Project title:	**On site detection of Tomato brown rugose fruit virus: validation of ToBRFV diagnostics (LAMP) for use by UK growers**
Project number:	PE035
Project leader:	Dr Adrian Fox, Fera Science Ltd
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Key staff:	Yue Lin Loh Anna Skelton
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Industry Representative:	Dr Philip Morely, TGA
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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Dr Adrian Fox

Senior Plant Virologist

Fera Science Ltd

Signature ..... Date .....

Yue Lin Loh

Molecular Biologist

Fera Science Ltd

Signature ..... Date .....

Report authorised by:

[Name]

[Position]

[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

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GROWER SUMMARY

Headline

Tomato brown rugose fruit virus is a tobamovirus of regulatory importance. LAMP approaches have been evaluated and validated for environmental swab sampling to detect the virus. This work has included comparative testing between LAMP and RT-qPCR, optimisation of extraction and sampling (swabbing), generation of validation data on sensitivity, specificity and matrix effects. These data indicate that LAMP will reliably detect ToBRFV from an RNA extraction to a dilution of 10^{-4}, compared to 10^{-6} for qPCR. The primers do not cross-react with either common tomato viruses or other members of the same genus. Initial testing has indicated some surfaces are challenging for the recovery of virus, likely due to their physical properties e.g. the porosity of concrete. Whilst there may be some applications for infield use, the use of swabs for inspector and grower testing cannot be supported due to the loss of sensitivity from non-extracted samples.

Background

Tomato brown rugose fruit virus has rapidly become the major virus of concern for the tomato industry. Surveillance for this virus involves sampling large numbers of plants, from a glasshouse which is time consuming for the inspector, costly, and only gives a 'snapshot' of the of a single point in the season. The current approach is to take leaf samples from 200 plants from a glasshouse of 10s if not 100s of thousands of plants. Whilst these numbers are statistically robust in line with ISPM 31, these levels of testing are set to give adequate levels of detection within the limits of inspector and laboratory resources. Work recently completed at Fera indicates that the timing and point of sampling on individual plants can have a strong influence on the reliability of detecting the virus (AHDB PE 034).

Loop-mediated isothermal amplification (LAMP) is a form of PCR testing which uses multiple primer sets to detect the presence of a target. However, unlike conventional PCR chemistry methods which require multiple cycles of rapid heating and cooling, the reaction in LAMP works at a single temperature. This means the equipment for running the tests can be much smaller than traditional PCR machines, making the equipment portable. Additionally, LAMP chemistry is less prone to the influence of inhibitors interfering with the diagnostic reaction, which means it can be used with crude sample preparations, and so may be amenable for onsite testing with low equipment resource requirements.

An alternative approach using onsite testing to focus on screening through environmental monitoring rather than through testing would have the potential advantage of reducing resource inputs and allowing ongoing monitoring throughout the season. This environmental monitoring could be achieved by testing of critical points in the process, using LAMP diagnostics for testing swabs and irrigation water. This would potentially allow a more

comprehensive approach throughout the season for less cost. However, where and how to best apply this technology has not been explored. LAMP for ToBRFV has already been initially evaluated for swabs and water samples but this requires validation to be completed for this application.

To support this, a scope of validation needed to be established. This involved mapping glasshouse processes to identify critical points, such as high traffic areas to develop sampling guidelines focused on areas where virus is likely to concentrate. Once this is established, LAMP is validated for inspection and grower support testing by comparing the applicability of the test for onsite and laboratory-based testing of plant and non-plant samples, such as comparing the influence of swab types and different surfaces. Additionally swab samples were collected during a visit to an active outbreak and tested to compare current laboratory methods and the application of LAMP for outbreak and post-outbreak monitoring. The main aims of this project are to establish if there are there alternative (improved) approaches for surveillance of a virus in a controlled environment rather than “snapshot testing” of small proportion of plants. The two key questions to be answered are:

- What are the ways which LAMP could be used to support inspectors and growers in surveying for the presence of ToBRFV?
- Does LAMP offer sufficient sensitivity and specificity to allow for onsite testing for the presence of ToBRFV?

Summary

The principal objectives of the project were:

1. Map glasshouse processes to identify key areas of virus concentration to fix a scope of validation
2. Validation of ToBRFV LAMP against current “gold standard” methods (real-time qPCR
    a. Establish sensitivity and specificity (inclusivity and exclusivity) characteristics for the test
    b. Determine any “matrix” effect from choice of swab or swabbed surface

To identify the key areas where an environmental monitoring process could focus sampling effort growers, inspectors and researchers attended an online workshop. The aim of the workshop was to identify the areas where virus residues were likely to accumulate within a

glasshouse production system. To achieve this discussion was based around the general schematic process shown in Figure 1.

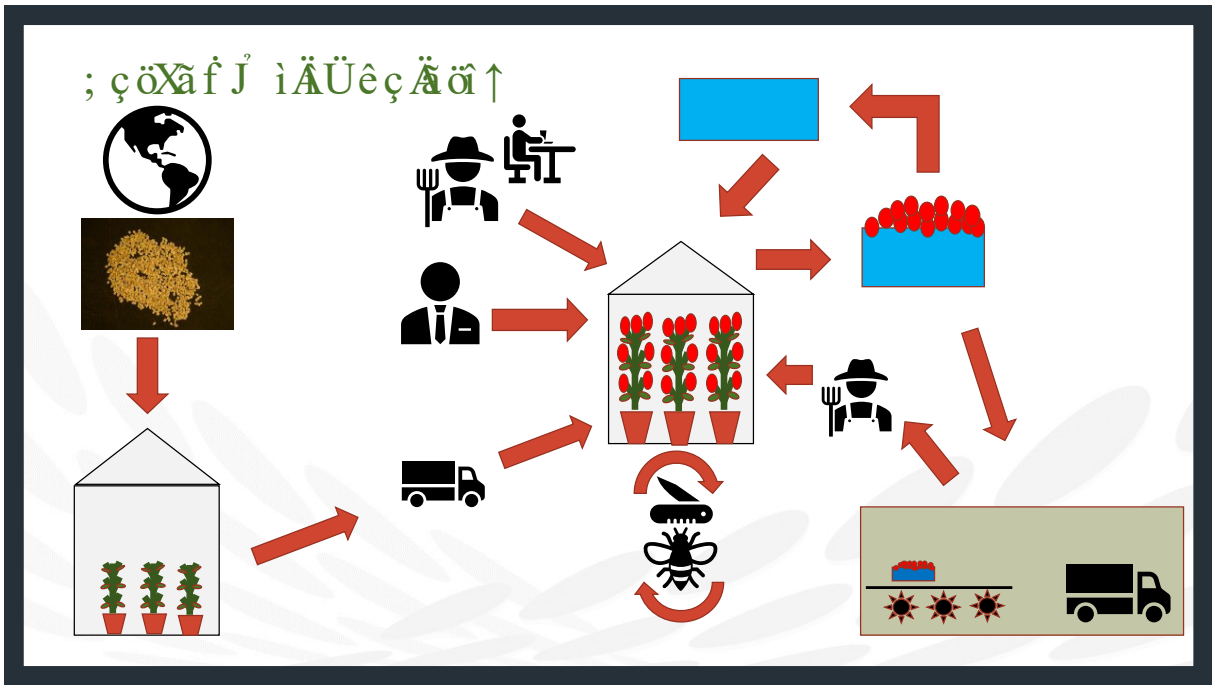

Figure 1. Potential risk points in the tomato production chain showing (left to right) global seed introductions into propagation, transport, inputs into the production system including staff, management, glasshouse practices (cutting, pollination), and via the packhouse.

Discussions based around this schematic helped to identify the key areas where process and diagnostic controls were currently in place (e.g. seed testing and checking propagation plants prior to despatch), and also areas where no controls were currently in place but would be potential points of concentration of virus residues Figure 2. These were considered to be key areas for where environmental swabbing could be used for monitoring for the presence of ToBRFV. These included surfaces in the glasshouse including both fixed and mobile equipment, PPE and peripheral equipment such as computer terminals. Other areas which were identified were areas outside the main glasshouse, such as the in the canteen, onsite accommodation and other surfaces such as mobile phones and surfaces in cars where staff (especially managers) may move between glasshouse sites. It was agreed that for the main validation work the surfaces used for previous survival and disinfection work would give a good representative cross section of surfaces, namely: glass, stainless steel, aluminium, hard plastic (picking crate), soft plastic (polythene sheeting), and disposable gloves (nitrile type).

