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Serodiagnostics For Plant Virus Detection

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Abstract
Plant pathogenic viruses are the major constraints to the global agriculture and account for significant yield loss each year. In order to protect the crops from viral diseases, practice of stringent and reliable disease management approaches is essential. Accurate detection of virus in infected plants is one of the most important steps in management of viral diseases. Based on antibodies specificity towards antigen, serodiagnostics offer many advantages in disease detection include robustness, field compatibility, ease in handling, cost effective, high sensitivity and specificity. In this review we have discussed some of the widely using serological techniques for detection of plant viruses.
Keywords:
Keywords
Plant virus, serodiagnosis, Enzyme linked immunoassay, antibody.

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INTRODUCTION
It is well known that global agriculture is severely suffering from plant viral diseases. It was estimated that annual global economic loss due to plant virus infection was more than $30 billion [1]. There are many reasons associated with global spread of plant viral diseases, these include i) global exchange and marketing of plants, seeds through multinational companies, ii) climate instability due to global warming iii) introducing new crop plants or contaminated seeds into new countries or continents without following quarantine regulations, iv) lack of on-site, rapid and reliable detection techniques for large scale screening for infected plant materials, v) monoculture practice for economically important crops production and many others. In order to control viral diseases, stringent disease management approaches must be implemented. The rapid spread of virus can be arrested by eradicating the source of infection, controlling the insect vector movement, selection of virus free planting material and usage of virus resistant plants for cultivation [2]. Hence, foremost step is required for the detection of disease-causing virus. The virus infected plants are not always symptomatic, and hence, symptoms-based virus detection is not a reliable approach. Sometimes host plants are infected by multiple viruses and express similar symptoms and sometimes the climatic conditions delay or masks expression of symptoms. *Cucumber mosaic virus* (CMV) and *Bannana streak viruses* (BSV) are two distinct viruses infects banana and expresses similar chlorotic streaks on the plant leaves [3]. To avoid confusion in virus detection, specific and sensitive diagnostics are requiring. To develop such diagnostics, broad knowledge on biological, biophysical, molecular, and serological properties of the pathogen is essential. Diagnostics for virus detection must be extremely sensitive, cost effective, simple, field applicable, rapid, and reliable. Several approaches like biological, cytopathological, serological and molecular approaches have been developed to diagnose the viruses in infected or suspected plant materials and insect vectors [4]. However, serodiagnostics are at the forefront in large scale screening of plant viruses. The sensitivity and reliability of serodiagnostics rely on the specificity and affinity between antigen and antibody.

Antisera to plant virus detection

Antigen is a foreign particle which can induce immune response (humoral and cell mediated immune response) in animals. Rise of effective immune response depends on the chemical nature, complexity and foreignness of the antigen. For example, high immune response can be seen with proteins or peptides followed by oligosaccharides, lipids, and nucleic acids. Plant viruses are nucleoproteins, their genome is covered and protected by protein shell or coat protein, however, some viruses belong to the *Rhabdoviridae* family are enveloped. Antibodies of humoral immune response are being used in diagnostics for detection of antigen. Depending on the requirement they were produced in experimental animals such as rabbits, guinea pigs, chickens, sheep, goats, horses, and camelids. All the immunodiagnostics rely on the interactions between specific antiserum and virus proteins. Well optimized procedures have been established for production of antibodies such as polyclonal, monoclonal and recombinant antibodies. Polyclonal antibodies (PAbs) are a heterogeneous mix of antibodies raised against to the multiple epitopes of the antigen. Quality of the antibodies depends on the purity of antigen (immunogen). The purified intact virus or the proteins of over expressed virus genes (eg. coat protein, replicase and movement protein genes) can be used to immunize the experimental animal for PAbs production. However, the latter one is preferred when the virus purification is difficult from plant material. Some of the plants are with hard tissue and rich with polyphenols such as phenolic acids, flavonoids, lignans and stilbenes, which hinder the virus purification process from plant material. For example, *Sugarcane mosaic virus* (SCMV) from sugarcane, *Banana bract mosaic virus* (BBrMV), *Banana buchytop virus* (BBTV), Banana streak virus (BSV) from *Musa* sp. and Dasheen mosaic virus (DsMV) from *Colocasia*. To overcome such difficulty, CP gene of several potyviruses including *Banana bract mosaic virus*, *Watermelon leaf mottle virus*, *Tuberose mild mosaic virus*, *Sugarcane streak mosaic virus* and *Ryegrass mosaic virus* [5-9] have been cloned and expressed in *E. coli* for production of polyclonal antibodies. These antibodies can bypass the false positive results as observed with conventional antibodies in ELISA. Further, these are more sensitive than to the conventional antibodies toward detection of antigen.

