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VIRAL AND VIROID DISEASES

**Nario Kusano · Keita Hirashima · Minoru Kuwahara
Kenji Narahara · Tadashi Imamura · Tomohiro Mimori
Ken Nakahira · Kuniaki Torii**

Immunochromatographic assay for simple and rapid detection of *Satsuma dwarf virus* and related viruses using monoclonal antibodies

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Abstract A simple and rapid immunochromatographic assay (ICA) to detect *Satsuma dwarf virus* (SDV) was developed using colloidal gold conjugates of anti-SDV monoclonal antibodies. Of six homogenization buffers tested, 0.1 M citrate buffer (pH 7.0) gave the best results for the ICA. In the ICA, addition of 0.1% thioglycolic acid in the homogenization buffers that have been widely used in enzyme-linked immunosorbent assays (ELISA) was deleterious to the reaction because of undesirable coagulation of the colloidal gold. ICA using the anti-SDV monoclonal antibodies was 8 times and 16 times more sensitive than double antibody sandwich-ELISA and ICA using the anti-SDV polyclonal antibody, respectively. The analysis is complete in only 15 min. Furthermore, ICA using the anti-SDV monoclonal antibodies could also detect SDV-related viruses.

Key words *Satsuma dwarf virus* · Detection · Immunochromatographic assay · ELISA

Introduction

Satsuma dwarf virus (SDV), a graft-transmissible pathogen of citrus trees, causes Satsuma dwarf disease, which reduces tree vigor and fruit yield in Japan (Yamada and Sawamura 1952). This disease is characterized by a dwarfed tree, boat-shaped or downward-cupped leaves, and fruit with lower sugar content and higher juice acidity than uninfected fruit (Shimizu et al. 2004). Based on field observations, SDV is transmitted through a soil source, but no vector has been identified (Koizumi et al. 1988; Katagi and Ushiyama 1990; Iwanami and Koizumi 2000).

N. Kusano (✉) · K. Hirashima · M. Kuwahara
Fukuoka Agricultural Research Center, 587 Yoshiki, Chikushino, Fukuoka 818-8549, Japan
Tel. +81-94-372-2243; Fax +81-94-372-4660
e-mail: nkusano@farc.pref.fukuoka.jp

K. Narahara · T. Imamura · T. Mimori · K. Nakahira · K. Torii
Mizuho Medy Co., Ltd., Tosu, Japan

During the 1960s, a group of SDV-like viruses, citrus mosaic virus (CiMV), natsudaidai dwarf virus (NDV), and navel orange infectious mottling virus (NIMV) were also reported in Japan. Based on a comparison of symptoms on various citrus plants and herbaceous indexing plants, these viruses seemed to be related to SDV and were thus classified as SDV-related viruses (SDV-RVs).

Recently, novel strains of SDV-RVs (e.g., Az-1, LB-1) were reported in Japan. Iwanami et al. (1993, 2001) suggested an alternative classification for SDV-RVs based on serology, nucleic acid sequence, and the symptomatology of citrus and herbaceous plants. Iwanami suggested that SDV-RVs could be classified into three groups, SDV (S-58, MIE-88), CiMV (Ci-968, LB-1, Az-1, ND-1), and NIMV (NI-1). Based on serological relationships, viruses of the CiMV group are relatively close to the SDV group, while NIMV is a distinct virus.

Satsuma dwarf disease has usually been diagnosed using an enzyme-linked immunosorbent assay (ELISA), although ELISA needs expensive equipment such as a microplate reader, uses many reagents, and is time consuming. Therefore, we focused our attention on developing rapid, simple detection methods for SDV. A rapid immunofilter paper assay (RIPA) has been developed and is often used to detect plant viruses (Tsuda et al. 1992; Ohki and Kameya-Iwaki 1996). Although we tried this method for SDV, we could barely detect the virus in 1:10 diluted extracts of SDV-infected plants. An immunochromatographic assay (ICA) was also developed to detect pathogens of humans such as hepatitis B virus, influenza virus, and others (Sato et al. 1996; Vaughn et al. 1998; Guan et al. 2004; Weinberg and Walker 2005). Recently, ICA has been used in plant detection devices, the Pocket Diagnostic (Central Science Laboratory, York, UK) and ImmunoStrip (Agdia, Elkhart, IN, USA), for viruses of vegetable and ornamental plants. ICA is a very simple and rapid test with a one-step sample application, as opposed to ELISA with multiple steps such as washing, incubation, and secondary color development. Therefore, we developed a new system and a versatile plastic assay device for ICA for plant viruses, and we report here on the first application of the ICA system in detecting SDV in the field.

