Virus Research 186 (2014) 20–31

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Methods in virus diagnostics: From ELISA to next generation sequencing

Neil Boonham[a], Jan Kreuze[b], Stephan Winter[c], René van der Vlugt[d], Jan Bergervoet[d], Jenny Tomlinson[a], Rick Mumford[a,*]

[a] The Food & Environment Research Agency (FERA), Sand Hutton, York YO41 1LZ, UK
[b] International Potato Center (CIP), PO Box 1558, Lima 12, Peru
[c] Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; in German), Plant Virus Department, Inhoffenstrasse 7B, 38124 Braunschweig, Germany
[d] Plant Research International, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

ARTICLE INFO

Article history:
Available online 19 December 2013

Keywords:
Molecular diagnostics
Real-time PCR
LAMP assays
Multiplex, Luminex
Next generation sequencing
Field testing

ABSTRACT

Despite the seemingly continuous development of newer and ever more elaborate methods for detecting and identifying viruses, very few of these new methods get adopted for routine use in testing laboratories, often despite the many and varied claimed advantages they possess. To understand why the rate of uptake of new technologies is so low, requires a strong understanding of what makes a good routine diagnostic tool to begin. This can be done by looking at the two most successfully established plant virus detection methods: enzyme-linked immunosorbant assay (ELISA) and more recently introduced real-time polymerase chain reaction (PCR). By examining the characteristics of this pair of technologies, it becomes clear that they share many benefits, such as an industry standard format and high levels of repeatability and reproducibility. These combine to make methods that are accessible to testing labs, which are easy to establish and robust in their use, even with new and inexperienced users. Hence, to ensure the establishment of new techniques it is necessary to not only provide benefits not found with ELISA or real-time PCR, but also to provide a platform that is easy to establish and use. In plant virus diagnostics, recent developments can be clustered into three core areas: (1) techniques that can be performed in the field or resource poor locations (e.g., loop-mediated isothermal amplification LAMP); (2) multiplex methods that are able to detect many viruses in a single test (e.g., Luminex bead arrays); and (3) methods suited to virus discovery (e.g., next generation sequencing, NGS). Field based methods are not new, with Lateral Flow Devices (LFDs) for the detection being available for a number of years now. However, the widespread uptake of this technology remains poor. LAMP does offer significant advantages over LFDs, in terms of sensitivity and generic application, but still faces challenges in terms of establishment. It is likely that the main barrier to the uptake of field-based technologies is behavioural influences, rather than specific concerns about the performance of the technologies themselves. To overcome this, a new relationship will need to develop between centralised testing laboratories offering services and those requiring tests; a relationship which is currently in its infancy. Looking further into the future, virus discovery and multiplex methods seem to converge as NGS becomes ever cheaper, easier to perform and can provide high levels of multiplexing without the use of virus specific reagents. So ultimately the key challenge from a routine testing lab perspective will not be one of investment in platforms–which could even be outsourced to commercial sequencing services–but one of having the skills and expertise to analyse the large datasets generated and their subsequent interpretation. In conclusion, only time will tell which of the next-generation of methods currently in development will become the routine diagnostics of the future. This will be determined through a combination of factors. And while the technology itself will have to offer performance advantages over existing methods in order to supplant them, it is likely to be human factors e.g., the behaviours of end users, laboratories and policy makers, the availability of appropriate expertise, that ultimately determine which ones become established. Hence factors cannot be ignored and early engagement with diagnostic stakeholders is essential.

Abbreviations: PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbant assay; LAMP, loop-mediated isothermal amplification; LFD, lateral flow device; NGS, next generationsequencing; NASH, nucleic acid spot hybridisation; FRET, fluorescent resonance energy transfer; NASBA, nucleic acid sequence based amplification; RPA, recombinase polymerase amplification; HDA, helicase dependent amplification; RFLP, restriction fragment length polymorphism; ICAN, isothermal and chimeric primer-initiated amplification of nucleic acids; RCA, rolling-circle amplification; MIA, microsphere immune assay; TSPE, target specific primer extension.

* Corresponding author. Tel.: +44 1904 462140.
E-mail address: rick.mumford@fera.gsi.gov.uk (R. Mumford).

0168-1702/$ – see front matter.
http://dx.doi.org/10.1016/j.virusres.2013.12.007

1. Introduction

The publication of an enzyme-linked immunosorbent assay (ELISA) method for the detection of Plum pox virus (PPV; genus *Potyvirus*, family *Potyviridae*) and Arabis mosaic virus (ArMV; genus *Nepovirus*, family *Secoviridae*) by Clark and Adams (1977) was a breakthrough in virus diagnostics, ushering in a new era of testing methods and technologies that have come to define modern phytodiagnostics. Prior to this, virus diagnosis was the preserve of specialists with years of experience in the recognition and description of virus symptoms on hosts, supplemented with complex and cumbersome methods such as bio-assays on indicator plants and the use of elaborate techniques like transmission electron microscopy. Serological methods had been in use for some time before ELISA was developed, either for diagnostic purposes (e.g., immuno-specific electron microscopy; Derrick, 1972) or to determine virus taxonomic relationships (e.g., Ouchterlony gel diffusion assay; Bercks, 1967). However these methods had a range of drawbacks e.g., using large amounts of crude antiserum or being limited by their format, and hence only very small numbers of samples could be tested. The introduction of ELISA then revolutionised virus diagnosis by simplifying detection and shortening the time required to reach conclusive results (Torrance and Jones, 1981). Further improvements came through the use of monoclonal antibodies, which improved assay sensitivity and specificity and through modifications of the test format, making ELISA the most versatile assay for simple and sensitive virus testing. ELISA quickly left research laboratories and became the major tool of virus testing in areas such as breeding (e.g., assessing virus resistance), quarantine (e.g., screening of imported material) and certification (e.g., to ensure planting material is virus-free). It has become established as the most widely used method for the detection of viruses in crops, replacing other more involved methods. For example, ELISA has largely replaced graft-inoculation testing for viruses infecting fruit trees including citrus (EPPO, 2004a) or stone fruits (EPPO, 2004b, 2005). Due to its speed, its ease of implementation, use and interpretation of results, ELISA is highly suited to high-throughput testing. For example in 2005 over 1 million prunus samples were tested by ELISA, as part of Canada's PPV eradication campaign (Thierry Poire, CFIA, personal communication) Key to this has been the commercial availability of equipment, reagents and antisera, allowing for a high degree of standardisation and comparability. This is further aided as serological reagents are provided through a limited number of commercial and public providers, allowing for consistency and transferability of protocols. These aspects, supported by a long track record of experience using ELISA, allow the diagnostic parameters of the technique, i.e., sensitivity, specificity, reproducibility and repeatability, to be evaluated (Vidal et al., 2012). Similarly they provide effective validation, allowing tests to be used reliably and with providing confidence in the sanitary status of crops tested, helping to prevent spread of virus diseases. Given that so much routine virus indexing in major crops around the world is currently based on ELISA, this has given rise to accepted standard operating procedures for many viruses and crops that make the virus testing process comparative and transparent. As a result ELISA has become established as the global industry standard for detection of viruses in agricultural crops.

However, while ELISA has many advantages for routine virus testing (Torrance and Jones, 1981), being cost effective, robust, easy to use and scalable to testing large numbers of samples, it also has several pitfalls that limit its use as a universally applicable test for plant virus diagnosis. Firstly, ELISA and its various formats require high-quality antisera, to permit the sensitive and specific binding to viral antigens. The production of such antisera requires virology expertise and the ability to purify virions or viral coat/other proteins as antigen. This is a lengthy process, which despite many technological advances, is still a highly unpredictable and costly process. As a result, the production of antisera has to be conducted in specialised laboratories. This is in sharp contrast to nucleic acid-based detection methods, where viral genome sequences are targeted using generic molecular approaches, that are accessible to many and at relatively low cost. The development costs for antisera, especially when expressed and purified recombinant virus proteins are used as antigens or, when monoclonal antibodies are to be prepared, are significantly higher and often do not match the projected market size for the putative ELISA reagent or test kits.

Secondly, the ELISA method detects viral antigens. In general is efficient and sensitive and even where viruses that cannot be purified, sensitive and specific virus detection by ELISA can be achieved, using antisera raised against recombinant virus proteins (e.g., Steel et al., 2010). However antisera often lack the resolution to correctly identify virus strains, which are closely related but which do have a distinct phenotype, often because the strain-determining character is not reflected in a coat protein variation e.g., PVY^{ntn} – the tuber necrosis strain of *Potato virus Y* (PVY; genus *Potyvirus*, family *Potyviridae*) or because the virus coat proteins are so highly conserved in a particular genus (e.g., genus *Begomovirus*, family *Geminiviridae*) that antibodies cannot be used to discriminate between species within it. Therefore, in a number of situations, for example where identification of specific viral species/strains is required, ELISA is often not appropriate.

Thirdly ELISA is widely used as a diagnostic tool to assess the phytosanitary status of plants, for quarantine purposes or virus certification, and has become an integral part of pathogen indexing. When a group of diverse pathogens needs to be tested for in one crop, e.g., screening seed potatoes for viroid, viral, bacterial and fungal pathogens, ELISA lacks the flexibility and compatibility that is inherent to some molecular methods. Nucleic acid-based methods allow several assays to be performed on a single plant nucleic acid preparation; either in parallel or as multiplex assays (Dovas et al., 2002; Wintermantel and Hladky, 2010; Papayiannis et al., 2011; Panno et al., 2012). Thus while ELISA is still a highly appropriate method for many situations, including surveillance, eradication, certification of mother plants, sanitation and quarantine, especially when specific viruses need to be reliably detected, nucleic acid-based methods are more generic and provide a platform that is more flexible to address a broader range of diagnostic questions.

