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PCR-based diagnostic methods for '*Candidatus* Liberibacter solanacearum' – Review

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Abstract: '*Candidatus* Liberibacter solanacearum' is an economically important pathogen in the Americas, New Zealand and Europe. The primary objective of this review is to systematically investigate the polymerase chain reaction (PCR)-based methods used for its detection in plant samples. Several databases were searched from the inception of the relevant literature up to August 2018. This review identified 53 studies that met all the inclusion criteria. The performance of the different methods was also compared, however due to data heterogeneity and insufficient evidence on the sensitivity of all assays used, a meta-analysis of the data was not possible. Nonetheless, the review indicates that the rtPCR designed to the 16S ribosomal RNA gene can be routinely employed as a fast, cost-effective, and reliable detection technique in diagnostic laboratories.

Keywords: Lso; leaf stunting; early detection; identification; *in planta*; molecular methods

'*Candidatus* Liberibacter solanacearum' (Lso), an uncultured phloem-limited Gram-negative *Proteobacterium*, is consistently associated to economically important diseases such as; zebra chip (ZC) in potatoes (*Solanum tuberosum*); psyllid yellows in tomatoes (*Solanum lycopersicum*) and vegetative disorders in carrots (*Daucus carota*), celery (*Apium graveolens*) and other species of the Apiaceae family

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(Hansen *et al*. 2008; Liefting *et al*. 2009a; Secor *et al*. 2009; Munyaneza *et al*. 2010a; Pitman *et al*. 2011; Teresani *et al*. 2014; Monger & Jeffries 2016; Hajri *et al*. 2017). Recently, Lso was also found in the Apiaceae species in old commercial seeds from 1973 in countries not reporting the presence of this bacterium (Monger & Jeffries 2018). The bacterium is also associated with significant crop losses in tobacco (*Nicotiana tabacum*), peppers (*Capsicum annuum*) and other solanaceous plants (European and Mediterranean Plant Protection Organization 2013). It is transmitted by insects from the Triozidae family, which feed on the host plant phloem sap. The main vector for the potato is the psyllid *Bactericera cockerelli* (Munyaneza *et al*. 2007), while in the Apiaceae, Lso is vectored by *Bactericera trigonica* (Alfaro-Fernández *et al*. 2012b; Teresani *et al*. 2015), *Trioza apicalis* (Munyaneza *et al*. 2010b, 2015), *Trioza* spp. and *Accizia* spp. (Scott *et al*. 2009). However, the risk of Lso transmission from the Apiaceae to the potato by *B. trigonica* appears to be negligible as observed by Antolinez *et al*. (2017). Several experiments on the seed transmission in carrots were conducted. The results of Bertolini *et al*. (2015), supporting the seed transmission, were not confirmed in more recent experiments reported by Loiseau *et al*. (2017a, b). Reduction in the incidence of Lso symptoms in carrots was found in areas only using the seeds that tested negative for Lso (Cambra 2014), this would support the seed transmission as being important in the epidemiology of the pathogen. However, this reduction could also be due to the reduction of the incidence of the vectors (European and Mediterranean Plant Protection Organization 2017). Furthermore, the studies show that Lso is not transmitted through the potato true seed from the infected plants (Munyaneza 2012). The economic impact of a Lso outbreak on the potato and tomato production was estimated at 338 million EUR/year on the European Union scale. Since the pathogen is not widely distributed and controlled, a decision to categorise this organism as a quarantine pest was supported (Soliman *et al*. 2013). '*Candidatus* Liberibacter solanacearum' (Solanaceae haplotypes) and its vector *B. cockerelli* were added to the EPPO A1 List of pests recommended for regulation as quarantine pests (Version 2017-09) (https://www.eppo.int/QUARANTINE/listA1.htm).

