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SYPHILIS TESTING: WHAT'S NEW IN THE LABORATORY?

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[video transcript]

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All right. So I'll get going. And I've been asked to talk today about what's new in the laboratory for syphilis testing. And essentially, I would respond to this as there's not a lot that's new. What is new is automation in the laboratory. So my speaker disclosures I don't have any. And the learning objectives that I'd like to present today are to review how diagnostic tests for syphilis are increasingly automated, but otherwise remain largely unchanged. To summarize how the reverse testing algorithm exploits increased sensitivity and automation in new assays, and to discuss how automated RPR assays may be available in some laboratories. So to begin, I just want to go through some background about syphilis that I'm sure you're all very familiar with. Again, Syphilis is caused by the Treponema pallidum spirochete bacterium, which was an organism that was first identified in 1906, and so named because of shape under the microscope, where it has the sort of spiral or more accurately known as a helical shape. And the name treponema comes from the Greek to turn a NEMA the thread so turn through it, and I think you can appreciate that based on its morphology. So Syphilis is usually a sexually transmitted disease that is also transmitted congenitally. And basically transmission occurs when the spirochete come into contact with the mucous membranes or abraded skin. And something that's really quite unique about syphilis as is it as a low infectious dose, with as few as 10 organisms being able to cause an infection, an infection. Once syphilis invades the epithelial layer that occurs within hours, and it can then disseminate throughout the body through the blood and lymphatic system. And like I'm showing in this image here, you can see these organisms growing to high numbers in tissue, using methods such as silver staining, or immunohistochemistry. Syphilis continues to be STI that is increasingly seen in both New York State and nationally, for syphilis, for syphilis that is sexually transmitted. Currently, there it is on the increase in New York State and other places around the world. And a lot of these are actually cases that are seen in men. This is basically data from New York State's surveillance report from 1936 to 2019. And you can basically see that over time it has waxed and waned, particularly with some public health interventions and with the advent of penicillin. So for example, here you can see in this sort of World War Two era, the advent of penicillin caused the number of syphilis diagnoses to decrease significantly. So congenitally transmitted Syphilis is also something that is increasingly seen and congenitally transmitted means that vertical transmission during pregnancy, and that is can result in fetal or perinatal death and morbidity and surviving newborns. And just something to mention, as I'm sure you're all very familiar with, although laboratory diagnosis is very helpful, it's very important to be have an accurate stage of syphilis, so that you can interpret the laboratory diagnostics correctly. So syphilis does have primary, secondary, latent and tertiary stages. And the primary is sort of characterized by the appearance of the chancre. The secondary stage of Syphilis is sort of notable for the appearance of rashes and mucous membrane lesions. The latent stage is typically asymptomatic. And the tertiary stage includes things like making name, late neurosyphilis, gumma and tabes dorsalis, and so many unsuspected cases of syphilis are discovered by laboratory testing. But the complexity of syphilis serology means that the services of both laboratories and clinical experts are often needed to stage correctly and come up with a plan.

So I'll talk briefly about some of the direct and molecular methods for the detection of syphilis. So direct observation methods include things like dark field microscopy, which is something that is less frequently seen now in both clinical laboratories and in point of care testing system point of care testing. So these are basically when you would take exudates from chancre and you would perform dark field microscopy to look for the presence of the bacteria in those specimens. In past times, culture has been used, but it's not practical anymore and not practical for diagnosis, because it typically relies on infection of epithelial cell cultures, or what's known as a rabbit infection test, where you take inoculation of your patient specimen into a rabbit testicle, but again, this is very specialized and not used a lot. The third method that is a molecular method is PCR. But that's also not something thing that is done with frequency because it usually requires a tissue biopsy.