Following the development and wide scale deployment of ELISA, new method development has been focused on the detection of viral RNA and DNA. While methods based on nucleic acid spot hybridisation (NASH) have been heavily used for some viruses and particularly for viroids, the technique that has been most successfully exploited is based on the Polymerase Chain Reaction (PCR). PCR methods for virus detection were first published in the early 1990s (Vunsh et al., 1990) and theoretically offered the user exquisite levels of specificity and sensitivity. Whilst the former can often be achieved, the latter is frequently below expectation, especially for conventional PCR-based assay (utilising gel-electrophoresis for resolution of the results). Whilst many PCR assays for the detection of plant viruses have been published, very few conventional PCR methods have been used routinely in diagnostic laboratories, due to a range of practical issues but in particular problems with post-PCR contamination. Effectively the sensitivity that attracted many users to PCR became its biggest problem as the small amounts of DNA liberated into the laboratory environment following the opening of the tubes after thermal cycling could eventually be detected by the PCR method, resulting in recurring problems with false positive results. These problems were in the main solved by the use of closed-tube, homogeneous PCR assays commonly known as real-time PCR or quantitative

PCR (q-PCR). Initially developed for quantification in gene expression studies, where the time at which amplification is observed to occur is related to the inverse Log of the quantity of target being amplified, this method was quickly adopted for diagnostic applications (Mumford et al., 2000). A key reason is that the fluorescent signal was generated within the closed PCR tube and could be detected either during amplification ('real-time') or at the end of it ('end point') without opening the tube; effectively sealing in the contamination risk. Like conventional PCR many different incarnations of real-time PCR were developed and evaluated for virus testing, predominantly the main differences were around the different ways signal could be generated within the reaction as it progressed. These were either probe-based methods (e.g., TaqMan™, molecular beacon, scorpion probes) or non-probe methods (e.g., SYBR green, LUX primers). Arguably the most widely used in virus diagnostics has been TaqMan™ probes based on the 5′–3′ exonuclease activity of Taq polymerase and Fluorescent Resonance Energy Transfer (FRET) activity of pairs of fluorescent dyes termed reporters and quenchers. Probe based methods have the added advantage that three (or more) independent oligonucleotides need to bind to the target within the reaction to generate signal reducing the likelihood of unspecific signal. In non-probe chemistry such as SYBR green, melting profiles are often used to discriminate primer artefacts from actual amplification, which adds steps to the test being performed, which are not needed in assays based on probe-based chemistries. Of the probe-based methods explored, TaqMan™ probes appear to be the most established, although this is probably due to them being one of the earliest approaches adopted and hence the most familiar technique used, rather than possessing any specific performance advantages over other probe systems. Real-time PCR techniques for virus detection were initially developed for applications which needed increased sensitivity (Boonham et al., 2009) or where antibody methods could not be used (Boonham et al., 2004). However, more recently real-time PCR methods have been developed for targets where ELISA could be used based purely on the performance (i.e., sensitivity/specificity) criteria needed and for three major practical reasons. Firstly, real-time PCR is quicker to establish compared with developing antibodies for newly described viruses. Secondly, the technique is of more generic applicability than ELISA, especially in routine laboratories looking to perform tests for bacteria, fungi and potentially invertebrate identification, where ELISA methods are less well established. Finally, whilst the per-test cost of ELISA is less than real-time PCR (per test reagents costs are typically 50% higher than for ELISA), the set-up cost of making an antibody is considerably more, thus the long term cost benefit needs to be significant to make the generation of a new antibody a viable proposition. Thus it seems that real-time PCR is here to stay; it enables high-throughput testing at a relatively low per-sample cost, unrivalled speed to set up a new assay and its success has driven down the capital cost of the equipment to less than one tenth of that around ten years ago.

The remainder of this review will focus on some of the new methods that are being developed now and which may become established in future years. This will build on the lessons learned during the development and subsequent establishment of ELISA and real-time PCR as successful routine diagnostic methods, accepting that the challenges faced by these technologies – speed, sensitivity, specificity, robustness and cost effectiveness – are just as relevant to the subsequent success of these new methods, as they were in the preceding decades. Hence if we consider the major challenge of moving diagnostics away from centralised reference laboratories into the field or more specifically to the point of decision making, then we must consider the same performance and quality assurance characteristics as we do for laboratory-based methods. These points are considered and discussed in the following section.

2. Isothermal amplification and field detection

Isothermal amplification methods share with PCR the central concept of the extension of target-specific primers by a DNA polymerase (or in some cases, an RNA polymerase). In general terms, the challenge of isothermal amplification is therefore to enable primer binding, such that amplification can occur without the repeated cycles of denaturation and annealing required for PCR. There are a number of approaches to the generation of single-stranded primer binding sites without thermal cycling, including methods based on: non-thermal methods of template denaturation; transcription of RNA; strand displacement around a circular template; nicking or partial degradation of primer extension products to allow extension or further rounds of priming; and formation of secondary structure containing single-stranded primer binding sites. Whilst isothermal methods (e.g., Nucleic acid sequence based amplification, NASBA) were initially investigated as alternatives to PCR or real-time PCR (Leone et al., 1997), there has been renewed interest in investigating these methods for applications in poorly resourced laboratories or for in-field testing. Since these methods rely on amplification at a single temperature, rather than thermal cycling, they can be performed using much simpler, less power-demanding equipment.

2.1. Established isothermal methods

A conceptually simple approach to achieving isothermal amplification of DNA is to separate the strands of the double-stranded template by non-thermal means. Helicase dependent amplification (HDA) (Vincent et al., 2004) and Recombinase Polymerase Amplification (RPA) (Piepenburg et al., 2006) are two examples of this approach. HDA uses a helicase to separate the strands of double-stranded DNA allowing primer binding and extension by DNA polymerase at a constant temperature of approximately 65 °C. Reaction times for HDA are generally in the range 30–90 min. This method sustains amplification of relatively short products of approximately 70–120 bp. HDA can be performed at a single temperature, but the inclusion of a brief incubation at 95 °C prior to the addition of the HDA enzymes has been shown to increase sensitivity.

RPA uses a recombinase which forms a complex with primers to initiate amplification without thermal denaturation. RPA does not require an initial denaturation step but sensitivity is increased if the reaction is agitated after 5 min of incubation. The reaction is incubated at a low reaction temperature (between 37 and 42 °C) which can easily be sustained by a low power instrument. However, the use of a low reaction temperature can result in the generation of more non-specific amplification artefacts than are typically observed in isothermal amplification methods, which use higher reaction temperatures. The major advantage of RPA is its short reaction times, which are typically <30 min.

NASBA is a method for isothermal amplification of RNA based on transcription (Compton, 1991). A modified primer is used to incorporate the sequence of the T7 RNA polymerase promoter into a double-stranded DNA intermediate, functionalising the promoter and resulting in transcription of a single-stranded RNA product at a reaction temperature of 41 °C. NASBA has been used for the detection of a number of plant pathogens in conjunction with molecular beacon probes, in a format sometimes referred to as AmpliDet (Klerks et al., 2001). This format, in which fluorescence is monitored in real time to detect hybridisation of the probe to the single-stranded amplicon, is a closed-tube system and allows quantification of the target sequence, but requires the use of an instrument with real-time fluorescence monitoring capability. NASBA requires denaturation of the template to allow primer annealing prior to the addition of non-thermostable enzymes,

making reaction set-up a two-stage process. NASBA is considered to be a highly sensitive detection method but with relatively long reaction times (typically 90 min).

Both NASBA and HDA perform optimally with a two-step thermal profile and as such are not truly isothermal. RPA additionally requires the tubes to be manually agitated, after several minutes of amplification, to achieve optimum performance. Each of these characteristics makes these chemistries less suitable for on-site use, increasing the time and complexity of amplification.

2.2. Other isothermal amplification methods

Two further isothermal amplification methods, which have been applied to the detection of plant pathogens, are rolling-circle amplification (RCA) and Isothermal and Chimeric primer-initiated Amplification of Nucleic acids (ICAN). In its simplest format, RCA is used to amplify circular nucleic acids utilising the strand displacement activity of Phi29 DNA polymerase. RCA followed by restriction fragment length polymorphism (RFLP) analysis has been used for diagnosis of geminiviruses which have small single-stranded circular DNA genomes (Haible et al., 2006). More complex methods based on RCA make use of circularisable padlock probes (Banér et al., 1998) to provide templates for amplification. ICAN uses chimeric (5′-DNA-RNA-3′) primers with a thermophilic RNase H which introduces a nick at the junction between the DNA and RNA portions of the primers, and a DNA polymerase with strand displacing activity which continues extension from the nick site (Mukai et al., 2007).

2.3. Loop-mediated isothermal amplification (LAMP)

The isothermal amplification methods discussed so far each have various advantages. However, in the context of developing methods for on-site use, factors such as reaction time (>1 h in the case of HDA, NASBA, and ICAN) and complexity of assay design (RPA, RCA for non-circular targets) are potential disadvantages. An alternative isothermal amplification approach is to design primers such that the amplification products contain single stranded primer binding sites. LAMP is the most commonly used method to take this approach, using three pairs of primers (internal, external and loop primers), to generate an amplification product which contains single-stranded loop regions to which primers can bind without template denaturation (Notomi et al., 2000) at a reaction temperature of around 65 °C. The internal primers introduce self-complementarity into the amplification product, causing loops to form, while extension of the external primers causes displacement of the extension products of the internal primers. The products of LAMP reactions consist of alternately oriented repeats of the target sequence. The addition of loop primers accelerates amplification by priming at the loop regions that are of the incorrect orientation for the internal primers to bind. Loop primers increase sensitivity and reduce reaction times, and are required for acceptable performance of some assays. However, to accommodate loop primers requires a longer region of suitable sequence, such that design of two loop primers may not be possible, and many assays have been reported in the literature, which achieve acceptable performance without loop primers, or with only one loop primer. A recently described modification of the LAMP reaction incorporates one or more 'stem' primers, which bind to the double stranded central portion of each repeat of the amplified region, to further enhance assay performance and increase primer design options (Gandelman et al., 2011). As LAMP uses at least six primer binding regions, it is possible to design assays with high specificity by positioning each primer at the site of mismatches between the target and non-target species. LAMP assays have been reported with sensitivity approaching that of comparable real-time PCR assays and typically exceeding that of conventional PCR (Tomlinson et al., 2007).