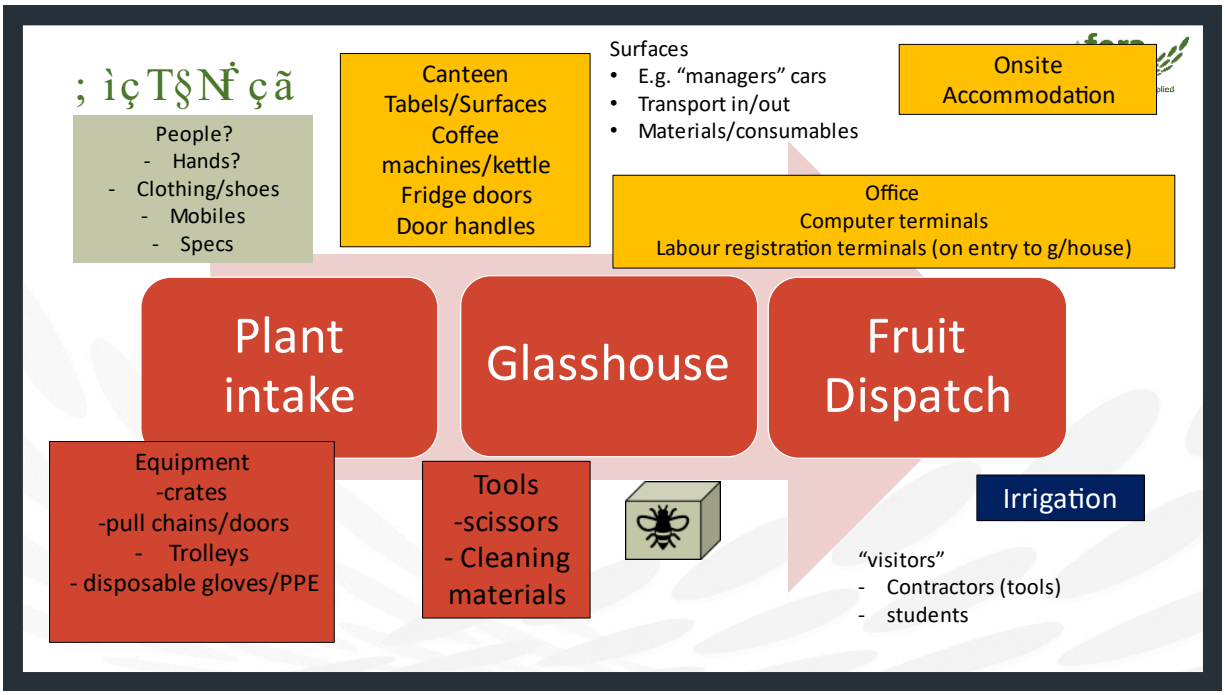

Figure 2 Areas for potential accumulation of environmental residues of ToBRFV identified during the stakeholder's workshop.

The current "gold standard" method, real-time RT-PCR, cannot be reliably used on samples using crude extractions, and requires RNA to be extracted from samples prior to testing. One consequence of this is that key aspects of test performance, such as analytical and diagnostic sensitivity should be compared with the current method to determine the relative limits of detection. For this reason, all validation was done in two stages to give a proxy measure of relative sensitivity. Initially the LAMP method was compared directly with existing real-time RT-PCR methods used in the laboratory on the same RNA extracts. Subsequently LAMP was trialled on crude preparations using leaf or swab samples diluted in a polyethylene glycol buffer. In this report real-time RT-PCR results are presented as the cycle threshold (Ct) which is the number of PCR heating cycles to register a positive result. LAMP results are presented as the time to positive amplification (Tp) and the annealing temperature of the LAMP product (Ta). The Ta measurement is specific to the target product of the LAMP reaction and serves as a secondary quality check on the test result.

A comparison of detection sensitivity between LAMP using and RNA extract (Figure 3) indicated that the limit of detection (LoD) was around 1 part in 1,000,000 ($1x10^{-6}$). Crude extractions only gave a LoD around 1 part in 10,000 ($1x10^{-4}$), a hundred fold less than extracted RNA. However, the impact on the time taken to register the sample as a positive reaction was impacted taking more than 10 minutes longer for the equivalent detection at $1x10^{-4}$. Due to the mechanics of the LAMP process, which require fluorescence to be emitted

during the reaction, the stronger preparations (neat and a 1:10 dilution) could not be read by the machine, registering a “negative” result.

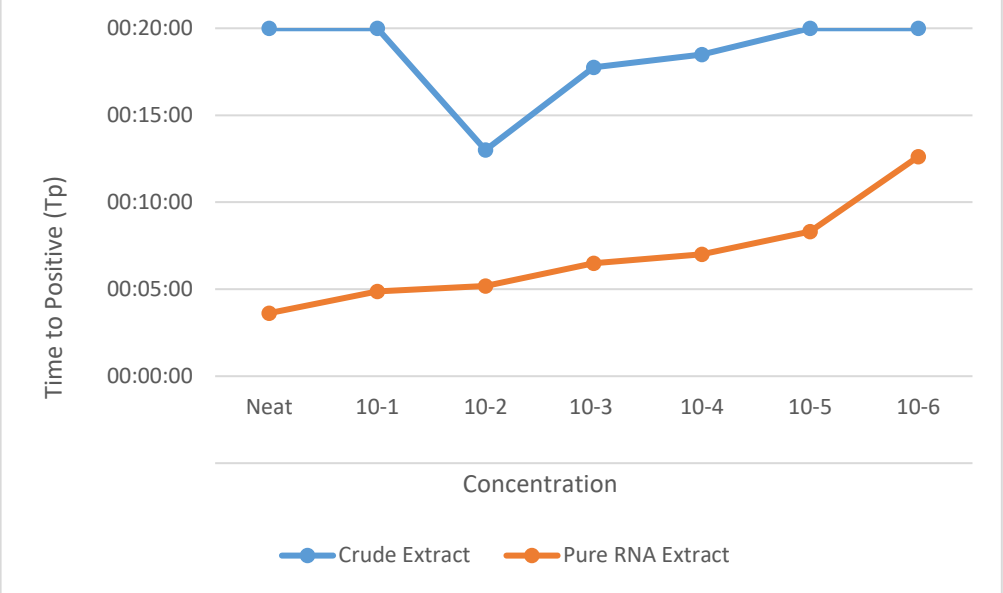

Figure 3 Comparison of detection of ToBRFV using Isothermal Amplification by 10-fold serial dilution. Each increment represents a further 1:10 dilution of samples plotted against the “time to detection”.

Ensuring a test detects the desired target, but does not detect non-targets is termed the specificity. When designing molecular primers sets (tests) such as for PCR, real-time PCR or LAMP, allowances are made for variations in the target genomes using a broad range of isolate genetic sequences as the basis for the design, during this phase a computer based “*in silico*” check is also carried out to ensure that the primers should not cross react with any other known virus sequences. As a confirmation of this a laboratory based specificity study was also carried out, this included regulated and non-regulated viruses and viroids which are either commonly tested for by laboratories (e.g. potato spindle tuber viroid, PSTVd), or which are commonly found in glasshouse samples (e.g. pepino mosaic virus, PepMV) or other viruses closely related to ToBRFV .

With the exception of one PepMV isolate, the assay detected all target isolates, and no non-target isolates, and performed as expected. In one case, an isolate of PepMV-Ch2 a positive result was obtained. This was further investigated with alternate test methods, and other isolates of this virus strain were also tested to ensure no cross reaction. The ToBRFV assay did not detect ToBRFV from any other isolate. However, further testing of the initial positive

isolate confirmed this result was the consequence of the isolate being contaminated with ToBRFV, likely the result of a co-infection in the glasshouse.

Table 1 Specificity testing of LAMP assay for ToBRFV showing negative reations with non-target viruses and viroids, and detection of ToBRFV test and controls. [(1)] PepMV sample tested positive, later shown to be cross-contamination of the sample. LAMP results are presented as the time to positive amplification (Tp) and the annealing temperature of the LAMP product (Ta).

Group	Target	Extracted RNA		
		Tp	Ta	Result
Viroids	PSTVd	/	/	Neg
	CLVd	/	/	Neg
	PCFVd	/	/	Neg
Tomato infecting viruses	PepMV Ch1	/	/	Neg
	PepMV Ch2	8:00	84.89	Contamination[1]
	PepMV Ch2 (isolate 2)	/	/	Neg
	PepMV EU	/	/	Neg
	STV	/	/	Neg
	TSWV	/	/	Neg
	TYLCV	/	/	Neg
	PVX	/	/	Neg
	PVY	/	/	Neg
Tobamoviruses	TMV	/	/	Neg
	ToMV	/	/	Neg
	PMMoV	/	/	Neg
	ToBRFV	3:00	84.96	Pos
	ToMMV	/	/	Neg
	UTobV1	/	/	Neg
Controls	No Template	/	/	Neg
	H2O 1	/	/	Neg
	H2O 2	/	/	Neg
	H2O 3	/	/	Neg
	Pos 1	5:45	84.97	Pos

	Pos 2	5:30	84.93	Pos
	Pos 3	5:45	84.93	Pos

Other aspects of test performance characteristics were investigated including the use of different swab types, and the influence of swabbing different surfaces Figure 4. The surfaces used were the same standard range of surfaces investigated in previous projects on survival and disinfection of ToBRFV (PE033/a). Swabs of sap contamination from inert surfaces, such as glass, steel and aluminium gave robust and rapid detection of ToBRFV. Similarly swabbing nitrile disposable gloves gave similar rapid detection of the virus. Plastics such as polythene sheeting and hard plastic (picking tray) may influence the detection of virus from a swab sample, possibly due to electrostatic charge in the plastic interacting with the virus or sap. Within the experimental work here, infected sap inoculated onto concrete could not be detected using a swab sample.

