Final Report

Project title (Acronym)
Comparison of multiple real-time PCR & real-time LAMP detection methods for the plant pathogen '*Candidatus* Liberibacter' spp. causing the Huanglongbing disease on *Citrus* spp. (HLBVALID)

Project duration:

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Contents

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2. Short project report

2.1. Short executive summary

The Huanglongbing (HLB / Citrus greening) disease is associated with three species of phloem restricted bacteria recognized as members of '*Candidatus* Liberibacter', namely 'asiaticus' (CLas), 'americanus' (CLam), and 'africanus' (CLaf). Although the causal agents of the disease have not been cultivated in axenic medium yet, they are inducing one of the most destructive and wide-spread disease of citrus across Asia, Africa, and America, but it was not identified in the EU or proximities. It is mainly affecting *Citrus* species, cultivars and hybrids and some other hosts within the *Rutaceae* family. This disease is associated with two main psyllids: *Diaphorina citri* and *Trioza erytreae*, and is also transmitted by propagative plant material, which can spread outbreaks on great distances.

As part of an initial diagnosis, the visual inspection of symptomatic plants is a routine method for the surveillance of HLB disease, but symptoms can be misinterpreted. Yellow shoots, leaf blotchy mottle, and lopsided fruits with colour inversion and aborted seeds are typical symptoms on HLB affected trees. However, symptoms alone are not enough to complete a diagnosis, as they can be confused with nutritional disorders (zinc, iron, manganese deficiencies) or with other diseases (Citrus tristeza virus, Stubborn, Citrus blight, Australian citrus dieback). The three species responsible for the HLB can also be present in the host plant at a very low concentration and as the disease develops irregularly, individual trees may show a mixture of normal and diseased parts; symptoms can appear up to 20 months after infection.

Although conventional PCR is a sensitive and specific method, the PCR tests can lead to false negative results due to the low titer and uneven distribution of the bacterium in the host plant, especially at the early stage of the infection (Jagoureix *et al*., 1994). Hence, conventional PCR method is not recommended for the detection of *Ca*. L. spp. responsible for the HLB disease in symptomless plants (Li *et al*., 2006), where bacterial use to be in low concentrations and in non-uniform distribution. Being more sensitive, real-time PCR tests may be useful in programs for the production of certified citrus nursery trees and in post-entry quarantine and are more adapted for early detection.

Various diagnostic real-time PCR tests were published in recent years and assessed for their performance, but with different procedures. Comparison of these protocols through the same procedure is hence required in order to fully compare their performance. The three main real-time PCR tests published that are routinely used are those from Bertolini *et al*. (2014); Li W. *et al*. (2006); and Morgan *et al*. (2012).

Proper HLB detection and identification is extremely important in preventing the entry of this disease in an area or in order to control the disease where it is already present. The recent (2015)[1] suspicious case of HLB declared by the Portuguese authorities and the absence of confirmation, underlines the need for a thorough and comparable assessment of detection tests to guarantee the reliability of the results obtained. Therefore, collaboration at an international level would be beneficial to compare the real-time PCR protocols for the detection of *Ca*. L. spp. responsible for the HLB disease in *Citrus* spp.

[1] https://gd.eppo.int/reporting/article-5204

2.2. Project aims

The project aimed to produce original data on the performance of three real-time PCR tests (Bertolini *et al*., 2014, Li, *et al*., 2006, Morgan *et al*., 2012) and one real-time LAMP test (Keremane *et al*. 2015) for the detection of *Ca*. L. spp. responsible for the HLB disease on *Citrus* spp.

Such validation will benefit to policy makers, National Plant Protection Organisations (including risk managers and diagnosticians), Regional Plant Protection Organizations, the citrus industry, and other stakeholders.

The project aimed to:

I. Build a DNA database of samples contaminated or non-contaminated by *Ca*. L. spp.;
II. Assess the intra-laboratory performance of proposed tests within a unique framework, following the EPPO validation protocol PM 7/098[2];
III. Organize a collaborative tests performance study, in order to validate the inter-laboratory reproducibility.