LAMP does not require initial template denaturation and more recently developed strand displacing DNA polymerases display faster reaction kinetics, such that LAMP reaction times can be reduced to <30 min. LAMP, in common with other isothermal DNA amplification methods, can be modified for detection of RNA targets by the addition of reverse transcriptase to the reaction. In RT-LAMP, reverse transcription and amplification of cDNA proceed concurrently at a single temperature of around 65 °C. Significantly for on-site testing, LAMP has been reported to be tolerant of some substances which are inhibitory to PCR (Kaneko et al., 2007), potentially allowing LAMP to be used in conjunction with simplified nucleic acid extraction methods.

In the specific context of on-site testing for plant pathogens, some assay characteristics are particularly desirable, including speed of amplification, simplicity of workflow (i.e., requiring few manipulations) and tolerance of inhibitors, all of which are exhibited by LAMP chemistry.

2.4. Detection platforms

A nucleic acid-based test for a particular target comprises not only the mechanism for nucleic acid amplification, but also a means of determining whether amplification has occurred. Some detection methods are broadly applicable and can be used to detect the products of diverse amplification methods including LAMP. The extremely high efficiency of LAMP results in the generation of sufficiently large amounts of amplification product to allow the use of relatively insensitive detection methods, which cannot be used with less efficient amplification chemistries. A common and broadly applicable method for detection of amplification products is gel electrophoresis; however, this is too cumbersome and time consuming for use outside the laboratory and in common with conventional PCR, opening tubes in a non-homogenous format provides a risk of contamination and false positive results.

2.5. Device free detection

One consequence of the high amplification efficiency of LAMP is that the generation of magnesium pyrophosphate (a by-product of DNA polymerisation) causes a measurable increase in turbidity as the reaction proceeds. This turbidity can be observed using the naked eye but only where the target concentrations are high and this tends to be subjective, not giving conclusive results for all assays (Wastling et al., 2010). Since no post-amplification manipulation is required, this method presents a lower risk of carry-over contamination than methods which require reaction tubes to be opened. A number of colour change reactions can be used for end-point detection, including the addition of intercalating dyes such as SYBR Green or PicoGreen at sufficiently high concentrations to produce a visible colour change, or the addition of fluorescent probes and cationic polymers. However, at the concentrations required these reagents are inhibitory to LAMP, so must be added at the end of the reaction, again requiring the tubes to be opened and thus creating a high risk of carry-over contamination and false positive results in subsequent tests. Alternative colour change reactions have been described using reagents, which do not inhibit amplification, allowing them to be used in a closed-tube format. These include calcein plus $MnCl_2$, which causes an orange-green colour change, and hydroxy naphthol blue (HNB), which results in a violet-to-blue colour change. The colour change with calcein and $MnCl_2$ has been reported to be more difficult to interpret than other methods and is best viewed under ultra violet illumination. Whilst the colorimetric change observed with HNB has been reported to be easily interpretable by end-users (Wastling et al., 2010), the

change can be subtle and clarity of the results may be somewhat assay-dependent (Tomlinson et al., 2010a). To prevent this problem of interpretation an alternative approach has been developed by incorporating ligands into amplification products, such that the products can be detected in an lateral flow device (LFD) immunoassay at the end of the reaction (James et al., 2010; Tomlinson et al., 2010b). The ligands can be incorporated during amplification using labelled nucleotides or primers, or via probes added at the end of the reaction. Amplicon detection using this method does have the drawback that it typically requires reaction tubes to be opened to allow the amplified product to be applied to the LFD. Devices are available which incorporate LFD detection in a closed cassette (Goldmeyer et al., 2008), but the per-device cost is higher than using LFDs in either a simple dipstick format or conventional open housing. All of these reporting methods do require a means of incubating the reaction at a temperature of 65 °C (such as a heat block or water bath) and as a result none are completely equipment-free.

2.6. Detection devices

Several different approaches can be used to monitor reactions in real-time. The first is to incorporate fluorescent, intercalating dyes or probes into the reaction mixture and monitoring the reaction using a fluorescent reader. The secondly is to continuously measure the turbidity generated by the precipitation of magnesium pyrophosphate. A third is by the incorporation of thermostable firefly luciferase into the reaction, enabling the pyrophosphate generation to be measured by an increase in bioluminescence in a technology referred to as BART (bioluminescent assay in real-time) (Gandelman et al., 2010).

These approaches based on detection devices have some fairly generic advantages over the device-free approaches described above, regardless of the reporting system used (i.e., luminescence, fluorescence or turbidity). Firstly, an electronic device can be used to both monitor the reaction, as well as incubate the reaction at a constant temperature. Thus amplification and detection are performed by a single instrument in real-time, without any post-amplification manipulations. Secondly, utilisation of an electronic device, however simple, can allow connectivity to the internet, permitting automated data storage and transfer, and enabling guaranteed data integrity, which is important for quality assurance systems. The advantages of performing LAMP using a device are epitomised in the Genie II instrument (Optigene, Horsham, Surrey, UK). Assays run on this type of platform are also cost effective, with an estimated reagent cost of around $USD 2–5 for a single test.

Real-time fluorescence monitoring of LAMP reactions, using intercalating dyes such as SYBR Green, offer further advantages over the detection of luminescence or turbidity. These methods allow further analysis of the product in terms of the temperature at which the amplified DNA melts or anneals. LAMP products contain structures of differing lengths containing catenated repeats of the target sequence which melt/anneal at a specific temperature, determined by the length and G/C content of the target. After amplification, reactions can be subjected to a gradual melting or annealing step with fluorescence monitoring, in order to discriminate specific amplification products from unexpected non-specific artefacts, and thus providing extra confidence in the result obtained.

In terms of applications, isothermal chemistry could be used in the laboratory and some of the advantages of methods such as LAMP may prove beneficial to the lab-based end users. For example tolerance of inhibiting compounds may enable the use of simple extraction techniques or may be beneficial when nucleic acid has been extracted from a difficult matrix, resulting in carry-over of inhibitory compounds. Furthermore the reaction kinetics of LAMP tend to give results that are easy to interpret; either amplifying very large amounts of DNA or none at all, unlike PCR where the amount of DNA amplified begins to decrease at very low titres of target. Of course the significant advantages of isothermal amplification are most likely to be realised in transferring diagnostics from well-equipped reference laboratories into poorly-resourced laboratories or even out into the field; performing diagnostics at the point of decision making. The methods are typically robust, require simple or even no nucleic acid extraction to be performed (aside from disrupting the tissue to be tested into a suitable buffer) and can be performed on relatively simple equipment. The methods have the potential to provide performance (sensitivity and specificity) similar to real-time PCR, yet using rudimentary equipment (e.g., simple hot-block heaters) and potentially by non-diagnostic specialists (i.e., those with basic lab skills, rather than trained molecular biologists). Other methods exist for field testing of viruses, namely serological-based immuno-chromatographic tests (i.e., LFDs). These devices are simple and inexpensive but the same parallels exist between isothermal methods and LFD as between ELISA and real-time PCR. Namely the cost associated with the generation of new serological reagents for emerging disease problems, or in situations where serological methods (LFD or ELISA) are not sensitive enough to detect the disease-causing pathogen.

3. Multiplex methods

So far in this review the focus has been on simplex assays, capable of reliably detecting a single target in each individual test. However, in many scenarios testing samples for multiple viral or even other pathogens is required. As previously discussed, the indexing of seed potatoes is one example where screening for multiple pathogens is routinely carried out. In these cases, the ability to conduct multiplex testing (i.e., the ability to detect more than one pathogen in a single assay) offers huge cost benefits, especially in a situation where large numbers of samples need to be tested. In this next section we describe and discuss the development of such methods; focusing on the latest developments that offer the potential for high-throughput testing.

3.1. Multiplex methods: serological-based

A method to expand the multiplex immunological detection capability of ELISA is the universal bead microsphere immune assay (MIA) (Joos et al., 2000; Vignali, 2000). This bead-based array allows simultaneous detection of a large number of pathogens (up to five hundred) and is comparable to the methods developed for ELISA. MIA is based on the universal bead-array (xMAP) produced by Luminex (Austin, Texas, USA). The beads in this array have a diameter of 6.5 μm and are internally stained with two fluorochromes. Currently there are over five hundred different beads colour-combinations available. These beads are paramagnetic and can be attracted by a magnet. This permits highly stringent washing procedures, which can significantly reduce background problems in the detection of plant pathogens in complex or difficult matrices. Beads can be covalently coupled with either proteins, peptides, antibodies, polysaccharides, lipids or oligonucleotides (Joos, 2004). As a result different virus-specific antibodies can be coupled to different internally dyed beads ('bead addresses') and these different beads can then be combined to create custom 'bead sets' for the detection of specific combinations of plant viruses. The bead-based immune assay procedure is identical to the standardised Double Antibody Sandwich (DAS)-ELISA and as such can be seamlessly integrated in existing workflows. In short, plant samples prepared in the standard ELISA buffer are transferred to a standard 96-well micro-titre plate and incubated with the antibody-coated bead set. During this incubation step the viruses are captured by the

