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Comparison of an Immunochromatographic Assay Kit with DAS-ELISA for Large-Scale Diagnosis and Molecular Discrimination of Satsuma Dwarf Virus Collected from Citrus Orchards

Mitsuhiro Kato ⊙[1]*, Kenta Tomimura ⊙[2], and Kanako Ishii[1]

[1]*Shizuoka Prefectural Research Institute of Agriculture and Forestry, Fruit Tree Research Center, Mobata, Shimizu, Shizuoka 424-0101, Japan*

[2]*Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization (NARO), Okitsu, Shimizu, Shizuoka 424-0292, Japan*

Satsuma dwarf virus (SDV) seriously damages citrus production by reducing the quality and yield of fruit. To avoid contamination with SDV, mother trees are checked to be SDV-free in advance of nursery tree distribution. In this study, we compared an immunochromatographic assay (ICA) kit with double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for large-scale diagnosis of SDV in orchard-grown trees in Shizuoka Prefecture, Japan. The two methods gave conflicting results for 11 of 1,705 samples, all of which were negative by DAS-ELISA but positive by ICA. The samples scored as positive by either DAS-ELISA or ICA were analyzed by reverse transcription polymerase chain reaction and all were confirmed to be positive. These results validate the use of ICA as a screening method for large-scale diagnosis. Strain discrimination revealed that 16 of 22 isolates belonged to SDV, while citrus mosaic virus (CiMV) infection only and co-infection (SDV and CiMV) were in a minority.

*Corresponding author.
Phone) +81-54-376-6154 , FAX) +81-54-376-5186
E-mail) mitsuhiro1_kato@pref.shizuoka.lg.jp
ORCID
Mitsuhiro Kato
https://orcid.org/0000-0001-6061-9807
Kenta Tomimura
https://orcid.org/0000-0002-3718-4523

Satsuma dwarf virus (SDV) seriously damages citrus production by reducing fruit quality and yield. Because it is a graft-transmissible disease, SDV prevalence has increased through the use of infected rootstocks and scions. SDV has been found in citrus production fields in Japan, China, Korea, and Turkey (Azeri, 1973; Kim et al., 2001; Ushiyama and Ogaki, 1970; Zhou et al., 1990). SDV-infected satsuma mandarin (*Citrus unshiu* (Swingle) Marcow.) forms boat- or spoon-shaped leaves (Ushiyama and Ogaki, 1970); however, depending on citrus cultivar, disease symptoms may not appear even if the material is infected. Therefore, it is important to diagnose SDV infection to enable the distribution of virus-free nursery plants.

Nomura et al. (2000) reported SDV diagnosis of young shoots collected from citrus orchards in Shizuoka Prefecture, Japan, using a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Voller et al., 1976). However, DAS-ELISA requires laboratory conditions and special equipment, uses many reagents, and is very labor intensive and time consuming. An immunochromatographic assay (ICA) kit has been developed that enables simple and rapid assays (Kusano et al., 2007) and has since been widely used for SDV detection.

Recently, SDV (order Picornavirales, family Secoviridae, genus Sadwavirus) was reclassified as the only one species of Sadwavirus (Thompson et al., 2017). Citrus mosaic

virus (CiMV) and navel orange infectious mottling virus (NIMV) induce similar symptoms on satsuma mandarin (Tanaka and Yamada, 1972). However, CiMV and NIMV were not approved as separate species by the International Committee on Taxonomy of Viruses, but considered to be distantly related strains of SDV owing to their biological, serological and genomic properties (Fauquet et al., 2005).

CiMV is widely distributed in citrus production fields, mainly in Wakayama Prefecture in the mid-western part of Japan (Inuma and Tomimura, 2016). The distribution of SDV and related strains in other areas, in particular in eastern Japan including Shizuoka Prefecture, has not been investigated.

Here, we assessed the diagnostic value of ICA as an alternative to DAS-ELISA for the large-scale detection of SDV in a multi-year survey. We compared both screening methods with reverse transcription polymerase chain reaction (RT-PCR), which is the most sensitive detection method (Iwanami, 2010; Shimizu and Miyoshi , 2002; Shimomura and Noguchi , 2003). Using positive samples detected by ICA in this study, we also assessed the population diversity of SDV and related strains in Shizuoka Prefecture.