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So serological testing is currently the best method for syphilis screening and diagnosis. And serological tests are usually broken up into treponemal tests and non treponemal tests and treponemal tests are so named because they actually detect treponema pallidum specific IgM and IgM-class antibodies. And so these treponemal tests include most of the conventional tests such as indirect IFA, which is shown in the screen picture here, where basically there are syphilis treponemes on a slide, that you then wash with the patient's antibody, and then you detect if the antibody is present using a fluorescent antibody that detects that patient antibody. There are now what's more common in clinical laboratories are these chemiluminescence and multiplex flow immunoassays, some of which I will talk about a bit later in the presentation. And then the second type of tests for syphilis are the non treponemal tests. And these are those that detect antibodies in serum or CSF against molecules such as cardiolipin. And so cardiolipin is not something that's specific to treponemal palllidum but it is detectable in most patients with syphilis. And because it's not specific for syphilis, it can result in biological false positives. And things like cardiolipin, and the release of them is thought to be a product of damaged host cells, when treponema and some other bacteria sort of invade through tissues. It's also important to note that some bacteria do actually have cardiolipin in their membranes. So that could be detecting the cardiolipin in treponema, or cardiolipin from the host resulting from damage. And so some of these non treponemal tests that are most common are RPRs and VDRLs, and I'm showing RPR on the right there, and I'll talk about that more a little bit later. So one of the reasons why there's been a lot of focus on the newer treponemal tests is because treponemal antibodies typically appear earlier than non treponemal antibodies. So as I'm showing here, in this diagram, on the y axis, it's the sort of percentage of patients testing positive. And then on the x axis, it's the number of weeks and years that you might be testing positive. So the first thing that is usually becomes positive is IgM antibodies for treponemal antigens. Whereas things like your treponemal IgG antibodies are a little bit later, and sort of coincide with non treponemal antibodies, which are those that would be detected in assays like the RPR and VDRL. So essentially, the point here is that some of these treponemal antibodies appear earlier than the non treponemal antibodies. And that's what some of the more modern treponemal chemiluminescent assays in clinical laboratories are designed to detect. So the RPRs, however, are still quite common. And they're these non treponemal quantitative tests. And that quantitative ability with these tests is what's particularly useful for clinicians. So RPR are a flocculation test that is based on the reaction of patient antibodies to a preparation of cardiolipin

that has been adhere to the side of a charcoal particle. And when a patient's antibody interacts with the cardiolipin on those charcoal particles, it sort of groups them together, it flocculates them together, causing these characteristic reactive patterns that you can see on the right there. And then the ability to quantitate by making dilutions of these allows you to get your titer in difference there which can be helpful to clinicians to assess differences between earlier and later tests. And again, here the quantitation is reported as the highest titer that produces a full reaction. And so this can be used to assess treatment response or to assess cases where reinfection might be considered. This is basically just a list of the current FDA approved manual non treponemal tests for syphilis. So as I've tried to illustrate here, I've split them up into primary, secondary and tertiary syphilis, and how well these tests perform. So the numbers here are showing the sensitivity for these assays for detecting syphilis in these different stages. So things like RPR may actually not perform particularly well in primary syphilis. But once you progress to secondary syphilis, they begin to work better and become at least up to 100% sensitive

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with respect to specificity. These tests generally have specificity between 93 to 99%. And most of them probably fall in the area of about 95%. The important thing to notice about these traditional non treponemal tests is that they are all manual. And the only one that sort of stands out here is the VDRL, which can also be used on CSF. The third type of test that is more recently on the scene in addition to the non treponemal, and the treponemal laboratory based tests, are these sort of rapid syphilis tests that have been FDA approved in recent years. And one that people often ask about is this syphilis health check. And this is what's known as a lateral flow assay that can be performed on fingerstick blood, which is approved for clear wave settings, which means it can be used at point of care, or in serum and plasma, which is performed in the laboratory. And this is basically for the qualitative detection of those treponemal antibodies. So this can be used as an initial screening test. And the manufacturers for these types of tests usually insist that these are followed by a non treponemal test performed in the laboratory. So with something like this, you'd always want to follow it up with an RPR. So the limitations of one of a test like this is that a positive result may not be useful for establishing a diagnosis of syphilis infection. And this is because they sort of lack specificity. And because they don't have an RPR, immediately performed after them, unless a sample is sent to a lab. So because they also tend to lack sensitivity in primary syphilis, they can also be falsely negative in early primary disease, when those treponemal antibodies have not yet been produced by your body. Another sort of new, rapid syphilis test that is on the market, is this DPP HIV syphilis system, which is approved for both HIV and syphilis. And something that is quite interesting to note about this is the technology that is used in this platform. And this is known as dual path. And this essentially is a lateral flow assay. So if you follow the picture down here, you would take the patient sample added to this pathway in the dual pathways test system, the antibodies in the patient's specimen would flow along into the test device, and they would bind the antigen on the test and control lines. Then a second step, where you basically add the reagents that detect the antibodies from the patient allows lines to form on the lateral flow assay that can then say, Oh, this test is positive for syphilis, or for treponemal antibody, or HIV antibody. And the reason why this dual pathway this dual path platform is being developed, is because assays like this are thought not to be influenced by what's known as the hook effect, where you can get