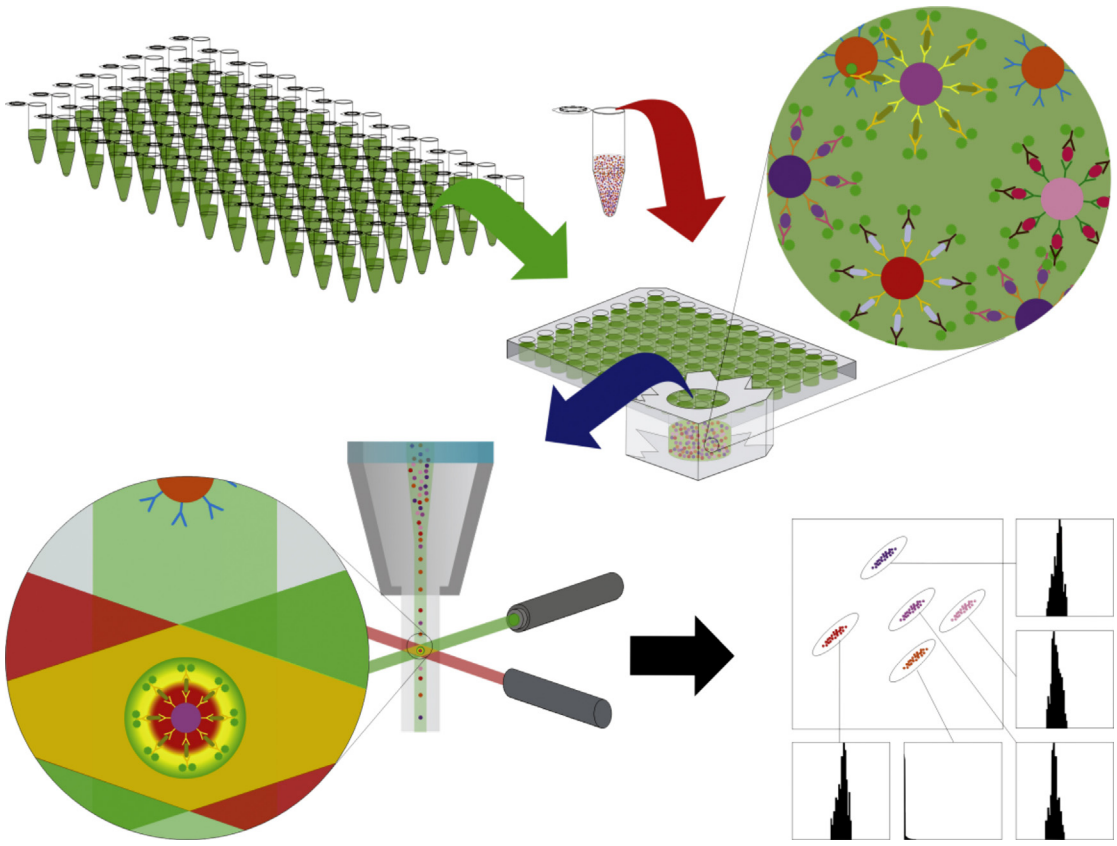

Fig. 1. Overview of the Luminex xMAP technology. Samples are prepared and transferred to a 96 well microtiter plate (top left) and a mixture of antibodies conjugated to the microsphere sets is added (top middle). During the incubation step, the pathogens bind to the capture antibodies and then secondary antibodies, conjugated with a fluorescent reporter, are added (top right). Samples are measured in a Luminex analyzer (bottom left) and the results are graphically presented (bottom right). If no pathogen is present no fluorescent reporter will be detected, resulting in no signal (as depicted in the lower middle panel). Positive samples are represented in the other 4 panels.

antibodies attached to the individual beads. After a short washing step, employing magnetic capturing of the beads, the samples are subsequently incubated with the appropriate mixture of secondary antibodies, all conjugated with the same reporter fluorochrome. After a second incubation step and a subsequent magnetic wash step, the samples are analysed. During the analysis the different beads and the attached reporter fluorochrome are detected using either a laser-based small flow cytometer or a LED-based image analyser. In this analysis a pre-defined number of individual beads of each specific address are interrogated by the analyser for both its internal colour (the 'bead-address') and the presence, or absence, of the fluorochrome attached to the secondary antibody. The presence of a particular virus results in a positive signal for the fluorochrome in combination with the virus-specific bead address (see Fig. 1). Similar to a standard DAS-ELISA the amount of virus present in the sample is correlated with the average (mean) level of fluorescence measured on the virus-specific beads (Mean Fluorescent Intensity, MFI).

In comparison to standard DAS-ELISA technology this procedure not only allows the serological detection of multiple analytes in a single well of a 96-plate but also is much faster. The whole procedure generally takes no more than 3–4 h, including sample preparation (by contrast typical DAS-ELISA protocols take 1–2 days). The major time-saving steps include the use of pre-defined bead mixtures, stable for a prolonged period of time, which abolishes the need for pre-coating of ELISA plates and improved fluid-dynamics allowing for much shorter incubation times. When compared to standard ELISA tests, a further advantage of Luminex xMAP technology is improved detection limits and a much higher dynamic range (Rao et al., 2004) and also considerable savings in costs, through significant reductions in labour and consumables (e.g., plates and reagents).

Overall this bead-based technology has already proven its value for true multiplex detection, allowing the detection of up to several hundred analytes in a single test, across a number of fields including human diagnostics (Kellar, 2003), food microbiology (Dunbar et al., 2003) and plant pathogen detection (Bergervoet et al., 2008).

3.2. Multiplex methods: nucleic acid-based

As discussed above multiplexing technologies that enable the simultaneous detection of multiple viruses in a single assay, can greatly reduce the time, cost and labour-input associated with conventional single reaction (simplex) detection technologies. A similar approach can be used with molecular techniques, to harness the flexibility achieved by detecting nucleic acid, whilst extending the multiplexing capability far beyond that achievable using real-time PCR. In terms of multiplexing, this technology has technical limitations, whereby accurately distinguishing the different fluorescent signals resulting from multiple reporter and quencher dyes used for detection, limits the number of assays which can be accurately detected in a single tube. This issue of 'cross-talk' theoretically limits current real-time PCR technology to four-fold multiplexing but in reality multiplexing beyond combining two assays is challenging (Mumford et al., 2006). The next generation TaqMan™ machines may raise this level of multiplexing but this is still a long way off from true multiplex detection of 10 or 20 viruses in a single test. Hybridisation-based array platforms (e.g., microarrays) do exist and offer the potential to extend the limits of multiplex testing into hundreds if not thousands of targets

(Boonham et al., 2007). However these platforms on not applicable to high-throughput testing but infact better suited to screening for new and unusual viruses (Boonham et al., 2007). This is further discussed in Section 5 of this review.

One platform that does offer a very high degree of multiplex nucleic acid detection is the Luminex MagPlex-TAG bead system. This technology has proven its value for the multiplexed detection of pathogens in clinical settings (Mahony et al., 2007; Lin et al., 2011; Liu et al., 2011) and the multiplex detection of viruses (Foord et al., 2013). This system incorporates the same 6.5 μm carboxylated, superparamagnetic polystyrene beads as the xMAp system (discussed above). These beads are also internally labelled with a spectrally distinct fluorescent dye and each distinct bead address is pre-coupled with a highly specific anti-MagPlex-TAG oligonucleotide sequence. Different beads can be distinguished by their spectral addresses and, when combined, up to 150 different nucleic acid sequence targets to be tested simultaneously in a single reaction.

The experimental approach of the xTAG assay (outlined in Fig. 2) involves a generic multiplexed Reverse Transcriptase-PCR step, followed by a multiplexed asymmetric PCR step termed Target Specific Primer Extension (TSPE). In this linear amplification step, one or more primers specific to the multiplexed amplification product are extended in the presence of biotinylated CTP. The 5′-end of the TSPE primer contains an additional 24nt sequence, the 'anti-TAG'. Following the TSPE reaction, products are then hybridised to the MagPlex-TAG bead mixture (MagPlex-TAG/anti-MagPlex-TAG hybridisation). Following a short washing step employing magnetic capturing of the beads/microspheres, a fluorescent reporter molecule is used to detect incorporated biotin. The bead-TSPE product complexes are detected on the Luminex instruments similarly as was described above for the serological xMAP method.

The specificity of this method is determined on two levels. First at the initial reverse transcriptase PCR step in which either the specificity of the primers or their generic nature determines whether a specific virus is amplified or a group (or particular genus) is amplified. Alternatively several loci from a particular target organism can be amplified generating more amplicons that can be detected in the subsequent TSPE reactions. The second level of specificity comes from the use of multiple TSPE primers directed against several targets amplified in the first step. Different strategies can be applied. Either different TSPE primers are directed against the same amplicon or more than one amplicon per target can be detected each by its own TSPE primer. Following these strategies (or a combination of both) means that always a particular and pre-defined pattern of TSPE positive signals is required for identification of a virus or viruses. The potentially high level of multiplexing can also be used to improve the reliability of the test. Including a plant-specific internal control can function as an RNA extraction control, while using both genus-specific and virus-specific markers in one test identifies virus-positive samples by at least two positive signals. A recently developed multiplex xTAG assay for all nine known pospiviroids (Table 1; Van Brunschot et al., manuscript in preparation) demonstrated the usefulness of this approach.

While multiplexing approaches offer real benefits in situations where high-throughput screening for multiple viruses or other pathogens, it is still limited to detecting 'known' targets. In common with ELISA, real-time PCR and other targeted methods, using specific probes, there is a limit to the degree of pathogen variation that can be detected and hence new pathogens or strains will not be detected and hence missed. Therefore virology diagnosticians also need tools for diagnosing these new and unusual pathogens. Again recent advances in molecular technology may provide the answer to this challenge.

4. Next generation sequencing in viral diagnostics

As in other fields of science the advent of next generation sequencing (NGS) technologies has led to a revolution in virus discovery and exciting new possibilities for diagnostics; the application of massively parallel sequencing approaches, and subsequent bioinformatics analysis for viral sequences, carries the promise of routine, generic detection of viruses and other pathogens alike. Indeed a number of approaches were first published in 2009 applying NGS to identify diverse plant viruses, using different sequencing platforms and nucleic acid preparations as starting material (Adams et al., 2009; Al Rwahnih et al., 2009; Kreuze et al., 2009) and many others have since followed. Whereas different sequencing platforms have been used (principally Roche 454 and Illumina) which can make a difference in cost, ease of sequence assembly and identification, the key distinctions lie in the nucleic acid purification techniques employed.

Whereas some approaches, such as rolling circle amplification (Ng et al., 2011a; Hagen et al., 2012) are clearly limited to certain types of viruses (i.e., circular DNA viruses), others are more broadly applicable to any virus type. Several studies have been successful simply by sequencing total mRNA (Al Rwahnih et al., 2009; Wylie and Jones, 2011; Wylie et al., 2012a, b, 2013). However, a drawback of this approach is that, particularly with low-titre viruses, a massive amount of sequence is 'wasted', since most sequence will be host RNA. Adams et al. (2009) addressed this problem by using an uninfected plant for subtractive hybridisation; thereby enriching for any non-plant RNA and limiting the amount of sequencing required. Despite enhancements like this, total mRNA sequencing may not optimally capture some viruses that lack the terminal polyA sequences used to enrich mRNA. Another frequently used method has been dsRNA isolation followed by random cDNA synthesis (Coetzee et al., 2010; Roossinck et al., 2010; Al Rwahnih et al., 2011). Given that endogenous plant RNAs do not form extensive double stranded structures, but replicative intermediates of RNA viruses do, this approach strongly enriches for viral nucleic

Table 1
Identification of pospiviroids in a multiplex xTAG assay. The presence of a particular pospiviroid is confirmed by both a generic pospiviroid TSPE product (PospiUni) and a viroid-specific TSPE product. NAD5 represents the RNA extraction control incorporated in the assay.

