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Confirmation of Insertion, Deletion, and Deletion-Insertion Variants Detected by Next-Generation Sequencing

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Guest: Dr. Linnea Baudhuin from the Department of Laboratory Medicine and Pathology at the Mayo Clinic in Rochester, MN.

Bob Barrett: This is a podcast from *Clinical Chemistry*, a production of the Association for Diagnostics & Laboratory Medicine. I'm Bob Barrett. Next-generation sequencing has revolutionized clinical molecular testing, offering higher throughput, decreased cost, and increased sensitivity relative to traditional Sanger sequencing. Sanger sequencing is still needed, however, in cases where next-generation sequencing read depth is low or if the DNA sequence of interest has substantial similarity with other areas of the genome. While these two uses of Sanger sequencing are unlikely to change anytime soon, a third application, confirmation of variants detected by next-generation sequencing, is more controversial. This is common practice in many laboratories, despite several studies showing that next-generation sequencing results generally do not require confirmation by an alternative method. As professional society guidelines offer conflicting recommendations, how should laboratorians approach this issue? When can next-generation sequencing results stand on their own and when is confirmation by another method required? A new article appearing in the October 2023 issue of *Clinical Chemistry* addresses these questions by comparing seven years' worth of next-generation sequencing results to corresponding Sanger sequencing performed on the same samples.

In this podcast, we are pleased to welcome the article's senior author. Dr. Linnea Baudhuin is a professor of laboratory medicine and pathology and a board-certified clinical molecular geneticist and laboratory director in the Department of Laboratory Medicine and Pathology at the Mayo Clinic in Rochester, Minnesota. Dr. Baudhuin's clinical and research interests are related to genomic medicine, including genomics technologies, cardiovascular and renal inherited disorders, and pharmacogenomics. Dr. Baudhuin, your article focuses on the quality of insertion and deletion variant results detected by next-generation sequencing and the need for confirmation of these variants prior to reporting. Can you just tell us a little bit about next-generation

sequencing technology and why it's an important tool for the clinical laboratory?

Linnea Baudhuin: Thank you. Next-generation sequencing, also called NGS, is a high-throughput gene sequencing technology that's used to detect genetic variants for a variety of clinical testing purposes. NGS is really a powerful technology because it has the capability to sequence gene regions at the rate of tens to hundreds to thousands of times in the same reaction, so it provides a lot of what's known as read depth to detect variants in a very sensitive way. And compared to the earlier sequencing technology known as Sanger sequencing, NGS has really enabled us to detect variants in a more efficient and sensitive manner, and at a lower cost, and it can also detect low level and complex variants. So, overall, NGS is a really great tool that can be used for diagnosis and precision medicine.

Bob Barrett: Doctor, you mentioned that NGS can detect some different variant types compared to Sanger sequencing. Can you expand on that a bit and explain why that is relevant to this study?

Linnea Baudhuin: Yes. Absolutely. Well, similar to Sanger sequencing, NGS can detect single nucleotide variants and another variant type known as insertion/deletion or indel variants, and unlike Sanger, NGS can also detect some larger copy number variants, some really more complex variants, and low-level variants. And the reason that NGS is good at detecting these other variant types compared to Sanger is because NGS is a highly sensitive method with multiple reads or read depth at each region as I just described. However, it is unfortunately the same high sensitivity that made the laboratory community a little bit concerned about the potential for a false positive call when NGS first came on the scene in the clinical laboratory. And because of this, laboratories were compelled to confirm all reportable NGS variant using another method, sometimes we call it an orthogonal method, and usually this other method has been Sanger sequencing.

However, as time has gone on, I mean we've been doing NGS now for over a decade, we and others have demonstrated that NGS detected single nucleotide variants, which are the most common type of variants, that clearly meet quality standards, really do not need confirmation. So, in other words, if we use appropriate bioinformatic and quality control measures, false-positive artifacts will be filtered out and we don't need to worry about those false positives. That being said, with the indel variant type, which is the second most common type of pathogenic variant, there have been a lack of publications providing guidance to the laboratories about whether or not to confirm that variant type.

So, the purpose of our research was to help provide evidence as to whether this orthogonal confirmation of indels is necessary.

Bob Barrett: Wouldn't you think it's advantageous to perform confirmation of the variants just in order to be truly sure of those results?