Monoclonal (MAbs) antibodies are monovalent, produced to a single epitope of an antigen. In order to produce MAbs, a single clone of B cell (antigen primed) is fused with myeloma cells of the mouse and after a scrupulous selection and screening picks up the correct hybridoma which produces monoclonal antibodies. However, this approach (hybridoma technology) is highly laborious, time consuming and expensive but more specific than to the conventional polyclonal antibodies for antigen recognition. Monoclonal antisera are much more specific than polyclonal antisera and can be used to differentiate strains of many pathogens. There are some examples of monoclonal antibodies that are virus group specific [4]. MAbs are produced successfully for the detection of certain potyviruses, for example, *Potato virus Y* (PVY), *Plum pox virus* (PPV), *Soybean mosaic virus* (SMV), *Lettuce mosaic virus* (LMV), *Maize dwarf mosaic virus* (MDMV) and *Bean yellow mosaic virus* (BYMV) [10-12]. Murine MAbs were raised to *Barely yellow dwarf virus* GAV strain (BYDV-GAV) and with these optimized two serological techniques viz., antigen coated plate ELISA (APC ELISA) and dot ELISA for their detection at field level in china [13]. Similarly, dot ELISA and DAS ELISA were established with *Potato virus S* (PVS) MAbs, which were detected PVS in infected tissue crude extracts upto 1:10 240 and 1: 163 840 (w/v, g/ml) dilutions, respectively [14].

There are recombinant antibodies such as ScFv antibodies (single chain variable fragment antibodies) produced *in vitro* through recombinant DNA technology can be used to detect and discriminate strains of potyviruses. ScFv antibody fragments have been selected from a synthetic phage-antibody library by affinity selection with purified *Potato virus Y* ordinary strain (PVYO) as an antigen. Such antibodies detected 7 out of 9 isolates of PVYO whilst it did not detect 15 isolates from the closely related necrotic strains PVYN and PVYNTN [15]. Anti-NIa scFv102 were produced against NIa of *Plum pox virus* which has the ability not only to detect PPV but also to detect several other potyviruses [16].

In general, preference is given to polyclonal antibodies for detection of plant viruses, because their production is simple and inexpensive. However, sometimes monoclonal antibodies are required to differentiate some of the closest virus strains. Based on the antigen and antibody interactions several techniques have been developed to detect plant viruses. However, some of the techniques like Enzyme linked immunosorbent assay (ELISA) and Tissue blot immunoassay (TBIA) are the most popularised in plant virus detection.

ELISA

It is a highly sensitive and widely preferred qualitative as well as quantitative diagnostic technique for plant virus detection. It was first

introduced and termed by Avrameas [17] and Voller et al, [18], respectively. The assay is cost effective, simple, sensitive and compatible for large scale screening of pathogens, and hence, it is advised to index plant viruses at field levels. Depending on the order of coating of antigen and antibodies into the 96 wells microplate, there are variations in ELISA. The direct and indirect ELISAs are frequently used in detection of plant viruses [19-21]. Coating or binding of antigen or antibody on to the polystyrene plate is purely hydrophobic interaction. In most of the studies carbonate buffer (100 mM) pH 9.6 was used as a coating buffer which favours the hydrophobic interactions between antigen or antibody to polystyrene plate. In the final stage of the ELISA, the enzyme alkaline phosphatase (ALP) hydrolyzes *para*-nitrophenylphosphate and release p-nitrophenol and inorganic phosphate (pi). The p-nitrophenol (p-nitrophenolate) at alkaline pH (pH>8) gives yellow colour with maximum absorption at 405 nm (E_{max}=405 nm). Based on the colour development in the well of the microplate, one can detect the pathogen of interest using ELISA reader.

