Questions & Answers with **Karolina Pusz-Bochenska**, first author of "A Rapid, Simple, Laboratory and Field-Adaptable DNA Extraction and Diagnostic Method Suitable for Insect-Transmitted Plant Pathogen and Insect Identification" published in *Plant Health Progress.*

How do you determine if a "positive" result generated by LAMP is a "true" or a "false positive"?

Assay validation data has been obtained for the phytoplasma assay (manuscript under review). Essentially, parameters such as analytical sensitivity (limit of detection); analytical specificity (ability of the assay to detect the target organism to the exclusion of nontarget); and comparison of methods—comparing assay results to an accepted "gold standard," which is nested PCR targeting 16S in the case of the phytoplasma assay. We found that samples testing positive by the gold standard nearly always test positive by LAMP, but some samples testing positive by LAMP test negative by nested PCR. Analyzing those samples with other assays (i.e., qPCR, nested PCR targeting other genes) revealed that those samples, in the large majority of cases, were false negative by nested PCR rather than false positive by LAMP. This is consistent with literature findings of increased analytical sensitivity of LAMP versus PCR, which we have seen in our other results as well.

Did you investigate portable PCR systems, and what are your thoughts on these for field testing?

We have not investigated the portable qPCR apparatus, as we do not have such a system. It certainly offers some advantages in theory, such as reliable quantification of target. LAMP is generally semi-quantitative, with an inverse relationship between time to positive and target amount, but not as linear as qPCR. For in-field work for which precise quantification is not essential, LAMP still offers more rapid detection versus qPCR. But other options such as insulated isothermal PCR are certainly possible. There is no reason why template DNA prepared in this way would not work for such detection methods, but we haven't tried them because we don't have the equipment available.

What wash buffer did you use for plant extraction?

For plant extraction, see Zhou et al., PLOS Biology, 15, e2003916, 2017 (https://doi.org/10.1371/journal.pbio.2003916). For the insects, we used Qiagen plant kit lysis buffer for lysis, then TE0.1 for washing—see our manuscript (https://apsjournals.apsnet.org/doi/10.1094/PHP-09-19-0063-FI).

Is it possible to adapt the technique to identify plant viruses transmitted by insects?

We have not tested whether the method would release nucleic acids from viruses. We did not have colonies of virus-infected insects available for such testing.

Nested PCR takes 2 days, not 1 week. We do nested PCR. Are FTA cards economically feasible?

True enough—the 1-week time frame was cited due to the fact that we commonly collect many samples to do over the course of a week or so and then do all of the samples at once. The FTA cards are not particularly expensive.

Can FTA extraction method be applied to ethanol- or formalin-preserved insects?

Ethanol, yes; formalin, not tested.

Any information on *Cydalima perspectalis* in Canada?

CFIA reported one introduction of box tree moth into Canada in 2018. No word on whether it established.

Could this method be used with plant tissue?

See Zhou et al., PLOS Biology, 15, e2003916, 2017 (https://doi.org/10.1371/journal.pbio.2003916). That method did not do a good job of homogenizing insect tissue.

In your experience, how difficult is the primer design for LAMP?

Primer design is complex to do by eye but relatively simple using readily available software. We have used LAMP Designer from Premier Biosoft, but there are free options available such as PrimerExplorer (https://primerexplorer.jp/e/).

For insect extraction, besides AP1 and 70% ethanol, did you use any washing buffer?

Just TE0.1 as per the protocol.

Any cost-effective alternative to FTA?

We have not found the FTA cards to be particularly cost-limiting. Due to the size of many of the insects, we can extract a number of insects on the same card, making sure to avoid cross contamination by not placing them too close together and by wiping the fabric punch and punching out paper blanks in between extractions.

Do we need to run sequencing for LAMP positive samples to confirm the identity of phytoplasma?

Once the assay has been validated, there is generally no need. The purpose of collecting assay validation data is to allow the proper interpretation of positive and negative results—things such as "which pathogens are detected?" "how much pathogen needs to be there to generate a postive signal?" and "how do the results generated by this assay compare to gold standards?" During assay validation, certainly there is some amount of sequencing confirmation and other validation approaches. But once the validation data are obtained, the assay can be used generally without the confirmatory steps.

