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.

Real-time reverse transcriptase polymerase chain reaction, or RT-PCR, is the principal diagnostic method applied in the worldwide struggle against COVID-19 and is capable of detecting a single molecule of a viral genome. Correctly designed and practiced RT-PCR assays for SARS-CoV-2 should not cross-react with similar but distinct viral pathogens, such as the coronaviruses associated with the common cold, and should perform with very high analytical sensitivity. Yet examples of false positive results have been routinely encountered. An Opinion paper appearing in the November 2020 issue of Clinical Chemistry provides some insights and recommendations regarding these false positive results. Titled “A Cautionary Note on Contamination of Reagents Used for Molecular Detection of SARS-CoV-2,” it was authored by nearly 20 experts in the area.

The lead author of this paper is Dr. Jim Huggett. He is a molecular biologist specializing in the application of measurement science to biomasurement, using methods like quantitative PCR and next-generation sequencing. He leads a team at the U.K.’s world-leading National Measurement Laboratory and has been working on SARS-CoV-2 molecular diagnosis to understand the analytical factors that may lead to performance issues such as the source of false positive results. Dr. Huggett is our guest in this podcast. So, doctor, in your Opinion paper you discuss false positive results in molecular diagnostic testing for SARS-CoV-2. Can you tell us some of the causes of false positives when using PCR?

Dr. Jim Huggett: Certainly. Yeah, so there could be a number of reasons you might get a false positive. There are a few situations which we can touch on where it’s a natural product of the actual measurement process. For example, if you’re looking at rare variants, you might be interested in genetic variants to perhaps guide cancer treatment or looking to genotype fetuses from maternal blood, from maternal plasma or serum, which is increasingly used. There are situations...
there where the assay may pick up the other, the wild-type sequence for example, or the more predominant variant, and so you will get this background signal when doing qPCR. It is normal. And so, that certainly can occur, and the situation sometimes in the past when people have looked at bacterial genome, like using 16S genes, the fact that the recombinant production is from the E. coli, you would often pick up the E. coli DNA as well, and that leads to probably the major source of false positive when performing PCR-based methods and other nucleic acid amplification technologies, is the contamination route, and that can occur from a number of causes.

You can contaminate a positive sample into your negative samples, but also the nucleic acid amplification technologies like PCR, they function by essentially generating billions of copies of the very thing that you’re interested in, and so they generate the perfect contaminant. And they also are able to measure very, very small numbers in a near single copy. And so, you have this prime route for potential contamination that occurs in that content that will give you these false positive results.

Bob Barrett: Which of these particularly apply to detection of SARS-CoV-2?

Dr. Jim Huggett: So, in SARS-CoV-2 detection, the false positives that occur due to cross-reactivity that I touched on in the earlier point does not really occur because there are no other variants certainly when you’re trying to identify the virus that may cause that. In some pathogens, there are potential false positives that could occur from other microorganisms or time that it may be present, but SARS-CoV-2 is fairly unique compared to the other pathogens that may be circulating, and so it’s quite difficult to align the genome, other causes of respiratory disease like a common cold or influenza, and so you wouldn’t expect, based on the design, to get cross-reactivity with this. The main cause we think that may occur with false positives for SARS-CoV-2 is this contamination route, and particularly from cross-reactivity, but also from the actual method and synthetically derived molecules.

Bob Barrett: So, doctor, why do you think contamination is a particular issue with SARS-CoV-2 testing?

Dr. Jim Huggett: So, we have a unique situation. Any viral pathogen measurement has the potential for cross-reactivity due to a sample with a lot of virus and some of that virus getting into the sample you’re processing that happens to be negative next to it. That is a common issue and it occurs in any sample, really, and it can be a particular case with viruses
because there can be quite a lot of them in a sample, you’ve got a lot of the virus in it.

Then, we touched on the fact that the PCR can contaminate itself, and so you can have this cross-reactivity from an older experiment, that has generated lots of material contaminating the next one you do. Generally, there are systems in place, that laboratories know about this, that is to have the unidirectional flow of the different stages of the PCR, to make sure that you do not contaminate the latest stage or with the earliest stage of the next experiment.