Variations of ELISA:

In ELISA, antigen is detected either by primary (direct ELISA) or secondary antibodies (indirect ELISA). The primary antibodies are raised against the antigen of interest in one animal for example rabbit, whereas the secondary antibodies are resulted by immunizing a goat or sheep with rabbit antibodies (Immunoglobulins). The primary antibodies are specific to antigen and the secondary antibodies universal which are specific to Fc region of the primary antibodies. There are two main variations of ELISAs namely antigen coated ELISA or antibody coated ELISA depending on the coating of antigen or antibody on ELISA microplate. If the antigen is detected directly by enzyme conjugated antigen specific antibody (primary antibody) named as direct ELISA, whereas the same is detected by enzyme conjugated universal secondary antibody designated as indirect ELISA.

Antigen coated ELISA.

It is also known as direct antigen coated ELISA (DAC ELISA). In this method first the antigen to be diagnosed is allowed to bind to ELISA plate and in the next step the enzyme conjugated primary antibodies binds to the antigen (Fig 2A). The antigen detection is made through the primary antibody, and hence, it is designated as direct DAC ELISA. However, difficulties are associated to produce enzyme conjugated primary antibodies to each virus. In order to overcome this difficulty, enzyme labelled secondary antibodies are used to detect the antigen via binding the primary antibodies (indirect DAC ELISA; Fig 2B).

Antibody coated ELISA.

Antibody sandwich ELISA

In this method, initially ELISA microplate is coated with primary antibodies and in the nest step these antibodies capture the antigen to be diagnosed. In the third step, primary antibodies bind to the entrapped antigen, and hence, the antigen became sandwiched between the two antibodies. There are two type of sandwich ELISAs namely double antibody sandwich ELISA (DAS ELISA) and triple antibody sandwich ELISA (TAS ELISA). First type is direct ELISA, where the antigen is directly detecting by the enzyme labelled antigen specific antibodies, whereas the latter one is indirect ELISA, because the enzyme conjugated secondary antibodies detects the antigen (Fig 2C&D).

In order to produce *in vitro* regenerated virus free potato plants, DAS ELISA was optimized to detect about seven viruses including PLRV, PVA, PVY, PVV, PVM, PVS and PVX. The technique was detected the above viruses even in asymptomatic plants [22]. ELISA variation like DAC, DAS- and TAS-ELISAs have used to detect *Banana bract mosaic virus* (BBrMV) in *Musa* sp. using with either polyclonal or MAbs in the Philippines, Australia and India [23-25].

Protein A sandwich ELISA

Protein A sandwich ELISA (PAS ELISA) is similar to indirect ELISA; however, antigen detection is confirmed not by the enzyme conjugated primary or secondary antibodies, it is by enzyme conjugated protein A. Protein A is a cell wall protein of staphylococcus *aureus* with a molecular weight ranges between 42 and 56 kD has the ability to bind Fc portion of the immunoglobulin, and hence, used for detection of pathogens especially in ELISA [26]. In PAS ELISA, initially the plate is coated with protein A followed by primary antibody, antigen, primary antibody, enzyme conjugated protein A and substrate (Fig 2E). PAS ELISA is more sensitive than DAS ELISA.

Immunochromatographic assay (ICA)

Another name of this technique is lateral flow immune assay (LFIA) is one simple, robust, and cost-effective immunodiagnostic technique for plant virus detection. The device of the technique is divided into five different segments including sample pad, conjugated pad, test line, control line and absorbent pad. All these segments are connected to each other and convenient to pass liquid sample from first segment to last segment of the device or strip (Fig 2). When a strip is dip into the plant extract is being analysed, the extract moves from sample pad to absorbent pad by the action of capillary flow and

