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A double antibody sandwich enzyme-linked immunosorbent assay for detection of soft-shelled turtle iridovirus antigens

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A double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for detection of the soft-shelled turtle iridovirus (STIV) was developed using a specific monoclonal antibody (mAb) against STIV and anti-STIV rabbit serum. Using DAS-ELISA, the detection limit of STIV was found to be 10^3 PFU/ml. The positive rate of 15 STIV samples was 100%, while the positive rate of 100 other aquatic virus samples was 0%. These data show that DAS-ELISA is highly specific and sensitive for the detection of STIV. In clinical tests, 128 samples isolated from pond-reared turtles were subjected to DAS-ELISA and PCR. The overall agreement between the results obtained by DAS-ELISA and PCR was 98.4%. The results indicate that the DAS-ELISA method could be used for diagnosing diseases caused by STIV.

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1. Introduction

"Red neck disease" is common in soft-shelled turtles. It has a mortality rate exceeding 40% and is caused by an iridovirus which was isolated originally in 1998 (Chen et al., 1999). Electron microscopy showed that this is an enveloped virus with icosahedral symmetry has and a diameter of 120–160 nm. On the basis of histopathological and morphological evidence, the virus is characterized as a member of the *Iridoviridae* family and is designated as soft-shelled turtle iridovirus (STIV) (Chen et al., 1999).

The Eighth Report of the International Committee on Taxonomy of Virus (ICTV) subdivides the family *Iridoviridae* into five genera: *Iridovirus* and *Chloriridovirus*, which infected mainly invertebrates, and *Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus*, which infect cold-blooded vertebrates (Chinchar et al., 2005). Phylogenetic analysis based on the major caspid protein (MCP) is considered to be an appropriate method for differentiating different iridovirus strains and species (Bollinger et al., 1999). Multiple alignment and phylogenetic trees of various STIV MCPs (major capsid proteins) show that STIV belongs to the genus *Ranavirus* (Zhao et al., 2007). The complete sequence of the STIV genome has been described (GenBank accession no. EU627010). The STIV genome is 105,890 bp in length and contains 105 potential open reading frames (ORFs), which encode polypeptides ranging from 40 to 1294 amino acids and 20 microRNA candidates. Comparative genomic analysis shows that STIV has a colinear arrangement of genes similar to that of frog virus 3 (FV3), which is a member of the *Ranavirus* (Huang et al., 2009).

STIV can cause cytopathic effects (CPE) at 15–30 °C in grass carp ovary (GCO) cells, fathead minnow (FHM) cells, carp kidney (CK) cells, and grass carp kidney (GCK) cells. Artificial infection showed that the mortality rate of turtles infected with STIV inoculated by injection was greater than 40% (Chen et al., 1999). Only a PCR-based method is available for the detection of STIV (Zhao et al., 2007). However, conventional PCR is neither sufficiently accurate nor sufficiently quantitative for routine diagnosis (Yue et al., 2008). The aim of this study was to develop a double antibody sandwich ELISA (DAS-ELISA) method for detection of STIV. Monoclonal antibodies against STIV were produced and used to develop the DAS-ELISA, which may prove to be useful for detecting STIV antigens in clinical samples.

2. Materials and methods

2.1. Virus strains

Nine virus strains were used in this study, including bovine immunodeficiency virus (BIV), frog iridovirus SSS0604 strain, soft-shelled turtle iridovirus (STIV) 9701 strain, spring viraemia of carp virus (SVCV), viral hemorrhagic septicemia virus (VHSV), infec-

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tious pancreatic necrosis virus (IPNV), grass carp hemorrhage virus (GCHV), infectious hematopoietic necrosis virus (IHNV) and viral nervous necrosis virus (VNNV), all of which were provided by the Shenzhen Exit-Entry Inspection and Quarantine Bureau. Grass carp gland cell line (CO), mouse myeloma cells SP2/0, and mouse anti-STIV monoclonal antibody cell lines 2-F9, 2G12, 3A6, and 4F1 were also provided by the Shenzhen Exit-Entry Inspection and Quarantine Bureau. Eight-week-old Balb/C mice were purchased from the laboratory animal center of Sun Yat-Sen University.