The Lso presence has been mainly detected in plants of the family Apiaceae in Europe and in the family Solanaceae in North America as well as New Zealand. Lso isolates from various geographic regions that exist as different haplotypes. These haplotypes are described by single-nucleotide polymorphisms (SNPs) across the gene regions of the partial sequences of 16S, the 16S/23S rRNA spacer region and the 50S rRNA subunits (Nelson *et al*. 2011, 2013). Based on the analysis of the SNPs, six Lso haplotypes, A, B, C, D, E and U have been determined in various plant species and in diverse areas of the world (Nelson *et al*. 2011, 2013; Teresani *et al*. 2014). The haplotypes A and B are only reported in solanaceous plants and described from Central and North America, New Zealand as well as Australia, whereas haplotypes C, D and E are found in the Apiaceae family in Europe, North Africa and Israel (Nelson *et al*. 2011, 2013; Alfaro-Fernández *et al*. 2012a, b; Munyaneza *et al*. 2012a, b; Loiseau *et al*. 2014; Tahzima *et al*. 2014; Munyaneza *et al*. 2015; EPPO 2017; Ben Othmen *et al*. 2018; Haapalainen *et al*. 2018; Thomas *et al*. 2018). Recently, a sixth haplotype of Lso, haplotype U, was found in stinging nettle plants (*Urtica dioica*) in Finland that belongs to neither Solanaceae nor Apiaceae but to the family Urticaceae (Haapalainen *et al*. 2018). In Apiaceae commercial seed lots, the Lso belonging to D and E haplotypes was detected in Italy, the UK and Tunisia (Ilardi *et al*. 2016; Monger & Jeffries 2016; Ben Othmen *et al*. 2018). Most of the liberibacters are unculturable bacteria, so that their identification must mainly rely on DNA sequence and phylogenetic analysis (Liefting *et al*. 2009a). The genetic variation among the Lso haplotypes was determined by the SNPs analysis (Liefting *et al*. 2009a; Secor *et al*. 2009; Wen *et al*. 2009; Nelson *et al*. 2011; Alfaro-Fernández *et al*. 2017b; Monger & Jeffries 2018) or by genotyping the PCR products amplified using a simple sequence repeat (SSR), and multi-locus sequence typing (MLST) markers (Glynn *et al*. 2012; Lin *et al*. 2012) which is costly and time-consuming. Normally, the 16S/23S rDNA of the bacteria exhibits greater variability in nucleotide composition compared to that of the 16S rDNA sequences and has been successfully used for PCR detection of plant-pathogenic bacteria (López *et al*. 2009; Ravindran *et al*. 2011).

The current detection methods for Lso rely primarily on the polymerase chain reaction (PCR)-based technologies, especially the real-time PCR (rtPCR) that allows for the detection of plant pathogenic bacteria with high sensitivity levels (Palacio-Bielsa *et al*. 2009). This technique provides a reliable estimation of the pathogen load (Mirmajlessi *et al*. 2015b) and since the amplicon detection is based on a specific

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fluorescent signal, it eliminates the requirements for the post-amplification processes reducing the time for large-scale analyses (Bustin *et al*. 2009). Since the effective detection depends on the pathogen occurrence along with its inoculum load in the host plants, a high sensitivity for the early detection of the pathogen is extremely important (Mirmajlessi *et al*. 2015b). Moreover, the identification of the appropriate target DNA regions is an important step in PCR-based assays. Depending on the sequences and the genomic regions chosen to design the PCR primer sets, highly accurate and sensitive diagnostic assays for the specific detection of the bacterial species can be achieved (Henson & French 1993). The primer sequences must be target specific to allow for the reliable identification of an organism (Hyndman & Mitsuhashi 2003). The 16S ribosomal RNA gene (16S rRNA), the 16S/23S intergenic spacer region and the the 50S ribosomal protein (*rpl*) genes along with other conserved housekeeping genes within the prokaryotic rDNA operons have been described as useful target genes for designing species-specific primers and probes for the detection of the plant pathogenic bacteria (Maiden *et al*. 1998; Maiden 2006; Lopez *et al*. 2009; Wen *et al*. 2009; Nelson *et al*. 2011). However, it is not always possible to design species-specific probes based on the very little sequence variation observed between the 16S rRNA genes (Ravindran *et al*. 2011).

As an important principle in plant disease control, detecting the infection sources using effective procedures is crucial to prevent the further spread and subsequent disease outbreaks. Consequently, the necessity of rapid, sensitive and specific techniques to detect the pathogens of interest is essential. The main objective of this systematic review is to provide a comprehensive overview of the current published literature available on the PCR-based techniques for the Lso identification and the detection in plant tissues. The efficiency of the pathogen transmission by insect vectors was not considered, however pre-analytical requirements such as: the sample preparation; the nucleic acid extraction; the selection of suitable genomic regions for designing the primers; the identification of items and steps that may affect the efficacy of the tests are also discussed.

The characteristics of the included studies

The papers included in this systematic review were published in English, in peer-reviewed journals up to August 2018 (Table 1). The review only focused on the PCR-based studies employed for the routine detection and/or identification of Lso, identifying 53 relevant publications. The relevant information was extracted from each paper and schematically outlined (Tables S1 and S2 in the Electronic Supplementary Material – ESM). They are indicated in the text by number [S1#].