A little divergence from plant pathological research or diagnostic—why do you think LAMP is not adopted for COVID testing, since it is sensistive and fast, based on your experience?

LAMP has absolutely been used for covid detection and with very good results. See Rabe, B. A. & Cepko, C. (2020) SARS-CoV-2 detection using isothermal amplification and a rapid, inexpensive protocol for

sample inactivation and purification. Proceedings of the National Academy of Science of the United States of America, 117, 24450-24458.

How much of your insect extract do you use for the LAMP reaction?

The whole thing!

Do you think the method is adaptable for RNA extraction from a bulk insect sample?

We have not examined this. It would be particularly useful for the detection of RNA viruses, but we haven't tested it.

What machine/tool did you use for the LAMP test? How much it cost?

We use a Genie instrument, available from OptiGene in the United Kingdom. It costs around $20K in Canada. There are many other options, some of them cheaper.

Can you indicate the reference for the extraction in 30 s from Zou et al.?

Zhou et al., PLOS Biology, 15, e2003916, 2017. https://doi.org/10.1371/journal.pbio.2003916

Can you describe how easy or difficult it was to develop four to six primer sets for LAMP? Have you considered comparing LAMP versus RPA, as I have heard its easier to develop RPA assays?

LAMP designer software facilitated primer design. We have also successfully used this extraction method to provide a template suitable for RPA (for the detection of Xylella fastidiosa*). In our limited experience, RPA primer design is not difficult, but it is iterative (need to test many different primer combinations in three rounds to get a set with maximal analytical senstivitiy). Moreover, the probes, while not difficult to design, must be tested empirically, and they are quite expensive; several probes may need to be tested to find one that works. We are not aware of software that can help with this.*

What are the costs of this methodology for insect DNA isolation and identification? Our resources are rather restricted, and we have standard PCR equipment. What specific consumables and equipment would we need to buy?

The consumables and equipment are detailed, along with the protocol, in the manuscipt. The method is designed to be as low-cost as possible. It might be useful to have a portable power supply, but these are not expensive—under $500 in Canada.

Was there a particular reason you went with a LAMP-based isothermal assay, as opposed to RPA?

*Just that we had more experience with LAMP (RPA was first described perhaps as far back as 2006 but I think has only been commercially available for a couple of years). We had equipment and experience. We have indeed used the method for providing a template for RPA (*Neokolla hieroglyphica *carrying* Xylella fastidiosa*), and it works well. That is in our upcoming manuscript in* Methods in Molecular Biology.

We might want to try the plant-based extraction method. Would you provide a link for the paper that describes that method so that we might try this?

Zhou et al., PLOS Biology, 15, e2003916, 2017. https://doi.org/10.1371/journal.pbio.2003916

Can you make a page of the protocol available?

https://apsjournals.apsnet.org/doi/10.1094/PHP-09-19-0063-FI

How effective is the paper-based DNA extraction method? Can it conveniently replace the conventional method? Are there specific requirements?

We tried to show in the manuscript that the method is effective for a wide range of insects in different conditions (live collected, ethanol, sticky traps). As a field-based method there will be trade-offs compared to lab-based methods in terms of DNA yield and purity, but we showed that the template provided is suitable to detect pathogen DNA in insects, in both field-collected insects and laboratory-reared infected colonies.

Do you think the LAMP method is oversensitive versus qPCR method?

Oversensitive. Good point. LAMP seems to have an increased analytical sensitivity versus PCR, but how sensitive does the method need to be? That depends on the pathogen—for some, any amount is too much; for others, there needs to be a certain threshold amount to cause disease. In the case of phytoplasma, any insect harboring the bacteria in its saliva, even at low levels, is a concern because the levels will increase and the insects are feeding on the plants and can transmit. The purpose is to know if there are insects with the pathogen in the field in order to time mitigative measures properly (insecticides). If you wait until symptoms appear, you should have sprayed weeks ago—nothing to be done to save that plant.