2.2. Cell culture and purification of STIV

The grass carp gland cell line (CO) was maintained at 25 °C in medium 199 (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal calf serum to propagate STIV. Infected cell cultures were freeze-thawed when cytopathic effects (CPE) were observed. Cell debris was removed by centrifugation at 4000 × *g* for 30 min at 4 °C. The supernatant was then ultracentrifuged at 45,000 × *g* for 70 min at 4 °C with a Beckman SW41 rotor. The virus pellet was resuspended in PBS (1.4 M NaCl, 0.014 M KH_2PO4, and 0.08 M Na_2HPO_4) and further purified by discontinuous sucrose (20% and 60%, w/v) gradient centrifugation at 45,000 × *g* for 70 min at 4 °C. The virus particle band was carefully collected and resuspended in PBS. The sucrose was removed by centrifugation at 45,000 × *g* for 2 h. The purified virus was resuspended finally in PBS and stored at 4 °C until use.

2.3. Monoclonal antibody production

Four eight-week-old female Balb/C mice were immunized intraperitoneally with a mixture containing 100 μl purified STIV (containing 100 μg viral protein) and an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO). Two eight-week-old Balb/C mice were immunized with only 100 μl Freund's complete adjuvant (Sigma, St. Louis, MO), for serum to be used as blank controls.

On days 14, 28, and 42 after the initial injection, booster immunizations were administered with a double amount of antigen in Freund's incomplete adjuvant. Three days before hybridoma fusion, a final booster was administered by an intrasplenic injection to each mouse (except blank controls) with 100 μl purified virus (containing 200 μg viral protein) without adjuvant. Hybridoma fusion was performed using the method described earlier (Zhou et al., 2006). Antibodies produced in hybridoma supernatants were measured by indirect ELISA, using purified STIV (1:100 diluted in PBS) as an antigen and CO cell debris as negative control.

ELISA plates were coated overnight at 4 °C with 100 μl of purified STIV (concentration, 5 μg/ml) per well diluted in bicarbonate coating buffer (pH 9.6) and the wells of negative control were coated with CO cell debris. The ELISA plates were then blocked with 5% skim milk in PBS for 90 min at 37 °C. The wells were drained and incubated with 100 μl hybridoma supernatant per well for 90 min at 37 °C. After endogenous peroxidase activity was blocked with 1% H_2O_2 for 15 min at room temperature and after three washes with PBS-T containing 0.05% Tween-20, peroxidase-conjugated goat anti-mouse IgG (Fab specific, 1:10,000 diluted in PBS-T, SIGMA, USA) was added. The plates were incubated for 90 min at 37 °C. After washing again with PBS-T, a substrate solution (0.1 M citrate/phosphate buffer, pH 5.0; 0.04% OPD, and 0.14% H_2O_2) was applied for 5 min at room temperature. Reactions were stopped by adding 150 μl 2 M H_2SO_4 per well, and optical densities (ODs) were measured at 450 nm with a thermo microplate reader (Multiskan ascent, USA). Absorbance values twice higher than the background level reactivity on CO cell line debris were considered to be positive.

Positive hybridomas were cloned subsequently three times by limiting dilution with one cell distributed per well. They were then amplified and cultured until the cell concentration was greater than 5×10^5/ml. The hybridoma clones were screened by indirect ELISA by the method described above in order to select supernatants with the highest titer.

2.4. Indirect immunofluorescence assay (IFA) of cells infected with STIV

For indirect immunofluorescent staining, paraformaldehyde-fixed CO cells grown on glass coverslips were first incubated with the original mAbs, stained subsequently with FITC-conjugated anti-mouse IgG antibody (Sigma, USA) diluted 1:200 in PBS. After washing, the stained slides were mounted inbuffered glycerin and examined under a fluorescence microscope (Olympus IX81S1F3, Japan).