Pre-PCR processing

Plant sampling, especially from newly developed young leaves, is a critical pre-analytical step and should be considered for the improved detection

Table 1. The publications included in the systematic review: the host plants, the geographic distribution, the PCR methods used for the detection of '*Candidatus* Liberibacter solanacearum'

Reference	Host plant	PCR-based method	Origin
Secor *et al*. (2009)	potato	cPCR	USA, Mexico, Guatemala
Munyaneza *et al*. (2009a)*	pepper	cPCR	Mexico
Munyaneza *et al*. (2009b)	tomato	cPCR	Mexico
Li *et al*. (2009)*	potato	cPCR + mrtPCR (TaqMan)	USA
Liefting *et al*. (2009a)*	tomato	cPCR	New Zealand
Wen *et al*. (2009)*	potato	cPCR + rtPCR (TaqMan) + mPCR	USA
Sengoda *et al*. (2010)	potato	cPCR	USA
Munyaneza *et al*. (2010a, b)	carrot	cPCR	Finland
Rehman *et al*. (2010)	potato	cPCR	Honduras
Munyaneza *et al*. (2011)*	carrot	cPCR	Finland
Levy *et al*. (2011)	potato, tomato	cPCR + rtPCR (SYBRGreen)	USA

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Reference	Host plant	PCR-based method	Origin
Nelson *et al.* (2011)*	carrot	cPCR	Finland
Pitman *et al.* (2011)	potato	cPCR + nPCR + mPCR	New Zealand
Ravindran *et al.* (2011)*	potato	cPCR	USA
Ling *et al.* (2011)	tomato	cPCR	Mexico
Crosslin *et al.* (2012a, b)	potato	cPCR	USA
Lin *et al.* (2012)*	potato	SSR	USA, Mexico
Munyaneza *et al.* (2012a)	carrot	cPCR	Norway
Munyaneza *et al.* (2012b)	carrot	cPCR	Sweden
Munyaneza *et al.* (2012c)	potato	cPCR	USA
Wallis *et al.* (2012)	potato	cPCR + rtPCR (SYBRGreen)	USA
Glynn *et al.* (2012)*	potato	MLST	USA, Mexico, New Zealand
Ravindran *et al.* (2012)	potato	LAMP + cPCR	USA
Alfaro-Fernández *et al.* (2012a, b)	carrot	cPCR	Spain
Aguilar *et al.* (2013)	tomato	cPCR	Honduras
Bextine *et al.* (2013)	tomato	cPCR	El Salvador
Wen *et al.* (2013)	potato	SSR + cPCR	USA
Beard *et al.* (2013)	potato	cPCR + nPCR + Semi-nested rtPCR(SYBRGreen) + rtPCR (TaqMan)	New Zealand
Munyaneza *et al.* (2014)	pepper	cPCR	Honduras
Nissinen *et al.* (2014)	carrot	rtPCR (SYBRGreen)	Finland
Teresani *et al.* (2014)*	celery, carrot	cPCR + rtPCR (TaqMan)	Spain
Loiseau *et al.* (2014)	carrot	cPCR + rtPCR (TaqMan)	France
Tahzima *et al.* (2014)	carrot	cPCR	Africa
Bertolini *et al.* (2015)	carrot	rtPCR (TaqMan)	Spain
Munyaneza *et al.* (2015)	carrot	cPCR	Germany
Cating *et al.* (2015)	potato	cPCR	USA
Ilardi *et al.* (2016)	carrot seeds	cPCR + rtPCR (TaqMan)	Italy
Satta *et al.* (2016)	carrot	cPCR + Virtual RFLP	Spain
Fujiwara and Fujikawa (2016)*	carrot seeds	cPCR + rtPCR (SYBRGreen-TaqMan)	Japan
Monger and Jeffries (2016)	parsley seeds	rtPCR (TaqMan)	UK
Hajri *et al.* (2017)	apiaceous crops	cPCR + rtPCR (TaqMan)	France
Haapalainen *et al.* (2017)	carrot, parsnip, *Anthriscus sylvestris*, *Solanum nigrum*, *Urtica dioica*	cPCR	Finland
Alfaro-Fernández *et al.* (2017a)	apiaceous crops	cPCR	Spain
Alfaro-Fernández *et al.* (2017b)	celery, carrot	rtPCR (TaqMan)	Spain
Antolinez *et al.* (2017)	apiaceous crops, potato	rtPCR (TaqMan)	Spain
Loiseau *et al.* (2017a)	carrot	rtPCR (TaqMan)	France
Monger and Jeffries (2018)	apiaceous crops	cPCR + rtPCR (TaqMan)	UK
Haapalainen *et al.* (2018)	carrot	rtPCR (TaqMan) + MLST	Finland
Ben Othmen *et al.* (2018)	carrot	rtPCR (TaqMan)	Tunisia
Thomas *et al.* (2018)	carrot	cPCR + nPCR	Australia

