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Rh Blood Group D Antigen Genotyping Using a Portable Nanopore-based Sequencing Device: Proof of Principle

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Guest: Dr. Tracey Madgett from the University of Plymouth in the United Kingdom.

Bob Barrett: This is a podcast from *Clinical Chemistry* sponsored by the Department of Laboratory Medicine at Boston Children's Hospital. I am Bob Barrett.

At the start of the 20th century, it was discovered that there were three blood types in humans, the A, B, and O blood groups, and that mixing blood from different types caused an immune response that resulted in clumping and was a reason for death in early attempts at blood transfusion. In the 1940s, another blood group system was discovered that classified blood groups by the presence or absence of the Rh antigen, or Rh factor, on the cell membranes of the red blood cells.

The RHD antigen is the most clinically significant in the Rh system due to its high immunogenicity, and the main cause of hemolytic disease of the fetus and newborn. A large number of RHD genetic variants have been reported and these encode different variants of RHD proteins that can result in a number of different clinical outcomes, and it has been proposed to perform RHD genotyping for all pregnant women. For that to become a reality, a cost-efficient sequencing needs to be available.

A paper appearing in the September 2022 issue of *Clinical Chemistry* examined and a small, portable nanopore-based sequencing devised to help address that very issue. We are pleased to have the senior author of that paper with us as a guest in this podcast. Dr. Tracey Madgett is a Lecturer in Molecular Biology at the University of Plymouth in the United Kingdom. And her research focuses on detection of blood group variation using sequencing technologies.

So first of all, Dr. Madgett, what are blood groups and why is the Rh blood group so important?

Tracey Madgett: Blood consists of red blood cells, white blood cells, platelets, and plasma. And so when we talk about blood groups, what we're interested in is categorizing blood based on the molecules that can be found on the surface of the red blood cells. We term these molecules "antigens" and they can be

either carbohydrate or protein in nature. These molecules are capable of eliciting an immune response in an individual who doesn't carry the structures. And so the genes we inherit from our parents determine our individual blood type, either by coding for particular proteins, or coding for enzymes involved in the synthesis of complex carbohydrates.

There are currently 43 recognized blood group systems according to the International Society of Blood Transfusion. These 43 blood group systems are determined by 48 genes and there are over 300 antigens that can be found on the surface of red blood cells. The most clinically significant blood group from a transfusion perspective is the ABO blood group, which was discovered in 1901. If a patient is given an incorrectly matched ABO blood transfusion, it can lead to a hemolytic transfusion reaction. However, this is incredibly rare, as the testing procedures for blood grouping are very safe. The second most clinically significant blood group is the Rh blood group. This blood group is highly polymorphic, meaning there is a lot of variation in the Rh blood group genes and this blood group is a major cause of hemolytic disease of the fetus and newborn. This is where a mother and her baby show Rh incompatibility and this situation can potentially have very serious consequences for the baby.

Bob Barrett: So, if the Rh blood group is so clinically significant, what causes RHD blood group incompatibility and what are RHD variants?

Tracey Madgett: So, the Rh blood group system consists of several antigens, such as D, C, and E. But you may have come across the term, rhesus positive, rhesus negative, or rhesus factor, and these only refer to the RHD antigen. If a mother does not express the RHD antigen on her red blood cells, she is RHD negative. But if the baby does express the RHD antigen on their red blood cells, i.e., they are RHD positive due to inheritance from the father, it means that any leakage of blood from the baby into the mother, either before or during birth, can trigger an immune response in the mother and lead to the generation of antibodies against the RHD antigen.

In subsequent pregnancies of the mother, where there is a similar blood group incompatibility, antibodies from the mother may pass across the placenta into the baby and cause problems, i.e., hemolytic disease of the fetus and newborn. In severe cases, this may even lead to the death of the baby. Nowadays though, this scenario is managed carefully using routine antenatal anti-D prophylaxis. Basically, this is where mothers who are RHD negative are given an antibody injection and this prevents harm to the baby.

So why might the mothers RHD negative? The most common mutation for the RHD gene is a complete deletion of the RHD

gene. With about 18% of Caucasians being RHD negative in this manner, in known Caucasian populations, other Rh variants are more common but may still result in a mother being RHD negative.

In the situation we have just been discussing, the baby will express the RHD protein if they have the RHD gene present. However, mutations in the RHD gene can give rise to variation in the RHD protein present on the surface of the red blood cells and therefore affect the immune response of the mother. Sometimes the mutations in the RHD gene may be small and just affect a single base of DNA. But in other cases, the mutations may mean an entire section of the resulting RHD protein is different. These differences in the RHD protein will affect how the mother's immune system sees the red blood cells and consequently affect the antibodies she produces.

So, individuals who are RHD negative may produce antibodies to the RHD protein if they come into contact with RHD positive red blood cells. This can obviously be a major cause of transfusion incompatibility as well as in the pregnancy complications described earlier. This means that variation or mutations in the RHD gene can cause potential problems with blood transfusions. It isn't just the ABO blood group that is important for transfusion. It's vital that blood is typed accurately so that a patient who is needing a transfusion is given the correct blood. RHD negative individuals need to receive blood that comes from RHD negative donors.