Linnea Baudhuin: Yes. It is absolutely advantageous to perform confirmation of the variants if you are not confident in the NGS results. However, every clinical lab test undergoes rigorous test development and validation to ensure the accuracy and reproducibility of its results. So, if a test is validated properly, which it should be in order to be a clinical laboratory test, and has appropriate bioinformatic and quality control measures, there should only be a really limited need for orthogonal confirmation. Additionally, there is a drawback to orthogonal confirmation because it adds to the workload, the turnaround time, and the expense. So if it's not necessary to do, we would prefer not to do it.

Also, an important point that sometimes lost is that with the indel variant type, they're actually much more difficult to read and call in the Sanger sequencing data, and NGS is a better technology for accurately calling this variant type. So, if we utilize Sanger as the confirmation method for indels and it's probably really the only option to use as a confirmation method, it can make the situation actually more confusing. So, what we found is that NGS, as a technology for looking at indels and calling them, is really a great technology.

Bob Barrett: Okay. So how do you go about determining whether indels detected by NGS did or did not need orthogonal confirmation?

Linnea Baudhuin: Yeah. So, this really gets to the heart of our study and our study was a retrospective review of 492 indel variants detected by clinical NGS testing, and all of these variants had either orthogonal confirmation or were assessed during verification studies, and they came in all different types of indel variants. So, the majority or about 75% were deletions, but we also had duplications, what's known as a more complex type of indel variant, a delins, and we had insertions. And we had a range of lengths from 1 to 68 base pairs, and we had heterozygous and homozygous variants, and we had good sequencing depth of coverage ranging from 62-fold to about 13,000-fold.

We also looked indels that were a little bit more what's known to be problematic in that they were known homologous or repetitive regions. But, overall, what we observed was that all the indel variants that we looked at all 490 plus were detected and called correctly by NGS and did not need orthogonal confirmation.

Bob Barrett: Okay. Well, given that all the variants you looked at did not need that orthogonal confirmation, how should laboratories handle NGS-detected indel variants going forward?

Linnea Baudhuin: Yeah. That's a great question. So, from our perspective based on our results, our laboratory feels comfortable going forward with not performing Sanger or orthogonal confirmation on most indels that fall between that range of lengths that we looked at, 1 to 68 base pairs, but we always review the BAM files to confirm their presence and the accuracy of their call and if they're not clear in the BAM files, we would then confirm them. There are, of course, other reasons to confirm. For example, if we see an indel in a more complex region that were just not quite comfortable with, and also for regulatory purposes in some cases, for example some regulatory bodies like New York State require confirmation.

So, that's what our laboratory is going forward with, but our recommendation for other clinical laboratories would be that they have their own strong understanding of all the genes included in their NGS panels, that they establish appropriate quality metrics, and that they look at the highly homologous genes and think about analyzing those by alternative methods because that's where you can run into the false positive or false negative situation more, and really have experienced laboratory staff to review and determine which variants truly need orthogonal confirmation.

Bob Barrett: Well finally Dr. Baudhuin, what are the overall takeaways from this study?

Linnea Baudhuin: Well, overall, we demonstrated that NGS is sufficient for detecting and calling the vast majority of indels observed and that orthogonal confirmation of this variant type is generally not necessary. Some parameters to take a look at to determine whether or not a variant needs to be confirmed include depth of coverage, reproducibility during the studies of the NGS, the length of the variant, and the genomic context, taking into consideration those more complex regions. And per regulatory requirements, clinical laboratories that perform both diagnostic and population screening tests need to have their own variant confirmation policy and so they may wish to perform an internal evaluation of their data similar to what we did in our study, and implementing a data-driven variant confirmation policy. What we found is that it's really helpful to reduce unnecessary expenses and time involved in orthogonal confirmation, and review and improve turnaround time, so very helpful for getting those patient results out much more quickly and meeting the needs of the patient.

Bob Barrett: That was Dr. Linnea Baudhuin from the Mayo Clinic in Rochester, Minnesota. She and her colleagues published a

study describing the accuracy of next-generation sequencing with the detection of insertion and deletion variants in the October 2023 issue of *Clinical Chemistry*, and she has been our guest in this podcast on that topic. I'm Bob Barrett. Thanks for listening.