Materials and methods

Virus isolates and antibodies

SDV and SDV-RV isolates were collected in Japan and maintained by grafting on trifoliate orange or rough lemon rootstocks under greenhouse conditions. Three isolates (MH, 97032YO, and HV) were supplied by Dr. T. Ito (National Institute of Fruit Tree Science), two isolates (HS-1 and AZ-1) were supplied by Dr. T. Iwanami (National Agricultural Research Center for Kyushu and Okinawa Regions), one isolate (SW-41) was supplied by K. Shimazu (Wakayama Research Center of Agriculture, Forestry, and Fisheries), and four isolates (CS-1, Ku-1, SA-7, and Cik-2) were collected in Fukuoka Prefecture. Three clones of monoclonal antibodies (Mabs) that recognize a different epitope on SDV (Hirashima et al. 1994) and a polyclonal antibody (Pab) (ELISA kit; Japan Plant Protection Association; JPPA) against SDV were used. The Pab against SDV (S-58) was supplied by Dr. Iwanami (National Agricultural Research Center for Kyushu and Okinawa Regions).

Double antibody sandwich enzyme-linked immunosorbent assay

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using an ELISA kit for SDV (JPPA) was performed according to the manufacturer's instructions with some modifications. The test samples were prepared from young shoots of infected citrus in mid-April and at the end of July. For the Mab, the biotinylated conjugate was modified and DAS-ELISA was carried out as in a previous report (Kusano and Shimomura 1995).

Immunochromatographic assay (ICA) and homogenization buffers

The underlying principle and the test device for our ICA system are illustrated in Figs. 1A,B and 2 respectively. The immunochromatographic devices were prepared as follows (Fig. 4): 1 mg/ml of anti-SDV monoclonal antibody (i.e., 1 µl/cm) was deposited onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) to make a test line. Similarly, 0.125 mg/ml of casein fluorescein isothiocyanate (FITC C0528, Sigma, St. Louis, MO, USA) was deposited to make a reference line. The membrane was then soaked in 0.5% casein (Kishida, Osaka, Japan). Finally, the membranes were dried at 50°C to finish the antibody-conjugated membrane.

The colloidal gold solution was prepared by reduction of tetrachloroauric acid with trisodium citrate (Frens 1973). The pH of the colloidal gold solution was adjusted to 7.0 with 0.2 M sodium carbonate buffer. Anti-SDV monoclonal antibody was added to the colloidal gold solution for a final concentration of 7.3 µg/ml. The mixture was incubated at room temperature with gentle stirring, and 1% bovine serum albumin (BSA) was added. The preparation was centrifuged at 12 000 rpm for 15 min, and finally the supernatant was removed to make anti-SDV monoclonal antibody–colloidal gold conjugate for the test line. Anti-FITC monoclonal antibody was conjugated as mentioned to make the anti-FITC monoclonal antibody–colloidal gold conjugate for the reference line.

The anti-SDV monoclonal antibody–colloidal gold conjugate and anti-FITC monoclonal antibody–colloidal gold conjugate were mixed at a concentration of optical density at 520 nm of 0.3 and 0.6, respectively. The preparation was deposited at 6 µl/mm onto glass fiber (Whatman, Middlesex, UK). Then the prepared glass fiber was dried in a vacuumed desiccator to make a conjugate pad.