Young shoots were collected from 1,705 citrus trees (600 in 2011, 736 in 2012, and 369 in 2019) from commercial orchards in Shizuoka Prefecture, Japan. The tested citrus species and cultivars are listed in Table 1. Each sample was assayed by DAS-ELISA and ICA. The samples collected in 2012 and 2019 that were scored as positive by either of these methods were then tested by RT-PCR. DAS-ELISA was performed with a commercial antibody (Japan Plant Protection Association, Tokyo, Japan) according to the manufacturer's instructions and published methods (Clark and Adams, 1977; Takahashi, 1988). Briefly, all DAS-ELISA tests were carried out in 96-well microtiter plates (Thermo Fisher Scientific Inc., Roskilde, Denmark). Sample preparation for DAS-ELISA tests were carried out as follows. Three 5 mm stainless beads and 3 ml of 0.1 M sodium citrate buffer (pH 7.0) were previously prepared in 15 ml polypropylene tubes. Citrus leaf tissue (0.3 g) and 10 mm stainless beads were added. The leaf material was disrupted using a Shake Master (Bio Medical Science, Tokyo, Japan) with the disruption condition of 110 rpm, 2 min. After the disruption of citrus leaf tissue, the grinding fluid were removed into a 2 ml microtube, and centrifuged at approximately 15,000 ×g, 10 min, 10°C. The resultant supernatant were kept at 4°C until ELISA test.

Plates were coated with the antibody by incubating them with a solution (200 μl per well) containing 10 μg antibody/ml in carbonate buffer (pH 9.6) for 4 h at 30°C and then at 4°C overnight. Plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST) three times. The samples were added to individual wells; the plate was then incubated at 30°C for 2 h and afterward 4°C overnight. Wells were again washed three times with PBST. Alkaline phosphatase–conjugated antibody was then added to each well and the plate was incubated at 30°C for 4 h. After three washes with PBST, alkaline phosphatase substrate (p-nitrophenyl phosphate) at a concentration of 1 mg/ml in diethanolamine buffer (pH 9.8) was added to each well and the mixture was incubated for 1 h at room temperature. The optical density of each well was measured with a Sunrise Remote microplate reader (Wako Chemicals, Osaka, Japan) at 405 nm. A sample was scored as positive if its absorbance value was ≥2 times that of the healthy controls, and as weakly positive if it was 1.5-2 times that of the healthy controls.

ICA assays were carried out using an SDV chromato kit (Mizuho Medy Co., Ltd., Saga, Japan) (https://www.mizuho-m.co.jp/en/) according to the manufacturer's instructions. Each 0.1 g sample was homogenized in 500 μl of 0.1 M sodium citrate buffer (pH 7.0) containing 0.1% sodium azide with an automatic macerating machine (Ide et al., 2011). Then, 3 drops of the extract were applied to the test plate. After 15 min, positive bands were identified visually.

Total RNA was extracted from 0.05 g of young leaves using ISOGEN (Nippon Gene, Tokyo, Japan) and an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Young citrus leaves were ground with 1 ml ISOGEN solution or 450 μl Buffer RLT containing 1% β-mercaptoethanol using a mortar and pestle. RT-PCR for detection SDV species was carried out using a Qiagen OneStep RT-PCR kit (Qiagen). Amplification was carried out in a 15-μl total reaction volume with 1 μM forward and reverse primers (FW146 and RV488) (Iwanami, 2010) and 1 μl of RNA extract according to the manufacturer's protocol. Primer pair of FW146 and RV488 could detect all SDV species including CiMV and NIMV strains. Amplification conditions were as follows: reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 15 min, and 40 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 90 s, and a final extension at 72°C for 10 min. Amplification products were analyzed by electrophoresis on a 2% agarose gel for 1 h at 50 mA and visualized with GelRed (Biotium, Fremont, CA, USA).