initiates sequential immunochemical reactions resulting coloration at test and reference lines. The virus specific antibodies (conjugated with colored colloidal gold particles, fluorescent dyes) on the conjugate pad interact with the viral proteins of the plant extract and move towards the test and reference lines where they interact with the immobilized antibodies and generates visible colour signals. If colour develops on both test and reference lines indicates the presence of virus of interest whereas only at reference line absence of virus. The test is also developed for detection of multiple viruses in a single attempt which saves time and cost of the experiment. Byzova et al. [27] developed express ICA test strip and simultaneous detected viruses of different shapes such as rod shaped (*Tobacco mosaic virus*; TMV), spherical (*Carnation mottle virus*, *Bean mild mosaic virus*) and filamentous (*Potato virus Y* and *X*) viruses. Similarly, in India, CPRI was developed ICA test device for detection of five viruses of potato including PVX, PVY, PVA, PVS and PVM [28]. Recently, the same approach was used to detect Grapevine leaf roll-associated virus 3 (GLRaV-3) [29], TMV and CMV [30]. There are companies such as Agdia Inc. (United States), Bioreba AG (Switzerland), and LOEWE Biochemica GmbH (Germany) commercially provide ICA test strips for detection of plant viruses.

Immunoblotting methods:

These methods are like indirect ELISA, samples to be tested for antigen detection are blotted onto the nitrocellulose or nylon membranes and allowed to react with specific antibodies followed by enzyme conjugated secondary antibodies and substrate for their confirmation. It develops colour on the membrane at the place where the antigen is located or entrapped. All these methods are qualitative only and further not require any reader for antigen detection. Based on the application of antigen on the membranes these methods are divided into three categories: 1. Western blot immunoassay, 2. Tissue blot immunoassay (TBIA) and 3. Dot blot immunoassay (DBIA).

Western blot immunoassay.

Usually, this method is widely used in characterization of viral proteins rather than large scale detection of plant viruses. In this method of detection, initially the infected plant leaf extracts or pure viral proteins are resolved on SDS PAGE and later they were blotted on to the nitrocellulose membrane. The immobilized antigens are allowed to react with primary as well as enzyme linked secondary antibodies and detected by a chromogenic substrate reaction**.** Usually, the antibody conjugated enzymes are alkaline phosphatase (ALP) or horse radish peroxidase (HRP). It is time consuming and labour-intensive approach and further incompatible for large scale screening for virus detection. In characterization of *Sorghum mosaic virus* (SrMV), SDS–PAGE and western immunoblotting indicated that the virus coat protein consisted of multiple copies of a peptide with a molecular weight of 35.68 kDa (31)

Tissue blot immunoassay (TBIA).

This method shows greater benefit over ELISA in terms of detection time, cost, sensitivity, and convenience. TBIA can be used to detect virus antigen in plant tissues such as leaf, stem, bulb, tuber, root and fruit or insect vectors [32]. To detect a virus of interest, suspected plant tissues are cut with a razor blade and pressed on to the nitrocellulose membrane (NCM), sap of the tissue contain viral particles blot on NC membrane which further detected by virus specific antibodies as similar in ELISA. This technique is simple, inexpensive, and highly suitable to field application for virus detection. The International centre for agriculture research in the dry areas (ICARDA) is commercially providing TBIA kits for detection of various viruses infecting legume crops including *Cucumber mosaic virus* (CMV), *Alfalfa mosaic virus* (AMV), *Bean yellow mosaic virus* (BYMV), *Chickpea chlorotic stunt virus* (CpCDV), *Pea seedborne mosaic virus* (PSbMV), *Beal leaf roll virus* (BLRV), *Soybean dwarf virus* (SbDV) [33-36].

Dot blot immunobinding assay (DBIA).

This blotting assay is also like ELISA requires antigen specific antibodies for correct detection of plant virus. In this method, initially the crude plant extract or the virus antigens spot as small dots on NC or nylon membrane and the remaining membrane is blocked with a neutral protein solution. After a wash with tris-buffer, the membrane is sequentially dip in the primary (specific) and secondary enzyme conjugated antibody buffers and finally the substrate develops colour if antibodies react with antigens of the dots. The technique can be used to discriminate the virus strains. In china, of the four strains of *Barley yellow dwarf virus* (BYDV), the BYVD GAV was detected in wheat and viruliferous aphid vectors with monoclonal antibodies [13]. DBIA was eight times more sensitive for detection of *potato virus X potyvirus* (PVX) and four times more sensitive for detection of *potato virus S Carlavirus* (PVS) and *potato virus Y potyvirus* (PVY) than DAS-ELISA [37].

