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Evaluation of a rapid diagnostic field test kit for identification of *Phytophthora* species, including *P. ramorum* and *P. kernoviae* at the point of inspection

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Plant health regulations to prevent the introduction and spread of *Phytophthora ramorum* and *P. kernoviae* require rapid, cost effective diagnostic methods for screening large numbers of plant samples at the time of inspection. Current on-site techniques require expensive equipment, considerable expertise and are not suited for plant health inspectors. Therefore, an extensive evaluation of a commercially available lateral flow device (LFD) for *Phytophthora* species was performed involving four separate trials and 634 samples. The assay proved simple to use, provided results in a few minutes and on every occasion a control line reacted positively confirming the validity of the test. LFD results were compared with those from testing a parallel sample, using laboratory methods (isolation and real-time PCR). The diagnostic sensitivity of the LFD (87·6%) compared favourably with the standard laboratory methods although the diagnostic specificity was not as stringent (82·9%). There were a small number ($n = 28$) of false negatives, but for statutory purposes where all positive samples must be identified to species level by laboratory testing, overall efficiency was 95·6% as compared with visual assessment of symptoms of between 20-30% for *P. ramorum* and *P. kernoviae*. This work demonstrates the value of the LFD for diagnosing *Phytophthora* species at the time of inspection and as a useful primary screen for selecting samples for laboratory testing to determine the species identification.

Keywords: bleeding cankers and lesions, on-site testing, plant health surveillance, ramorum dieback, sudden oak death

Introduction

Phytophthora ramorum is a recently described pathogen that is causing extensive damage to trees, woodland and ornamental plants in North America and Europe (Werres *et al*., 2001; Rizzo *et al*., 2002). Symptoms of the disease were first seen in California in 1993 where an unexplained rapid mortality of tanoaks (*Lithocarpus densiflorus*) and several oak species (*Quercus agrifolia* and *Q. kelloggii*) was reported and where it was referred to as sudden oak death (Rizzo *et al*., 2002). However, it was not until 2000 that the cause of the problem was identified as a species of *Phytophthora*. Parallel to this, a new but undescribed species of *Phytophthora* was found causing an aerial dieback principally of rhododendrons, but also viburnums, in Germany and the Netherlands (Werres & Marwitz, 1997). In late 2000, the causal agent of the disease in California and the rhododendron dieback in Europe was identified and formally described as *P. ramorum* (Werres *et al*., 2001). The Department of Environment, Food and Rural Affairs (Defra) Plant Health and Seeds Inspectorate (PHSI) initiated a survey for the presence of the pathogen in England and Wales in autumn 2001. In April 2002, *P. ramorum* was found for the first time in the UK and elsewhere in Europe since the initial reports in the Netherlands and Germany (Lane *et al*., 2003a; Lane *et al*., 2003b). Subsequently, *P. ramorum* has been reported on a wide range of other ornamental plants in the UK such as *Pieris* spp. (Inman *et al*., 2003), *Camellia* spp. (Beales *et al*., 2004a), *Hamamelis virginiana* (Virginian witch-hazel) (Giltrap *et al*., 2004), *Syringa vulgaris* (lilac) (Beales *et al*., 2004b) and *Taxus baccata* (yew) (Lane *et al*., 2004a) and in many other European countries on rhododendron and viburnum (Žerjav *et al*., 2004). In October 2003, *P. ramorum* was found causing bleeding cankers for the first time in Europe on mature trees both in the UK (*Q. falcata* – Southern red oak) (Brasier *et al*., 2004) and the Netherlands (*Q. rubra* – Northern red oak) with further limited findings on other trees in both countries. Due to the initial absence of the organism in the UK, emergency phytosanitary legislation was introduced in June 2002 to prevent its introduction or spread which was soon followed by emergency EC

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legislation coming into effect in November 2002. In autumn 2003, another new species of *Phytophthora* was discovered that caused symptoms on rhododendron and beech trees in trees in southwest England that were similar to those caused by *P. ramorum*. This organism was originally referred to as *Phytophthora* taxon C but more recently the species was formally described as *Phytophthora kernoviae* (Brasier *et al*., 2005; Brown & Brasier, 2007). In the UK, quarantine measures similar to those applied to *P. ramorum* have been implemented for *P. kernoviae* (Beales *et al*., 2006).