RT-PCR for strain discrimination was carried out with strain-specific primers (Table 2) (Inuma and Tomimura, 2016) using a PrimeScript OneStep RT-PCR kit ver.2

Table 1. List of citrus variety and the number of trees used for SDV detection in this study

Sampling year	Citrus species or cultivar	No. of trees tested[a]
2011	Satsuma mandarin (*Citrus unshiu* (Swingle) Marcow.)	456 (58)
	Hyuga-natsu (*C. tamurana* hort. ex Tanaka)	22 (2)
	Shiranui [(*C. unshiu* (Swingle) Marcow. × *C. sinensis* L. Osbeck) × *C. reticulata* Blanco]	4
	Harumi [(*C. unshiu* (Swingle) Marcow. × *C. sinensis* L. Osbeck) × *C. reticulat*a Blanco]	1
	Unknown	117
	Sub-total	600 (60)
2012	Satsuma mandarin (*C. unshiu* (Swingle) Marcow.)	406 (46)
	Hyuga-natsu (*C. tamurana* hort. ex Tanaka)	21 (1)
	Konta (*Fortunella crassifolia* Swingle)	11
	Suruga Elegant (*C. natsudaidai*)	9
	Shiranui [(*C. unshiu* (Swingle) Marcow. × *C. sinensis* L. Osbeck) × *C. reticulata* Blanco]	1
	Unknown	288 (1)
	Sub-total	736 (48)
2019	Satsuma mandarin (*C. unshiu* (Swingle) Marcow.)	323 (22)
	Harumi [(*C. unshiu* (Swingle) Marcow. × *C. sinensis* L. Osbeck) × *C. reticulata* Blanco]	23
	Shiranui [(*C. unshiu* (Swingle) Marcow. × *C. sinensis* L. Osbeck) × *C. reticulata* Blanco]	12
	'Lisbon' lemons (*C. limon* L. Burm. f. cv. Lisbon)	3
	Kawano natsudaidai (*C. natsudaidai*)	2
	Setoka {[(*C. unshiu* (Swingle) Marcow. × *C. sinensis* L. Osbeck) × (*C. nobilis* Lour. × *C. deliciosa* Ten.)] × Murcott}	2
	Suruga Elegant (*C. natsudaidai*)	2
	Lemon (*Citrus* sp.)	1
	Ohta Ponkan (*C. reticulata* Blanco)	1
	Sub-total	369 (22)
Total		1,705 (130)

[a]Number of citrus trees judged satsuma dwarf virus (SDV) positive by double-antibody sandwich enzyme-linked immunosorbent assay and/or immunochromatographic assay are in parentheses.

(Takara, Shiga, Japan) according to the manufacturer's instructions. Amplification conditions were as follows: reverse transcription at 50°C for 30 min, initial denaturation at 94°C for 2 min, followed by 35 amplification cycles of denaturation at 94°C for 30 s, annealing at 60°C (SDV) or 58°C (CiMV and NIMV) for 30 s, and extension at 72°C for 90 s, and a final extension at 72°C for 10 min. Amplification products were separated by electrophoresis, and strains were identified by amplicon size. Strains SDV (S-58) and CiMV (NG2) were used as positive controls.

Among the 600 samples tested in 2011, 47 samples (7.8%) were scored as positive by both DAS-ELISA and ICA, and 540 (90.0%) were scored as negative by both methods (Table 3). Another 8 samples (1.3%) were scored as weakly positive by DAS-ELISA and positive by ICA.

The two methods gave conflicting results for 5 samples (0.8%), all of which were negative by DAS-ELISA but positive by ICA.

Among the 736 samples tested in 2012, 34 samples (4.6%) were scored as positive by both DAS-ELISA and ICA and 688 (93.5%) were scored as negative by both methods (Table 3). Another 9 samples (1.2%) were scored as weakly positive by DAS-ELISA and positive by ICA. The two methods gave conflicting results for 5 samples (0.7%), all of which were negative by DAS-ELISA but positive by ICA.

Among the 369 samples tested in 2019, 21 samples (5.7%) were scored as positive by both DAS-ELISA and ICA, and 347 (94.0%) were scored as negative by both methods (Table 3). The two methods gave conflicting re-

Table 2. Summary of primer combination and expected DNA band size in SDV detection by strain specific RT-PCR

Strain	Primer combination		Expected DNA band size[a]
	Forward primer	Reverse primer	
SDV	SDR2CPL1P	DT30	1,075
CiMV	CIR2CPL3P	DT30	1,623
NIMV	NIR2CPL1P	DT30	1,127

RT-PCR, reverse transcription polymerase chain reaction.
[a]Expected DNA band sizes were estimated from the sequences of satsuma dwarf virus (SDV; accession no. AB009959), citrus mosaic virus (CiMV; accession no. AB465581) and navel orange infectious mottling virus (NIMV; accession no. AB465583) strains (Iwanami, 2010).