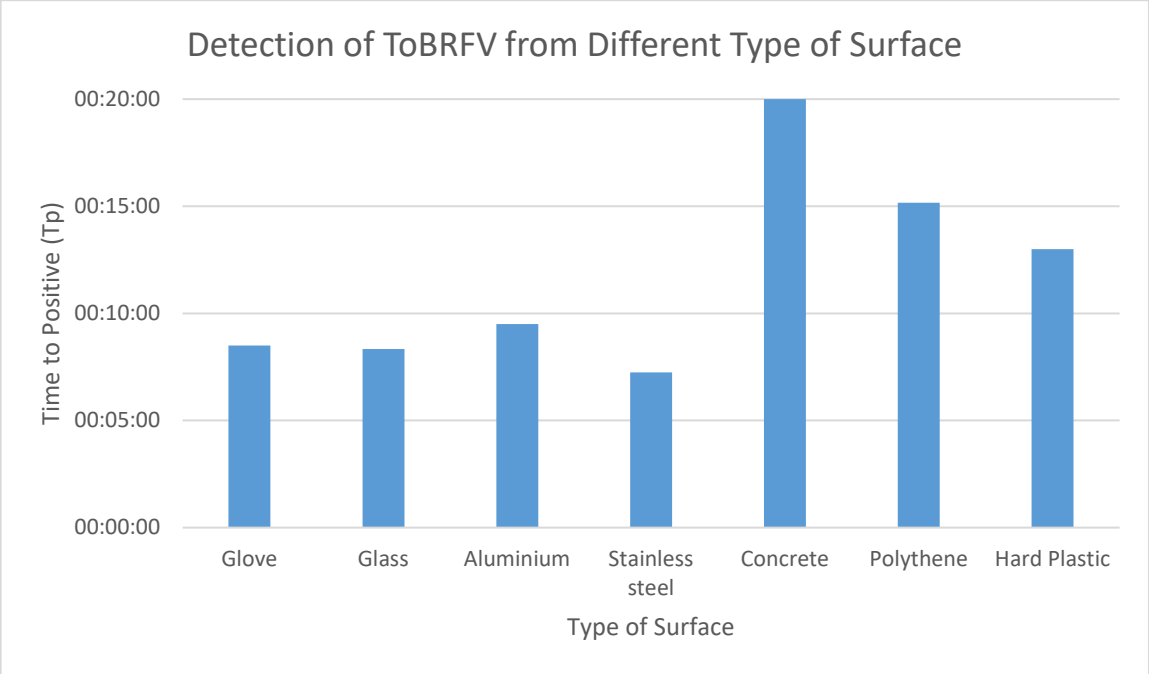

Figure 4 Detection from different surface types showing time to detection

Following validation work to define the performance characteristics of the test, and to optimise the sampling process, the swab sample method was trialled in two glasshouse scenarios. One of these was as part of the statutory plant health action following a recurrent outbreak in the UK, the second scenario was swabbing the glasshouse cubicle at Fera which has been used for containing previous work conducted on ToBRFV including work reported in PE 033/a

and PE 034 (Table 2). This work was crucial to understanding the limits of detection of the investigated methods with comparison to the current standard method.

Table 2 Swab results from the experimental glasshouse at Fera, showing a direct comparison of rates of detection with real-time RT-PCR, LAMP on extracted RNA and LAMP on a crude (PEG) extract. Real-time RT-PCR results are presented as the cycle threshold (Ct) which is the number of PCR heating cycles to register a positive result. LAMP results are presented as the time to positive amplification (Tp) and the annealing temperature of the LAMP product (Ta).

	Real-time RT-PCR RNA extract	LAMP			
		RNA Extract		Crude (PEG)	
Sample type	Avg Ct	Tp	Ta	Tp	Ta
window 1	28.43	00:10:45	84.51	/	/
window 2	28.27	00:11:15	84.52	/	/
window 3	29.11	00:11:15	84.86	/	/
Bench edging - face out	34.37	/	/	/	/
Bench edging - face in	32.24	/	/	/	/
ladder	25.93	00:08:30	84.66	00:10:45	84.61
wall 1	34.88	/	/	/	/
wall 2	28.15	00:12:15	84.72	/	/
floor	26.96	/	/	00:04:45	84.46
plant pot 1	26.93	00:06:30	84.77	/	/
plant pot2	26.77	00:06:45	84.58	/	/
plant pot black tray	21.08	00:05:45	84.66	00:14:45	84.61
Stand - leg	32.25	/	/	/	/
Stand - middle bar	29.47	00:18:15	84.44	/	/
Stand - grid panel	18.89	00:05:30	/	00:06:30	84.26
Glove	36.70	/	/	/	/
Tyvek sleeve	36.60	/	/	/	/
Plastic apron	31.06	00:16:15	84.79	/	/
H2O	40.00	-	-	-	-
ToBRFV + (avg)	22.98	00:06:00	85.3	00:06:23	85.15

This testing indicated that the comparative limit of detection of the LAMP when testing extracted RNA is equivalent of a real-time PCR result of 30-31Ct (Table 2). This means that this testing is approximately the same sensitivity as conventional PCR methods for ToBRFV

testing. However, this comparative lack of sensitivity meant that the LAMP method only detected 60% of the swab samples which would have been considered positive by real-time RT-PCR. Testing crude extractions did detect the presence of ToBRFV in some samples, however this was poor by comparison to RNA extraction LAMP and real-time RT-PCR, only detecting around 25% of the samples that the current standard method would have detected.

The results presented here indicate that LAMP testing does have potential for screening testing for ToBRFV, and when used on extracted sample RNA gives comparable levels of detection to conventional PCR methods. However, the loss of sensitivity incurred when using the approach on crude preparations from swabs means that this approach cannot be currently recommended as an infield test. The testing carried out in glasshouses as part of this project indicates the high risk of contamination following an outbreak of ToBRFV with environmental residues of the virus being present in areas where there had been no direct contact with the virus. Work is ongoing at Fera and with international partners to better understand the sources of, and risks associated with, these environmental residues.

Financial Benefits

There are no direct financial benefits to growers from this work, however, these data have validated the use of swab testing by both LAMP and real-time RT-PCR. These results indicate that laboratory based testing of environmental swabs could provide a way of monitoring glasshouses for the presence of ToBRFV allowing early intervention in an outbreak. However, due to the persistence of the virus, swab testing by molecular diagnostic methods does not provide an effective way to demonstrate freedom from the virus.

Action Points

- Swab testing with laboratory based testing can be used to monitor for the presence of ToBRFV
- In the event of a ToBRFV outbreak the virus can rapidly contaminate surfaces and these may form a source for future carry over infections
- LAMP testing provides another potential diagnostic method in the battle against ToBRFV, and could be used in conjunction with RNA extraction procedures, however cannot be recommended for testing crude sample preparations at this time.

SCIENCE SECTION

Introduction

Tomato brown rugose fruit virus has rapidly become the major virus of concern for the tomato industry. Surveillance for this virus involves sampling large numbers of plants, from a glasshouse which is time consuming for the inspector, costly, and only gives a 'snapshot' of a single point in the season. The current approach is to take leaf samples from 200 plants from a glasshouse of 10s if not 100s of thousands of plants. Whilst these numbers are statistically robust in line with ISPM 31, these levels of testing are set to give adequate levels of detection within the limits of inspector and laboratory resources. Work recently completed at Fera indicates that the timing and point of sampling on individual plants can have a strong influence on the reliability of detecting the virus (AHDB PE034). An alternative approach using onsite testing to focus on screening through environmental monitoring rather than through testing would have the potential advantage of reducing resource inputs and allowing ongoing monitoring throughout the season.

This environmental monitoring could be achieved by testing of critical points in the process, using LAMP diagnostics for testing swabs and irrigation water. This would potentially allow a more comprehensive approach throughout the season for less cost. However, where and how to best apply this technology has not been explored. LAMP for ToBRFV has already been initially evaluated for swabs and water samples but this requires validation to be completed for this application.

To support this, a scope of validation must be established. This has involved mapping glasshouse processes to identify critical points, such as high traffic areas to develop sampling guidelines focused on areas where virus is likely to concentrate. Once this is established, LAMP is being validated for inspection and grower support testing by comparing the applicability of the test for onsite and laboratory-based testing of plant and non-plant samples, such as comparing the influence of swab types and different surfaces. Additionally swab samples were collected during a visit to an active outbreak and tested to compare current laboratory methods and the application of LAMP for outbreak and post-outbreak monitoring.

The main aims of this project are to establish if there are there alternative (improved) approaches for surveillance for a virus in a controlled environment rather than "snapshot testing" of small proportion of plants. The two key questions to be answered are:

- What are the ways which LAMP could be used to support inspectors in surveying for the presence of ToBRFV

- Could this same technology be used by growers for early detection of ToBRFV throughout the season

The principal objectives are:

1. Map glasshouse processes to identify key areas for swabbing due to being points of virus concentration
2. Optimise and validate ToBRFV LAMP against current “gold standard” methods (real-time qPCR)
    a. Establish sensitivity and specificity (inclusivity and exclusivity) characteristics for the test
    b. Determine any “matrix” effect from choice of swab or swabbed surface
3. If available, try swabbing approach in a “live” outbreak scenario

Objective 1. Mapping glasshouse processes for identifying potential sampling areas

Background

The purpose of assay validation is to understand the performance characteristics of a given test. This should include an assessment of the scope of validation i.e. the test should be validated for the specific application for which it is intended, including assessing the impact of the host plant material, other pathogens which might be encountered during testing, and the “matrix” which is to be tested. In conventional testing approaches, this would generally be plant material such as leaf, fruit and seeds. However, in this case, where the test is intended to be used for testing environmental residues such as sap or trace contamination on surfaces there are other matrices to be considered.