Effective disease management and implementation of plant health legislation is reliant upon rapid and accurate disease diagnosis based upon recognition of symptoms in the field and identification of causal agent. Typically only 20–30% of suspect samples test positive for *Phytophthora* in the laboratory (Lane, 2006). This is due in part to the fact that *P. ramorum* and *P. kernoviae* are recently encountered organisms, so knowledge of symptoms and host range are incomplete, making reliable and effective inspection difficult. Other diseases and disorders can cause similar symptoms. For these reasons many negative samples are submitted which must undergo labour and time-intensive laboratory testing including isolation onto semi-selective agar medium and morphological characterization, screening by enzyme-linked immunosorbent assay (ELISA) and molecular methods based on conventional (Lane *et al*., 2003a; Martin *et al*., 2004; Anonymous, 2006; Schena *et al*., 2006;Tooley *et al*., 2006) and real-time polymerase chain reaction (PCR) (Hayden *et al*., 2004; Hughes *et al*., 2006a). At the Central Science Laboratory (CSL), direct testing of the same plant material by real-time PCR when compared with the combination of isolation and real-time PCR of suspect positive cultures has resulted in a very accurate and reliable diagnostic test for *P. ramorum* with a diagnostic specificity of 99·3% and diagnostic sensitivity of 92·3% (Hughes *et al*., 2006a,b). However, these techniques are not suitable for identification at the time of inspection. The ability to detect *Phytophthora* in the field would eliminate a large proportion of samples and only those positive samples would need to undergo further testing for species identification.

Molecular methods have been developed successfully for on-site testing (Tomlinson *et al*., 2005) and have been used in the field both in the UK and California for identifying *P. ramorum* and *P. kernoviae* within 2 h of sampling. However, this method requires considerable technical knowledge and expensive specialized equipment not currently suited for phytosanitary inspectors. Serological assays can be formatted to offer a simpler and more rapid test for field use (Danks & Barker, 2000). Antisera specific to *P. ramorum* or *P. kernoviae* are not available, but polyclonal and monoclonal antibodies that detect *Phytophthora* spp. have been commercially available (Neogen Corporation, USA) both in ELISA format for large-scale screening in the laboratory (Agriscreen®) and also as a multi-step flow-through assay designed for field use (Alert®). The latter requires storage at 4°C, takes 10–15 min to complete the five steps and is relatively intricate for field use. However, 'point-of-care' test kits using single-step lateral flow device (LFD) formats have been successfully used in clinical situations for at least 15 years. The most famous use of this technology is the home pregnancy test first introduced by Unipath in 1988 (May *et al*., 1991.). On-site detection using LFDs have been developed for various uses including plant pathology (Danks & Barker, 2000; Thornton *et al*., 2004), mycotoxin detection in foodstuffs (Danks *et al*., 2003) and indicators of stress in animal welfare (Lane *et al*., 2004b).

The Central Science Laboratory has successfully developed a LFD for detection of *Phytophthora* using antisera supplied by the Neogen Corporation. Therefore, the objective of this work was to evaluate the *Phytophthora* genus-specific LFD for diagnosis of *P. ramorum* and *P. kernoviae* during routine plant health inspection and investigations at two known outbreak sites.

Materials and methods

Lateral flow device

Phytophthora LFD kits designed to recognize all species of *Phytophthora*, including *P. ramorum* and *P. kernoviae*, were supplied by Forsite Diagnostics Ltd, York, UK.

Trials, samples and LFD testing

Trial 1

Six plant health inspectors experienced with recognition of phytophthora diseases in the field were supplied with LFD kits and asked to test a range of plants for *P. ramorum* during their routine inspection work.