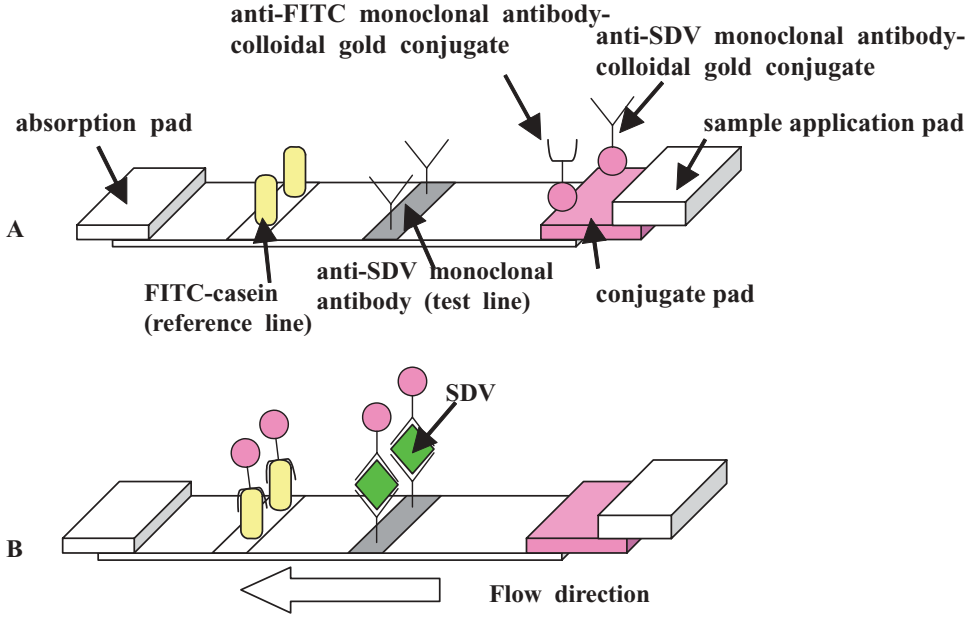

Fig. 1A, B. Principle of immunochromatographic assay (ICA) for *Satsuma dwarf virus* (SDV). **A** Prepared device before sample application, **B** prepared device after application of extract from plant infected with SDV. *FITC*, fluorescein isothiocyanate

Filter paper (the adsorption pad), the membrane, the conjugate pad, and glass fiber (the sample application pad) were assembled on an adhesive plastic sheet. The sheet was cut into 6-mm wide strips with a hand cutter, and every strip was inserted into a plastic device (Fig. 2). Finally, the assembled devices were sealed with a desiccant in moisture-resistant protective foil.

Six homogenization buffers for ICA were prepared: 0.1 M citrate buffer pH 7.0 (0.1 M CB), 0.01 M CB, 0.1 M tris buffer pH 7.5 (0.1 M TE), 0.01 M TE, 0.1 M phosphate-buffered saline pH 7.4 (PBS), and 0.02 M PBS. Effects of thioglycolic acid, which is often added to the homogenization buffer in ELISA, on ICA were tested. ICA was simply performed by the addition of 100 µl of supernatant of plant extract directly onto the sample application pad. After the sample application, the result was read by visual inspection for staining of the test and reference lines. A positive result was indicated by a typical purple line, which is produced when virus particles are trapped on the test line. The reference line ensures that the test result is valid. The intensity of any line was scored as negative (Fig. 3A), weak positive (Fig. 3B), moderate positive (Fig. 3C), or strong positive (Fig. 3D).

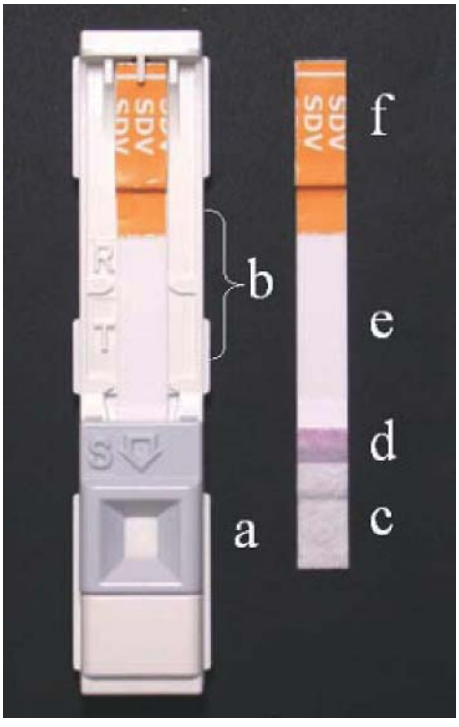

Fig. 2. The immunochromatographic assay system for *Satsuma dwarf virus* (SDV). Composite assay strip (*right*) attached to a plastic assay device (*left*). The assay device has a square sample application pad (*a*) and a test (*T*) and a reference (*R*) area (*b*). The assay strip consists of a sample application pad (*c*), a conjugate pad (*d*) containing dried detection reagent, a detection strip (*e*) with distinct test and reference lines, and an absorption pad (*f*)