Target	TSPE										
	NADS	Pospi Uni	PSTVd	CSVd	CEVd	CLVd	IrVd	MPVd	TASVd	TCDVd	TPMVd
Potato spindle tuber (PSTVd)	√	√	√								
Chrysanthemum stunt (CSVd)	√	√		√							
Citrus exocortis (CEVd)	√	√			√						
Columnea latent (CLVd)	√	√				√					
Iresine (IrVd)	√	√					√				
Mexican papita (MPVd)	√	√						√			
Tomato apical stunt (TASVd)	√	√							√		
Tomato chlorotic dwarf (TCDVd)	√	√								√	
Tomato planta macho (TPMVd)	√	√									√

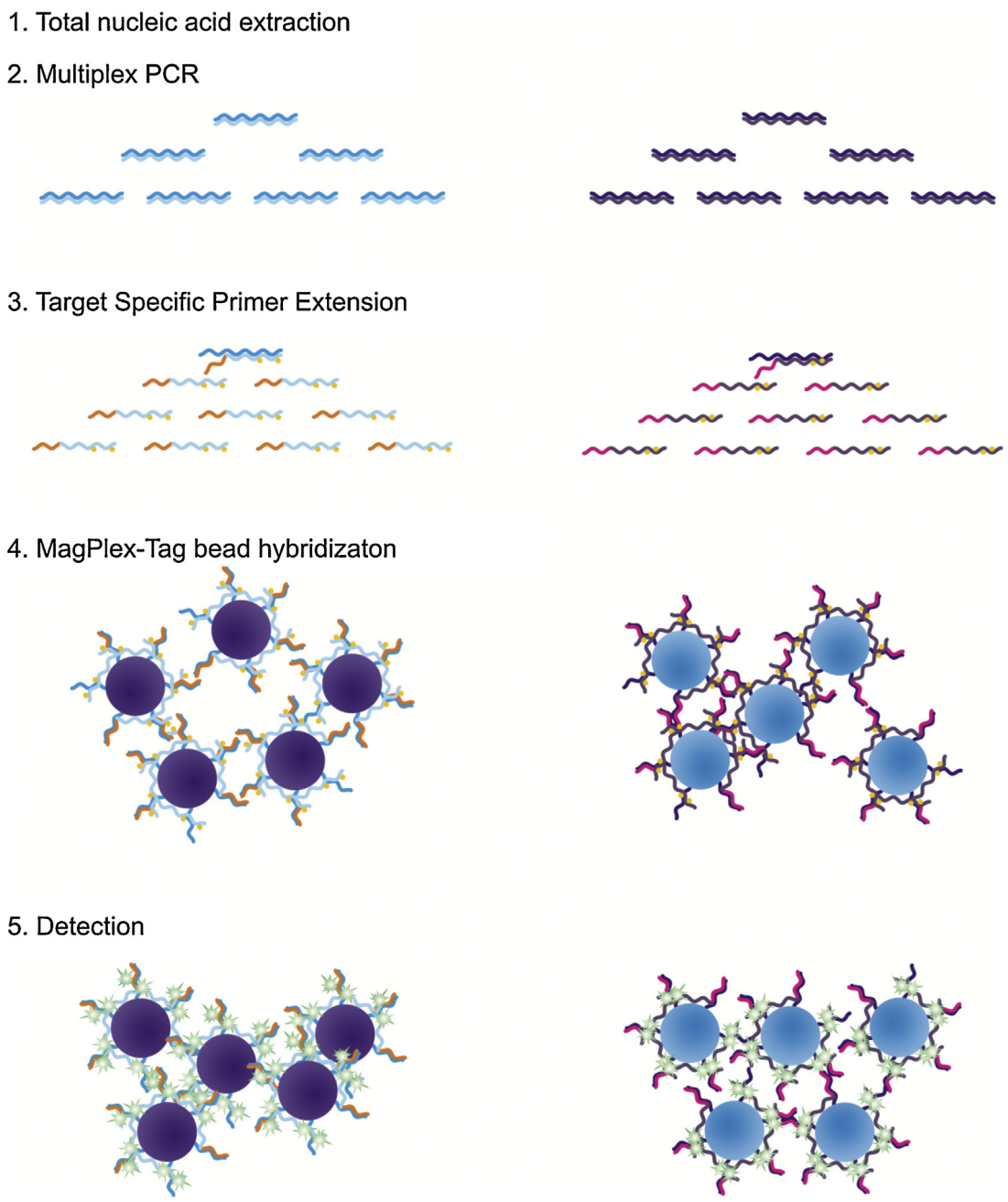

Fig. 2. Summary of the Luminex xTAG technology. Following a total nucleic acid extraction (1) a multiplex reverse transcriptase PCR is performed to amplify the desired viral targets (2). After removal of excess primers a Template Specific Primer Extension (TSPE) reaction in the presence of biotinylated dCTP generates labelled virus-specific amplicons with a 5′-xTAG sequence (3). These xTAG are hybridised to their specific beads (4) and the TSPE products detected by fluorescent labelled Streptavidin in combination with the specific bead address (5).

acids. Nevertheless, some viruses, such as DNA viruses, produce little or no dsRNA and again may be missed using this approach. Indeed Roossinck et al. (2010) in a dsRNA enrichment based eco-genomic survey identified almost exclusively RNA viruses with a particular bias for dsRNA viruses. An alternative approach to enrich for viral sequences in an unbiased way has been to combine simple purifications to enrich for virus-like particles (VLPs) followed by nucleic acid extraction and high throughput sequencing (Ng et al., 2011a, b; Adams et al., 2013a). It is however probably still too early to say if the VLP purification protocols are truly universal, being sufficiently capable of capturing the real diversity of all plant viruses. Another tactic that is gaining in popularity has been to sequence, and assemble, plant small RNAs (Kreuze et al., 2009). Small RNA sequencing and assembly (sRSA) taps into the natural eukaryotic anti-viral defence system based on RNA silencing, which generates small 21–24 nt small RNA (sRNA) molecules corresponding to invading viruses (Mlotshwa et al., 2008) and thus enriching for viral sequences. The approach makes optimal use of sequencing platforms that produce massive amounts, but relatively short sequences and has been used successfully to identify many different plant viruses (Kreuze et al., 2009; Untiveros et al., 2010; Cuellar et al., 2011; Zhang et al., 2011; Bi et al., 2012; Fuentes et al., 2012; Kashif et al., 2012; Li et al., 2012; Loconsole et al., 2012a, b; Sela et al., 2012; Wu et al., 2012; De Souza et al., 2013; Hwang et al., 2013; Kreuze et al., 2013), but is also efficient with viroids (Li et al., 2012; Wu et al., 2012) and viruses of invertebrate and vertebrate animals (Isakov et al., 2011; Ma et al., 2011; Wu et al., 2012), demonstrating the universal utility of sRSA. However, while sRSA

has proven its worth in the identification of new and known viruses from infected organisms, the short length of sRNA sequences poses particular challenges to assembly of full genome sequences, especially when samples are infected with several closely related strains or defective sub-viral molecules are present.

Regardless of which sample preparation method is applied, any method will only become broadly utilised if it is easy to use, relatively fast and not too costly. Whereas rapid and easy protocols have been published for most of the sample purification techniques mentioned, commercial kits are only available for total RNA, mRNA and small RNA purification. Nucleic acid preparation steps prior to sequencing, which include serial steps of adapter ligations and PCR enrichment can be entirely outsourced to sequencing providers or performed in-house to save costs. To make maximum use of the ever increasing throughput of NGS sequencers, and to bring down the cost per sample, samples are often 'tagged' by including a short DNA 'barcode' during sample preparation and combined into one sequencing run. Kits for barcoding and bulking up to 96 samples are available, and each sample can then be de-convoluted after a run with a combined sample using specifically designed computational algorithms. Whereas each of the previous steps may have been more-or-less standardised, assembly and identification of viral contigs is not yet straightforward and poses its own set of challenges, particularly concerning new viruses and virus variants and when incomplete sequences are obtained. Different solutions have been presented for specific situations (Isakov et al., 2011; Li et al., 2011; Vodovar et al., 2011; Wu et al., 2012) and several software packages have been developed for analysis of human viruses (Wang et al., 2013). Nevertheless, none of them present an integrated solution addressing each of the different issues. The development of automated and universally applicable bioinformatics pipelines for the analysis of NGS data specifically for virus discovery and diagnosis remains a challenge, which needs to be met before these technologies can become part of mainstream viral diagnostics.

One particularly interesting application of NGS-based virus identification has been vector-enabled metagenomics (VEM) where insects are sampled to identify viruses present in the environment (Ma et al., 2011; Ng et al., 2011a, b; Rosario et al., 2012, 2013). Ng et al. (2011a) using a VEM approach on whiteflies, were subsequently able to confirm a newly identified begomovirus in *Chenopodium ambrosiodes* with viral symptoms, which was growing in the sampled eco-system and thus demonstrating the effectiveness of the technique for proactive monitoring of plant viruses. Rosario et al. (2013) in a more comprehensive adaptation of the VEM approach targeted their survey to the top insect predators in the ecosystem, dragonflies. Using this approach they were able to identify new mastrevirus and alphasatellite-like genomes. Thus insects can effectively be used as sentinel species to monitor particular environments for the presence or introduction of new viruses.