Trial 2

Laboratory staff experienced in the recognition of disease symptoms and LFD used the LFD kits at an outbreak site in South Wales where both *P. ramorum* and *P. kernoviae* were known to occur.

Trial 3

Laboratory staff experienced in the recognition of disease symptoms and LFD used the kits at an outbreak site in Cornwall, England where *P. kernoviae* had been found previously.

Trial 4

Plant health inspectors from across the whole of England and Wales (90 inspectors) experienced in recognition of phytophthora diseases in the field were supplied with LFD kits. Detailed instructions on LFD use were supplied. In summary, several small pieces of leaf material showing symptoms were placed in a plastic bottle containing five small (~3 mm) ball bearings and extraction buffer. In Trials 2, 3 and 4 the pieces of suspected diseased tissue were broken up between the thumb and fingers before transfer into the extraction bottle. Gloves were used when testing more than one sample to prevent the possibility of carry over. The bottle was shaken vigorously for 60 s and then the

extract taken up in a small disposable dropper. Two to four drops were placed onto an absorbent pad within the kit and left for at least 2 min but no longer than 10 min before reading (see Fig. 1). A single blue line developed to indicate the test kit was working (control line) whilst the development of a second blue (target line) indicated the presence of *Phytophthora* spp.

A larger sample from the same part of the plant with identical symptoms was submitted for laboratory testing. The extraction bottle and LFD device were returned to CSL and kept at 4°C until the remains of the tissue were tested in the laboratory. The type and host distribution of samples tested during this trial was representative of material submitted during the UK national survey for *P. ramorum*. The majority of samples tested were rhododendrons ($n = 147$), but many other ornamental genera (e.g. *Camellia*, *Pieris*, *Viburnum*) on which *P. ramorum* has been recorded previously were included. Leaves from a number of trees (e.g. *Aesculus hippocastanum* (horse chestnut), *Taxus baccata* (yew)) that are known foliar hosts for *P. ramorum*, in addition to a number of other hosts on which *P. ramorum* has not been detected in the UK (e.g. *Rubus*, *Populus*) were also tested. LFD tests were completed by six plant health inspectors based in different parts of England (data not shown).

Laboratory-based identification

Samples collected were tested according to a protocol developed at CSL and now part of the EPPO Diagnostic protocol (Anonymous, 2006). Small pieces of tissue were excised aseptically from the leading edge of four lesions. These were placed in a small plastic bag to which 50 mL of distilled water was added. The samples were left to soak for up to 2 h to aid rehydration, shaken vigorously for 30 s to remove any debris before plating out onto a *Phytophthora* semi-selective medium containing pimaricin, ampicillin, rifamycin, PCNB and hymexazol (P_5ARP[H]) (Jeffers & Martin, 1986). Plates were incubated in the laboratory at approximately 20°C with natural daylight for 6 days then examined under the compound microscope at ×100 magnification to check for characteristic features of *P. ramorum* (Werres *et al*., 2001) or *P. kernoviae* (Brasier *et al*., 2005). Suspect positive cultures were tested by real-time PCR using *P. ramorum* or *P. kernoviae*-specific primers and probes (Hughes *et al*., 2006a, and unpublished data). All laboratory work was carried out under strict quarantine conditions under Plant Health Licence Number PHL 251A/4736.

LFD sensitivity

The sensitivity of the LFD was evaluated using naturally infected rhododendron leaves submitted as part of routine plant health surveillance and previously tested as positive for *P. ramorum* by isolation and real-time PCR. A small square of necrotic tissue (approx. 12 × 12 mm) was excised from the leaf and the wet weight determined. It was then dissected further into smaller portions, the wet weights determined and then tested with an LFD. A similar piece of known healthy leaf tissue (12 × 12 mm) was tested in addtition to neat buffer solution. The presence of a test line was visually scored after 5 min in addition to quantification of the intensity of the test result using an optical reader (Chromatoreader Type 2, Otsuka Electronics). Using the optical reader, a negative result was recorded as zero. Lines were visible at between optical reader values of 4–10, but were easily seen in excess of 15 and could rise to over 100. The experiment was repeated with three unrelated samples. Results are presented in Table 1. The experiment was then repeated again but the total volume of tissue tested was maintained by the addition of healthy rhododendron leaf pieces. Results are presented in Table 2.