2.5. Production of anti-STIV rabbit serum

Two six-month-old female New Zealand White Rabbit (NZWR) were immunized intraperitoneally with a mixture containing 500 μl purified STIV (containing 500 μg viral protein) and an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO). On days 14, 28, and 42 after the initial injection, booster immunizations were administered with a double amount of antigen in Freund's incomplete adjuvant. A final booster was administered by an intrasplenic injection to each rabbit with 500 μl purified virus (containing 1 mg viral protein) without adjuvant. Three days later, the serum was collected and detected by indirect ELISA described in Section 2.3. The positive serum was used as a detection antibody for DAS-ELISA.

2.6. Indirect competitive double antibodies sandwich ELISA

The development and optimization of DAS-ELISA were carried out using the method described previously (Zhang et al., 2007; Wang et al., 2009). To develop a highly sensitive and specific DAS-ELISA, assay conditions needed to be optimized, including the concentration of coating mAb 2F9, dilution of anti-STIV rabbit serum and HRP-IgG, and so on. The assay conditions were optimized according to the checkerboard titration method (Lorenzen et al., 1990). The optimal concentration of the coating mAb 2F9 was found to be 5 μg/ml. The best dilution of anti-STIV rabbit serum was 1:5000 and a suitable dilution of anti-rabbit IgG-HRP was 1:5000.

In the indirect competitive ELISA procedure, the mAb 2F9 as capture antibody was first diluted with carbonate buffer (pH 9.8) to 5 μg/ml and distributed into the wells of a 96-well microtiter plate (100 μl/well). The plate was incubated at 37 °C for 1 h and then incubated at 4 °C overnight. After thrice washing with PBS-T, a blocking buffer (250 μl/well) was added to the plate for 4 h at 37 °C. This plate was stored at 4 °C until use. After the plate was washed using a method described before, standard solutions or samples (100 μl/well) were then added to the plate, which was subsequently incubated for 30 min at 37 °C. The plate was removed from the incubator, washed and then treated with rabbit anti-STIV (1:5000) at 100 μl/well. The plate was again incubated for 30 min at 37 °C. The plate was removed from the incubator, washed, and then treated with anti-rabbit IgG-HRP (1:5000 diluted in PBS-T, SIGMA, USA) at 100 μl/well. The plate was incubated once more for 30 min at 37 °C. The plate was again removed from the incubator and then treated with the substrate solution (200 μl/well). After incubation in the dark for approximately 10 min, a stopping solution (2 mol/l H_2SO_4, 50 μl/well) was added. The absorption was measured at 450 nm using a microplate reader, and calibration curves were constructed.