Table 3. Comparison of detection performance using DAS-ELISA and ICA in satsuma dwarf virus

Year	ICA test result	No. of DAS-ELISA results (%)[a]		
		Positive	Weakly positive	Negative
2011	Positive	47 (7.8)	8 (1.3)	5 (0.8)
	Negative	0	0	540 (90.0)
2012	Positive	34 (4.6)	9 (1.2)	5 (0.7)
	Negative	0	0	688 (93.5)
2019	Positive	21 (5.7)	0	1 (0.3)
	Negative	0	0	347 (94.0)

[a]Percentage of citrus trees judged by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and immunochromatographic assay (ICA) are in parentheses.

sults for 1 sample (0.3%), which was negative by DAS-ELISA but positive by ICA.

Samples collected in 2012 and 2019 that were scored as positive by either DAS-ELISA or ICA were tested by RT-PCR (Table 4). All of the 48 such samples in 2012 and 22 in 2019 were positive by RT-PCR, including the 5 samples in 2012 and 1 sample in 2019 scored as negative by DAS-ELISA. We also tested 22 samples in 2019 that were scored as negative by ICA and found all of them to be also negative by RT-PCR.

Amplicons were detected in all the 22 SDV-positive samples from the ICA test in 2019 with RT-PCR primers FW146 and RV488, which could amplify all SDV species including CiMV and NIMV strains (Iwanami, 2010). Analysis of these 22 samples with primers specific for SDV, CiMV, or NIMV detected SDV in 16 samples, CiMV in 2 samples, and co-infection with SDV and CiMV in 1 sample (Fig. 1). NIMV was not detected (data not shown). In three samples, no virus strain was detected with any of the strain-specific primers; a common-sequence amplicon was directly sequenced using both amplification primers, and CiMV was detected in all of them. In a previous study by Iwanami et al. (2001), isolates that could not be char-

Table 4. Correlation between ICA and RT-PCR results in satsuma dwarf virus detection

Year	ICA test result	RT-PCR result[a]	
		Positive	Negative
2012	Positive	48	0
	Negative	-[b]	-[b]
2019	Positive	22	0
	Negative	0	22

[a]Samples positive by immunochromatographic assay (ICA) were analyzed by reverse transcription polymerase chain reaction (RT-PCR).
[b]Not tested.

acterized by biological properties would be identified as CiMV by analyzing sequence diversity. Considering the fact that we could not discriminate three samples when we used CiMV specific primer pair in this study and the report of Iwanami et al (2001), the diverse population of CiMV may reflect. The understanding of CiMV diversity would be revealed the demarcation of SDV species.

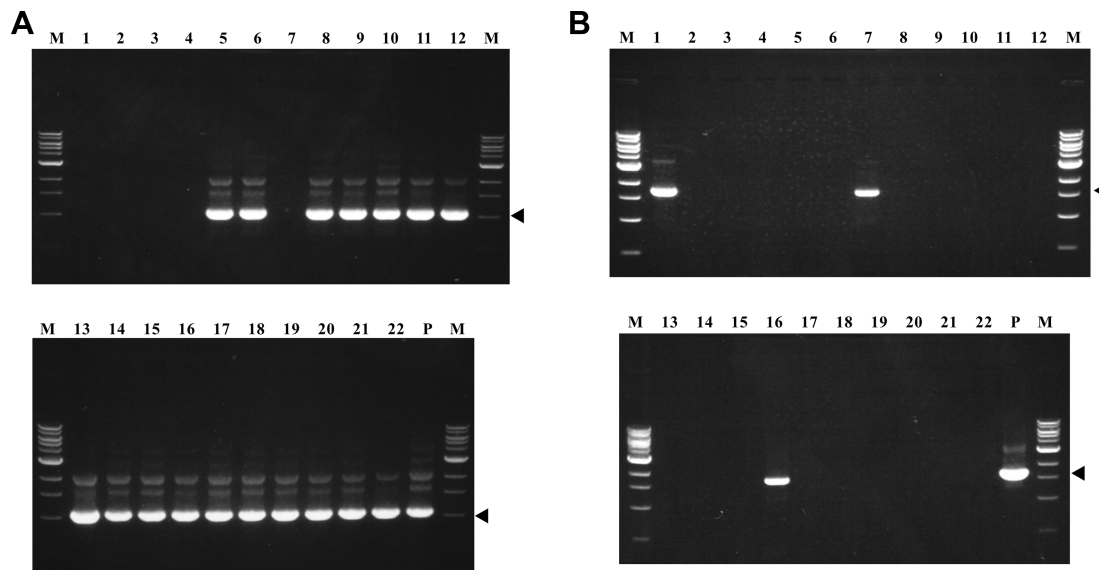Since ICA was launched commercially in 2008, it has