Figure 1 Lateral flow devices (positive result: upper LFD; negative result: lower LFD) and extraction bottle.

Table 1 Sensitivity of lateral flow device for detecting the presence of *Phytophthora ramorum* in naturally infected rhododendron leaves. The presence of a test line was detected by visual examination and using an optical reader. Results are the mean of three replicates

Wet weight (mg)	Line visible	Optical reader
26·8	Y[a]	85·7
21·4	Y	73·5
7·5	Y	28·3
3·4	Y	27·0
1·6	(Y)	12·4
0·9	N	4·3
0·3	N	4·1
0·1	N	0
Buffer	N	0
Healthy rhododendron leaf	N	0

[a]Y = yes, N = no.

Table 2 Sensitivity of lateral flow device for detecting the presence of *Phytophthora ramorum* in naturally infected rhododendron leaves mixed with healthy leaf tissue. The presence of a test line was detected by visual examination and using an optical reader

Wet weight (mg)					
Necrotic tissue	Healthy tissue	Total weight	%Necrotic tissue	Line visible	Optical reader
0	36·4	36·4	0	N[a]	0
1·5	35·9	37·4	4·0	Y	29
3·0	37·7	40·7	7·4	Y	37·5
5·7	29·6	35·3	16·1	Y	53·1
20·5	32·1	52·6	39·0	Y	84·9
32·7	24·4	57·1	57·3	Y	113·1
30·8	0	30·8	100	Y	127

[a]N = no, Y = yes.

LFD specificity

The specificity of the LFD was evaluated using a range of cultures (see Table 5). A small piece of agar (1 cm^2) was excised from the centre of the colony, placed in an extraction bottle, shaken vigorously for 10–15 s and then tested with an LFD as described above. Devices were read after 5 min and scored visually as either negative or positive.

Interpretation and analysis of results

The diagnostic sensitivity and specificity were calculated using a 2 × 2 contingency table used initially in clinical medicine to assess diagnostic tests (Alberg *et al*., 2004). Where both methods resulted in a positive result, samples were allocated the letter A, both negative the letter D, where the LFD was positive but the comparative method was negative (false positives) the letter B, and when the LFD was negative but the presence of *Phytophthora* spp. was detected using the comparative method (false negative) the letter C. The diagnostic sensitivity ($A/(A + C)$) as a measure of a diseased specimen testing positive, and specificity ($D/(D + B)$) as a measure of a disease-free sample testing negative were calculated using these formulae combined with adjusted 95% confidence intervals (Agresti & Coull, 1998).

Results

Trial 1

The results for 252 LFD devices used from April to August 2004 to test 20 different plant genera are detailed in Table 3. On every occasion the LFD control line developed, although the intensity of the test line was variable. Five out of six inspectors recorded false positives and four recorded false negatives. The diagnostic sensitivity (72·0% with an adjusted confidence interval of 60·7–81·1%) and specificity (83·6% with an adjusted confidence interval of 77·3–88·5%) were determined as described in Table 4. There was agreement between the LFD and isolation result on 202 occasions (80·2%) with a large proportion of negative samples (148/252). A *Phytophthora* species other than *P. ramorum* occurred on 11 occasions for the

Table 3 Range of plant material tested in Trial 1 showing a comparison of the lateral flow device (LFD) as a method for detection of *Phytophthora* spp. with isolation. Numbers in brackets refer to the number of *Phytophthora* species other than *P. ramorum*