false negatives due to high concentration of antibodies that essentially prevent the antibody from binding to the antigen test line. So an essay like this can also be used as a first tier essay in the reverse algorithm. And I'll be talking about the reverse algorithm in a bit more detail later. So here is the traditional reverse and what I call the European screening algorithms. So these algorithms continue to be controversial because they have several interpretations. So the traditional algorithm usually begins by screening with a non treponemal test. And in most cases, this is RPR. And if you're nonreactive, then basically the APHL reporting language here is such that you can say no laboratory evidence of syphilis detected. If the RPR is reactive, many labs will do a treponemal test like FTA ABS or one of the more modern chemiluminescent immuno assays. And if it's reactive, then the reporting language is consistent with past or current, potentially early syphilis. If it's nonreactive, syphilis, unlikely, biological false positive possible.

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In comparison, the reverse algorithm begins with a treponemal test. And this is usually done using an automatic automated immuno assay in the clinical laboratory. And if you're nonreactive in this first screening test, then the reporting language is no laboratory evidence of syphilis. If you are reactive by your first treponemal test, that's followed up with a non treponemaltest, which is RPR. And if it's reactive, the reporting languages consistent with current or past syphilis. But if the non treponemal test is nonreactive, then a laboratory should perform a confirmatory treponemal test. And the sort of tests that the CDC prefers or mentions most often is the TPPA. And if it's nonreactive, here, then it's inconclusive for syphilis. But if it's reactive, then this could be consistent with past or current on potentially early syphilis. And I just wanted to show some of the sort of differences in the language here. So for the reverse algorithm, if it's reactive by the second treponemal test, they usually put the past first here, so it's consistent with past or current, sort of emphasizing that this might be a result, you might see if you've had treatment, or if the syphilis infection was, was in many years ago, okay. And then the European algorithm is just slightly different, because it doesn't really use RPR or oh, RPR is done standalone, and this uses two treponemal tests in succession. So essentially, the CDC recognizes both the traditional and reverse approaches. But what do clinical laboratories do? I think in recent years, there has been this trend towards laboratories adopting the reverse algorithm, particularly if those laboratories are busy reference labs. So from College of American Pathologists, surveys in 2016 at least 63% of laboratories said that they were still using the traditional algorithm, where a 16% said that they were using the reverse algorithm, but at least 10% of laboratories were anticipating changes in the coming year. And the thought was that this would represent a shift towards the reverse algorithm. So what are other benefits and drawbacks of the traditional reverse syphilis serological algorithm. So for the traditional algorithm, some of the problems with these non treponemal tests, like RPR, is that they can be subjective, and also have less specificity than treponemal tests. And this may result in false positive results, especially in areas with low disease prevalence. And this is actually true of the treponemal tests in the reverse algorithm as well. What's more of a problem is that even though RPRs are inexpensive, they are manual tests, and they require manual processing. And that is a significant cost and limitation for high volume clinical laboratories. Because it's manual that requires a lot of technologists time, and a lot of people working on that to have a high throughput. And then something that is commonly associated with beginning your traditional algorithm with an RPR is that false negative results may also occur with some of those tests due

to the prozone effect. That would be where you have excess antibody that prevents the test from working as it should. With respect to the reverse algorithm, sometimes the results from these automated treponemal tests are more objective than manually interpreted essays, because they're detecting the production of light or fluorescence. An instrument is basically responsible for interpreting those. So that's why those results are considered more objective. And because it's detecting the production of light or so on in these assays, they are also thought to have increased sensitivity. And they also have increased sensitivity because they're detecting treponemal antibodies, which appear earlier than non treponemal antibodies. And with this increased sensitivity, the CDC estimates that an additional 3% of patients can be diagnosed with this algorithm compared to the traditional algorithm. The other thing that's also helpful is that automation enables high throughput testing. And so for the larger population, this causes faster turnaround times to be possible. And the other thing that the reverse algorithm has often been touted for is that it may be useful for detection of patients with untreated latent syphilis, who may be RPR negative. The other thing that I wanted to mention is that there are now FDA approved automated RPR assays. And so the manufacturers have brought these assays up because