Results

Comparison of the sensitivities of ICA and DAS-ELISA

Out of the three combinations of using three Mabs for ICA, clone 2G2 and clone 4E6, which had a different paratope group (PG), were more sensitive and suitable to the colloidal gold conjugate and coating antibody on the nitrocellulose strip for detection of SDV, respectively (data not shown). To determine which of the six homogenization buffers was the most suitable for ICA, the ICA results using four different virus isolates and the buffers were compared. Of the six buffers, 0.1 M CB gave the strongest, most stable staining (Table 1). When using 0.1% thioglycolic acid in the homogenization buffers, none of the virus isolates produced any positive test lines and the intensities of all reference lines were very weak. Sensitivities of ICA and DAS-ELISA were compared using young shoots from an SDV-infected tree at the end of July (Table 2). ICA using Mabs remained sensitive up to a 1:640 dilution, while DAS-ELISA remained sensitive to a dilution of 1:80, indicating that ICA using Mabs is eight times more sensitive than DAS-ELISA.

Comparison of sensitivity of monoclonal and polyclonal antibodies in ICA

The amount of colloidal gold conjugate in ICA using Pab was optimized for the highest sensitivity (Table 3, cases 3-1, 3-2, 3-3). Suspensions of colloidal gold conjugate at various concentrations were applied to the reagent pad, and ICA

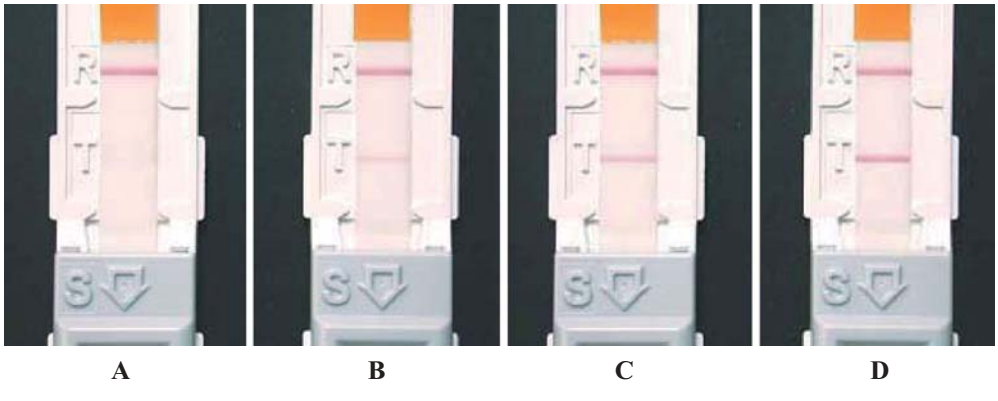

Fig. 3A–D. Reaction results in a representative immunochromatographic assay. **A** Negative (–), **B** weak positive (±), **C** moderate positive (+), **D** strong positive (++)

Table 1. Comparison of homogenization buffers for immunochromatographic assay (ICA) with monoclonal antibodies for detecting *Satsuma dwarf virus* (SDV) in extract of young shoots of citrus sampled in mid-April

Buffers	Isolates			
	SDV		SDV-RV	
	CS-1	Ku-1	AZ-1	MH
Water	++[a]	++	±	–
0.1 M CB	++	++	++	++
0.01 M CB	++	++	+	++
0.1 M TE	++	++	±	+
0.01 M TE	++	++	±	++
0.1 M PBS	+	+	–	±
0.02 M PBS	++	++	±	±