		Agreement		Disagreement	
Host	Number	Both negative (D)	Both positive (A)	False positives (B)	False negatives (C)
LFD		–	+	+	–
Isolation		–	+	–	+
Acer platanoides	1			1	
Aesculus hippocastrum	1	1			
Arbutus	1	1			
Azalea	2	2			
Camellia	11	8	3		
Fagus sylvatica	1	1			
Kalmia	3	3			
Laurus	5	4			1
Leucothoe	4	3			1
Pieris	13	4	2 (1)	6	1
Polyplody	1	1			
Populus	1	1			
Quercus	2	1			1
Rhododendron	147	75	40 (9)	17	15 (7)
Rubus	2	1		1	
Salix	2	1		1	
Syringa	4	2	1 (1)	1	
Taxus	2	2			
Vaccinium	1	1			
Viburnum	48	36	8	2	2
Total	252	148	54	29	21

Table 4 Comparison of the lateral flow device with isolation for detection of *Phytophthora* spp. illustrating diagnostic sensitivity ($A/(A+C)$) and specificity ($D/(D+B)$) for all four trials

Trial	A (both positive)	B (false positive)	C (false negative)	D (both negative)	Diagnostic sensitivity (%)	Diagnostic specificity (%)
					($A/(A+C)$)	($D/(D+B)$)
1	54	29	21	148	72·0	83·6
2	21	2	0	12	100	85·7
3	18	3	0	33	100	91·7
4	104	36	7	146	93·7	80·2
Total	197	70	28	339	87·6	82·9

Table 5 Specificity of lateral flow device (LFD) against a range of cultures of *Phytophthora*, *Pythium* and seven fungal spp.

Isolate	Reference	LFD result
P. cactorum	CSL2499[a]	+
P. cambivora	CBS 376·61[b]	+
P. cinnamomi	PD 93/1389[c]	+
P. citricola	FR P1013[d]	+
P. cryptogea	SCRI P521[e]	+
P. foliorum	LT1222[f]	+
P. fragariae var *rubi*	PFR-163[g]	+
P. ilicis	P3939[h]	+
P. kernoviae	CSL 2169[a]	+
P. kernoviae	CSL 2306[a]	+
P. lateralis	P1728[h]	+
P. pseudosyringae	P10444[h]	+
P. ramorum	BBA 12/98[i]	+
P. ramorum	BBA 15/01-18[i]	+
P. ramorum	BBA 9/3[i]	+
P. ramorum	CSL 1684[a]	+
P. ramorum	P1349[h]	+
P. syringae	CBS 364·52[b]	+
Agar (unamended)		–
Alternaria alternata	CSL 325[a]	–
Botrytis cinerea	CSL 507[a]	–
Cylindrocarpon sp.	CSL 1543[a]	–
Monilinia laxa	CSL 782[a]	–
Pleospora herbarum	CSL 553[a]	–
Pythium sp.	CSL 21675[a]	–
Pythium intermedium	CSL 35[a]	–
Pythium ultimum	CSL 33[a]	–
Pythium debaryanum	048558[j]	–
Rhizopus sp.	CSL 1725[a]	–
Trichoderma harzianum	CSL 1465[a]	–

[a]CSL, York, UK.
[b]CBS, Utrech, the Netherlands.
[c]H de Gruyter, Dutch Plant Protection Services, Wageningen, the Netherlands.
[d]Forest Research, Alice Holt, Farnham, UK.
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[f]University of Tennessee.
[g]Laboratoire National de la Protection des Vegataux, Nancy, France.
[h]M. Coffey, University of California, USA.
[i]S. Werres, BBA, Braunschweig, Germany.
[j]CABI, Egham, UK.

54 positive samples. False positives (LFD positive, but no *Phytophthora* isolated) occurred for 29 samples (11·5%) and false negatives (LFD negative, but a *Phytophthora* isolated – most commonly *P. citricola* or *P. cactorum*) at a slightly lower rate (21 samples; 8·3%). False negatives occurred with both *P. ramorum* and *Phytophthora* spp.