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if you're if people say that the reverse algorithm allows labs to automate, then these allow labs who still use the traditional algorithm to automate the first step there as well. So these are the three FDA approved automated RPR assays that I'm familiar with. And the first is the BioPlex syphilis total RPR. So this actually performs both the treponemal test and an RPR at the same time, the ASI evolution and the AIX 1000. And these have all been approved in very recent years in 2016 and 2018. In terms of their sensitivity, they all seem to range between 85% and 95%. And maybe a bit better than that. So this is less than the treponemal antibody assays. In terms of their specificity, they all seem to hover around 98 to 99%. So their specificity is quite good, but their sensitivity is not as good as treponemal antibody tests. These are the sort of comparison automated treponemal tests that are somewhat widely used in laboratories. And again, I've sort of tried to show these by their sensitivity in the different stages of syphilis. So what you can see here is compared to something like an RPR, these assays do have greater sensitivity, in general in primary syphilis, and in syphilis in general. And they also tend to have greater specificity. And part of this greater specificity is because these assays tend to use sort of recombinant antigens, that are sort of cause these assays to have a greater ability to be specific, as opposed to whole treponemal or antibodies to cardiolipin which are not specific in those non treponemal tests. And as you can see, for laboratories that process a large number of specimens, these allow labs process a large number of specimens relatively quickly. So they all have very convenient runtimes. And they can be used on a variety of different specimen types that are sort of compatible with VRP, RS and downstream assays. So this is one of those assays that I wanted to mention, it's one of the things that has been more recently approved. And it's an assay that can do both the first treponemal antibody step, or the RPR, assay and titering all on one platform. And this is important because it can be used with both the traditional and reverse algorithms. So this allows laboratories extra flexibility. So the Bio Plex is what's known as a multiplex flow analyzer. And I'll just go through how it works very quickly. So I'm showing here, what are supposed to be these sort of unique antigen coded microparticles. So there's one bead with cardiolipin. On it, there's one bead that has treponemal fusion proteins on

it. And then there's other beads that are internal standards and so on, you would then add your patient sample to those beads. And if the patient sample had anticardiolipin, or reaginic antibodies present, they would bind the cardiolipin and if the patient had treponemal antibodies present, they would bind a treponemal fusion protein, and then you would add what's known as your conjugate antibody, which is bound to a fluorescent molecule. And if those antibodies for cardiolipin or treponemal, were present, they would bind to those the conjugate antibody would bind to those antibodies. And then you can excite those with the laser, and then they would produce essentially light. And then basically, you have a pool of all these beads, and it passes through a multiplex flow detection system. And that allows the instrument to count the different beads, and to determine which of the analytes was positive or had a detection on it. And this is how the instrument basically allows you to say, Oh, I'm RPR positive, or I'm positive for treponemal antibody at the same time. And this instrument is also useful because it can automatically titer up to one and 64. And if you want to titer above that, you'd have to perform a manual titering. So like but part of the difficulty with, you know, incorporating something like this is how to work this in with new algorithms. So that's something that in the future may have to be considered if you do two tests at the same time up front. And this is the test that had recently been in the news because there's an FDA alert about it with false positive RPR reactivity, potentially caused by administration of a COVID vaccine. So those of you who frequent the CDC website may have seen this.