The purpose of this initial objective was to get growers and inspectors to think about what surfaces would be suitable for ongoing monitoring via swabbing.

Methods

Workshops were held with growers, Inspectors and diagnosticians to consider which areas within the glasshouse production system would be likely to be “concentration areas” where high traffic would lead to build up of virus on surfaces. Using Figure 5 as a starting point, the end-to-end process of the tomato production chain was discussed in terms of physical movements of equipment and people. These considerations included the tomato seeds and

propagation plants as likely sources of infection, but focused more on physical areas and processes which were likely to result in an accumulation of virus.

The overall process was divided into plant reception/intake, glasshouse, and fruit dispatch. Additionally, there were several peripheral operations areas highlighted including offices, social spaces such as the canteen, and onsite accommodation for workers. These were identified as areas where cross contamination events could occur, or where people congregated and there was likely to be a concentration of virus as environmental residues.

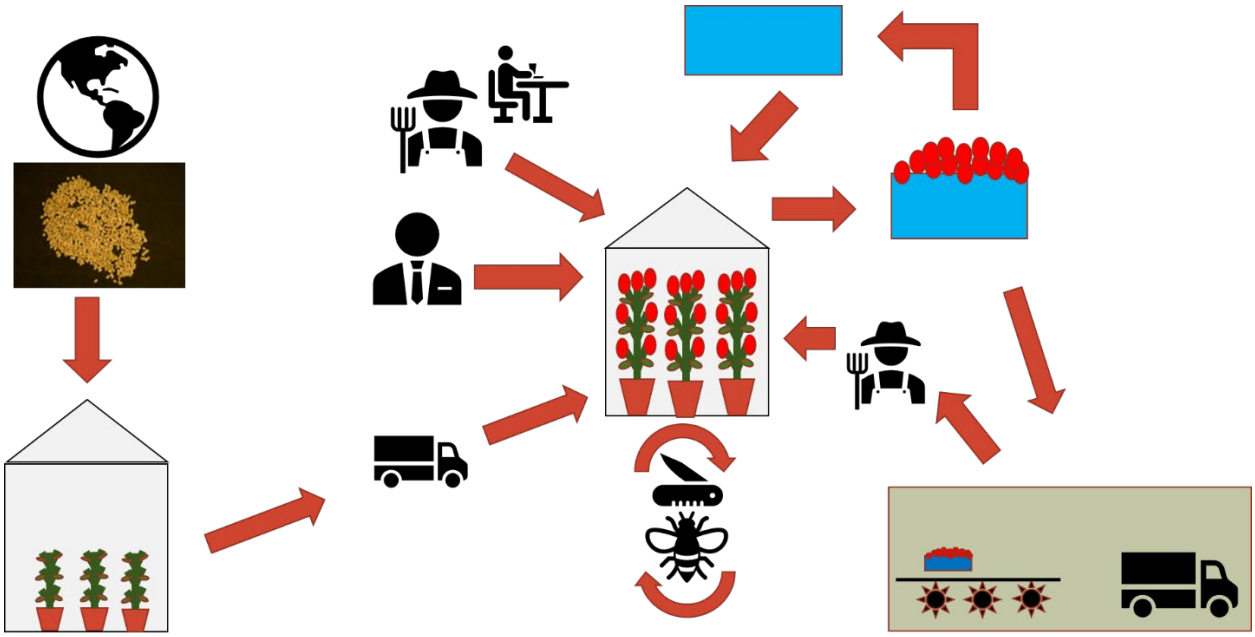

Figure 5. Overview of the end to end flow of the tomato production process encompassing global seed production

Results

Following a stakeholder workshop, the following areas of focus for potential swabbing sites were identified (Figure 6). Discussions on equipment initially focused on the items which are essential for glasshouse operations such as fixed items (door handles, pull chains) and moveable items (e.g. Picking crates, platform lifts, cutting tools). However other items which were identified for swabbing were operations-based items such as computer terminals, or work/labour recording devices (e.g. RFID loggers), but also items in social areas such as coffee machines and canteen surfaces. Other items which were highlighted were items which would be touched by people moving between glasshouse sites, such as production managers e.g. plastics inside cars and mobile phones. Whilst these items are complex in nature and made up of multiple components, in principle, most of the items listed in Figure 6 were considered to be comprised of the same basic materials used in glasshouses. Therefore, stakeholders agreed that for initial validation the “representative” surfaces which had been

used in previous disinfection work (PE033/a) would be suitable as a broad representation of surface types namely glass, steel, aluminium, concrete, polythene, hard plastic (picking tray) and disposable gloves. Whilst this wouldn't cover all the potential surfaces, this would cover the basic families of surfaces and indicate potential issues such as dealing with porous surfaces or surfaces such as plastics which may carry an electrostatic charge.

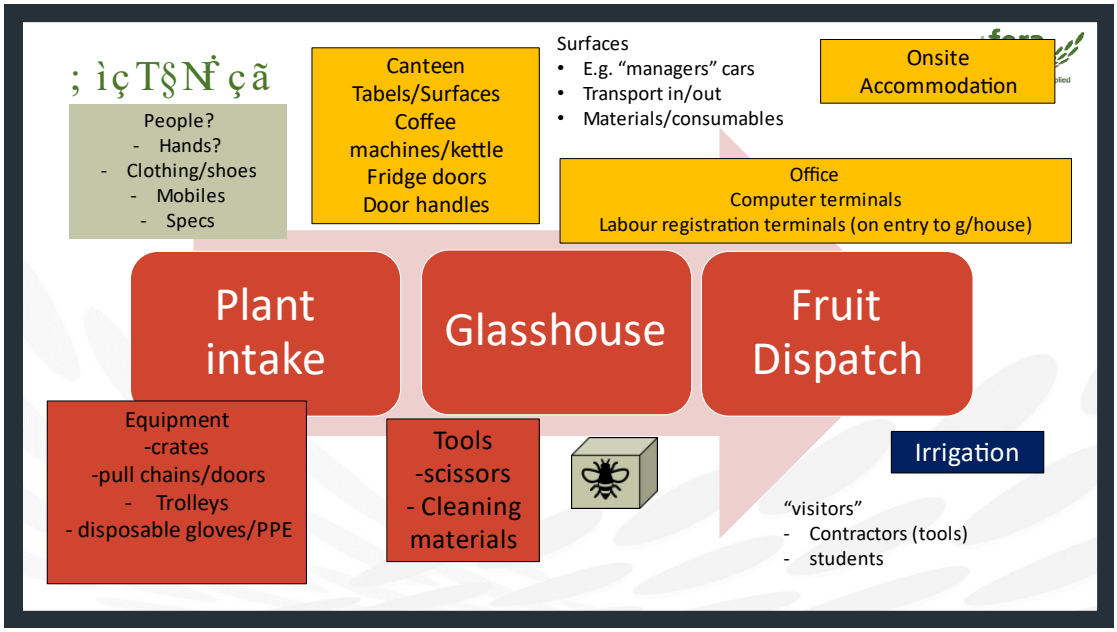

Figure 6. Output process map highlighting areas where swab sampling could be advantageous for maintaining biosecurity.

Objective 2: Optimisation and Validation of ToBRFV LAMP against current "gold standard" methods (real-time qPCR)

Background

As stated under objective 1, the purpose of assay validation is to understand the performance characteristics of a given test. This should include an assessment of the scope of validation i.e. the test should be validated for the specific application for which it is intended, including assessing the impact of the host plant material, other pathogens which might be encountered during testing, and the "matrix" which is to be tested.

The general approach to validation testing in plant health is set out in international standard EPPO PM 7/98 (OEPP/EPPO 2018), This standard recommends approaches for

determining the specificity and sensitivity of a test as well as the repeatability and reproducibility criteria of the test. Due to the intended application, knowledge of the analytical sensitivity (e.g. limit of detection following a dilution series) is of limited practical use, and so within this exercise the analytical sensitivity and diagnostic sensitivity will be determined (i.e. performance relative to the current “gold standard” test). The aim of this is to allow a better understanding of how the test would perform for the intended use, as opposed to in “ideal” conditions.

Specificity of the test will be determined within practical limits. Whilst a test should be validated to ensure there is no risk of cross reaction with similar pathogen/targets, and that all known isolates of the desired pathogen/target are detected, in practice this is not feasible as hosts such as tomato can host a huge number of viruses and viroids, and isolates of many of these are unavailable to test. (Roenhorst et al. 2018). Therefore, many of these cross-reactions are accounted for during assay design, and primers are checked for potential cross reactions through *in silico* analysis. Practical validation work can then focus on a broad, but reduced suite of representative pathogens, such as those closely related to the target pathogen, and those likely to be encountered in the same environment such as common glasshouse/tomato infecting viruses.

LAMP is a form of PCR chemistry which works on an isothermal basis. Additionally, the chemistry used for LAMP testing is less prone to the influence of reaction inhibition than real-time RT-PCR, the current standard test method. The simplicity of the kit used, combined with this robustness, means that LAMP may be amenable for in-field use by growers and inspectors. However, to assess this the performance characteristics of the test need to be established. As the intended purpose of the test in this scenario is to monitor for environmental residues of ToBRFV infection, the matrices considered relevant need to be included in the validation testing. This includes the influence of the swab used as a well as the potential influence of the surfaces being swabbed.