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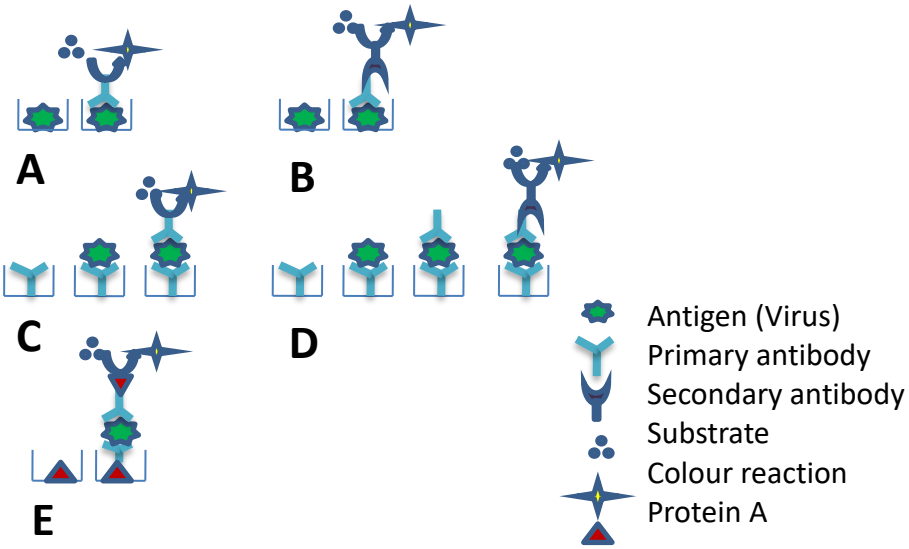

Fig 1. Variations of ELISA for virus detection

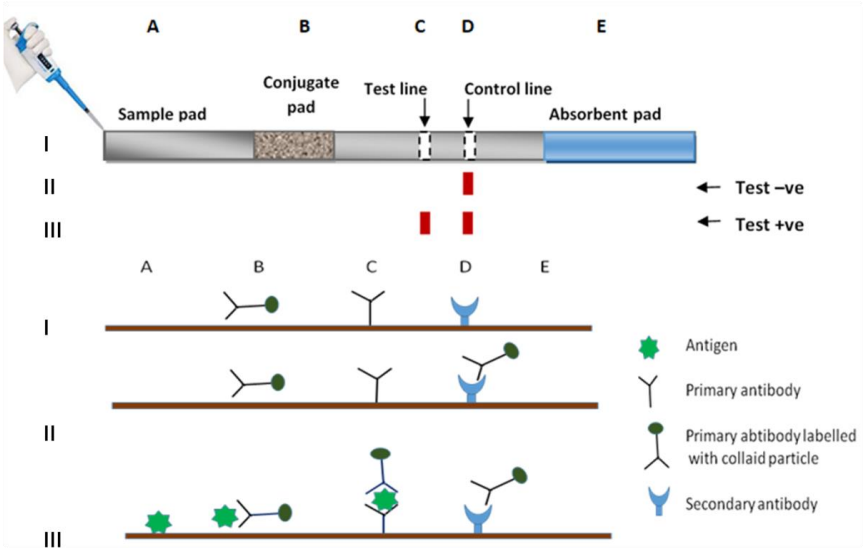

Fig 2. Immunochromatographic assay: I. ICA strip is divided into five segments (A-E), where A- sample pad, B-conjugate pad (colloid labelled primary antibodies), C-test line (antigen specific antibodies), D -control line (secondary antibodies) and E-absorbent pad. II. Test negative (colour developed only in control): In absence of antigen, the colloid labelled primary antibodies unable to bind to primary antibody at test line (no colour) but these are entrapped by secondary antibodies at control line (colour). III. Test positive (colour developed both in test and control): Presence of antigen, the colloid labelled antibodies bind to antigen and this complex react with test line antibodies (colour), the excess or unbounded colloid labelled antibodies bind to secondary antibodies at control line (colour).

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