Fig. 1. PCR detection of (A) satsuma dwarf virus (SDV) and (B) citrus mosaic virus (CiMV) strains using specific primers. The 22 citrus samples collected in 2019 were detected both immunochromatographic assay and reverse transcription polymerase chain reaction (FW146 and nRV488 primer pair) test. Citrus isolates of S-58 and NG2 were positive control of SDV and CiMV strains, respectively. Expected amplified products are indicated by arrowheads. Lane M, 1 kb DNA ladder (New England Biolabs, Ipswich, MA, USA); lane P, PCR-positive control.

been widely used for SDV diagnosis in citrus orchards (Iwanami, 2010). The method is easy and rapid, and assays can be performed within the orchard. In this study, we verified the effectiveness of the ICA kit for conducting large-scale diagnosis of orchard-grown trees in Shizuoka Prefecture. The positive scores obtained by ICA in 2012 and 2019 were fully consistent with those obtained by RT-PCR analysis (Iwanami, 2010). In the present study, the sensitivity of ICA was higher than that of DAS-ELISA, which missed 11 positive cases. The relative difference in sensitivity (i.e., the percentage of cases negative by DAS-ELISA but positive by ICA) was 0.8% in 2011, 0.7% in 2012, and 0.1% in 2019. All the ICA-negative samples, which were collected in a region in which SDV is widespread, were also negative by RT-PCR analysis. Thus, the results of ICA were entirely consistent with those of RT-PCR.

Both DAS-ELISA and ICA have advantages and disadvantages. DAS-ELISA has been widely used for diagnosis of SDV in large-scale surveys, but it is time consuming. In the present study, when 736 samples were tested in 2012 by 5 people, it took 2 days for them to perform the assay by ICA, but 7 days to perform DAS-ELISA. The ICA kit is also easy to use in the field, but is more costly than DAS-ELISA: it costs about 6 USD per sample, whereas DAS-ELISA costs about 1 USD. Considering labor costs can be

considerably reduced by using the ICA kit, ICA would be useful as an alternative tool for the detection of SDV.

The specificity of the ICA kit is sufficient for the detection of various SDV strains (SDV, CiMV and NIMV) (Kusano et al., 2007). Thus, ICA can be used for large-scale surveys of complex populations of SDV species in citrus orchards. Here, we detected a population containing SDV and CiMV strains in Shizuoka Prefecture. Overall, it can be concluded that ICA is a useful tool for the diagnosis of SDV in large-scale surveys. Indeed, in Shizuoka Prefecture, ICA has been adopted for large-scale diagnosis since 2014 owing to its advantages, and is now widely used for routine SDV diagnosis.

This study revealed that the SDV strain is predominant in Shizuoka Prefecture; this population structure is different from that in Wakayama Prefecture, a major citrus production area, where CiMV is prevalent (Inuma and Tomimura, 2016). Most isolates tested positive by both ICA and RT-PCR were identified as SDV strains. In 3 samples, RT-PCR failed to detect SDV, CiMV, or NIMV, but direct sequencing using common RNA-2 sequences (Iwanami, 2010) detected CiMV in all 3 samples. These results suggest that CiMV is more diverse than SDV. To analyze CiMV diversity, more samples from citrus orchards need to be collected and analyzed. Interestingly, the 3 isolates identified as divergent CiMV were collected from the same

citrus orchard. The coexistence of SDV and CiMV strains in the same citrus orchard indicates that such orchards would be sources of dispersal of more than one SDV strain. Further precise analysis is expected to reveal interesting genetic diversity of SDV species, including the divergent CiMV strain.

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