*original PCR method used; cPCR – conventional PCR; mrtPCR – multiplex real-time PCR; rtPCR – real-time PCR; mPCR – multiplex PCR; nPCR – nested PCR; MLST – multilocus sequence typing; LAMP – loop-mediated isothermal amplification; SSR – simple sequence repeat; RFLP – restriction fragment length polymorphism

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(Levy *et al*. 2011; International Plant Protection Convention 2017), as this phloem-limited bacterium is detected in symptomless plant tissues where the bacterium may be present at a very low concentration. Geographically extensive sampling and a proper sampling size are also necessary pre-analytical factors to evaluate the genetic structure of the pathogen populations in different regions, and to reconstruct an evolutionary scenario (Haapalainen *et al*. 2018). Generally, plant sampling can be done using a variety of methods, which are essentially related to the type of plant materials (International Plant Protection Convention 2008). The presence of the PCR inhibitors in the plant tissues can frequently reduce the reaction sensitivity, and a low copy number of the target DNA may cause false-negative results due to a non-optimal DNA extraction procedure, its degradation or the presence of the PCR inhibitors (Schena *et al*. 2013). Consequently, an internal positive control such as the amplification of a conserved DNA segment or a house-keeping gene (Li *et al*. 2009) can be included in the assay. DNA extraction is a critical pre-analysis step for the PCR-based experiments that could have a major impact on the result of diagnostic tests and, therefore, is an important variable. Properly purified DNA for the PCR analyses can be obtained with the use of the commercially available extraction kits as shown through several studies (Table S1 in ESM). Occasionally, a modified lysis step like the homogenisation in a cetyltrimethylammonium bromide (CTAB) extraction buffer is recommended for the plant tissues that contain high levels of polyphenolic compounds and polysaccharides (Green *et al*. 1999). The inhibitory substances can also be removed using lyophilisation, template dilution and other column-based protocols (Multinu *et al*. 2018). Overall, the simplicity and rapidity as well as the removal of the inhibitory compounds are the main advantages of the commercial kits. Nevertheless, the commercial kits are frequently not cost-effective when analysing large numbers of samples (Stöger & Ruppitsch 2004). However, Teresani *et al*. (2014) interestingly reported that, in their experience, there is no difference between the DNA extraction by a kit and the CTAB methods when applying the rtPCR assay. The use of plant sap membrane spotting was successfully tested to minimise the effects of the PCR inhibitors and is recommended for large-scale screening purposes (de Boer *et al*. 2012): the total nucleic acid is obtained by spotting the crude extract from the plant tissues onto special membranes like FTA®. Furthermore, the spotted membranes can be used to transport the samples from the field to laboratories without compromising the reliability of the test (Osman & Rowhani 2006). However, for Lso detection, the use of purified DNA as a template showed a higher sensitivity than direct spotting in celery (Teresani *et al*. 2014) and carrots (Bertolini *et al*. 2015). Using direct spotting, on Whatman 3 mm paper, no amplification was obtained with the cPCR while the rtPCR assay detected the bacterium down to a 10^{-2} dilution (Teresani *et al*. 2014). Generally, the DNA-based methods are also able to detect dead microorganisms causing false positive results for studies wishing to identify viable cells. Specialised methods should be taken into consideration to discriminate between viable and dead cells. This may include the use of propidium monoazide (PMA), a DNA-intercalating agent that only penetrates into the bacteria with compromised/dead cell membranes and efficiently suppresses the rtPCR signals of the dead cells (Nocker *et al*. 2009). The effectiveness of PMA was shown by Bertolini *et al*. (2015) and Ben Othmen *et al*. (2018) where a large number of quantified viable Lso cells were detected in carrot seed lots using rtPCR.

Detection of the pathogen

The conventional PCR. The conventional PCR is the most widely used technique described to detect '*Candidatus* Liberibacter solanacearum' and distinguish the closely related species. Detection protocols defined for cPCR were mainly based on the use of primers designed for the 16S rDNA sequence. However, different primer sets show different levels of detection sensitivity by different laboratories using various protocols and reagents (Tables S1 and S2 in ESM). For instance, the cPCR protocol with the primer set LsoF/OI2c was 10-fold more sensitive than the OA2/OI2c-based assays targeting 16S rRNA (Liefting *et al*. 2008; Li *et al*. 2009). Ravindran *et al*. (2011) found that when using the primer sets Lso TXF/R or Lp FragF/R, targeting 16S-23S-ITSrDNA, the detection rate increased from 49.1 to 69.5% when compared to the primer set LsoF/OI2c targeting 16S rDNA. Recently, Fujiwara and Fujikawa (2016) designed six new primer sets to amplify the Lso-specific regions in the 3′-terminal region of the 16S rDNA. Among them, four primer sets Lso-972F/LsoLSS, Lso-931F/LsoLSS, Lso-807F/LsoLSS and Lso-786F/LsoLSS could effectively detect