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But that's not to say that you don't get false positives with some of the other assays. And so false positives with RPR essays in particular, are particularly well known. And these have been observed in people with systemic infections that are unrelated to syphilis, including things like TB, HIV, Lyme, malaria and so on. Also, sometimes common following immunization. I guess now we know that may be true with COVID vaccine as well. And false reactivity is sometimes seen during pregnancy and with IV drug use and As part of this is sort of mathematical because these people may be screened more frequently. But there may also be a biological reason behind this. And so part of the reasons for the CDCs guidelines which recommend screening reactive results, or confirming reactive RPR results with a treponemal test is because of these known fast reactivities. And as I mentioned before, the CDC recommends using TPPA. And these are basically in a group that are known as the conventional treponemal tests for syphilis. And again, these are all manual assays, even though they're technically simple to perform, they are labor intensive, and they do have subjective interpretation. So, in the reverse algorithm algorithm, the TPPA would be used as the third step, if you want to if you if you had the situation, where you were reactive by your first year by your treponemal test. And then if you had done an RPR, and that RPO was negative, then you would go on to do a TPPA to confirm the results. So these can assist in determining if the first two results are truly or falsely positive. So the one I already mentioned was the sort of fluorescent treponemal antigen, indirect immunofluorescence assay. And the one that I'll talk about next is the TPPA. And this is an agglutination type assay that detects IgM and IgG. And it is thought to have good sensitivity, but more particularly, it has excellent sensitivity. And that's kind of the feature that you're looking for in your second treponemal antibody test. So the TPPA test is the triple aim of Pallidum particle agglutination. And the CDC considers this to be the most specific manual treponemal assay. And basically how it works is gel particles are sensitized, with sonicated treponemal pallidum

spirochetes. And this basically means that antigens from that bacteria on the outside of the gel particles, you then add the patient's serum. And if it contains antibody to T pallidum, you essentially get a result like this, where you have a smooth matte of agglutinated gel forming in the well. But if there aren't antibodies present the particle sail to the bottom, forming a compact button. And you can also use this assay to titer and quantitate. So even though TPPA is commonly used in many clinical laboratories around the country, some laboratories may actually choose to use other treponemal antibody assays in the third tier of their reverse algorithm. And so for example, one that seems to be used with frequency is this TrepSure enzyme immuno assay, which can either be used as an initial screening test, or as a confirmatory diagnostic test in the third tier. And again, one of the features about this assay is it is thought to have 99.8% specificity. So it has good specificity, on par with that of TPPA. And that would be a feature that you would look for in this third step algorithm. And the other thing about it that I'd like to mention is that even though that this is the specificity that the manufacturer has set forth, it has been shown in clinical trials that this can actually be less depending on the patient population that you're using it in.

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And so this is my last slide. And I just wanted to go over why sensitivity and specificity is important in these testing algorithms. And essentially, the reason is, is because they are in they are used to drive the positive predictive value. And that's something that is highly dependent on the specificity of the assay and the prevalence of the disease in the population. And I'll try and show you what happens when you use two tests with these different sensitivities and specificities into different population, one with a prevalence of 0.0386% versus a population with a prevalence of 1%. And I chose this point 038 6% Because that's what New York State had said. The number of cases of early syphilis were occurring per 100,000 residents. So if you have a test that is 97.5% sensitive, and 98.4% specific in a population, where Syphilis is prevalent, prevalence is only point zero 3%. The positive predictive value is only 2.3%. So this means that if you tested 100 patients, only two of them would be true positives and the rest 98% would be false positives. However, it does work well at saying that people are truly negative. And likewise, if you had a test that was a little bit more specific than the first test, your positive predictive value would increase to about 15%. So this means that about 15 out of 100 tests would be true positives, and 85% would be true would be false positives. If you put the two tests together and recalculate the positive predictive value, combined, the positive predictive value for those two tests increases to about 92%. So that means for 100 people that are tested, 90 to 92 would be true positives, and only eight would be false positives. And then if you sort of increase the prevalence of syphilis in the population to 1%, now you basically improve the positive predictive value for each test separately. And combined, the positive predictive value increases to 99.7%. So this slide here is just to show the importance of knowing the population that you're testing, if they have risks that sort of increase the prevalence of syphilis in their patient population. And also understanding how the different tests can affect, you know, the ratio of true positives to false positives that you might expect. And this also shows the importance of these different algorithms in ensuring that results are accurate. So I'd like to conclude by just saying that the diagnosis of Syphilis is helped by evidence of exposure and characteristic symptoms and signs which allows you to stage syphilis accurately, alongside with the laboratory tests, of which the serological ones are the mainstay. The complexity of syphilis, serology means that

the services of both laboratories and clinical experts are often needed to interpret them correctly. And the reverse algorithm is increasingly used by clinical laboratories to take advantage of automatic assays. But also because there are now automated assays for RPR. This may cause some laboratories to remain traditional. So in conclusion, it can be important to understand your laboratories algorithm, and how it might perform in different people, different patient populations, or at different stages of syphilis. And I think at this point, I can take any questions that people might have

[End Transcript]