5. Discussion

Although traditional methods (in particular host plant inoculations and electron microscopy) are still used for virus diagnostics in routine testing laboratories, the worldwide adoption of ELISA in the 1970–80s was a massive advance and changed the face of viral diagnostics. It provided a platform that can be used generically for a range of different viruses with very little modification. It is robust and easy to perform; requiring little training and expertise to generate constantly high levels of performance. It piggybacks onto an industry standard testing format, namely the 96-well plate, which brings with it standardised equipment e.g., pipettes, tips, plate readers, etc.; which are all formatted to the same generic footprint. This has helped to drive down cost and make materials readily available to routine testing laboratories across the world and has undoubtedly contributed to the continued success of the method. Of the raft of post-ELISA methods developed, NASH and conventional PCR have both been used successfully and their adoption has been reasonably widespread. However, real-time PCR is the nucleic acid-based method that has really become established in testing laboratories across the world. It is perhaps not surprising then that it shares many of the characteristics of ELISA, which have led to this broad establishment. Like ELISA it is based on an industry standard format (again the 96- and more recently the 384-well plate) in the same footprint for high-throughput testing. It has had good generic uptake in many fields outside of phytodiagnostics that has driven competition between instrument manufacturers and reduced the price of reagents, plasticware and equipment. Like ELISA, real-time PCR works based upon a generic base protocol, including the reaction cycling conditions, used for every test, making it simpler to establish testing for many targets using the same basic method. Finally the overall levels of basic expertise required to run ELISA and real-time PCR are similar; with operators needing to be competent lab technicians rather than expert molecular biologists. In recent years real-time PCR has even started being used as a first choice method compared with ELISA, something that would be unthinkable 10 years ago. The reasons for this are several fold but probably one of the most significant is predictability. Whilst antibody manufacture is often time-consuming, expensive and unpredictable, development of PCR-based assays is fast, inexpensive and rarely fails. Added to this up-front saving in time (and therefore money) the price differential, both per-test and for equipment, has shortened considerably. Thus unless very large sample sizes are to be tested, if antiserum is not available, PCR-based methods (increasingly real-time PCR) are becoming the first choice for new and emerging virus threats. Where monoclonal antibody cell lines assure a long term supply of reagent and where ELISA performs to an adequate level of sensitivity and specificity, it seems unlikely that it will be dropped from most testing laboratories in the near future. However, for newly emerging threats the investment required to make new reagents, suggests that real-time PCR will gradually become the most widely used diagnostic in testing laboratories around the world. Many of the new methods 'on the block' have been described in this review. Time will tell which of these will become established in the next 10 years and which will be resigned to history.

Some of the methods under development and evaluation offer unique capabilities, for example the field testing methods, such as LFDs and isothermal amplification methods such as LAMP, and the parallel testing methods, such as arrays and NGS. Field testing methods enable end users to expedite their decision making by generating results at the point at which the material is sampled. The success and establishment of these methods will be largely dependent upon several factors, either independently or more likely acting in concert. Firstly, the genuine need for the advantages generated by rapid decision making. This may be linked to the ability of the user to make those decisions effectively, since testing laboratories often provide not just results, but also provide advice and assume liability for the results generated. Thus they may become more established with specialist remote staff, such as agronomists or phytosanitary inspectors, who are better able to deal with the results produced. Secondly, with the speed and ease with which results can be attained, do the methods fit as part of an efficient inspection process or do they create a distraction to the process of inspection. Finally the per test and platform cost may play a significant role; potentially not individually, since many LFDs are cheaper (typically $USD 4–8 per test) than laboratory tests (typically $USD 10–50 per test depending on type and volume; the increased costs of the latter supported by the value of the advice, support and assurance provided), but in combination with the other factors listed

above will combine to determine establishment of the methods. Of course the key factor is that this approach is a significant change from the norm and it may take some time for the confidence of the end user to build and the maturity of the testing laboratories to grow, accepting that some samples will no longer be sent to the laboratory but that they will continue to provide a supporting role, where the speed of field testing provides a significant advantage.

The parallel or multiplex testing methods (arrays, Luminex and NGS) seem to converge considerably in performance when looking into the medium to longer term, yet are currently being separated purely on price. Some of the factors that will make parallel testing methods successful in the short term are the need to provide a work flow as simple as simplex testing (e.g., using ELISA or real-time PCR) but provide significant cost savings over simplex tests being performed side by side. This price advantage typically comes with the scale of multiplexing achieved, i.e., the greater the multiplexing required the cheaper the per target cost becomes. This probably also provides interplay with simplicity: the greater the cost saving, the more likely a routine laboratory is to invest in a more complex method. Whilst significant multiplexing appears on the surface to be an obvious step forward performance wise, significant applications for heavily multiplexed tests (say in excess of 10 targets) do not abound in the routine laboratory. Thus establishing simple work flows (potentially like those achieved with bead arrays) become significant in order to achieve the modest cost saving associated with low levels of multiplexing (1–10 targets).

Very highly multiplexed array techniques have been developed (e.g., www.bio-chip.co.uk) and these arrays are usually targeted at virus discovery and resolution of complex disease problems. Despite the hype surrounding some significant diagnoses achieved using the techniques (Wang et al., 2003), these examples are fairly few and far between. This is the area where NGS is already eclipsing the array techniques, predominantly because the mode of action is simply better suited to the task in hand. Virus discovery arrays are reliant effectively on the cross-hybridisation of probes, designed to known viruses, to enable detection of new viruses. Whilst probes can be designed in conserved regions and many variants of probes can be deployed on high density arrays, the design of these probes is a complex prospect. Most array design and experimental protocols are constructed to get good detection and discrimination of closely related sequences, and the development of probes and methods to achieve this is relatively straightforward and the bioinformatics is relatively well understood. Conversely, the development of probes and low-stringency protocols that will cross-hybridise with as yet unknown virus sequences, yet alone discriminate them from cross hybridisation events between probes and an abundance of host sequence, is an experimentally challenging proposition. NGS on the other hand uses bioinformatic search tools to look for sequence similarities between the generated sequences and an ever growing, freely available database of known sequences. Not only does this make the methods more effective right now, but also they will become ever more effective as the database of known sequences increases over time.

In the longer term however NGS and parallel testing methods will begin to converge considerably. The main driver in the development of NGS is personalised medicine, specifically the sequencing of entire human genomes at a very low cost. Hence as time goes on the cost per base pair is dropping at an incredible rate. Linked to this decrease in consumable costs are other improvements, namely simpler, faster protocols and decreases in the costs of the sequencing platforms. An Illumina Mi-Seq is already a third of the price of a Roche 454-FLX. Yet it generates approximately 10 fold more data, in one half of the time and at one tenth of the cost. Together this suggests that in the future generating large amounts of sequence data from a sample will continue to become easier, quicker and cheaper. Already the costs of NGS is making virus discovery and disease resolution a realistic proposition in the routine laboratory (Monger et al., 2010a, b; Adams et al., 2013a, b; Harju et al., 2012), with rapid development and deployment of a high-throughput detection technique, such as real-time PCR, as a follow-on (Adams et al., 2012). Since the ability to multiplex samples on the sequencing platforms is very high, as the throughput of the platforms becomes bigger there is a very real possibility that, in the medium term, the per sample cost of NGS will become affordable in the routine diagnostic situations, for samples where there is the need to test for multiple targets; effectively eclipsing some of the multiplexing platforms such as arrays. Looking further ahead it can be envisaged that one day the prices will drop to a point where providing an NGS result for a sample will be lower than the price of a single ELISA or PCR test. Even at current costs, while a single NGS run might cost up to $USD 8000 (including all reagents, sample prep and staff time), the huge sequencing capacity available could allow for up to 24 samples to be combined, making the per sample cost less than $USD 350. In a diagnostics context, where typically samples are subjected to multiple, parallel ELISA, PCR or other tests, this cost is highly comparable. Not only that, but the method will be completely generic ('non-targeted'), requiring no target specific reagents and will be capable of detecting any virus present in the sample, either expected or not (e.g., proving the role of new viruses in disease aetiology). Of course as the technology reaches this point, it will generate its own challenges. In order to turn this vision of the future into a reality there will be the absolute need to characterise the natural viral communities that do exist in healthy plants and be able to separate these from the presence of disease-causing viruses. Ultimately Koch's postulates are still highly relevant in this respect. It seems likely that this determination of aetiology is the step that will cause the real delays in progress, rather than developments in the technology itself.

In conclusion, only time will tell which of the next-generation of methods currently in development will become the routine diagnostics of the future. This will be determined through a combination of factors. The first obvious consideration is that the technology itself will have to offer performance advantages over existing methods in order to supplant them and this review demonstrates the potential to achieve this for a number of emerging technologies. The second consideration will be access to resources and expertise. Technologies such as NGS require major investment: considerable upfront capital spending, ongoing running cost expenditure and significant investment in molecular biology and bioinformatics expertise. This makes NGS technology (as it currently exists) only suitable for well-funded, centralised laboratories. However, this situation is not new and the same criteria once applied to techniques such as real-time PCR. Commercial market forces have changed that situation, where capital and reagent costs have plummeted for real-time PCR. This has greatly reduced the entry-level for laboratories to adopt this technology and it has become established in many laboratories now. Furthermore techniques such as LAMP, designed as field-testing technologies, offer huge potential for laboratories where resources and expertise are in short supply. Hence it is possible to envisage a future where an even greater number of laboratories around the world are capable of carrying out target-specific, routine molecular diagnostics. These will be linked to larger centres, which act as reference laboratories; providing expertise to support existing diagnostics and develop new ones, increasingly based on non-targeted approaches to identify new and emerging threats. However while factors linked to the technology itself will play a role in future adoption, it is probably human factors which are likely to be the most significant in deciding which new methods become widely adopted and which ones fail. Ultimately the behaviours and influence of end users, laboratories and policy makers will be crucial. Hence these factors cannot be ignored and early engagement with diagnostic stakeholders is essential.

References

Adams, I.P., Glover, R., Souza-Richards, R., Bennett, S., Hany, U., Boonham, N., 2013a. Complete genome sequence of arracacha virus B: a novel cheravirus. Arch. Virol. 158, 909–913.