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Lso in the range of 10^2 to 10^8 Lso cells/µl in carrot seeds. Because of the variable parameters in the cPCR assays, optimisation of the conditions is always challenging and time consuming. These assays may also provide reduced sensitivity compared to other PCR-based methods such as rtPCR (Beard *et al*. 2013). In comparison with rtPCR, cPCR is also more prone to contamination since the amplified material must be manipulated for its visualisation (Mirmajlessi *et al*. 2015a). Nonetheless, the sensitivity of cPCR assays can be further increased by using high-fidelity enzymes [S1#50]. A high-fidelity enzyme has been used to increase detection specificity and to amplify the target sequence in the presence of large amounts of non-target DNA and/or PCR inhibitors. The low error rate DNA polymerase provides a high degree of accuracy in the replication of the DNA of interest, and the sensitivity of the cPCR protocol can further be increased. Cating *et al*. (2015) compared high-fidelity and cPCR-based techniques using the primer pair Clipo-F/OI2c targeting 16S rDNA and demonstrated that the detection of Lso from symptomatic potato tubers was significantly increased by 30–40% over the cPCR. The ability of a high-fidelity PCR to detect latent infections and low titres of pathogens directly from the plant tissue, make it a suitable tool for diagnostic laboratories.

The multiplex PCR. This technique has been reported [S1#9, 20] as being able to detect Lso in symptomatic and asymptomatic plants. The multiplex PCR, allows for the amplification of several different DNA sequences simultaneously by using multiple primers in one reaction mixture and was also applied for Lso detection in plants. Wen *et al*. (2009) developed a multiplex PCR assay using different primers including Btub1F/R plus ZCf/OI2c, and Btub2F/R plus OA2/OI2c under the same conditions of the ZCf/OI2c or OA2/OI2c primer sets as described by Liefting *et al*. (2009b), allowing for the detection of Lso strains in different solanaceous plants. Indeed, the DNA quality was verified by reliable amplification of the potato β-tubulin (Btub) DNA region with two specific primer sets, Btub1F/R and Btub2 F/R, which can eliminate the false negatives attributable to the poor DNA template quality. A multiplex PCR could improve the cPCR reliability by decreasing the false negative results, however, due to the competition between the different amplification products it requires tedious and time-consuming optimisation processes to reach the sensitivity of the cPCR (Elnifro *et al*. 2000). In this regard, the mPCR protocol developed by Li *et al*. (2009) is used in many studies as a singleplex PCR.

The nested PCR. A less investigated test, the nested PCR [S1#19, 39, 70], can be applied to increase the detection sensitivity and reduce the effect of the PCR inhibitors. This technique with both internal and external primers targeting the 16S rDNA was described to increase the detection sensitivity when the titre of Lso in the potato plants was unevenly low (Pitman *et al*. 2011). Transmission of Lso without an insect vector from mother tubers of a potato into the foliage and the daughter tubers was also investigated using nPCR and mPCR based on the specific primers targeting 16S rDNA and plant *β*-tubulin gene (Pitman *et al*. 2011). The nested PCR provides significant increases in sensitivity over cPCR, but it increases the risk of false positives due to the cross-contamination of reaction mixtures. To circumvent this risk a nPCR technique known as a single-step semi-nested PCR [S1#37, 40] was applied by Beard *et al*. (2013). This method uses multiple primers targeting 16S rDNA for the detection of Lso in field samples and were 50- and 20-fold more sensitive than the cPCR and nPCR respectively. In this type of PCR, a single tube contains a primer pair flanked by a third primer (forward or reverse) allowing the reaction to progress in a single round of amplification reducing the risk of contamination and the reaction time. An increase in the sensitivity compared to the traditional two-step nested or semi-nested PCR was reported (Prariyachatigul *et al*. 2003; Saini *et al*. 2009).