Plant extract was prepared at 1:10 dilution
CB, Citrate buffer pH 7.0; TE, tris buffer pH 7.5; PBS, phosphate-buffered saline pH 7.4
[a] ICA reaction time: 15 min. Test line intensities: ++, strong (positive); +, moderate (positive); ±, weak (positive); –, negative

Table 2. Comparison of sensitivity of immunochromatographic assay (ICA) and DAS-ELISA for detecting *Satsuma dwarf virus* (SDV) in various dilutions of extracts of young shoots of citrus sampled at the end of July

	Dilution rate	ICA (Mab)	DAS-ELISA (JPPA kit)[a]
Noninfected	1:10	–[b]	–[b]
SDV infected	1:10	++	++
	1:20	++	++
	1:40	++	+
	1:80	++	+
	1:160	++	–
	1:320	+	–
	1:640	±	–
	1:1280	–	–
	1:2560	–	–

DAS-ELISA, Double antibody sandwich enzyme-linked immunosorbent assay; Mab, monoclonal antibody
[a] ELISA kit was supplied by Japan Plant Protection Association
[b] Test line intensities for ICA and color intensities for DAS-ELISA: ++, strong (positive); +, moderate (positive); ±, weak (positive); –, negative

Table 3. Comparison of monoclonal (Mab) and polyclonal (Pab) antibodies in an immunochromatography assay (ICA) to detect *Satsuma dwarf virus* (SDV) in extracts from young shoots of citrus sampled at the end of July

	Dilution rate	Case 1 ICA (Mab) OD_{520} = 0.305	Case 3-1 ICA (Pab[a]) OD_{520} = 0.208	Case 3-2 ICA (Pab[a]) OD_{520} = 0.416	Case 3-3 ICA (Pab[a]) OD_{520} = 0.833
Noninfected	1:10	–[b]	–	±	+
SDV infected	1:10	++	+	++	++
	1:20	++	+	++	++
	1:40	++	±	+	++
	1:80	++	–	+	++
	1:160	++	–	±	+
	1:320	+	–	±	+
	1:640	±	–	±	+
	1:1280	–	–	±	+
	1:2560	–	–	±	+

The immunochromatographic devices were prepared under the following conditions:
Case 3-1, 1 mg/ml of anti-SDV polyclonal antibody was deposited onto a nitrocellulose membrane to make a test line. The anti-SDV polyclonal antibody–colloidal gold conjugate suspension was prepared at a concentration of optical density at 520 nm (OD) of 0.208, and was deposited onto glass fiber; Case 3-2, The immunochromatographic device conditions were as described in Case 3-1, except that the concentration of optical density at 520 nm (OD) was 0.416; Case 3-3, The immunochromatographic device conditions were as described in Case 3-1, except that the concentration of optical density at 520 nm (OD) was 0.833; Case 1, The immunochromatographic device conditions were as described in Case 3-1, except that all the antibodies were monoclonal and the concentration of optical density at 520 nm (OD) was 0.305
[a] S-58 polyclonal antibody
[b] Test line intensities for ICA: ++, strong (positive); +, moderate (positive); ±, weak (positive); –, negative

was performed. The concentrations of the conjugate in cases 3-1, 3-2, and 3-3 were adjusted to give absorbances of 0.208, 0.416, and 0.833 at 520 nm, respectively. In case 3-1, the ICA detected SDV up to a dilution of 1:40 of the infected young shoot and was negative for the uninfected shoot, while ICA in cases 3-2 and 3-3 produced false-positive lines for the uninfected shoot. On the other hand, ICA using the Mabs detected SDV up to a dilution of 1:640 and produced no false-positive lines for the uninfected shoot. This indicated that the sensitivity of ICA using Mabs was 16 times higher than ICA with the Pab.

The effect of the concentration of the anti-SDV monoclonal antibody on the nitrocellulose membrane was also evaluated. From 0.25 to 2.0 mg/ml, the sensitivity reached a plateau at 1 mg/ml. Therefore, we selected 1 mg/ml as the optimum concentration (data not shown).