Can you do multiple reactions with LAMP like multplex PCR?

Yes. One example is DARQ probes—see Tanner, N. A., Zhang, Y. & Jr., T. C. E. (2012). Simultaneous multiple target detection in real-time loop-mediated isothermal amplification. BioTechniques, 53, 81-89.

Did you use conventionally purified DNA extracts or the rapid paper-based extracts for the sensitivity comparison of LAMP and other methods?

Paper extracts so that all methods were compared on the same templates.

Can we differentiate the plant and insect microbiome in your total plant extract?

There is no reason that the template would not work using universal PCR primers targeting 16S, ITS, or other taxonomic markers.

Do insects caught on sticky traps present any problems for your extraction methods?

We successfully extracted DNA from insects on sticky traps. They were removed with forceps and placed on the FTA matrix cards.

Can we use the LAMP for fungal pathogens? Have you done any experiments on that?

Absolutely. We have developed LAMP assays for many different fungal pathogens, and there are many other examples in the literature. The method will simply detect the presence of DNA of a particular sequence. For DNA extraction from fungi using the FTA cards, we have not examined this.

How much is the cost of this primer?

LAMP primers are not expensive—a complete set of six can be under $100 in Canada. We typically have the longer FIP and BIP primers HPLC purified (to avoid truncated primers), which adds to the cost but we feel provides better results. For us, a complete set of primers is around $200 and is good for many hundreds of reactions.

Does LAMP work with maise mollicutes?

LAMP will work with any DNA or RNA (RT-LAMP) organism.

Did you extract DNA from individual insect or pool of insects with this method?

*Typically, individual insects. We did experiments using up to five AY leafhoppers (*Macrosteles quadrilineatus*) in one spot, and it worked fine as these insects are quite tiny. It has the advantage of screening more insects in a single extraction (as you could see, with field-collected insects, most are typically negative).*

How much does it cost per sample?

This will depend on your region. The method is inexpensive from a consumables point of view. You need lysis buffer from the Qiagen plant kit, FTA cards, TE0.1, and ethanol. And a hammer!

Would it be possible to use this method for early detection of plant pathogens? (For example, as a supplement to state early detection programs for *Phytophthora ramorum*.)

Potentially—LAMP or RPA, for sure. The most suitable nucleic acid purification method will depend on the pathogen and if applicable its carrier. We needed a method for insects because our pathogen of interest is carried in insect salivary glands. The FTA cards could be used for plant tissue as well, but for our purposes we did not need to detect it in plants since we wanted to provide data allowing intervention prior to plant infection.

Can you help explain how this method was validated. In the paper, it appears that testing with using lab reared was only with phytoplankton-positive insects. Also, any indication of how stable that sample is—how long can this stay on the FTA card?

We validated the method using nested PCR targeting 16S (accepted gold standard for phytoplasma detection) and other PCR-based assays (a nested PCR targeting rp gene; qPCR targeting cpn60). We used lab colony phytoplasma positive insects (to get sufficient numbers of positives) but also field-collected insects, most of which were negative by both methods. FTA cards are made for long-term storage of

samples, and the nucleic acids are quite stable, but we have not done experiments to detemine how long the NA is stable. For us, the key is speed, so we do not usually wish to store the nucleic acids.

Can asymptomatic plants also be detected?

With the 30-s extraction method (Zhou et al.) that is highly unlikely, since the titre of the phytoplasma would likely be low. We have found that the rapid plant extraction method was able to detect phytoplasma DNA in samples with high levels of phytoplasma, but lower levels would not likely be detected. I would guess that most asymptomatic plants would test negative with NA prepared using that method, whether LAMP or nested PCR was used.

Does aster yellow disease affect a specific type of plant?

A huge variety of plants can be infected with phytoplasmas, including the group I phytolasmas (aster yellows). More than 1,000 plant species, including essentially all known crop species. For a review, see Namba, S. (2011). Phytoplasmas: A century of pioneering research. Journal of General Plant Pathology, 77, 345-349.