The collaborative work will help to harmonize operating procedures throughout routine laboratories. Additionally, the performance values obtained will help laboratories applying for accreditation for such analysis.

Finally, this project aimed to build trust and expertise both in the research and the diagnostic fields for the management of the HLB disease along with the international scientific effort.

2.3. Description of the main activities

The project was organized into three main activities:

Activity 1: Production of a DNA collection

The first activity of the project was to build a DNA library from various plant species of the *Rutaceae* family with the contribution of partners. DNA samples gathered from project partners and other sources originated from HLB diseased trees and HLB-free trees, in order to respectively constitute a DNA collection of target and non-target DNA samples. DNA samples from close related species was also considered for inclusion into the collection as non-target DNA samples (e.g. *Ca*. L. solanacearum responsible for the potato zebra chip disease). The status of DNA for HLB (positivity and species typing) was checked and the target and non-target DNA samples to be used for the subsequent project activities were selected.

Activity 2: Intra-laboratory performance assessment

The second activity of the project was to obtain validation data from different tests in order to validate their performance and check the fitness of the tests with their intended purpose. The intra-laboratory performance assessment study focused on 3 main real-time PCR tests published by Bertolini *et al*. (2014); Li *et al*. (2006); and Morgan *et al*. (2012). Unlike it was planned by the consortium, the Keremane *et al*. (2015) test was unfortunately not assessed and was removed from the project.

[2] https://onlinelibrary.wiley.com/doi/epdf/10.1111/epp.12508

Two modalities of the protocol from Morgan *et al*. (2012) were included, comprising the TaqMan and SYBR GREEN modalities. Also, two modalities of the protocol from Bertolini *et al*. (2014) were included: the original Probes/Primers from the above cited publication and the dehydrated mastermix modality from the commercial PlantPrint kit ("*Candidatus* Liberibacter" spp. associated to Citrus HLB disease - Rapid screening test by real-time PCR).

A total of five modalities were assessed (Li / Morgan TaqMan / Morgan_SYBR / Bertolini / PlantPrint), involving the evaluation of the analytical specificity, the analytical sensitivity, and the repeatability of the real-time tests on different DNA panels constituted as described in Activity 1.

Each DNA sample was tested for two wells per run for a given repetition. If at least one of the two wells was positive, the run was considered positive for the detection of *Ca*. L. spp. responsible for the HLB disease. A total of 3 repetitions was done for every run, yielding a total of 6 results per DNA sample.

DNA controls were added to each repetition of tests in order to validate the repetition: 1 Positive CLas DNA control, 1 positive CLaf DNA control, and 1 Negative Mastermix control.

Activity 3: Collaborative test performance study

The third activity consisted of performing a collaborative tests performance study (TPS). The TPS involved 8 laboratories from 7 European countries and occurred from 17th October 2018 to 4th December 2018. The goal was to determine the interlaboratory variability of the tests selected. During this TPS and following the EPPO recommendations, the participating laboratories had to analyze identical sets of blind samples according to the same five real-time PCR modalities, along with the reference conventional PCR, and to report their results on standardized forms. A panel of 20 DNA samples was selected, comprising 10 CLas, 3 CLaf, and 7 Non-Targets DNA samples. Only the sample named T+ was of known status (positive) for the TPS participants and was used as a positive control. A dilution was done on original DNA samples by the organizer in order to produce large batches of homogenized tubes. Validation of the status of target and non-target DNA samples was performed with the Li modality. To evaluate the analytical sensitivity, repeatability, and reproducibility, 6 target DNA samples were duplicated. Analyses were performed by all the participants under their usual working conditions and in the same manner as other samples, which are routinely analyzed in the laboratory. Final acceptance of laboratory results was under the condition that conformity was confirmed for DNA control amplifications with expected results.

2.4. Main results

Intra-laboratory performance

The detection of CLas and CLaf in all samples was not possible by a unique test, either real-time or conventional PCR. This was mainly due to the low bacterial concentration in some samples that were below the limit of detection (LOD) for most of the tests.

