

Analysis is based on a single microliter (µl) of whole blood, such that blood spotted on a Gutherie Card can be eluted and analyzed with ease.

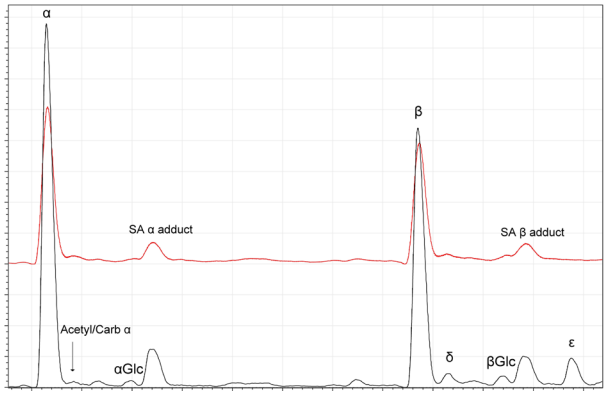
- Samples can be collected from rural villages as part of a Government initiative or sent by individuals sampling in their own homes from a kit consisting of single use lancet, Gutherie card on which to blot and an addressed envelope.
- Actual reagents costs are miniscule to the point of less than a penny per sample.

- Samples received at a central laboratory are quickly analysed by direct MALDI-ToF after minimal proprietary chemical preparation and results generated in minutes. In addition, loading on a MALDI-ToF plate varies from 96 to 386 sample spots, so throughput is high.
- The results are then interpreted as indicative of Sickle Cell Disease or other Haemoglobinopathy based on characteristics of the spectra as illustrated in Figure 3.
- Processing to result reporting time is in minutes and not days as for conventional Hb analysis.
- Confirmation of results can be carried out via genetic analysis as is current standard practice.
- Detecting the mass spectral profile changes of globin proteins in a 1ul blood sample not only increases the impact of population screening for haemoglobinopathies because of better analytical sensitivity and specificity, but will also be due to better compliance: Sampling is simple, non-arduous and can be conducted entirely by adult subjects themselves. Furthermore, the major attraction to health providers is that the entire logistic, from sample to results, is inexpensive.

Panel A



Panel B



Panel C

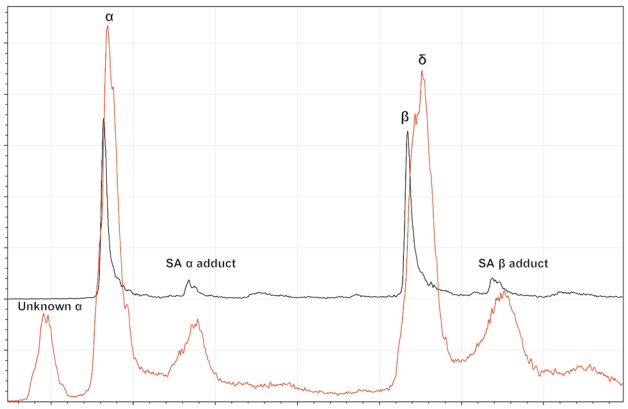


Figure 3: Example MALDI-ToF MS spectra of aberrant globins detected in pin-prick blood samples of: (a) a Sickle Cell Disease sufferer with SC genotype, (b) a beta-thalassemia trait carrier expressing delta and fetal globins, and (C) an asymptomatic carrier of a complex alpha-globin variant expression and high-level expression of delta globin, alongside beta. Off-set in each panel is the profile of a phenotypically normal control blood sample.

Conclusion

The robust nature, ease of operation of MALDI-ToF mass spectrometry combined with the wealth of data that is generated, makes this technique attractive as a clinical diagnostic tool. Furthermore, the non-invasive, small sample volume requirement and affordable consumable costs, makes this approach revolutionary. Here we have demonstrated its application to screening for haemoglobinopathies, in the next decade many more biomarkers and disease will be evaluated by this technique [9,10].

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