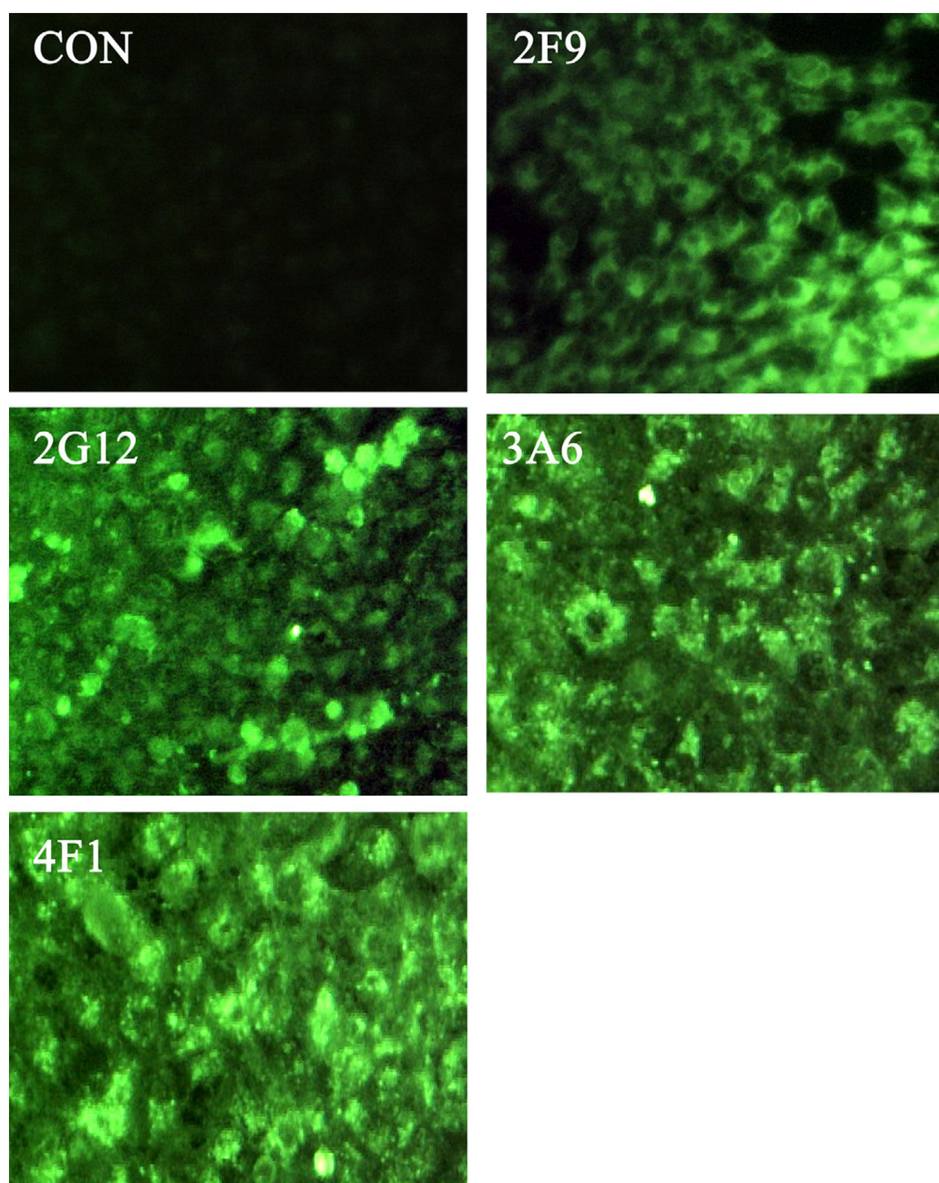

Fig. 1. Immunofluorescence staining of the CO cells infected with STIV with different mAbs. Con: uninfected CO cells. Photographs were taken at 24 h post-infection.

2.7. Detection limit and cross-reaction

To define negativity and positivity, 30 negative samples of uninfected CO cell lines described before were analyzed by DAS-ELISA. The means (X) and standard debytion (SD) of the absorbance values of the 30 samples were calculated, and the critical value ($X + 2SD$) of negative serums was used to define negativity and positivity.

Cell cultures infected with STIV and with complete CPE ($TCID_{50} = 10^{-6.5}$/0.1 ml, about 10^7 PFU/ml) were serially diluted 10-fold until a dilution of 1×10^{-6} was reached. The diluents were then detected by DAS-ELISA to define the detection limits.

The cross-reactivity (CR) of the mAb produced was also investigated. Fifteen positive samples from STIV viral suspensions and 80 other virus samples (the suspensions of BIV, FV, IHNV, SVCV, VHSV, IPNV, GCHV, and VNNV) were selected for testing. Twenty negative samples from non-infected (mock) cell debris were evaluated by the DAS-ELISA method.

2.8. Sample detection

A sample of 128 one-month-old turtle seedlings were collected from farms in Shen Zhen, China. Samples of the homogenate supernatants of turtle livers were screened by DAS-ELISA.

The samples were also subjected to tissue culture detection, which was the “gold standard” for iridovirus, and to PCR. Homogenate supernatants were transferred on to 24-h-old monolayers of CO cells overlaid with cell culture medium containing 10% fetal calf serum (FCS) and suitable buffer (HEPES [N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid]) in two dilutions, final dilutions of tissue material in cell culture medium were 1/100 and 1/1000 respectively. The ratio between inoculums size and volume of cell culture medium was 1:10. Each dilution was inoculated in two wells of 24-well cell culture tray. The samples without CPE were negative. The inoculated cell cultures examined for CPE were screened by PCR detection secondly, as CO cell line is not only sensitive with STIV.