Adams, I.P., Miano, D.W., Kinyua, Z.M., Wangai, A., Kimani, E., Phiri, N., et al., 2013b. Use of next-generation sequencing for the identification and characterization of *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* causing maize lethal necrosis in Kenya. Plant Pathol. 6, 741–749.

Adams, I.P., Abidrabo, P., Miano, D.W., Alicai, T., Kinyua, Z., Clarke, J., et al., 2012. High throughput real-time RT-PCR assays for specific detection of Cassava brown streak disease causal viruses, and their application to testing of planting material. Plant Pathol. 62, 233–242.

Adams, I.P., Glover, R.H., Monger, W.A., Mumford, R., Jackeviciene, E., Navalinskiene, M., et al., 2009. Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. Mol. Plant Pathol. 10, 537–545.

Al Rwahnih, M., Daubert, S., Golino, D., Rowhani, A., 2009. Deep sequencing analysis of RNAs from a grapevine showing Syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Virology 387, 395–401.

Al Rwahnih, M., Daubert, S., Urbez-Torres, J.R., Cordero, F., Rowhani, A., 2011. Deep sequencing evidence from single grapevine plants reveals a virome dominated by mycoviruses. Arch. Virol. 156, 397–403.

Banér, J., Nilsson, M., Mendel-Hartvig, M., Landegren, U., 1998. Signal amplification of padlock probes by rolling circle replication. Nucleic Acids Res. 22, 5073–5078.

Bergervoet, J.H.W., Peters, J., van Beckhoven, J.R.C.M., van den Bovenkamp, G.W., Jacobson, J.W., van der Wolf, J.M., 2008. Multiplex microsphere immunodetection of potato virus Y, X and PLRV. J. Virol. Methods 149, 63–68.

Bi, Y., Tugume, A.K., Valkonen, J.P., 2012. Small-RNA deep sequencing reveals *Arctium tomentosum* as a natural host of Alstroemeria virus X and a new putative Emaravirus. PLoS One 7, e42758.

Boonham, N., Laurenson, L., Weekes, R., Mumford, R., 2009. Direct detection of plant viruses in potato tubers using real-time PCR. In: Burns, R. (Ed.), Methods in Molecular Biology No. 508: Plant Pathology: Techniques and Protocols. Humana Press, New York, USA, pp. 249–258.

Boonham, N., Pérez, L.G., Mendez, M.S., Peralta, E.L., Blockley, A., Walsh, K., et al., 2004. Development of a real-time RT-PCR assay for the detection of *Potato spindle tuber viroid*. J. Virol. Methods 116, 139–146.

Boonham, N., Tomlinson, J., Mumford, R.A., 2007. Microarrays for rapid identification of plant viruses. Annu. Rev. Phytopath. 45, 307–328.

Bercks, R., 1967. Methodiche Untersuchungen uber den serologichen Nachweis pflanzen pethogener Viren mit dem Bentonit-Flockungstet, dem Latex-Test und dem Bariumsulfat-test. Phytopath. Z. 58, 1–17.

Clark, M.F., Adams, A.N., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34, 475–483.

Coetzee, B., Freeborough, M.J., Maree, H.J., Celton, J.M., Rees, D.J., Burger, J.T., 2010. Deep sequencing analysis of viruses infecting grapevines: virome of a vineyard. Virology 400, 157–163.

Compton, J., 1991. Nucleic acid sequence-based amplification. Nature 350, 91–92.

Cuellar, W.J., Cruzado, R.K., Fuentes, S., Untiveros, M., Soto, M., Kreuze, J.F., 2011. Sequence characterization of a Peruvian isolate of *sweet potato chlorotic stunt virus*: further variability and a model for p22 acquisition. Virus Res. 157, 111–115.

De Souza, J., Fuentes, S., Savenkov, S., Cuellar, W., Kreuze, J., 2013. The complete nucleotide sequence of sweet potato C6 virus: a carlavirus lacking a cystein rich protein. Arch. Virol. 158, 1393–1396.

Derrick, K.S., 1972. Immuno-specific grids for electron microscopy of plant viruses. Phytopathology 62, 753–754.

Dovas, C.I., Katis, N.I., Avgelis, A.D., 2002. Multiplex detection of criniviruses associated with epidemics of a yellowing disease of tomato in Greece. Plant Dis. 86, 1345–1349.

Dunbar, S.A., Vander Zee, C.A., Oliver, K.G., Karem, K.L., Jacobson, J.W., 2003. Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP system. J. Microbiol. Methods 53, 245–252.

EPPO, 2004a. Diagnostic protocol for regulated pests. Citrus tristeza virus. OEPP/EPPO Bull. 34, 239–246.

EPPO, 2004b. Diagnostic protocol for regulated pests. Plum pox potyvirus. OEPP/EPPO Bull. 34, 155–157.

EPPO, 2005. Diagnostic protocol for regulated pests. Tomato ringspot nepovirus. OEPP/EPPO Bull. 35, 271–273.

Fuentes, S., Heider, B., Tasso, R.C., Romero, E., Zum Felde, T., Kreuze, J.F., 2012. Complete genome sequence of a potyvirus infecting yam beans (*Pachyrhizus* spp.) in Peru. Arch. Virol. 157, 773–776.

Foord, A.J., White, J.R., Colling, A., Heine, H.G., 2013. Microsphere suspension array assays for detection and differentiation of Hendra and Nipah viruses. BioMed. Res. Int. 2 [Article ID 89295].

Gandelman, O., Jackson, R., Kiddle, G., Tisi, L., 2011. Loop-mediated amplification accelerated by stem primers. Int. J. Mol. Sci. 12, 9108–9124.

Gandelman, O.A., Church, V.L., Moore, C.A., Kiddle, G., Carne, C.A., Parmar, S., et al., 2010. Novel bioluminescent quantitative detection of nucleic acid amplification in real-time. PLoS One 5, e14155.

Goldmeyer, J., Li, H., McCormac, M., Cook, S., Stratton, C., Lemieux, B., et al., 2008. Identification of *Staphylococcus aureus* and determination of methicillin resistance directly from positive blood cultures by isothermal amplification and a disposable detection device. J. Clin. Microbiol. 46, 1534–1536.

Hagen, C., Frizzi, A., Gabriels, S., Huang, M., Salati, R., Gabor, B., et al., 2012. Accurate and sensitive diagnosis of geminiviruses through enrichment, high-throughput sequencing and automated sequence identification. Arch. Virol. 157, 907–915.

Haible, D., Kober, S., Jeske, H., 2006. Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. J. Virol. Methods 135, 9–16.

Harju, V., Skelton, A., Forde, S., Bennett, S., Glover, R.H., Monger, W.A., Adams, I.P., Boonham, N., Fox, A., 2012. New virus detected on *Nasturtium officinale*, watercress. New Dis. Rep. 25, http://dx.doi.org/10.5197/j.2044-0588.2012.025.001.

Hwang, Y.T., Kalischuk, M., Fusaro, A.F., Waterhouse, P.M., Kawchuk, L., 2013. Small RNA sequencing of *Potato leafroll virus*-infected plants reveals an additional subgenomic RNA encoding a sequence-specific RNA-binding protein. Virology 438, 61–69.

Isakov, O., Modai, S., Shomron, N., 2011. Pathogen detection using short-RNA deep sequencing subtraction and assembly. Bioinformatics 27, 2027–2030.

James, H.E., Ebert, K., McGonigle, R., Reid, S.M., Boonham, N., Tomlinson, J.A., et al., 2010. Detection of *African swine fever virus* by loop-mediated isothermal amplification. J. Virol. Methods 164, 68–74.

Joos, T.J.G., 2004. Protein microarray technology. Expert Rev. Proteomics 1, 1–3.

Joos, T.O., Schrenk, M., Hopfl, P., Kroger, K., Chowdhury, U., Stoll, D., et al., 2000. A microarray enzyme-linked immunosorbent assay for autoimmune diagnostics. Electrophoresis 21, 2641–2650.

Kaneko, H., Kawana, T., Fukushima, E., Suzutani, T., 2007. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J. Biochem. Biophys. Methods 10, 499–501.

Kashif, M., Pietilä, S., Artola, K., Jones, R.A.C., Tugume, A.K., Mäkinen, V., et al., 2012. Detection of viruses in sweetpotato from Honduras and Guatemala augmented by deep-sequencing of small-RNAs. Plant Dis. 96, 1430–1437.

Kellar, K.L., 2003. Applications of multiplexed fluorescent microsphere-based assays to studies of infectious disease. J. Clin. Ligand Assay 26, 76–86.

Klerks, M.M., Leone, G.O., Verbeek, M., van den Heuvel, J.F., Schoen, C.D., 2001. Development of a multiplex AmpliDet RNA for the simultaneous detection of *Potato leafroll virus* and *Potato virus Y* in potato tubers. J. Virol. Methods 93, 115–125.

Kreuze, J., Koenig, R., De Souza, J., Vetten, H.J., Muller, G., Flores, B., et al., 2013. The complete genome sequences of a Peruvian and a Colombian isolate of Andean potato latent virus and partial sequences of further isolates suggest the existence of two distinct potato-infecting tymovirus species. Virus Res. 173, 431–435.

Kreuze, J.F., Pérez, A., Untiveros, M., Quispe, D., Fuentes, S., Barker, I., et al., 2009. Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: a generic method for diagnosis, discovery and sequencing of viruses. Virology 388, 1–7.

Leone, G., van Schijndel, H.B., van Genien, B., Schoen, C.D., 1997. Direct detection of potato leafroll virus in potato tubers by immunocapture and the isothermal nucleic acid amplification method NASBA. J. Virol. Methods 66, 19–27.

Li, R., Gao, S., Hernandez, A.G., Wechter, W.P., Fei, Z., Ling, K.S., 2012. Deep sequencing of small RNAs in tomato for virus and viroid identification and strain differentiation. PLoS One 7, e37127.

Li, S.C., Chan, W.C., Lai, C.H., Tsai, K.W., Hsu, C.N., Jou, Y.S., et al., 2011. UMARS: un-mappable reads solution. BMC Bioinform. 12, S9.