The real-time PCR. As mentioned above, the real-time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases as the reaction proceeds if the target DNA is present. Real-time PCR protocols can be used to confirm the presence or absence of pathogens, or can be designed to quantify the number of bacterial cells in a sample (i.e., quantitative PCR or qPCR). From the results of the currently available published literature, two main reporter systems were studied for the Lso detection. Levy *et al*. (2011) developed a SYBR Green rtPCR assay that could detect Lso at a very low concentration in newly developing leaves of asymptomatic potatoes in which the bacterium was not detectable by cPCR until three weeks after the infection. However, the TaqMan-based rtPCR assays [S1#5, 8, 41, 45, 47, 51, 54, 57–59, 63, 64, 66, 67, 69] were used more often compared to the SYBRGreen-based rtPCR assays [S1#16, 28, 43, 57]. Bertolini *et al*. (2015) could quantify a

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low number of bacterial cells (1.4×10^4 cells/g seed) in carrot seeds using the TaqMan rtPCR method, which was previously modified by Teresani *et al*. (2014). In this compilation, 6 rtPCR studies, targeting the 16S RNA gene, are presented on the potato (Li *et al*. 2009; Wen *et al*. 2009; Levy *et al*. 2011; Wallis *et al*. 2012; Beard *et al*. 2013; Antolinez *et al*. 2017), 13 on the carrot (Loiseau *et al*. 2014; Nissinen *et al*. 2014; Teresani *et al*. 2014; Bertolini *et al*. 2015; Fujiwara & Fujikawa 2016; Ilardi *et al*. 2016; Alfaro-Fernández *et al*. 2017b; Antolinez *et al*. 2017; Hajri *et al*. 2017; Loiseau *et al*. 2017a; Ben Othmen *et al*. 2018; Haapalainen *et al*. 2018), 2 on celery (Teresani *et al*. 2014; Alfaro-Fernández *et al*. 2017b), 1 on the tomato and parsley (Levy *et al*. 2011; Monger & Jeffries 2016) and 3 on other Apiaceous crops (Antolinez *et al*. 2017; Hajri *et al*. 2017; Monger & Jeffries 2018). Although most of those studies have not implemented the same method, the rtPCR protocol developed by Li *et al*. (2009) was used in the largest number of studies and on a range of different plant materials showing the versatility of this protocol (Table S1 in ESM).

The current study shows that the 16S rRNA gene region is the most commonly used one to design primers and probes for the rtPCR assays for the detection of Lso in the plant tissue prior to and during the appearance of the symptoms. The chemistries used in the rtPCR systems make them more suitable for the multiplex detection purposes to differentiate among the closely related sequences, facilitating the development of the multiplex rtPCR protocols whereby different targets can be co-amplified within a single reaction (Smith & Osborn 2009). Li *et al*. (2009) described a fast, specific and reliable TaqMan rtPCR method multiplexed with the plant cytochrome oxidase (COX)-based probe-primer set as internal control primers, in which the low detection limit was about 20 copies of Lso 16S rDNA templates per reaction for field-collected plant samples. This low detection limit showed that the multiplex rtPCR was at least 10- to 100-fold more sensitive than the cPCR assays with the LsoF/OI2c primer pair, and it was consistent to the previous comparison between the real-time and conventional PCR assays for the '*Candidatus* Liberibacter' species assotiated with citrus huanglongbing (Li *et al*. 2007).

The loop-mediated isothermal amplification. Another rapid, efficient and labour-saving technique for the detection of bacterial pathogens is the loop mediated isothermal amplification (LAMP), which is only reported in one study (Ravindran *et al*. 2012). It is described as a fast and cost-effective assay for the reliable detection of Lso in infected potato plants [S1#30]. Specific primers targeting several housekeeping genes (i.e. *rpoB*, *rpoD*, *gyrB*, *adk*, *recA*, *dnaG*, *fumC*, *pyrE*, *kdo*, *ftsA*, *gnd*, *mdh* and *sucC*) along with the 16S/23S rRNA and 16S rRNA genes were selected as the potential target sequences. However, only the primers specific for the 16S rDNA gene reliably amplified Lso (Ravindran *et al*. 2012). Although LAMP, as a non-quantitative molecular method, does not need a thermalcycler or post-PCR analysis, its detection sensitivity was comparable with those of the PCR assays. The LAMP detection sensitivity has the potential to be approximatly 100 times more sensitive than the cPCR, being, therefore, able to detect positive samples containing 100 to 0.01 ng of DNA while the bands were clearly visible for the samples containing 100 to 1 ng of DNA using the cPCR with LsoTX16/23 F/R primers. The specific primers designed for the 16S rRNA gene are used for both the LAMP and cPCR assays (Ravindran *et al*. 2012; Wallis *et al*. 2012).