Table 1
Antibody titers of hybridoma cell culture supernatant by indirect ELISA.

Hybridoma cell	Isotypes	Titer of supernatant[a]	Negative control[b]
2-F9	IgG2b	1:640	–
2G12	IgG2b	1:160	–
3A6	IgG2b	1:320	–
4F1	IgG2b	1:160	–

[a] The secondary antibody used in ELISA was horseradish peroxidase-conjugated goat anti-mouse IgG (Fab specific, 1:10,000 diluted in PBS-T, SIGMA, USA).
[b] The wells of negative control were coated by CO cell line debris.

PCR detection is the most common method for the detection of STIV. PCR was performed using the method described previously (Zhang et al., 2007). On the basis of the conserved sequence of STIV MCP, a pair of primers located at the 5′ and 3′ flanking region of the MCP was designed (forward: 5′-ATGTCTTCTGTA ACT GGT TCA-3′, reverse: 5′-TTACAAGAT TGGGAATCCC-3′). The viral DNA was extracted from samples using a Mini BEST Viral RNA/DNA Extraction Kit (TaKaRa, Japan). PCR was carried out with approximately 10 ng of the viral DNA template and 0.25 μl LA Taq (TaKaRa, Japan) each in a 25-μl reaction mixture under the following conditions: 35 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 2 min), and extension (72 °C for 1 min). The PCR results were used to evaluate the coincidence rate compared with the DAS-ELISA results.

3. Results

3.1. Hybridoma stability and mAb titer against STIV

Approximately 95% fusion rate (120 hybridoma cultures) was achieved in seeded wells. Four hybridomas (2-F9, 2G12, 3A6, and 4F1) producing antibodies against STIV were detected by indirect ELISA. A 96-well-ELISA plate was coated with purified STIV at 0.5 μg/well. The titers of hybridoma supernatants detected with indirect ELISA are shown in Table 1. All of the four mAbs were of the subclass IgG2b. The titers of hybridoma supernatants were in the range of 1:160–1:640. The 2-F9 mAb was selected for coating onto 96-well plates used in sandwich ELISA because its titer was higher compared to those of the others.

3.2. Immunostaining of CO cells infected with STIV

All of four mAbs showed positive reaction by IFA of virus infected CO cells. The specific fluorescence signals dispersed in the cytoplasm and granular structures around the peripheral zone of cells cytoplasm were observed. No fluorescence staining was observed in the control cells (Fig. 1).

3.3. Detection limit and cross-reaction

The mean (X) was 0.165, and the standard debytion (SD) was 0.021, so the critical value ($X+2SD$) was 0.228, which was used to define negativity and positivity.

After DAS-ELISA detection limit experiments, the values of 10^6–10^3 PFU/ml were above 0.228, and others were less than this (Fig. 2). Thus, the detection limit of DAS-ELISA was 10^3 PFU/ml.

The results of cross-reactivity (CR) of the mAb 2F9 with STIV and eight other viruses (BIV, FV, IHNV, SVCV, VHSV, IPNV, GCHV, and VNNV) in DAS-ELISA are shown in Fig. 3. Except for STIV, the mAb 2F9 did not cross-react with any other virus strain. These findings indicate that the present DAS-ELISA method has a high specificity for STIV.

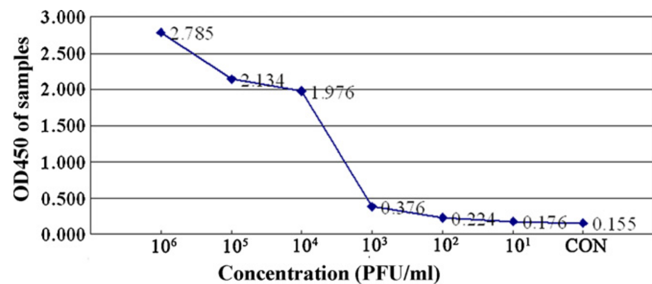

Fig. 2. Detection limit of double antibodies sandwich ELISA coated by mAb 2F9. CON was negative control of uninfected CO cell debris. The samples infected with STIV were diluted 10-fold to 10^6–10^1 PFU/ml serially. The values of 10^6–10^3 PFU/ml were above critical value 0.228, and two times higher than negative control (0.155). It showed the detection limit of DAS-ELISA was 10^3 PFU/ml.