The inclusivity assessment of CLas through the real-time PCR tests was at least as good in terms of inclusivity as the conventional PCR test. The Bertolini test showed 100% of detection, but presented serious exclusivity issues, highlighting a stringency problem during the amplification. The lower scores for repeatability among all real-time PCR tests could be

explained by both, the low relative concentration of some samples, and the higher analytical sensitivity, as compared to the conventional PCR.

Relatively to the detection of CLaf, although inclusivity assessment scores were higher than for the conventional PCR for the tests Li, Bertolini, and PlantPrint, they were drastically lower for the Morgan TaqMan and Morgan SYBR tests. This result revealed the incompatibility of the Morgan *et al.* tests with a reliable detection of the CLaf species, although a strong capacity to detect the CLas species. The highest score for the inclusivity of CLaf was for the Li test (100.0%), followed by the PlantPrint (93.3%) and the Bertolini (86.7%) tests, although the latest showed serious exclusivity issues.

The exclusivity assessment was interesting, relatively to the performance comparison of the different real-time PCR tests, with the reference conventional PCR. The results showed that the Li, Morgan TaqMan, and Morgan SYBR tests, along with the reference conventional PCR reached the maximum score of 100%, meaning a perfect exclusivity on this panel of DNA samples. The PlantPrint test showed a slightly lower score of 91.7%, mainly explained by the expected positive amplification signal of the '*Candidatus* Liberibacter solanacearum' (CLso) DNA sample, as this test is designed to amplify all the '*Candidatus* Liberibacter' species. Nevertheless, as a pathogen of potato, CLso is unlikely to be present on plants of the Rutaceae family, but still raises suspicion of host-jump out of its usual host range as previously discussed for the HLB associated species. The same positive amplification of CLso was expected and observed for the Bertolini test, but in our experimental conditions, this test showed a drastically low exclusivity score of 25.0%.

It is worth noting that no cross-reactions were observed with the non-target sample contaminated by *Xanthomonas citri* pv. *citri* (Xcc) with all tested modalities, beside inclusivity issues for the Bertolini modality. This bacterial plant pathogen can be found on citrus leaves and co-infecting HLB diseased tree. Absence of cross-reaction with Xcc as well as negative results from HLB free plants that contained the regular microbiota are strong indicator of the modalities´ specificity. It has been reported in the literature that the Li modality could cross-react with '*Ca*. Liberibacter' associated with the Australian eggplant psyllid *Acizzia solanicola*. This novel candidate species of the *Liberibacter* genus '*Candidatus* Liberibacter brunswickensis' (CLbr) has been amplified from DNA extracts using the HLBas and HLBaf primers; but not from the HLBam primers. Nevertheless, this psyllid has not been reported on citrus.

The analytical specificity score was calculated as a summary of the inclusivity and the exclusivity scores for each species, showing the strength and the weakness of each test. The Li test was the only test to show higher scores for both the CLas and the CLaf species, as compared to the reference conventional PCR. At the exception of the expected amplification of the '*Candidatus* Liberibacter solanacearum' DNA sample, we could have drown the same conclusion for the PlantPrint test. On the contrary, the Bertolini test was showing a lower analytical specificity score for both HLB tested species, which was drag down by its exclusivity score. In order to investigate the false positive tendency observed in the Bertolini modality, Sanger sequencing (Genewiz, Takelay, UK) was performed on amplification obtained on non-target DNA samples (data not shown). Results showed that best bi-directional hits were associated with *Methylorubrum* spp. (*M. rhodinum*; *M. aminovorans*; *M. extorquens*) with 87% homology, but did not match any CLas or CLaf sequences (Blastn on nr/nt database NCBI).

The Morgan TaqMan and Morgan SYBR tests only showed higher scores than the reference conventional PCR test for the CLas detection and these tests are hence recommended for the sole detection of the CLas species. Repeatability for this set of experiments was generally high for all tests and panels (Li and PlantPrint tests showed the highest scores than other real-time PCR tests), except for the Morgan SYBR test on CLaf and CLas.