SARS-CoV-2 has a unique component, however. One of the things we would do, and many other laboratories would do, wanting to design a new assay to a serious pathogen like SARS-CoV-2 where we don’t necessarily have the ability to handle it, is to synthesize the molecule. We would make the target molecule that we’re interested in part of the viral genome to get our PCR assay up and running.

Now, this happens all over the world for a variety of different situations, but crucially we use the same, generally use the same chemistry that we used to make the primers that are also used in the assay, but the trouble is when we make these molecules, we make huge amounts. A basic synthesis is able to make a thousand trillion molecules in a single go, and so it’s a very sensible practice to not make the molecule that you’re interested in detecting in the same place as the reagents that you’re going to use to detect it. Because it doesn’t matter how careful you are with your method, a thousand trillion molecules, it’s so easy to contaminate them and bearing in mind the methods, the nucleic acid amplification technologies, were able to measure very small amounts. So, it doesn’t take much.

If you imagine you have a thousand trillion molecules, we put that in an Olympics-sized swimming pool and mix it around a bit. You’re going to pick them up when you take another microliter out. It’s just physics and the numbers. So, normally, we’d make sure we didn’t make the molecule in the same place as we get our reagents from, but this is different. Everybody is making these molecules now across the world. And so, it may no longer be as easy to choose a particular manufacturer of your oligos, or your primers, of the reagents, because they may well have made the molecules for the assay, the whole gene that you’re interested in already, and what it may also be the case is they may not know that they’ve done it, because people may be asking for these products to be produced without necessarily telling the manufacturer what they are.

And so, it’s a unique situation, and so we’ve seen this and in the opinion piece we wrote, we’ve demonstrated this has
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occurred until you may be ordering assays for something completely different, but it will have SARS-CoV-2 DNA in there simply because of this mass production globally of these molecules, whereby as I say, you’re making a thousand trillion molecules in a single run to the extent that we may be in the same – SARS-CoV-2 is an RNA virus, but it’s possible although discussion that there are now more DNA gene molecules because of the synthesis that we’ve done on earth, than there are RNA of the actual pathogen itself. And so a very unusual situation and this opinion piece essentially wanted to flag that to the community, to make sure they are aware that this is a potential problem.

Bob Barrett: Are other molecular tests susceptible to the same false positive concerns?

Dr. Jim Huggett: If you broadly split the three molecular tests into three different categories of hybridization or arrays, sequencing, and nucleic acid amplification technology, and these are not mutually exclusive, it is really the nucleic acid amplification technologies that are the ones that are at most susceptibility to this, and this is because they are, they work by finding the molecules of interest that could be fairly dilute in the sample, and generating the lots and lots of them. So, they’re both quite analytically sensitive--measuring hundreds, tens, even single molecules, the digital PCR technology is measuring single molecules as a rule, and so because it has this exquisite sensitivity, it’s particularly sensitive to this. Other molecular methods, the sequencing-based strategies, or array-based strategies are perhaps less so because they’re not as sensitive. However, many of them will use a nucleic acid amplification technology as the front end to improve their sensitivity, and then you use an array or a sequencing-based strategy to then measure that, and so yes, in that context, they may well be.

So, it’s dependent on the nucleic acid amplification stage of it. And so, PCR is an obvious one, but there are other isothermal nucleic acid amplification technologies which work in a similar way, and so they will also be sensitive potentially to background contamination that may be occurring and also other sources that we described earlier.

Bob Barrett: So, what can be done to prevent such positives now and in the future?

Dr. Jim Huggett: So, if we think about the earlier causes like the cross-contamination from high samples and synthetic sources from the PCR reaction itself, essentially lab practice, more careful lab practice to stop cross-contamination and not having this unidirectional workflow where you separate your new experimental preparation from the post analysis place where you’re maybe handling the PCR products or other
nucleic acid amplification products that have generated after the experiment. Those are common procedures we’re very familiar with, and the other thing we must do to consider this is control. This is very, very important, is to have controls throughout the experiments.