Methods

General Methodology

The sap was prepared from ToBRFV infected tomato leaves, ground with 10X phosphate buffer. The sap was diluted to the concentration of $1x10^{-3}$, which equals to a detection in the 20-23 Ct by real-time reverse transcription PCR (real-time RT-PCR), used as the standard inoculum for all the swab testing. The surface of various materials (glass, aluminium, stainless

steel, concrete, hard plastic, polythene and glove) were prepared with an inoculation of 25 µl sap, the inoculated surface area was left to air dry.

RNA extraction was carried out according to standard Fera SOPs using Qiagen RNeasy Plant Mini kit, the swab was dipped into a tube containing 1 ml phosphate buffer to pre-wet the swab tip. The pre-wet swab was used to swab across the inoculated surface area and then re-deposited into the tube. The buffer RLT (a component from the Qiagen kit) was added to the tube and the mixtures were processed following the kit manufacturer protocol. The RNA extracts were tested using LAMP and real-time RT-PCR.

Real-time RT-PCR comparisons were carried out in accordance with Fera standard SOPS and the EPPO diagnostics standard on detection of ToBRFV using the primers from Menzel and Winter (EPPO PM7/146; Appendix 5).

For crude extraction (using PEG buffer), the swab was dipped into a tube containing 0.5 ml PEG buffer to pre-wet the swab tip. The pre-wet swab was used to swab across the inoculated surface area and then re-deposited into the PEG-tube containing one large steel ball bearing. The swab sample was shaken by hand for about 1 minute. The addition of ball bearing is to improve the recovery of the virus from the swab into the PEG buffer and the PEG buffer which is alkaline helps with the cell lysis. The extract was diluted 1 in 10 in nuclease-free water, the diluted extracts are then ready to be tested using LAMP.

Establish sensitivity and specificity (inclusivity and exclusivity) characteristics for the test

Analytical Sensitivity

Leaf samples from *Solanum lycopersicum* infected with ToBRFV were used to prepare infected leaf sap. The leaf sap was used to prepare ten-fold dilution series up to eight dilution per sample. A total of 9 sap samples (1 leaf sample x 9 concentrations) were tested in triplicate using loop-mediated isothermal amplification (LAMP). Each dilution was aliquoted to undergo RNA extraction to yield pure RNA extracts. The RNA extract of each dilution was tested by both LAMP and real-time reverse transcription PCR (real-time RT-PCR). The lowest virus dilution in which the virus could be detected in all replicates will be referred as the Limit of Detection (LoD).

The RNA extraction was performed to determine the concentration of the ToBRFV infected leaf material and the concentration of the ToBRFV from each dilution.

The aim of this experiment was to demonstrate the comparison of the LoD for LAMP to the LoD for real-time RT-PCR and therefore is able to detect the virus at low concentration by using swab and crude extraction method followed by LAMP detection.

N.B. Approximate 2 g of ToBRFV infected leaf was ground by using Homex, then added with 10X phosphate buffer to produce the 'test' leaf sap. RNA extraction was performed by using KingFisher sample purification systems.

Additional experiments to select the optimum run conditions and most appropriate enzyme reagents were also carried out following the method detailed above but with and without an RT step.

Analytical Specificity

ToBRFV isolates from the UK and Netherland were selected for the test using LAMP assay.

For non-target virus, a range of common and regulated tomato viruses and related tobamovirsues were screened to ensure no cross reaction would occur.

Repeatability

To minimize the variable of the LAMP testing, ToBRFV RNA extracts were chosen to determine the LoD and the repeatability of the LAMP assay. The LoD (based on RNA extract) was determined from the data generated. The LoD is observed at the concentration of $1x10^{-4}$ to $1x10^{-5}$ (equals to Ct 23-26 and Ct 27-29, correspondingly, by real-time PCR). ToBRFV RNA extract at lower concentration ($1x10^{-3}$ to $1x10^{-6}$) was tested 8 times each dilution to establish the consistent and reliable detectable LoD and its repeatability. (Data not presented)

Reproducibility

A reproducibility experiment was performed using the method that was purposely developed for this project, i.e. using a swab to sample a ToBRFV inoculated glass slide followed by crude extraction and LAMP detection.

Selectivity: Determine any “matrix” effect from choice of swab or swabbed surface

Swab selection

a) Type of Swab

In addition to the data generated for analytical sensitivity, instead of the detection of the virus directly from the leaf material, the test material was substituted by using a cotton bud swab sampling on a known concentration of ToBRFV inoculum (test sap) on a surface, follow by nucleic acid extraction and LAMP detection as well as real-time RT-PCR. There are two types of cotton bud swab, a commercially available household use cotton bud swab and a sterile sample collection swab (manufactured in clean room), were used to compare the effect of different types of swab for the swab testing. The test sap was inoculated on a glass surface and tested in triplicate (2 types of swab x 2 inoculum concentrations of sample x 3 replicates = 12). A blank glass surface was also tested for any negative matrix effect.

b) Crude extraction

Once the type of swab was selected, the selectivity was repeated using household-use cotton bud swab (choice of swab), follow by crude extraction using PEG buffer and LAMP detection. The number of swabs per sample collection tube, volume of PEG buffer and number of ball bearing were investigated to optimise crude extraction method using PEG buffer.

Effect of Surface

The type of swab and the optimal crude extraction method were determined in the determination of selectivity.

In addition to a glass surface, various surface materials including aluminium, stainless steel, concrete, hard plastic, polythene,and nitrile glove were tested using the same swab testing method followed by crude extraction and LAMP detection.

N.B

The sap was prepared from ToBRFV infected tomato leaves, ground with 10X phosphate buffer. The sap was diluted to the concentration of $1x10^{-3}$ and $1x10^{-4}$ (equals to Ct 20-22 and Ct 23-26, correspondingly, by real-time RT-PCR), as the two selected inoculum concentratiosn, to be used as a standard inoculum for all the swab testing.

The surface of various materials including glass, aluminum, stainless steel, concrete, hard plastic, polythene and glove were prepared with an inoculation of 25 µl of the test sap, the inoculated surface area was left to air dry.

For nucleic acid extraction (using Qiagen RNeasy Plant Mini kit), the swab was dipped into a tube containing 1 ml phosphate buffer to pre-wet the swab tip. The pre-wet swab was used to swab across the inoculated surface area and then re-deposited into the tube. The buffer RLT (a component from the Qiagen kit) was added to the tube and the mixtures are processed following the kit manufacturer protocol. The RNA extracts were tested using LAMP and real-time RT-PCR.

For crude extraction (using PEG buffer), the swab was dipped into a tube containing 0.5 ml PEG buffer to pre-wet the swab tip. The pre-wet swab was used to swab across the inoculated surface area and then re-deposited into the PEG-tube containing one large steel ball bearing. The swab sample was shaken by hand for about 1 minute. The addition of ball bearing is to improve the recovery of the virus from the swab into the PEG buffer and the PEG buffer which is alkaline helps with the cell lysis. The extract was diluted 1 in 10 in nuclease-free water, the diluted extracts were then ready to be tested using LAMP testing.

Swabbing in “outbreak” scenarios

The approaches trialled experimentally were also trialled in real scenarios within contaminated glasshouses. The first of these was during a recurrent ToBRFV outbreak in the West Midlands. Laboratory staff visited the outbreak site and took swabs of a range of surfaces. Surfaces were deliberately selected to represent areas which would be contaminated with environmental residues from plant infections rather than from direct contact with infected plants. These environmental residues will include “dust” arising from pollen, broken leaf trichomes etc. In the second scenario, the Fera glasshouse cubicle previously used for a series of ToBRFV experiments, including those reported in PE033/a and PE034, was swabbed. This cubicle had been subjected to previous disinfection treatments with protocols in compliance with PE033/a, but is currently housing a ToBRFV host range and seed transmission experiment, therefore both current and “ghost” nucleic acid from prior work would be present in the cubicle.

In both glasshouses, swabbing was carried out with supermarket-type cotton swabs pre-wetted with buffer. An area approximately 2cm x 2cm was swabbed using a rolling motion and the contaminated swab placed with remaining buffer into a screw top tube.

Due to the outbreak glasshouse being the subject of ongoing plant health containment and eradication action, results from this testing were to be fed back to plant health risk managers.

Therefore, swabs had to be processed using nucleic acid extraction to allow for real-time RT-PCR detection, and crude extraction could not be trialled in this scenario.

In the contaminated experimental glasshouse duplicate swabs were taken to allow a comparison of real-time RT-PCR with LAMP on the same RNA extracts, and a duplicate swab could be tested using the crude extraction protocol.

Results

In this report real-time RT-PCR results are presented as the cycle threshold (Ct) which is the number of PCR heating cycles to register a positive result. LAMP results are presented as the time to positive amplification (Tp) and the annealing temperature of the LAMP product (Ta). The Ta measurement is specific to the LAMP product for each test target and serves as a secondary quality check of the test reaction.

Analytical sensitivity: Comparison of LAMP detection from crude preparation and extracted nucleic acid

Following enzyme selection both extracted viral RNA and crude extract of leaf material were assessed for analytical sensitivity through a comparative 10-fold dilution series. These results are presented in Figure 7.For samples which had been through the standard nucleic acid (RNA) extraction, pure extract was detected after 3minutes 15 seconds. Even after 1000-fold dilution virus was detected within 5 minutes (4m 20s). At the limit of detection of $1x10^{-6}$, virus was detected in around 16 minutes (15m 55s).