Trial 2

Samples for Trials 2–4 were disrupted to a greater extent by breaking up leaves between the thumb and forefinger before placing in the extraction bottle than for Trial 1.

The results for 35 samples collected in the second trial are given in Table 4. The majority of samples tested from this *P. kernoviae*–woodland outbreak site were *R. ponticum* leaves, but leaves from beech (*Fagus sylvatica*), sycamore (*Acer platanoides*) and oak (*Quercus* sp.) were also tested. Laboratory testing confirmed the presence of *P. kernoviae* only and no other species of *Phytophthora* was detected. There were no false negatives and only two false positives, with agreement between the remaining 33 samples. The diagnostic sensitivity and specificity was 100% (adjusted confidence interval of 81·8–100%) and 85·7% (adjusted confidence interval of 58·2–97·4%) respectively.

Trial 3

The results from a large public garden and country park with both *P. ramorum* and *P. kernoviae* present are summarized in Table 4. Again the majority of samples tested were leaves of *R. ponticum* but other hosts included *Corylus* ($n = 1$), *Magnolia* ($n = 2$), *Parrotia* ($n = 1$), and *Syringa* ($n = 1$). The LFD successfully identified a *Phytophthora* sp. on *Parrotia* and *Magnolia* that were subsequently confirmed by isolation and TaqMan® PCR to be new host records for *P. ramorum*. There were no false negatives and only three false positives with agreement between the remaining 51 samples. The diagnostic sensitivity was 100% (adjusted confidence interval of78·7–100%) and specificity 91·7% (adjusted confidence interval of 77·0–98·0%).

Trial 4

A total of 293 samples were tested between January and July 2005 from a wide range of ornamental hosts by many plant health inspectors across England and Wales (data not shown). The LFD performed well in comparison with isolation, with 250 out of 293 samples (85·3%) giving the same result. False positives occurred on 36 occasions (12·3%) and a few false negatives (2·4%) were recorded. The diagnostic sensitivity was 93·7% (adjusted confidence

interval of 87·2–97·2%) and the diagnostic specificity was 80·2% (adjusted confidence interval of 73·7–85·5%).

All trials

Across all four trials a total of 634 samples were tested with agreement on 536 occasions (84·5%). False positives and negatives were encountered on 70 (11·0%) and 28 (4·4%) occasions respectively. The diagnostic sensitivity was 87·6% (adjusted confidence interval of 82·4–91·4%) and the diagnostic specificity was 82·9% (adjusted confidence interval of 78·8–86·3%) (Table 4).

Sensitivity

A positive reaction was clearly obtained with just a few mg of necrotic rhododendron leaf tissue (equivalent to a few square millimetres) either alone or when mixed with healthy leaf tissue, permitting detection in leaf tissue which was less than 1% infected by *P. ramorum*.

Specificity

The data are presented in Table 5. The negative control (agar plug), true fungi (*Alternaria alternata*, *Botrytis cinerea*, *Cylindrocarpon* sp., *Monilinia laxa*, *Pleospora herbarum*, *Trichoderma harzianum*, *Rhizopus* sp.) and isolates of the oomycete *Pythium* also all tested negative. All 13 species of *Phytophthora*, including *P. ramorum* and *P. kernoviae* tested positive.

Discussion

A commercially available LFD for *Phytophthora* (Forsite Diagnostics Ltd, York) identified the presence of *P. ramorum*, *P. kernoviae* and other *Phytophthora* species on a wide range of plant material as part of plant health inspection and disease management work. The assay was demonstrated to identify a broad range of *Phytophthora* species and did not cross-react with other true or lower fungi. The LFD was shown to be very sensitive and able to detect *P. ramorum* in less than 1% infected rhododendron leaf tissue. The assay was simple to use, provided results in 3–5 min and on every occasion a control line appeared confirming the validity of the test. LFD results were compared with those from testing a parallel, but not always identical, sample using well-established laboratory methods. For *P. ramorum* this has been extensively evaluated with a diagnostic specificity of 99·3% and diagnostic sensitivity of 92·3% when isolation was compared with direct real time PCR for a large number of samples (Hughes *et al*., 2006a).