Pathogen identification and haplotyping

Sequencing. Several PCR-based protocols have been developed by targeting different loci in the genome to identify Lso. Species-specific primer sets targeting the 16S rRNA gene, the 16S-23S-ITS rRNA genes, the *adk* genes and the 50S *rplJ-rplL* ribosomal protein genes were applied for the identification of Lso haplotypes using the PCR-based assays [S1#17, 45, 48, 68, 69]. The identification of the haplotypes can be achieved by sequencing several of these genomic regions. For a sequence to be identified as Lso, it will have a high similarity (99–100%) to the reference sequence when analysed using the basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blasgt.ci). A single nucleotide polymorphism in the OA2 primer binding site on the 16S rRNA gene of the Lso haplotypes C and B was found which reduced the detection specificity and sensitivity of the OA2/OI2c primer set when used in the cPCR. This led to the development of the haplotype C specific primer Lsc2 used in conjuction with OA2 (Haapalainen *et al*. 2017). The 16S rDNA gene has been used to design primer sets to distinguish inter- and intraspecific genetic variation. However, due to the low genetic diversity within this gene, the fine-scale genetic relationship of the closely related species could not be defined (Page & Holmes 1998).

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Developing primers designed for the target 16S/23S rDNA gene is another approach which was applied in several studies (Rehman *et al*. 2010; Levy *et al*. 2011; Nelson *et al*. 2011; Ravindran *et al*. 2011, 2012). The 16S-23S intergenic spacer has a higher variation in length and sequence than the 16S rDNA, and can be analysed along with the 16S region to determine the phylogenetic relationships between the closely related species (Martinati *et al*. 2007). Teresani *et al*. (2014) identified the Lso haplotype E, in carrots and celery using the primer pairs OA2/OI2c, LsoTX F/R and CL514F/R targeting the 16S-23S-ITS rDNA gene and the 50S rRNA gene for which the primer pair LsoTXF/R was the most sensitive. Recently, the sensitivity of Lso TX primer set for the ribosomal 16S-23S-ITS has been confirmed in comparison with OA2/OI2c and the CL514F/R primer sets by Monger and Jeffries (2018). The haplotypes D and E were identified in carrots from France by sequencing and analysing the 16S rRNA gene sequences (primer set OA2/OI2c) and the 50S *rplJ-rplL* ribosomal gene sequences (primer set CL514F/R) to confirm the positive rtPCR results with cycle threshold (Ct) values ranging from 17 to 32 (Hajri *et al*. 2017). According to Ben Othmen *et al*. (2018), the new primer pair CaLsol 50S-F/R designed for the amplification and sequencing of the 50S *rplJ-rplL* ribosomal protein genes gave stronger positive amplicons than the primers previously described by Munyaneza *et al*. (2009b). Analysis of the Lso positive samples revealed, for the first time, the co-infection of haplotypes D and E in a carrot plant, whereas only haplotype D was present in the carrot seeds. Similarly, the co-infection of haplotypes A and B has been reported in potato samples (Wen *et al*. 2013). In Finland, Haapalainen *et al*. (2017, 2018) investigated the frequency and occurrence of the Lso haplotype C in carrot plants and D in carrot seeds using primer pairs designed for the 16S-23S-ITS rRNA and 50S rRNA genes. Furthermore, conserved housekeeping genes from Lso such as *adk*, *grpE*, *kdo*, *fumC* and *gdh* have been used as phylogenetic markers and allow for the differentiation of the '*Candidatus* Liberibacter' species (Ravindran *et al*. 2011; Glynn *et al*. 2012). The highly conserved *rplL* genes are the most variable among the markers studied as shown in Table S2 in ESM.

Simple sequence repeat. SSR [S1#24, 35] was used for detecting and genotyping closely related Lso strains infecting solanaceous plants (Lin *et al*. 2012; Wen *et al*. 2013). This allows for the simultaneous detection and differentiation of Lso haplotypes associated with zebra chip disease. Potato plants with Lso symptoms were analysed using SSR markers and were shown to be infected with either haplotype A, haplotype B or both. In the infected potato samples, the detection sensitivity of the SSR PCR was similar to the 16S ribosomal gene-based cPCR assay with a detection limit of 100 copies for haplotype A and 10 copies for haplotype B. SSR assays are less expensive and faster compared to conventional genotyping methods as no special equipment is required (Wen *et al*. 2013). However, it is not able to distinguish all Lso haplotypes. A combination of phylogenetic analyses of the Lso haplotypes using the 16S rRNA genes, the 50S *rplJ-rplL* ribosomal protein genes together with virtual restriction fragment length polymorphism (RFLP) were used to confirm the '*Candidatus* Liberibacter solanacearum' haplotype D in the carrot samples (Satta *et al*. 2016). So, an RFLP analysis might be applicable for Lso as an alternative tool for the haplotype discrimination.