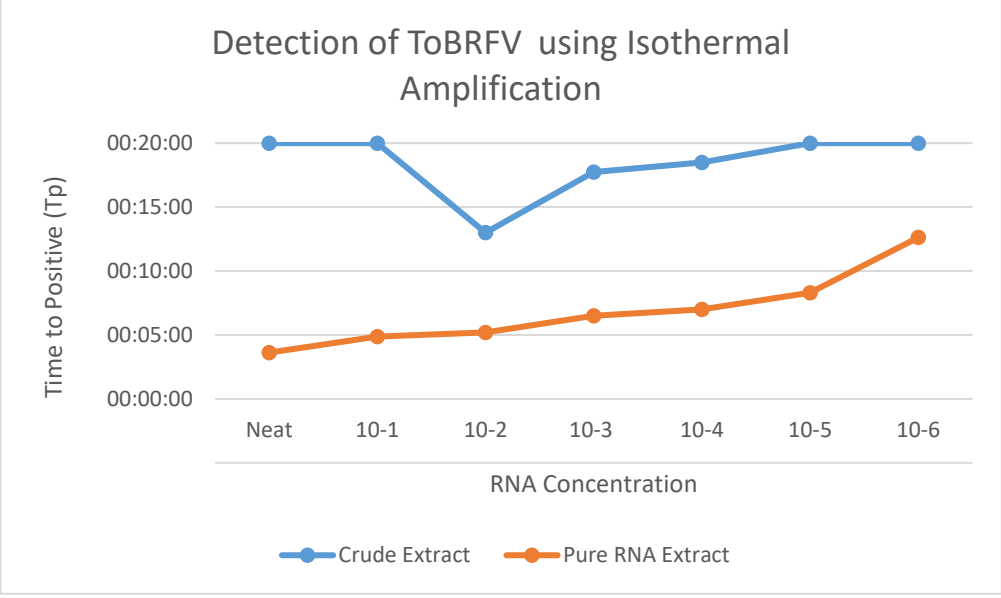

Figure 7 Detection of ToBRFV in a comparative 10-fold dilution series with crude extract (homogenised sample in PEG buffer) and extracted RNA

Crude extracts were prepared with leaf homogenised 1 to 10 weight to volume of PEG buffer. The analysis presented in Figure 7 shows the comparative detection of these crude preparations against the industry standard RNA extraction method. Preparations of “neat” homogenate and a further 1 x 10^{-1} dilution were not detected. This is a consequence of the functionality of the LAMP machine, where fluorescence is read from the reaction but depends on light produced from an LED being read by an optical sensor, therefore the deep green colour from plant sap in the stronger concentrations were preventing the proper operation of the analytical equipment. This is particularly relevant for the neat, and 1x10^{-1} dilutions. Where comparative data are available, viral target was detected significantly more quickly from extracted RNA than from crude preparations. The limit of detection (LoD) is determined by multiple extracts, in this case eight repetitions. at these dilutions. This determined the maximum limit of detection of LAMP on RNA extractions at 1x10^{-6}, as opposed to LAMP on a crude preparation giving detection to 1x10^{-4}. However, repeatability testing (testing 8 repeat samples at each dilution) indicated the robust limit of detection of RNA extraction LAMP was 1x10^{-4}, as some positive samples were missed at lower concentrations.

Analytical Specificity: Testing ToBRFV LAMP primers against a range of target and non target viral pathogens

Table 3 Specificity testing of LAMP assay for ToBRFV showing negative reations with non-target viruses and viroids, and detection of ToBRFV test and controls. (1) PepMV sample tested positive, later shown to be cross-contamination of the sample. LAMP results are presented as the time to positive amplification (Tp) and the annealing temperature of the LAMP product (Ta).

Group	Target	Extracted RNA		
		Tp	Ta	Result
Viroids	PSTVd	/	/	Neg
	CLVd	/	/	Neg
	PCFVd	/	/	Neg
Tomato infecting viruses	PepMV Ch1	/	/	Neg
	PepMV Ch2	8:00	84.89	Contamination[1]
	PepMV Ch2 (isolate 2)	/	/	Neg

	PepMV EU	/	/	Neg
	STV	/	/	Neg
	TSWV	/	/	Neg
	TYLCV	/	/	Neg
	PVX	/	/	Neg
	PVY	/	/	Neg
Tobamoviruses	TMV	/	/	Neg
	ToMV	/	/	Neg
	PMMoV	/	/	Neg
	ToBRFV (UK)	3:00	84.96	Pos
	ToMMV	/	/	Neg
	UTobV1	/	/	Neg
Controls	No Template	/	/	Neg
	H_2O 1	/	/	Neg
	H_2O 2	/	/	Neg
	H_2O 3	/	/	Neg
	Pos 1 (DSMZ)	5:45	84.97	Pos
	Pos 2 (DSMZ)	5:30	84.93	Pos
	Pos 3 (DSMZ)	5:45	84.93	Pos

The specificity of the LAMP primers used in the test methods is checked as part of the initial assay design phase. By cross-checking the primer sequences against complimentary sequences deposited in public sequence databases (e.g. NCBI GenBank) a process termed *in silico* analysis. To further investigate the specificity (inclusivity and exclusivity) of the test method a range of tomato infecting viruses and viroids were tested (See table 3). Different isolates of ToBRFV were tested to ensure that these can be detected (Inclusivity), including a bought in positive control (DSMZ, Germany) and an isolate from a UK outbreak site known to have some genetic divergence. To determine exclusivity, non-regulated viruses which commonly occur in tomato crops were tested using the method, such as strains of pepino mosaic virus (PepMV), southern tomato virus (STV), tomato spotted wilt virus (TSWV), potato virus X (PVX) and potato virus Y (PVY). Regulated viruses and viroids which are commonly tested for in statutory screening were also included in the suite of pathogen targets including viroids (potato spindle tuber viroid (PSTVd), columnea latent viroid (CLVd), pepper chat fruit viroid (PCFVd)) and tomato yellow leaf curl virus (TYLCV). Additionally, viruses from the same genus (*Tobamovirus*) were included in the suite of pathogen targets checked, as these

closely related viruses are most likely to lead to cross reactions. This suite included viruses such as tobacco and tomato mosaic virus (TMV, ToMV), pepper mild mottle virus (PMMoV); tomato mottle mosaic virus (ToMMV), and the recently discovered Ullucus tobamovirus-1 (UTobV1). To ensure no cross reaction with the other test reagents or non-specific amplification a “no template” control (reagent master mix only) and water controls (H_20) were also included in the suite of testing.

With the exception of ToBRFV test and positive control, no positve reactions were observed with any other target (cross reactions). In the case of PepMV strain Ch2, the initial positive reaction (as presented in Table 3) was investigated further. Following additional testing this was shown to be sample contamination in the glasshouse, in effect a “real” positive reaction. Several other isolates of PepMV Ch2 were tested with no evidence of cross reaction.

Selectivity: influence of swab selection

The results presented in Figure 8 show the comparative average performance of two different swab types, a “common swab”, of a cotton bud type, bought from a local supermarket, and a laboratory swab from a specialist supplier, at a standard dilution rate of $1x10^{-3}$. Similar results from lower concentrations are not presented. Both swabs detected all samples with comparable efficacy. At a standard dilution of $1x10^{-3}$ the cotton swabs gave a more rapid detection of extracted RNA, but a slightly slower detection rate from crude (PEG) prepared samples. Over a range of dilutions these results were inconsistent, but when considered as a qualitative test, similar levels of detection were achieved with either swab.

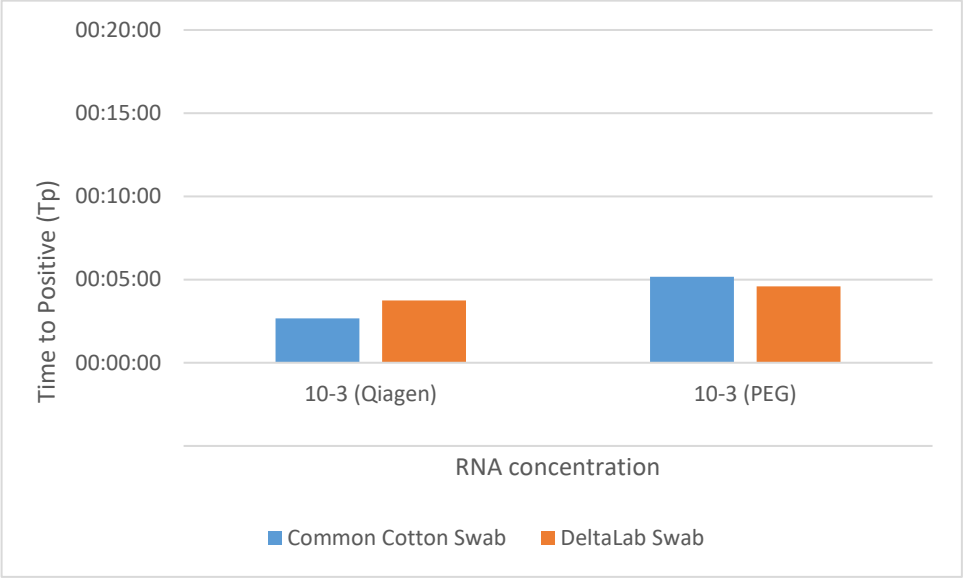

Figure 8 Comparison of different swab types for the detection of ToBRFV (RNA extract) from an inert swabbed surface (Glass)

Selectivity: The influence of surfaces

Previous studies (PE033/a) have shown that survival and disinfection of ToBRFV can be influenced by the type of surface. Following the initial workshops with industry to identify potential surfaces for swabbing, initially the same range surfaces used for the survival and disinfection studies were used as "standard" surfaces with a standardised inoculum. For each surface the "best" (quickest detection), "worst" (slowest detection) and the average are presented in Figure 9. Inert surfaces such as glass, aluminium and stainless steel showed reliable detection from swabbed samples with detection between 6m15s and 10m00s. Detection from nitrile disposable gloves was in a similar range to glass, steel and aluminium, indicating these could all be reliable surfaces for the focus of swab testing, with minimal influence on the outcomes of testing. Detection from plastics was both slower on average and much more variable, indicating that these surfaces may have characteristics which interfere with detection. As this is evident on both hard plastic and polythene this is unlikely to be a physical characteristic (e.g. surface pores/pitting) and more likely an inherent characteristic of this type of surface such as the effect of electrostatic charge from the plastic leading to erratic adherence of the virus or sap sample. This would require further work to investigate the actual cause of this effect.