In the initial trial, there was an acceptable level of agreement between the LFD result and subsequent laboratory testing. Greater physical disruption of the sample prior to placing in the extraction bottle led to a reduction in the level of false negatives and a statistically significant improvement in the diagnostic sensitivity due to non-overlapping 95% confidence intervals from 72·0 (Trial 1) to 93·7% (Trial 4) equivalent to the sensitivity obtained during the comparison of isolation to direct PCR (92·3%) mentioned above. Further improvements in performance were achieved when the LFD assays were done by experienced staff (Trials 2 and 3) with no false negatives and fewer false positives recorded. The confidence intervals were higher than those of inexperienced users in Trial 4 although there is some degree of overlap due to the small number of samples tested in Trials 2 and 3. In all four trials (634 samples) the diagnostic sensitivity (87·6%) was comparable to the laboratory test (92·3%), although the very high diagnostic specificity (99·3%) of the laboratory test was not achieved using LFDs (82·9%). The overall level of agreement for the *Phytophthora* LFD (84·5%) compares favourably with other LFDs for fungal plant pathogens. Thornton *et al.* (2004) have developed an LFD for detection of *Rhizoctonia* that when compared with conventional methods resulted in agreement of 86·1% (31/36) with five false negatives. The agreement levels for CSL's other LFDs for EC listed plant pathogenic bacteria and viruses are typically in excess of 96% (C Danks, CSL, York, unpublished data). However, these assays have been developed using antibodies developed specifically to detect these organisms, resulting in greater control over antibody selection. The reduction in agreement level was offset by the generic nature of the phytophthora LFD that allowed it to be implemented quickly for field diagnosis of *P. kernoviae*, a recently described species, without a time delay to develop a new assay.

The occurrence of false negatives and positives is not desirable but inevitable. However, it must be noted that since the testing is destructive it is not possible for the LFD and isolation to use exactly the same tissue pieces. For this reason some authors have referred to these discrepancies as 'unconfirmed' positives and negatives (MacDonald *et al*., 1990) and by considering how these occur it may be possible to mitigate them and improve the assay's efficiency and reliability. Incorrect reading of the device is always a potential problem which can be reduced by good training and experience as seen in Trials 2 and 3 when testing was carried out by staff familiar with the devices. Reading errors could be further reduced by the use of an optical reader to reduce observational error. However, the cost (approx. £300) and size of these, although hand-held, should be considered when deciding on use. The level of agreement was higher when samples for comparative testing were taken at the same time (Trials 2 and 3); therefore, some of the variation may be due to sampling errors cited as a common problem in other studies (MacDonald *et al*., 1990; Benson, 1991).

False negatives pose a problem resulting in an under estimation of pathogen levels. There are a number of reasons why these may occur – for example it could be a sensitivity issue due to very low levels of infection or poor extraction of the sample. When the contents of 10 buffer bottles from Trial 1 that resulted in false negatives were checked by PCR, *P. ramorum* was detected on eight out of 10 occasions (data not shown). Many of the hosts tested (especially rhododendron) produce thick, waxy leaves that are difficult to break down to release antigens. Physical

measures such as squashing, cutting or tearing of the plant material prior to placing in the sample extraction bottle would be expected to improve antigen release and reduce the number of false negatives. This effect was observed in subsequent trials with fewer false negatives and has been seen with other plant samples tested in general plant clinic work (AV Barnes, CSL, York, personal communication). If plant material remains largely intact following shaking, removing it from the bottle and breaking it down manually and testing it with a new device frequently results in more intense test lines. Hosts with softer leaves, e.g. lilac (*Syringa* spp.) pose fewer problems since the leaf is clearly broken down as shown by the strong green colour of the test fluid. The sensitivity of other *Phytophthora* serological assays using related antibodies, but in different formats, has been determined. MacDonald *et al.* (1990) and Benson (1991) found that the Agri-Diagnostics multiwell kits D and E were of sufficient sensitivity to detect at least 1% of infected roots from azalea and chrysanthemum respectively or 30–40 zoospores in irrigation water as found by Ali-Shtayeh, *et al.* (1991). In these studies, the LFD could detect the presence of *P. ramorum* in very small amounts of naturally infected rhododendron leaves.