It is also worth noticing that no negative interaction (additional amplification bands) through the conventional PCR modality or PCR amplification competition (data not shown) was observed from the GB1/GB3 primer pair from Teixeira *et al.* with the A2/J5 primer pair from Hocquellet *et al.* for the detection of CLas and CLaf samples.

Relatively to the analytical sensitivity, as expected, the real-time PCR tests showed a high performance compared to the reference conventional PCR test. The parameter employed here are showing the performance of a given test to deliver a 100% detection at a particular dilution factor. This approach is useful for laboratories willing to ensure its own results with a maximum confidence. As it could be interpreted as restrictive, the full results showed that the analytical sensitivity scores could be pushed somehow a little bit further than those found with this parameter. This approach is confirming that the reference conventional PCR also delivers reliable results.

Moreover, sensitivity of tests was not correlated with the molecular target type (16s rDNA, prophage, intergenes), but more with the different technology used (real-time vs conventional PCR). Analytical sensitivity was also depending on the sample, showing that some amplification may fail for a given test on a given sample or may be less sensitive, whereas other are performing well. Furthermore, no analytical specificity correlation was found among the different molecular target types.

Collaborative test performance study (TPS)

The collaborative TPS showed that the real-time PCR modalities Li and conventional PCR achieved the highest levels of performance for the detection of both CLas and CLaf samples. When only considering CLas samples, the real-time PCR modalities of Morgan (Taqman and SYBR) achieved the highest levels of performance, along with Li and the conventional PCR tested.

The seven laboratories that yielded consistent results produced a strong framework for assessing the different proposed protocols for the detection of CLas and CLaf samples. Even the lower number of participants (n = 4) that tested the PlantPrint modality was enough to characterize this modality through this TPS.

The first step analysis highlighted that the Li real-time PCR and the conventional PCR modalities showed an analytical specificity (ASP) ratio close to 1. Due to detection issues with CLaf DNA samples, the Morgan TaqMan and Morgan SYBR modalities showed a lower ASP ratio, but still represent a strong alternative when only dealing with CLas DNA samples.
Whereas Bertolini and PlantPrint are showing perfect-like score of 1 for their inclusivity, their exclusivity scores are again the lowest of all tested modalities; showing a lack of PCR stringency in the detection of either CLas or CLaf samples. This can lead to wrongly diagnose samples as positive results. This fact affects negatively the confidence on the performance of the test and usually a second step using three specific protocols for CLas,

CLaf, and CLam is performed in order to confirm a positive result obtained with the Bertolini or PlantPrint tests, thus increasing time of response.

The positive repetitions analyzed in this second step analysis allowed to assess repeatability, reproducibility, and concordance odds ratio that characterize the different modalities in an inter-laboratory environment testing.

All PCR reactions, both conventional and real-time, showed high performance for all the assessed criteria. This second step analysis showed that there are no significant differences between laboratories on results yielded by different modalities. This information is important; as those modalities are used across the world to detect HLB contaminated material. This is supported by the use of different thermocycler equipment by the different partners, which allowed to yield comparable results; highlighting the fact that the robustness of the different assessed tests will allow to compare results across different laboratories in the world, which are not influenced by the laboratory environment (equipment, manipulators, time and place…).

2.5. Conclusions and recommendations to policy makers

The evaluation of the analytical specificity showed different behaviours according to different tests: (i) the Li real-time PCR and the conventional PCR modalities showed the best overall performances considering both CLas and CLaf, and it is worth noticing that the exclusivity reached the maximum score (100%) with high repeatability. (ii) The Morgan SYBR test showed high analytical specificity performances, but low inclusivity for CLaf. This observation is even more relevant for the Morgan TaqMan test, which showed an even lower inclusivity score for CLaf. (iii) The Bertolini test showed high inclusivity but low exclusivity, yielding many false positive amplifications on non-HLB-contaminated samples, whereas the PlantPrint test showed better analytic specificity performance, although based on the same probe/primers Master Mix.