Lin, A., Nguyen, L., Lee, T., Clotilde, L.M., Kase, J.A., Son, I., et al., 2011. Rapid O serogroup identification of the ten most clinically relevant STECs by Luminex microbead-based suspension array. J. Microbiol. Methods 87, 105–110.

Liu, J., Kibiki, G., Maro, V., Maro, A., Kumburu, H., Swai, N., et al., 2011. Multiplex reverse transcription PCR Luminex assay for detection and quantitation of viral agents of gastroenteritis. J. Clin. Virol. 50, 308–313.

Loconsole, G., Onelge, N., Potere, O., Giampetruzzi, A., Bozan, O., Satar, S., et al., 2012a. Identification and characterization of citrus yellow vein clearing virus, a putative new member of the genus Mandarivirus. Phytopathology 102, 1168–1175.

Loconsole, G., Saldarelli, P., Doddapaneni, H., Savino, V., Martelli, G.P., Saponari, M., 2012b. Identification of a single-stranded DNA virus associated with citrus chlorotic dwarf disease, a new member in the family Geminiviridae. Virology 432, 162–172.

Ma, M., Huang, Y., Gong, Z., Zhuang, L., Li, C., Yang, H., et al., 2011. Discovery of DNA viruses in wild-caught mosquitoes using small RNA high throughput sequencing. PLoS One 6, e24758.

Mahony, J., Chong, S., Merante, F., Yaghoubian, S., Sinha, T., Lisle, C., et al., 2007. Development of a respiratory virus panel test for detection of twenty human respiratory viruses by use of multiplex PCR and a fluid microbead-based assay. J. Clin. Microbiol. 45, 2965–2970.

Mlotshwa, S., Pruss, G.J., Vance, V., 2008. Small RNAs in viral infection and host defense. Trends Plant Sci. 13, 375–382.

Monger, W.A., Adams, I.P., Glover, R., Barrett, B., 2010a. The complete genome sequence of Canna yellow streak virus. Arch. Virol. 155, 1515–1518.

Monger, W.A., Alicai, T., Ndunguru, J., Kinyua, Z.M., Potts, M., Reeder, R.H., et al., 2010b. The complete genome sequence of the Tanzanian strain of *Cassava brown streak virus* and comparison with the Ugandan strain sequence. Arch. Virol. 155, 429–433.

Mukai, H., Uemori, T., Takeda, O., Kobayashi, E., Yamamoto, J., Nishiwaki, K., et al., 2007. Highly efficient isothermal DNA amplification system using three elements of 5′-DNA-RNA-3′ chimeric primers, RNaseH and strand-displacing DNA polymerase. J. Biochem. 142, 273–281.

Mumford, R.A., Walsh, K., Barker, I., Boonham, N., 2000. Detection of *Potato mop top virus* and *Tobacco rattle virus* using multiplex real-time fluorescent RT-PCR assay. Phytopathology 90, 448–453.

Mumford, R.A., Boonham, N., Tomlinson, J., Barker, I., 2006. Advances in molecular phytodiagnostics – new solutions for old problems. Eur. J. Plant Pathol. 116, 1–19.
Ng, T.F.F., Duffy, S., Polston, J.E., Bixby, E., Vallad, G.E., Breitbart, M., 2011a. Exploring the diversity of plant DNA viruses and their satellites using vector-enabled metagenomics on whiteflies. Plos One 6, e19050.
Ng, T.F.F., Willner, D.L., Lim, Y.W., Schmieder, R., Chau, B., Nilsson, C., Anthony, S., Ruan, Y.J., Rohwer, F., Breitbart, M., 2011b. Broad surveys of DNA viral diversity obtained through viral metagenomics of mosquitoes. Plos One 6, e20579.
Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., et al., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, e63.
Panno, S., Davino, S., Rubio, L., Rangel, E., Davino, M., Garcia-Hernandez, J., et al., 2012. Simultaneous detection of the seven main tomato-infecting RNA viruses by two multiplex reverse transcription polymerase chain reactions. J. Virol. Methods 186, 152–156.
Papayiannis, L.C., Harkou, I.S., Markou, Y.M., Demetriou, C.N., Katis, N.I., 2011. Rapid discrimination of *Tomato chlorosis virus*, *Tomato infectious chlorosis virus* and co-amplification of plant internal control using real-time RT-PCR. J. Virol. Methods 176, 53–59.
Piepenburg, O., Williams, C.H., Stemple, D.L., Armes, N.A., 2006. DNA detection using recombination proteins. PLoS Biol. 4, e204.
Rao, R.S., Visuri, S.R., McBride, M.T., Albala, J.S., Matthews, D.L., Coleman, M.A., 2004. Comparison of multiplexed techniques for detection of bacterial and viral proteins. J. Proteome Res. 3, 736–742.
Roossinck, M.J., Saha, P., Wiley, G.B., Quan, J., White, J.D., Lai, H., et al., 2010. Ecogenomics: using massively parallel pyrosequencing to understand virus ecology. Mol. Ecol. 19, 81–88.
Rosario, K., Dayaram, A., Marinov, M., Ware, J., Kraberger, S., Stainton, D., et al., 2012. Diverse circular ssDNA viruses discovered in dragonflies (Odonata: Epiprocta). J. Gen. Virol. 93, 2668–2681.
Rosario, K., Padilla-Rodriguez, M., Kraberger, S., Stainton, D., Martin, D.P., Breitbart, M., et al., 2013. Discovery of a novel mastrevirus and alphasatellite-like circular DNA in dragonflies (Epiprocta) from Puerto Rico. Virus Res. 171, 231–237.
Sela, N., Luria, N., Dombrovsky, A., 2012. Genome assembly of bell pepper endornavirus from small RNA. J. Virol. 86, 7721.
Steel, E., Barker, I., Danks, C., Coates, D., Boonham, N., 2010. *Agrobacterium tumefaciens* mediated transient expression as a tool for antigen production for *Cucurbit yellow stunting disorder virus*. J. Virol. Methods 163, 222–228.
Tomlinson, J.A., Barker, I., Boonham, N., 2007. Faster, simpler, more-specific methods for improved molecular detection of *Phytophthora ramorum* in the field. Appl. Environ. Microbiol. 73, 4040–4047.
Tomlinson, J.A., Dickinson, M.J., Boonham, N., 2010a. Detection of *Botrytis cinerea* by loop-mediated isothermal amplification. Lett. Appl. Microbiol. 51, 650–657.
Tomlinson, J.A., Dickinson, M.J., Boonham, N., 2010b. Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. Phytopathology 100, 143–149.
Torrance, L., Jones, R.A.C., 1981. Recent developments in serological methods suited for use in routine testing for plant viruses. Plant Pathol. 30, 1–24.
Untiveros, M., Quispe, D., Kreuze, J., 2010. Analysis of complete genomic sequences of isolates of the *Sweet potato feathery mottle virus* strains C and EA: molecular evidence for two distinct potyvirus species and two P1 protein domains. Arch. Virol. 155, 2059–2063.
Vidal, E., Yokomi, R.K., Moreno, A., Bertolini, E., Cambra, M., 2012. Calculation of diagnostic parameters of advanced serological and molecular tissue-print methods for detection of *Citrus tristeza virus*: a model for other plant pathogens. Phytopathology 102, 114–121.
Vignali, D.A.A., 2000. Multiplexed particle-based flow cytometric assays. J. Immunol. Methods 243, 243–255.
Vincent, M., Xu, Y., Kong, H., 2004. Helicase-dependent isothermal DNA amplification. EMBO Rep. 5, 795–800.
Vodovar, N., Goic, B., Blanc, H., Saleh, M.C., 2011. *In silico* reconstruction of viral genomes from small RNAs improves virus-derived small interfering RNA profiling. J. Virol. 85, 11016–11021.
Vunsh, R., Rosner, A., Stein, A., 1990. The use of the polymerase chain reaction (PCR) for the detection of *Bean yellow mosaic virus* in gladiolus. Ann. Appl. Biol. 117, 561–569.
Wang, D., Urisman, A., Liu, Y.-T., Springer, M., Ksiazek, T.G., Erdman, D.D., et al., 2003. Viral discovery and sequence recovery using DNA microarrays. PLoS Biol. 1, e2.
Wang, Q., Jia, P., Zhao, Z., 2013. VirusFinder: software for efficient and accurate detection of viruses and their integration sites in host genomes through next generation sequencing data. PLoS One 8, e64465.
Wastling, S.L., Picozzi, K., Kakembo, A.S., Welburn, S.C., 2010. LAMP for human African trypanosomiasis: a comparative study of detection formats. PLoS Negl. Trop. Dis. 4, e865.
Wintermantel, W.M., Hladky, L.L., 2010. Methods for detection and differentiation of existing and new crinivirus species through multiplex and degenerate primer RT-PCR. J. Virol. Methods 170, 106–114.
Wu, Q., Wang, Y., Cao, M., Pantaleo, V., Burgyan, J., Li, W.-X., et al., 2012. Homology-independent discovery of replicating pathogenic circular RNAs by deep sequencing and a new computational algorithm. PNAS 109, 3938–3943.
Wylie, S.J., Jones, M.G.K., 2011. The complete genome sequence of a *Passion fruit woodiness virus* isolate from Australia determined using deep sequencing, and its relationship to other potyviruses. Arch. Virol. 156, 479–482.
Wylie, S.J., Li, H., Dixon, K.W., Richards, H., Jones, M.G.K., 2013. Exotic and indigenous viruses infect wild populations and captive collections of temperate terrestrial orchids (*Diuris* species) in Australia. Virus Res. 171, 22–32.
Wylie, S.J., Luo, H., Li, H., Jones, M.G.K., 2012a. Multiple polyadenylated RNA viruses detected in pooled cultivated and wild plant samples. Arch. Virol. 157, 271–284.
Wylie, S.J., Tan, A.J.Y., Li, H., Dixon, K.W., Jones, M.G.K., 2012b. Caladenia virus A, an unusual new member of the family Potyviridae from terrestrial orchids in Western Australia. Arch. Virol. 157, 2447–2452.
Zhang, Y., Singh, K., Kaur, R., Qiu, W., 2011. Association of a novel DNA virus with the grapevine vein-clearing and vine decline syndrome. Phytopathology 101, 1081–1090.