Obviously you have positive controls, but also considering of your negative controls to try and track the presence of potential contaminations and numbers of controls, not just one or two in a 96-well plate, a substantial amount of controls to look at the different scenarios. And maybe you don’t need to run them every time you do the experiment; you need to run controls every time you do the experiment but maybe you don’t need to run lots and lots of controls. However, when you have a new batch of reagents or something, it would be worth testing those just to ensure that there’s no background signal present in those.

One of the important things to consider with the controls is to have what we call carrier-containing controls. A non-template control is a common control you would use, and essentially that is the entire experiment with no nucleic acid, no RNA or DNA in it. However, it’s kind of well-known now that if you include a carrier, an RNA or DNA, it can improve the sensitivity of the method and the clinical samples tend to have carrier, they tend to have nucleic acid in them. So, it’s quite important to have a nucleic acid containing control that is like the clinical sample, that you know does not contain the target of interest, it doesn’t contain SARS-CoV-2, and that just allows you to really be sure that the low-level signals you may be getting are potentially, exactly that, that they’re real, that it’s not contamination.

Bob Barrett: Well, finally, doctor, given the current status of antibody, antigen, and molecular testing, does real-time PCR deserve its much-touted status as the gold standard for the SARS-CoV-2 diagnostics?

Dr. Jim Huggett: And that’s an amazing – a really good question, and we have -- how long have we got? So, first of all, I answer this question not as a clinician, having to handle this pandemic. I’m very busy at the moment but I’m rather fortunate that I am able to work on this in the lab without having those stresses. And so, real time PCR, or real-time RT-PCR, has allowed us to respond to the pandemic.

To try and imagine where we would be now if PCR didn’t exist in responding to this, it is almost rather frightening to think about that. However, we have chosen the method that is potentially the most analytically sensitive method we have at our disposal. And so we may find when we look back, in three, four, or five years when we’re able to review this, having much better knowledge of what the results
mean in terms of infection, in terms of infectivity, in terms of disease, we may look back and find that actually, the method was a little too sensitive, and certainly people are beginning to advocate this per se in the U.K., perhaps you hear it in the United States, the methods may be measuring dead RNA and essentially a patient may have had COVID-19 infection and they may have cleared it, but it is possible because of this exquisite sensitivity that the PCR is picking up these fragments of RNA that you’re no longer infected, so you’ve cleared the disease. And that may be complicating the response because the patient may be being called positive when perhaps they are not. However, I would say that the methods that have been used to look at that, the qPCR method itself, is highly variable. It’s quite a precise method to quantify, but the actual, the unit, the qualification cycle, or the cycle threshold, are highly variable. And so, using that as a metric to quantify is tempting, but we need to be very cautious.

In the context of antibody testing is a bit of a different measure, I guess, because it’s measuring the patient’s response to the infection, and that may have occurred afterwards, but in the case of antigen testing, that’s a very, very interesting query because the antigen testing may well be much less sensitive, analytically. So, if the PCR can measure near-single molecules in the reaction, which translate to hundreds of molecules per mil of, I guess, viral transport medium, what does that mean in the context of the antigen? Well, it’s possible the antigen will not be as sensitive — very likely, the antigen is not going to be as sensitive as that, because the methods are not as sensitive as the PCR, but what that means, we don’t really know yet. And so, I think we need to be very, very careful with calling PCR the gold standard and everything must be compared to that, and anything disagrees with it is not necessarily valid. I think we need to tread quite carefully because this pathogen has only really been -- well, in the U.K. and in the U.S., we’ve only been really working on it for what? Nine or ten months. And so, I think we need to be careful about using it, and so does it deserve its status as a gold standard? I don’t think so in terms of a diagnostic comparison study. However, we’re very lucky we have it.

Bob Barrett: That was Dr. Jim Huggett from the University of Surrey and the UK National Measurement Laboratory. He was lead author of a paper in the November 2020 issue of Clinical Chemistry on examining contamination as the cause of false positive SARS-CoV-2 results. I’m Bob Barrett, thanks for listening.