Is it possible to quantify the target DNA using this method? What will be the threshold for this pathogen to cuase disease?

Certainly the DNA can be quantified using nanodrop or Qubit. The threshold of transmissibility is a difficult question to answer. I'm not sure there is a good answer for that—we have proceeded on the assumption that any amount is bad, because the bacterium can certainly reproduce over time in the insect's salivary gland and transmission can occur.

Why is it that phytoplasma is limited to be transmitted by leaf hoppers and not by other insects?

That is a great question. There is an intricate connection among the leafhopper, the bacterium, and the plant. The bacterium does not cause any harm to the insect, and in fact "infected" insects have greater feeding activity and fucundity. The bacterium wants to get to the plant tissue where it can reproduce, so it modifies the plant so that the floral tissue becomes leaf-like—more food for the leafhoppers! It is a fascinating triumverate of interaction, with the ultimate loser being the plant (and producers!), since the floral tissue and seed set are so strongly affected.

How did you determine the limit of detection? What is the lowest concentration of target DNA in a sample that may be detected with this test?

Great question! We have used probit analysis to get at the LOD of the LAMP assay (manuscript under review). Essentially, you set up a series of replicate assays at various known quantities of template and determine the proportion of assays that test positive at each concentration. Then, using probit analysis or other mathematical modeling of the response, you can determine the C95, or the concentration of analyte that must be present to generate a positive result 95% of the time. An excellent description of this, along with software used to calculate LOD, can be found at Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., et al. (2020). Reporting the limits of detection and quantification for environmental DNA assays. Environmental DNA, 2, 271-282.

Is LAMP primer designing software available free of cost?

Yes—see PrimerExplorer (https://primerexplorer.jp/e/).

For the comparison of the diagnostic techniques (PCR, qPCR, LAMP), did you use the same primers?

Not the same primers. LAMP and PCR would use different sets of primers. For LAMP and qPCR, the same molecular target (cpn60) was used. For nested PCR, the 16S rRNA-encoding gene was used. For the other nested PCR, a published assay targeting the rp operon of phytoplasma was used.

I missed the plant-based wash buffer answer. Would you repeat the wash buffer?

For the plant DNA extraction, see Zhou et al., PLOS Biology, 15, e2003916, 2017. https://doi.org/10.1371/journal.pbio.2003916. It is open access.

Can you use your method to directly extract DNA from a diseased plant?

The FTA matrix cards were designed for nucleic acid extraction from plants, so yes. We have not tested it extensively, except for some high-titer plant material that tested positive. For us, the plants are a less desirable target for field-based testing because once they are infected, it is too late—should have sprayed for insects that were feeding a few weeks ago. To provide actionable information, you need to know when the transmitting insects appear in the field.

Your LAMP took 30 min to 2 h, how many cycles did you use?

The LAMP can take various times depending on two factors: the amount of target template DNA in the sample, and the detection method employed. For samples with the exact same amount of target template, using a commercial mastermix from OptiGene can generate a positive signal in under 10 min, while that same sample might take 25 to 30 min using calcein, HNB, or other detection systems. That is why we give a bit of a range. The LAMP is isothermal, so a single cycle at 63 ℃ is used (although some of the commercial mastermixes have a "warm start" at 40 ℃ for 10 s first). Can do LAMP in a qPCR thermocycler, in which case you set it up to do, say, 60 cycles at 30 s each (30 min total), all at the same T: 63 ℃.

Is this method suitable for mycorrhizal fungi detection?

We have not tested the method with fungi. They have cell walls with unique characteristics, but since the FTA cards can lyse the presumably even more difficult plant cells, we guess that it would work for fungi. One very interesting possibility is to use the cards to extract DNA from beetles that may carry Ophiostoma novo-ulmi*, which transmits Dutch elm disease. We did some very preliminary work with this a couple of years ago and got some positive results, but did not get very far with it due to a paucity of positive insects to work with. This made validation of the method very difficult. We think it would work.*

Read more about this research in *Plant Health Progress:*
https://apsjournals.apsnet.org/doi/10.1094/PHP-09-19-0063-FI