Bob Barrett: How is testing for blood groups currently performed and what can genotyping offer to the field?

Tracey Madgett: So, as I just mentioned, it's key that if blood is to be transfused into a patient, it needs to be correctly typed to avoid any potentially fatal transfusion reactions. Typing of blood is routinely managed through serological tests, which use antigen-antibody reactions to determine which antigens may be present on the red cells, and which antibodies may be present in the plasma. If anti-A antibodies are present for example, and there is A antigen on the surface of the red cells, you will see an agglutination reaction when serological testing is completed.

Serological testing is fast, and for many antigens inexpensive, though there can be limitations. If the situation is complex and it's hard to determine the phenotype of the blood by serology, then genotyping can be used to look for any variation in the blood group genes. Genotyping has used a variety of tools over the past decade or so, from microarrays through to sequencing. With microarray-based approaches, the mutations in the genes need to be already known, whereas sequencing-based approaches can discover novel mutations.

Bob Barrett: Your study uses a portable nanopore-based sequencing device to show proof of principle for blood group genotyping. A couple of questions, how does nanopore-based sequencing work and have you compared it to other methods in your studies and how else is nanopore-based sequencing used in research?

Tracey Madgett: Okay, thanks Bob, that's quite a few questions to answer there. I will try and approach all of them. So nanopore-based sequencing is where we are determining the bases that are present in DNA and it's often called single molecule sequencing. It allows fast and direct sequencing of single stranded DNA molecules using biological pores. It has the advantage over next generation sequencing methods of longer reads and the polymerase chain reaction, or PCR for short, free approach abolishes sequencing biases. Nanopore-based sequencing is able to reduce the time taken for library preparation.

In our study, we use MinION from Oxford Nanopore Technologies. MinION sequencing technology is based on a flow cell containing pores that are embedded in a synthetic membrane that submerged in ionic solution. By applying a voltage, a DNA molecule is driven through the pores, causing changes in the ionic current running through the pores in a distinctive manner described as squiggle. These changes are measured by a sensor thousands of times per second, which are then translated to nucleotides using software in a process known as base calling. Previously, the MinION sequencer has been used in infectious agent surveillance and clinical diagnosis, since these areas benefit the most from real-time sequencing technology. Studies have shown the great potential of the MinION, for example during the Ebola and Zika virus outbreaks, and the technology was also being used to sequence SARS-CoV-2 during the COVID-19 pandemic.

In 2018, the RHD gene was fully sequenced by our group using next-generation sequencing on the Ion Personal Genome Machine, where allele-specific reference sequences were established. In the current study, we tested the suitability and efficiency of the MinION sequencer in blood group genotyping by fully sequencing the RHD gene and comparing the results to those previously published. RHD genotyping using MinION proved to be successful in our study and the RHD alleles determined agreed with the ones identified using the Ion Personal Genome Machine.

There were three known variant RHD alleles: RHD*01W.02, RHD*11, and RHD*15, and six novel RHD variant alleles. Both sequencing platforms, the Ion Personal Genome Machine and the MinION, give added benefit over serology in that we determine specific variations in the blood group gene.

Bob Barrett: Finally, Dr. Madgett, in your opinion, what are the advantages of using nanopore-based sequencing for blood group genotyping and what challenges remain?

Tracey Madgett: The advantages of using MinIon sequencing, a next-generation sequencing for blood group genotyping, are the faster library preparation, real-time sequencing analysis, and sequencing of longer reads that allow for better assembly. The bioinformatics for base calling and determination of variants with the MinION sequencing takes approximately one to two days. And in the future, eliminating the need for PCR amplification should speed the library preparation process and enable easier allele phasing, which is important in blood group genotyping to allow assignment of alleles successfully when there are two copies of the RHD gene present.

Phasing is the task of assigning alleles to the paternal and maternal chromosomes and is important for the RHD gene, where mutations may occur along the length of the gene and have different implications depending on the combinations and mutations present. Single molecule sequencing is also important to identify novel deletions, insertions, or hybrid alleles. But there are some challenges that remain in the phase single molecule sequencing and these include data handling, storage, and analysis.

The evolving nature of the sequencing technology makes it difficult to establish a user-friendly software that would enable fast and accurate data analysis to make it suitable for clinical use in blood group genotyping. Currently, there are many steps involved between obtaining the base course and establishing which RHD allele or alleles may be present. However, we do feel that single molecule sequencing of the RHD gene has a place alongside serology in determining the genotype of rare Rh variants when the necessary serological reagents may not be available.

Bob Barrett: That was Dr. Tracey Madgett from the University of Plymouth in the United Kingdom. She has been our guest on this podcast on using a portable, nanopore-based sequencing device for Rh blood group D antigen genotyping. Her paper on that topic appears on the September 2022 issue of *Clinical Chemistry*. I'm Bob Barrett. Thanks for listening.