Multi-locus sequence typing. The polymorphic MLST marker uses DNA fragments of multiple housekeeping genes to genotype and evaluate the genetic diversity in the Lso populations [S1#29, 68]. The technique involves the PCR amplification followed by the sequencing of a series of housekeeping genes located throughout the genome. In brief, the MLST tests the alleles of the selected housekeeping genes by nucleotide sequencing a 500- to 600-bp segment of the gene. Then, the data are analysed to determine the genetic similarity of the strains (Noller *et al*. 2003). According to Glynn *et al*. (2012), the MLST analysis detected two sequence types (ST-1 and ST-2) for each of the 10 MLST markers developed, resulting in the genotyping and assessing the genetic diversity of the Lso populations. Recently, Haapalainen *et al*. (2018) identified a novel Lso haplotype, haplotype U, using a new MLST tool, indicating that the new haplotype was more closely related to haplotypes A and D than to C. Although MLST is a powerful tool for comparing the genetic structure of plant pathogen populations, the phylogenetic relationships can be masked by the use of housekeeping genes (Obert *et al*. 2007). Genotyping studies using SSR and MLST markers are enclosed as practical techniques for the estimation of the genetic diversity of Lso haplotypes in plants and broad epidemiological studies of diseases associated with this bacterium (Glynn *et al*. 2012; Lin *et al*. 2012; Wen *et al*. 2013; Haapalainen *et al*. 2018). An assessment of the bacterial genetic diversity is also achievable using other multilocus molecular markers such as the amplified fragment length polymorphism (Wittwer *et*

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al. 2005) or the random amplified polymorphic DNA (Chelossi *et al*. 2004). However, since these markers rely on pure genomic DNA, they cannot easily be used for this not yet cultured bacterium (Satta *et al*. 2016).

Further considerations

In comparison with cPCR, rtPCR has become a gold standard tool for the accurate, reliable and high throughput detection of the target DNA with a low level or latent presence of the pathogens (Mirmajlessi *et al*. 2015a, b). Real-time PCR technologies frequently amplify very short DNA fragments (70 to 100 bp) which have a higher level of efficiency compared to cPCR (Schena *et al*. 2013). Moreover, cPCR primers and protocols can be adjusted to be used in rtPCR-based detection if the amplicon size criteria are met (Okubara *et al*. 2005; Mirmajlessi *et al*. 2015a). In this respect, Fujiwara and Fujikawa (2016) designed six different primer sets and used them for the detection of Lso presence by cPCR. They showed that four of these primer sets were appropriate for rtPCR in which, the Lso-931F/LsoLSS primer set was the most specific and reliable in both cPCR and rtPCR with a SYBRGreen and fluorescent probe. This review identified four original rtPCR protocols [S1#5, 8, 45, 57] based on the TaqMan probes, which provide greater sensitivity and specificity, but higher costs than other PCR-based technologies. DNA-intercalating dyes can be a valid alternative to probe-based methods. SYBRGreen chemistry is a cost-effective alternative to TaqMan and is also one of the most widely used intercalating DNA dyes for rtPCR assays. Although there is no probe, it provides an additional quality control step using the melt curve analysis to confirm the specificity of the amplified products by detecting the primer-dimers as non-specific products which would otherwise result in the false-positive results (Okubara *et al*. 2005; Beard *et al*. 2013). Regardless of the chemistry used, the necessity for the specific amplification is the primary factor in the choice of the rtPCR systems.

CONCLUSION

There are several unclear issues in the comparability of the results of the selected studies that should also be taken into consideration. The sensitivity of the PCR diagnostic methods defined in the selected articles is provided in Table S1 in ESM. However, many studies, including short reports, did not provide data on the sensitivity levels of the used technique(s). The majority of the included studies used DNA isolation kits, a CTAB buffer, or both to extract DNA from the plant tissues making a direct comparison between the studies problematic. Therefore, a common harmonised DNA extraction protocol is strongly recommended. To compare the sensitivity and specificity of several techniques or protocols, a common set of samples and protocols must be tested in several laboratories (in ring tests or interlaboratory tests). Therefore, the capability of ranking PCR-based amplification assays along with pre-analytical requirements according to their suitability for routine analyses was restricted. Up to four types of original rtPCR studies are reported here for the Lso detection in plant materials. It should be noted that their number has been increased from only four between 2009–2012 to 17 between 2013–2018 (Table 1). So the rtPCR, inspite of the described limitations, can be used in high-throughput screening systems especially for disease forecasting or field surveys where speed, reliability, high sensitivity, and quantitative data are needed. From a systematic review of the published literature, a suitable PCR-based detection methodology combined with a better understanding of the pre-analytical requirements will considerably improve the ability to develop appropriate control strategies against the '*Candidatus* Liberibacter solanacearum' dissemination. Harmonisation of the laboratory methods together with inter-laboratory comparisons are essential steps to ensure that the procedures used by different laboratories give consistent results.

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