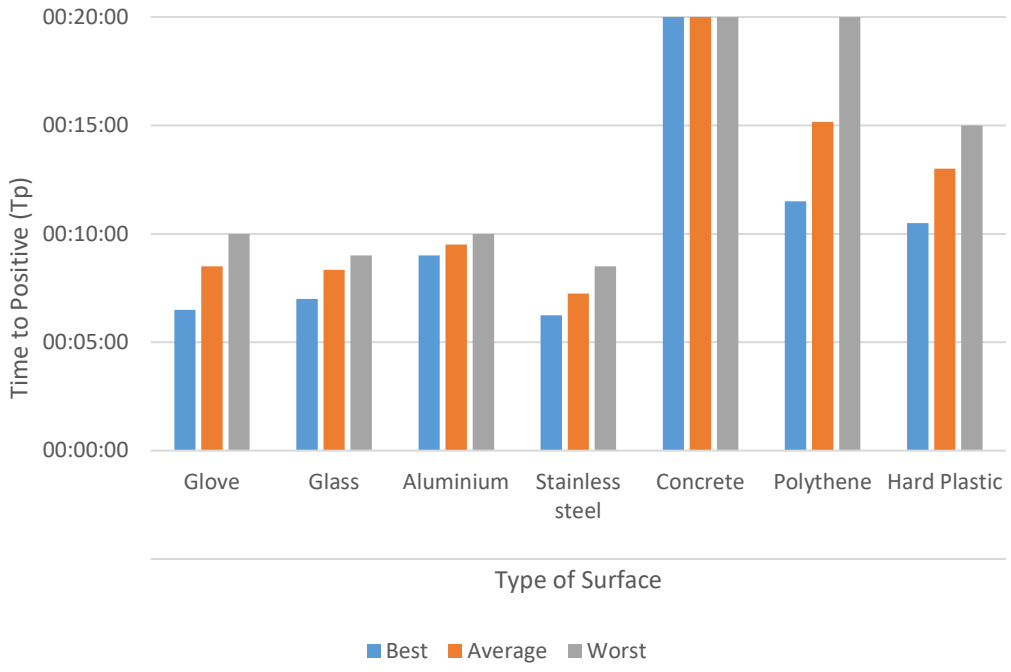

Figure 9 Detection of ToBRFV using swabs from different surfaces showing best (quickest), worst (slowest) and average detection for each surface

Objective 3. Applying ToBRFV LAMP testing in different glasshouse scenarios

Following swab selection and determination of sensitivity, specificity and selectivity the testing approaches were trialled in glasshouses with active infections of ToBRFV. One of these was a current active outbreak in a commercial glasshouse, the other was the experimental glasshouse at Fera where active ToBRFV work is currently being carried out.

Outbreak glasshouse tests

The results presented in Table 4 are from samples taken during an active outbreak in a UK glasshouse. Except for "Glasshouse 1/Bee Box 2", all results presented are a direct comparison between real-time RT PCR and LAMP detection on the same nucleic acid extracts. Using real-time RT PCR revealed environmental contamination from ToBRFV in all parts of the glasshouse. This included contamination onto single use items such as personal protective equipment (Tyvek suit and gloves), and also onto items which had not been exposed directly to the glasshouse environment (Grower Phone).

*Table 4 Results of swab testing from an outbreak glasshouse, comparing real-time RT-PCR and LAMP (RNA extracted samples). For real-time PCR detection a Ct value of 40 indicates no target amplification (negative). For LAMP a / denotes no target amplification (negative). * Crude extract in polyethylene glycol (PEG) buffer. Real-time RT-PCR results are presented as the cycle threshold (Ct) which is the number of PCR heating cycles to register a positive result. LAMP results are presented as the time to positive amplification (Tp) and the annealing temperature of the LAMP product (Ta).*

Source	Sample site	real-time PCR	LAMP		
		Ct Ave	Tp	Ta	LAMP Conclusion
Glasshouse 1	Top of light	35.36	19:15	84.45	ToBRFV
	Fan	22.09	6:45	84.3	ToBRFV
	Under Gutter	20.86	6:00	84.15	ToBRFV
	Behind whiteboard	33.55	/	/	Negative

	Top of Cable tray	20.25	6:30	84.53	ToBRFV
	Bee Box 1	22.91	8:30	82.52	ToBRFV
	Bee box 2 – PEG*	not tested	16:30	84.7	ToBRFV
	Inside panel door	23.39	8:15	82.82	ToBRFV
	socket lid	25.57	8:15	84.5	ToBRFV
	Pheromone trap	31.63	19:15	84.35	ToBRFV
Glasshouse 2	Roof structure	17.36	6:15	83.42	ToBRFV
	Hive	16.98	5:15	84.61	ToBRFV
	Top light	25.33	7:00	84.41	ToBRFV
	Fan	17.54	4:45	84.67	ToBRFV
	Under gutter	16.76	5:00	84.47	ToBRFV
People	Tyvek Suit	16.23	4:45	84.44	ToBRFV
	Grower Phone	31.18	/	/	Negative
	Grower Glove	19.80	5:15	84.52	ToBRFV
Irrigation Water	NFT Water	40	/	/	Negative
	Substrate Water	40	/	/	Negative
Controls	Neg - H_2O	40	/	/	Negative
	Pos - ToBRFV+	11.21	5:45	85.32	ToBRFV

The results presented here indicate that the approximate limit of detection for LAMP on RNA extracted samples equates to a real-time RT PCR value of 30Ct. Due to the nature of the outbreak duplicate samples were not taken. Two samples were taken from separate bee boxes in the roof space of Glasshouse 1. Swabs were taken by rolling the swab around the entrance hole of the bee box. These two were used as “similar” (not duplicate) samples and the sample from bee box 2 was used to investigate the use of swabbing and crude extraction. Although this is only a single datapoint, this indicates that bee boxes may provide a natural accumulation point of environmental residues of ToBRFV for surveillance sampling.

Experimental glasshouse testing

Duplicate swab samples were taken from one of the experimental glasshouse cubicles at Fera Science Ltd. This cubicle has been used for a series of ToBRFV experiments including the inoculation and detection work in PE 034, water detection studies, and it is currently in use for ToBRFV host range studies on a range of ornamental and at-risk solanum species.

These previous and current experiments mean that the glasshouse has been repeatedly exposed to contamination of environmental residues. Duplicate swabs were taken from a range of surfaces including windows, walls, metal benching, moveable equipment (aluminium ladders, plastic plant pots and trays), and also PPE (gloves, polythene apron and Tyvek sleeves). The results from this swab testing are presented in Table 5. Real-time RT-PCR indicated that all swabbed surfaces were positive for the presence of ToBRFV with a range of Ct values from 18.89 (bench grid) through to 36.70 (the glove used for sampling). As with the outbreak swab samples, the limit of detection of LAMP on extracted RNA was around 30Ct, with the plastic apron detected at 31.06 Ct and a time to detection on LAMP of 16m15s. This relative lack of sensitivity meant that LAMP on RNA extracts only detected 61% of the surfaces detected by real-time RT-PCR. These surfaces were both metal and plastics.

Crude extraction with LAMP was slower to detect the presence of virus than from RNA extracts. Fewer surfaces were shown to be positive due to the consequent loss of sensitivity with only 25% of surfaces positive by crude extraction-LAMP compared to the “gold standard” method. One anomalous result was from floor swabbing, where real-time RT-PCR and crude extraction LAMP both detected the presence of virus, however, LAMP on the RNA extracted sample did not. Given the RNA extracted LAMP and the real-time RT-PCR were tested from the same sample extraction this appears to be a “false negative” in the RNA extraction LAMP.

Table 5 Comparative results of glasshouse swabbing from a controlled environment (Fera experimental glasshouse). For real-time PCR detection a Ct value of 40 indicates no target amplification (negative). For LAMP a / denotes no target amplification (negative). Real-time RT-PCR results are presented as the cycle threshold (Ct) which is the number of PCR heating cycles to register a positive result. LAMP results are presented as the time to positive amplification (Tp) and the annealing temperature of the LAMP product (Ta).