False positives occurred in all trials which raises some interesting questions about interpretation of these results. Cross-reactivity is always an issue and of greater likelihood when broad specificity is sought. Related antibodies, when used in an ELISA test system, are known to react weakly with several species of *Pythium* and *Peronospora* (MacDonald *et al*., 1990, Pscheidt *et al*., 1992), although in practice this does not always cause difficulties (Benson, 1991) and was not encountered in this study. *Pythium* is not known to cause a foliar blight on common, *P. ramorum*-affected ornamental hosts such as rhododendron, viburnum, or camellia, although it has been associated with root decay and so may be more of a problem if testing roots. For statutory purposes the occurrence of false positives is not an issue as all positive samples require further testing to determine the species and for growers the cultural and control measures for these two organisms are the same. There are no records of *Peronospora* on these three hosts, although another downy mildew (*Plasmopara viburni*) is reported on viburnum in central and eastern United States (Farr *et al*., 2006). No cross-reactivity has been encountered when the *Phytophthora* LFD has been used to test downy mildewed plant samples from a range of genera including *Bremia lactucae*, *Peronospora farinosa*, *P. lamii*, *P. parasitica*, *P. radii*, and *P. violae*. A further explanation for false positives is that the LFD result is correct, the pathogen was present in the sample tested in the field but absent or could not be cultured from the sample submitted to the laboratory. The level of agreement between serological assays and isolation for *Phytophthora* spp. is variable, with some authors reporting no statistically significant difference between ELISA and isolation (MacDonald *et al*., 1990) whilst others found greater discrepancies (Pscheidt *et al*., 1992). In general, the greatest discrepancies were most commonly observed with low infection levels. High temperatures, desiccation or recent fungicide application could prevent successful culturing; however, this was found not to be a significant problem with similar plant material when isolation was compared with direct PCR of like-for-like samples (Hughes *et al*., 2006a). The recovery of *P. ramorum* from leaf material with symptoms can be quite variable as, for example, tissue taken from very similar leaf spots close to each other does not always result in isolation of *P. ramorum* and hence the need to try and isolate from at least four pieces of tissue. The results indicate that pooling several small pieces of tissue as opposed to testing one larger piece would be beneficial. The sensitivity of the LFD would permit pooling of tissue from several suspect lesions. Additionally, LFDs may provide a useful indication of the presence of the pathogen in substrates where isolation is notoriously difficult such as bark samples or plants in less temperate climates where desiccation of samples is more likely.

These trials demonstrate that LFDs offer a useful decision-support tool for the detection of *Phytophthora* spp. at the point of inspection. For statutory purposes, as positive-LFD results require laboratory testing to determine the species of *Phytophthora*, the presence of false positives is overcome. Therefore, in this study where all LFD positives were submitted for laboratory testing, an overall efficiency of 95·6% was achieved, which is a substantial improvement on relying on visual assessment alone. The LFD kits for detecting *Phytophthora* spp. cost from £6 per test so are significantly cheaper than laboratory testing. They have been of considerable value in instructing new plant health inspectors in disease recognition, helping to convince growers and land-owners of the need to sample and hold plants and have led to the discovery of several new host species (e.g. *P. ramorum* on *Parrotia*) (Hughes *et al*., 2006b). The simplicity and robustness of these kits makes them ideally suited for all skill levels and their size and weight ideal for varying sites and conditions to obtain a rapid assessment of whether *Phytophthora* may be present. They have the potential to assist plant health organizations manage their disease campaigns in a new way by helping to optimize and target the use of field inspectors, highly skilled diagnostic staff and centralized laboratory services.

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