Reactivity of SDV and SDV-RV in ICA and DAS-ELISA

The reactivity of three isolates of SDV and seven isolates of SDV-RV in ICA with Mabs and in DAS-ELISA using the JPPA kit and Mabs was studied. The results showed that ICA is superior to the commercial JPPA kit and ELISA with Mabs (Table 4). ICA and DAS-ELISA gave strong

positive reactions and high ELISA values, respectively, for three SDV isolates, CS-1, Ku-1, and SW-41. ICA produced higher positive reactions for SDV-RV isolates compared with the JPPA SDV ELISA kit and ELISA with Mabs. ICA detected HS-1 and AZ-1, while the JPPA kit did not. Furthermore, ICA was completed within 15 min, while DAS-ELISA required about 2 days.

Discussion

The addition of 0.1% thioglycolic acid into the homogenization buffer is known to stabilize the reaction in ELISA. However, the reagent should not be used in the homogenization buffer for ICA because it caused an undesirable coagulation of the colloidal gold, leading to inaccurate results. In our ICA system, SDV(-RV) is also stable in the plant homogenate without thioglycolic acid for at least several weeks at 4°C or several years at –30°C (data not shown). Homogenization buffers without thioglycolic acid have been reported by Salomone and Roggero (2002) and Salomone et al. (2004), who tested homogenization buffers (PBS pH 7.2 containing 0.05% Tween-20, 2% polyvinylpyrrolidone, 0.5% Triton-X100, and 10 mM sodium *N,N*-diethyldithiocarbamate trihydrate for *Pepino mosaic virus*; PBS pH 7.2 containing 0.02% Tween-20, 2% polyvinylpyrrolidone, and 0.5% Triton-X100 for *Citrus tristeza virus*) in ICA. Of the six homogenization buffers tested, 0.1 M citrate buffer (pH 7.0) gave the best results.

Citrus tristeza virus in ICA using polyclonal antibody returned 5% false positives in the total test results when compared with ELISA; thus, this level was acceptable only as a screening method (Salomone et al. 2004). Porter et al.

Table 4. Reactivity of isolates of *Satsuma dwarf virus* (SDV) and SDV-related viruses (SDV-RV) in an immunochromatography assay (ICA) and in DAS-ELISA

Group and isolates	ICA(Mab)[a]	DAS-ELISA			
		JPPA kit[b]		Mab	
Satsuma dwarf virus					
CS-1	++[c]	++[c]	1.375[d]	++[c]	1.206[e]
Ku-1	++	++	1.291	++	1.167
SW-41	++	++	1.180	++	1.341
SDV-related viruses					
SA-7	++	+	0.235	+	0.338
Cik-2	++	+	0.226	+	0.315
HS-1	±	–	0.120	–	0.116
AZ-1	++	–	0.103	–	0.057
MH	++	±	0.132	–	0.103
97032Y0	++	+	0.202	±	0.186
HV	++	+	0.152	+	0.522
Healthy	–	–	0.061	–	0.076

Young shoots of infected citrus were sampled in mid-April. Plant extract was prepared at 1:10 dilution
[a] ICA reaction time 15 min
[b] SDV ELISA kit supplied by Japan Plant Protection Association
[c] ++, strong (positive); +, moderate (positive); ±, weak (positive); –, negative
[d] ELISA values: absorbance at 415 nm after 2 h of incubation with substrate. Values are the means of four wells
[e] ELISA values: absorbance at 415 nm after 0.5 h of incubation with substrate. Values are the means of four wells