	Real-time RT-PCR				
	RNA extract	LAMP			
		RNA Extract		Crude (PEG)	
Sample type	Avg Ct	Tp	Ta	Tp	Ta
window 1	28.43	00:10:45	84.51	/	/
window 2	28.27	00:11:15	84.52	/	/
window 3	29.11	00:11:15	84.86	/	/
Bench edging - face out	34.37	/	/	/	/
Bench edging - face in	32.24	/	/	/	/

ladder	25.93	00:08:30	84.66	00:10:45	84.61
wall 1	34.88	/	/	/	/
wall 2	28.15	00:12:15	84.72	/	/
floor	26.96	/	/	00:04:45	84.46
plant pot 1	26.93	00:06:30	84.77	/	/
plant pot2	26.77	00:06:45	84.58	/	/
plant pot black tray	21.08	00:05:45	84.66	00:14:45	84.61
Stand - leg	32.25	/	/	/	/
Stand - middle bar	29.47	00:18:15	84.44	/	/
Stand - grid panel	18.89	00:05:30	/	00:06:30	84.26
Glove	36.70	/	/	/	/
Tyvek sleeve	36.60	/	/	/	/
Plastic apron	31.06	00:16:15	84.79	/	/
H2O	40.00	-	-	-	-
ToBRFV + (avg)	22.98	00:06:00	85.3	00:06:23	85.15

Discussion and Conclusions

The key purpose of validation is to ensure you understand the operating parameters (performance characteristics) of the test you are using. These performance characteristics can then be used to inform the use of the test in different diagnostic applications. The European Plant Protection Organisation has set out key parameters for validating tests for use in plant health (EPPO, 2018). The programme of validation presented here follows these parameters. Within the programme of validation, the analytical and diagnostic sensitivity of the test has been established by serial dilution and comparative testing with the gold standard assay. The specificity has been established across a arrange of target and non-target virus and viroid isolates, and additionally the test has been used in “real world situations” in both an active outbreak and an experimental glasshouse known to be contaminated with ToBRFV.

Two different core reagent enzymes were investigated for use in the test, one with a faster action and potentially greater sensitivity, and also two different sets of reaction conditions. To test an RNA virus using PCR or LAMP based methods it must first be converted to a DNA template, a process known as a reverse transcription step (RT step). Both enzymes were trialled with an RT step prior to the main diagnostic reaction, and with the RT step occurring simultaneously within the main reaction. With the separate RT and reaction steps the quicker acting enzyme gave enhanced sensitivity (increasing sensitivity by a further 10-fold dilution), however, these conditions gave non-specific amplification in the reaction, leading to a high

number of false positives including in no-template and water controls. When the RT step was integral to the main reaction the performance of the quicker enzyme was comparable to the performance characteristics of the slower reagent set, giving no real advantage in test performance.

One issue faced in validation for deployment of in-field testing is that the current laboratory standard real-time RT-PCR process requires extracted nucleic acid samples. LAMP is robust enough to use either extracted nucleic acids or crude extractions (in this case leaf or swab samples diluted in PEG). This means that it is challenging to get a direct comparison of sensitivity (limit of detection) of LAMP on a crude extract with the current method. For this reason, all testing reported here involved a comparison of the PCR method with LAMP on extracted RNA and a subsequent separate test of the LAMP on a crude preparation from the same source. Although this does not give a direct comparison, this allows for an indicative measure of comparative sensitivity (e.g. Figure 7).

For laboratory validation the selectivity of a test is a key parameter for understanding the reliability of the test method within the intended application. With plant testing this would include testing different plant parts or matrices (leaf, fruit, seed, calyx, roots, etc). However, the desired application of the test being investigated was to deal with environmental swabs as an in-field, on site diagnostic test. Therefore, key matrices which may influence the reliability of the sampling process in these applications are the type of swab used and the surface being swabbed. Two types of swabs were compared, a “supermarket swab” i.e. a traditional “cotton bud”, and a specialist laboratory swab. The results presented in Figure 8 indicated that there was a slight improvement in speed of detection with crude extracts with the laboratory swab, however this was the reverse of the result observed from RNA extractions. The supermarket swabs were more cost effective, and with the additional complications of securing supplies of laboratory swabs created by the ongoing COVID pandemic, supermarket swabs were selected for use in further validation testing. The type of surface tested has been shown to impact on the survival and disinfection of ToBRFV (PE033/a). The results of the validation work presented here indicate that the surface being tested may also influence the ability to recover virus during swabbing. Inert surfaces such as glass and metal allowed robust, repeatable detection of virus contaminated sap. However, surfaces such as plastics gave relatively poor detection, with greater variability in speed of detection. This is likely to be a consequence of these surfaces holding an electrostatic charge, interfering with recovery of the virus. No virus was recovered from contaminated concrete. As with the survival and disinfections experiments carried out in PE033/a, concrete is problematic for surface detection. With no detection of virus from this surface. As with the erratic survival results this is likely a consequence of the porous surface of concrete. This

unlikely to be a physiochemical effect of concrete (e.g. pH or similar surface influence) as results elsewhere in these trials have indicated strong results from this surface (see Table 5 – “floor” where swabbing of the glasshouse cubicle at Fera used for PE034 indicated a strong result for the presence of the virus). This may be either the consequence of greater inoculum levels on the floor of the active experimental cubicle or may be a consequence of foot traffic in the cubicle over 30 years rendering the floor “smooth” effectively reducing the porosity of the surface.

The specificity of the test was ascertained by testing a series of common and regulated tomato infecting viruses and viroids, and other viruses related to ToBRFV. An additional check on specificity is carried out using an *in silico* check of viruses on the public sequence database (NCBI GenBank). Given these checks, the “positive” reaction with an isolate of pepino mosaic virus-Chile 2 strain was unexpected. Further investigation showed that this isolate also cross-reacted with the EPPO standard listed real-time RT-PCR assays, whereas multiple other isolates of PepMV-Chile 2 strain did not react with either the LAMP or the real-time PCR assays, indicating this initial result was the result of a co-infection of the isolate.

Following the validation work described above the assays were trialled in two different “real world” scenarios. The first of these trials was as part of statutory follow up work at the only current UK outbreak site. RNA extracts from swab samples taken at the outbreak site were tested to give a direct comparison between LAMP and real-time PCR. The site was subject to recurrent infections with many thousands of plants infected leading to high levels of virus present in the environment. This testing indicated that LAMP testing of RNA extractions gave a comparable limit of detection approximately equivalent of a real-time RT-PCR result of 30Ct. In this environment, with high levels of environmental residues of ToBRFV present, this led to LAMP returning two false negative results from the 17 samples tested (12%). The second scenario, in the Fera experimental glasshouse, there was likely to be a lower level of environmental residues, as although the cubicle had been used for ToBRFV infection work, the density of plants was much lower than in a commercial planting. This second site allowed a cross comparison between real-time RT-PCR, LAMP on extracted samples, and LAMP on crude extractions. For the direct comparison of LAMP and real-time RT-PCR a similar limit of detection was observed, with the highest Ct value which was also detected by LAMP being 31Ct (Table 5). This limit of detection for LAMP on RNA extracted samples (Ct 30-31) is approximately equivalent to the limit of detection achieved with conventional PCR approaches.

As expected the general levels of environmental residues in this glasshouse cubicle were lower than the levels in the outbreak glasshouse, as observed by the higher overall Ct values on PCR from this glasshouse, indicating a lower concentration of viral RNA present in

samples. This lower concentration of virus in sample swabs led to a consequent higher number of “false negative” results on the comparative testing, with LAMP missing approximately 40% of the samples detected by real-time RT-PCR. Testing crude extracts only detected 25% of those samples detected by real-time RT-PCR. Crude testing did detect one sample which was detected by real-time RT-PCR, but not LAMP on extracted samples (Table 5 - Floor), supporting the hypothesis that the issues experienced with detection from concrete are a physical (porosity) rather than a chemical issue.

The outcomes of these validation trials indicate that LAMP testing has applications within plant health testing for the presence of ToBRFV. However, the significant reduction in analytical sensitivity when testing from crude extracts, mean that this approach would only be useful with an associated RNA extraction protocol, therefore this approach would not be applicable for onsite testing in its current form. Ongoing work at Fera will look at other applications for this work, with a primary focus on the application of the crude extract approach for screening import fruit samples, where the loss of sensitivity should not be a limiting factor in the testing.

The other unexpected outcome of this work is the demonstration of the prevalence of environmental residues of ToBRFV following an outbreak, as shown in the results of both the PCR and LAMP testing from glasshouse scenarios (Table 4 and Table 5). These residues may from the source of carry over infections in commercial glasshouses, or as demonstrated in PE033a, may confound the ability to use diagnostic solutions to demonstrate the efficacy of disinfection following an outbreak. There are multiple potential sources of such residues and work is ongoing to better understand the sources and implications of these sources of ToBRFV.

Knowledge and Technology Transfer

The progress towards the outcomes of this project have been regularly fed back to growers via both the TGA technical committee and the UK ToBRFV steering group. Additionally, the results from this project have also been presented at the following events:

- International Plant Virus Epidemiology Symposium: Poster presentation on detection of ToBRFV from plants and surfaces (AHDB PE034 and PE035). Marid, 5-8 June 2022
- ToBRFV Research Symposium, Ontario, Canada, 17-18 August 2022
- UK Tomato Growers conference, 22 September 2022

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