The evaluation of the analytical sensitivity also showed different behaviours according to the species considered and according to different tests. Overall, the tests gave lower analytical sensitivity scores for CLaf than for CLas.

Thus, we can conclude that: (i) the Li and the conventional duplex PCR tests are recommended for the detection of HLB. (ii) The Morgan test shows both high analytical specificity and high sensitivity, but only for CLas samples; CLaf samples are generally not detected. (iii) The Bertolini modality showed the lowest exclusivity score, although results on other performance criteria were comparable to the Li modality; a best alternative would be through the PlantPrint ready-kit. The exclusivity score for the Bertolini test represents a rejection reason for the final validation of the test because of an excessive risk of false positive amplification. We also have to keep in mind that both the Bertolini and PlantPrint tests can yield a false positive amplification for the detection of the HLB associated ‘Candidatus Liberibacter’ spp., as they are designed with universal primers for the global detection of all ‘Candidatus Liberibacter’ spp. This is not a flaw of the design, but laboratories should confirm results from these tests with other specific HLB detection protocols; and (iv) all tested protocols are robust and can be transferred to different laboratories that are willing to perform HLB detection and ensure to deliver comparable results among laboratories across the world.

We also confirm that the Li and Morgan TaqMan/Morgan SYBR modalities are well adapted for the detection of the HLB (respectively to their species performance) especially considering asymptomatic plant samples or early infection, as their analytical sensitivity is higher than for the conventional PCR tested.

The combination of Li real-time PCR and the conventional PCR tests can minimize the risk of releasing infected material when the tests are producing negative results, which is particularly important for the certification of citrus trees material. Especially, these tests will produce very few false positive results and will guarantee safe and justified destruction decisions for HLB suspected infected material.

2.6. Benefits from trans-national cooperation

The main benefit from this trans-national cooperation was to build trust among international laboratories in order to deliver recommendations to policy makers. The collaboration allowed to gather a representative collection of samples and an opportunity to test different test modalities within the framework of a collaborative test performance study.

Collaboration at every levels, from the technical to the editorial, led the consortium to submit results to an important scientific and technical community during the International Research Conference on the HLB in USA (Riverside, CA, March 2019), and to submit a manuscript to PLoS ONE journal, an open access rank A scientific journal. Also, future collaborations will be easier between those laboratories that worked together for more than 2 years.

Finally, this project demonstrates the usefulness of extensive comparative laboratory testing for protocol validation. Although not very widespread in the field of plant pathology, they are recommended by different regional and international organizations in plant health such as the European Plant Protection Organization, or the USA National Academies of Sciences. These studies are essential to provide guidance for reliable detection of a given pathogen.

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▪ Teixeira, DC, Danet JL, Eveillard S, Martins EC, de Jesus Junior WC, Yamamoto PT, Lopes SA, Bassanezi RB, Ayres AJ, Saillard C, & Bove JM. 2005. "Citrus huanglongbing in Sao Paulo State, Brazil: PCR detection of the 'Candidatus' Liberibacter species associated with the disease." *Molecular and Cellular Probes* 19 (3):173-9. doi: 10.1016/j.mcp.2004.11.002.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

Gilles Cellier, Cristina Redondo, Jaime Cubero, Montserrat Roselló, Eugénia de Andrade, Leonor Cruz, Elen Ince, H. Nilüfer Yildiz, Pakize Gök Güler, Anna Maria D'Onghia, Thaer Yaseen, Khaled Djelouah, Eveline Metz-Verschure, Francesca Gaffuri, Richard A. Gottsberger, Baldissera Giovani. Comparison of the performance of the main real-time and conventional PCR detection tests for '*Candidatus* Liberibacter' spp., plant pathogenic bacteria causing the Huanglongbing disease in *Citrus* spp. Manuscript submitted to PLoS ONE in May 2019.

4. Open Euphresco data

Data will be made open after publication of manuscript.