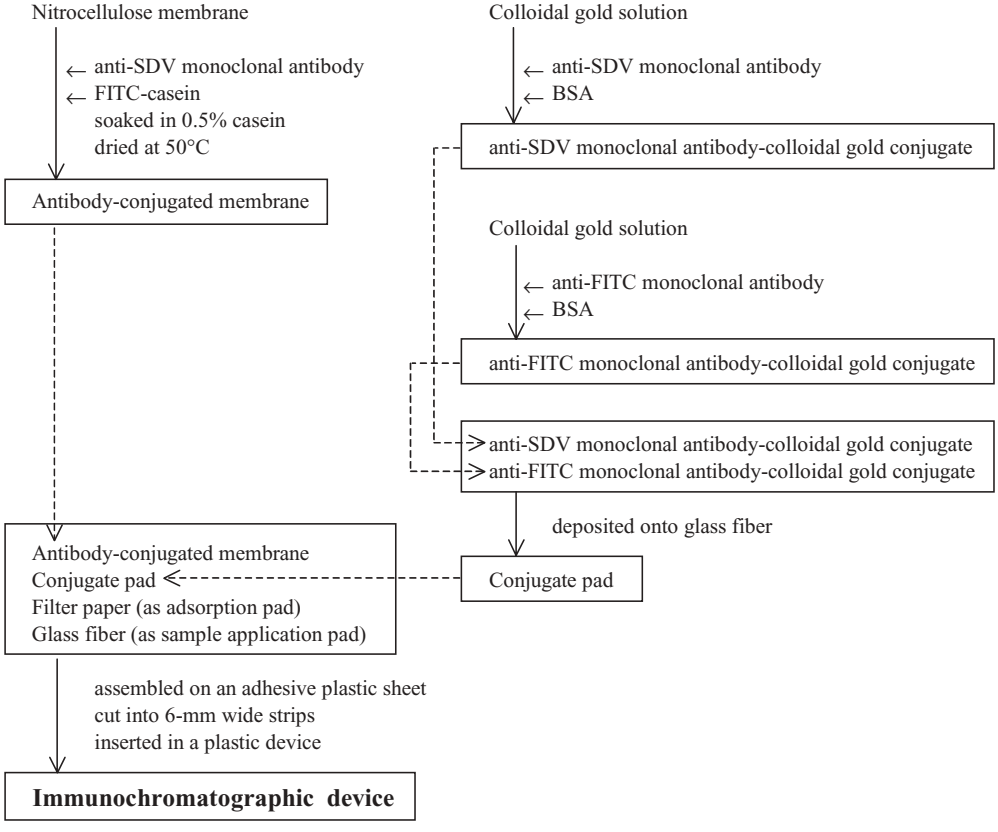

Fig. 4. Schematic chart of immunochromatographic device preparation. *BSA*, Bovine serum albumin

(1999) reported that ICA and ELISA were similar for sensitivity and specificity in the detection of dengue virus. Guan et al. (2004) developed ICA and ELISA to detect immunoglobulin G antibodies to severe acute respiratory syndrome (SARS) coronavirus in SARS patients with excellent correlation between ICA and ELISA.

We evaluated the sensitivity and specificity of ICA using ten virus isolates. CS-1, Ku-1, and SW-41 were shown serologically to belong to SDV by DAS-ELISA using Pab (Table 4). Iwanami et al. (1993) reported that AZ-1 belongs to the CiMV group. Furthermore, Ito et al. (2002) suggested that MH is a strain of NIMV, based on a sequence analysis of part of the coat protein region. Iwanami et al. (2001) indicated that SDV-related viruses (SDV-RV) could be classified as SDV, CiMV, or NIMV by sequence analysis. Ito et al. (2004) showed Hyuganatsu virus (HV) is a new SDV-RV by sequence analyses of the partial RNA-dependent RNA polymerase region in RNA 1 and the coat protein region in RNA 2 of the virus. Thus, the ten virus isolates used in this experiment are probably various types of SDV-RVs.

The higher sensitivity of ICA with Mabs over that with Pab or the ELISA kit (Tables 2, 3) for SDV is supported by previous results with different PG Mabs in ELISA, which was also more sensitive than with Pab to detect SDV (Hirashima et al. 1994). As for specificity, the reactivity of ICA was clearer than the ELISA kit for SDV-RV (seven virus isolates) detection and ELISA with the same combination of Mabs (Table 4). We think the 100% specificity (seven of seven samples) was the reason that ICA requires no washing process and that the complex of the colloidal gold conjugates and virus moves on the nitrocellulose strip by capillary action. ICA detecting SDV and SDV-RV offers advantages, such as the sensitivity and specificity, over ELISA.

In conclusion, ICA using Mabs is a simple, reliable, and rapid detection method for SDV and SDV-RV in citrus fields; results can be obtained within 15 min after dropping 100 µl of the sample homogenate on the nitrocellulose membrane.

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