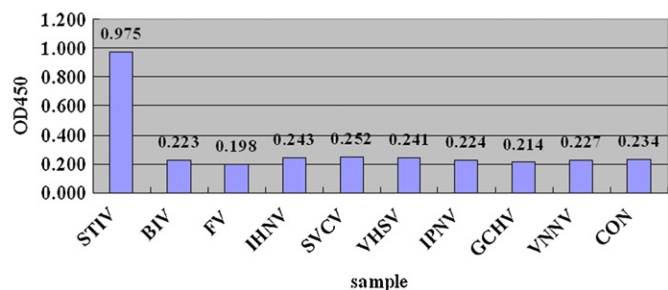

Fig. 3. Specificity of double antibodies sandwich ELISA coated by mAb 2F9. CON was negative control of uninfected CO cell debris. The average value of STIV samples was 0.975, above critical value and four times higher than the control's. The value of others samples were similar to control's. It indicated that the mAb 2F9 react with STIV specifically.

Table 2
Results of samples tested by DAS-ELISA and tissue culture-PCR.

	DAS-ELISA positive	DAS-ELISA negative	Total
Tissue culture-PCR positive	10[a]	0	10
Tissue culture-PCR negative	2[b]	116[b]	118
Total	12	116	128

[a] Positive on tissue culture.
[b] Negative on tissue culture.

3.4. Sample detection

A total of 128 collected clinical samples were screened by DAS-ELISA. Twelve positive samples and 116 negative samples were detected. On the other hand, tissue culture and PCR detected 10 positive samples and 118 negative samples respectively.

Ten STIV positive samples were both detected by DAS-ELISA and PCR. The correlation rate between two methods was 98.4% [(10 + 116)/128]. The data obtained by DAS-ELISA and PCR are shown in Table 2.

4. Discussion

STIV is the first Asian iridovirus isolated from reptiles, and the first iridovirus from *Tenodera sinensis* (Chen et al., 1999). It can lead to death of turtles. At present, a serological method that detects rapidly STIV is not yet available.

As with reptiles and poikilotherms, the immune systems of soft-shelled turtles are more highly developed than those of fish but lower than those of homeotherms (Borysenko and Cooper, 1972; Plant et al., 2009). They also have a high humoral immune response (Maung, 1963) but their ability to produce antibodies against pathogens is unstable and is affected by environmen-

tal temperatures and constitution (Coe, 1972; Cray et al., 2001; Munoz and De la Fuente, 2001). The soft-shelled turtle has different humoral immune responses to *Aeromonas hydrophilla* in different seasons (Jian et al., 1998). The titers of antibodies are higher during July to September, but no special antibodies are detected in spring and winter even after second immunization. These findings indicate that, in some seasons and environments, it is inaccurate to determine pathogens in turtles by examining their antibodies.

As a classical detection method, monoclonal antibody ELISA has been used in diagnosis of disease, antigen detection, and clinical diagnosis (Wolski et al., 1986; Zhou et al., 2006). Some studies have utilized the monoclonal antibodies to diagnose aquatic diseases, such as spring viremia of carp virus (Bing et al., 2007), lymphocystis disease virus (Cheng et al., 2006), infectious hematopoietic necrosis virus (Huang et al., 1994), and grouper iridovirus (Shi et al., 2003). The principle of DAS-ELISA involving monoclonal antibodies has been described by Engvall et al. (1971), the method was first used to detect viruses in 1976 (Michale and Richard, 1980; Voller et al., 1976). DAS-ELISA has been developed for the detection of many types of pathogens, such as the coronavirus associated with severe acute respiratory syndrome (SARS) (Chen et al., 2005), human immunodeficiency virus type 1/2 (HIV-1/2) (He et al., 2002), renal syndrome virus (Zhang et al., 2007), and influenza A virus (Watcharatanyatip et al., 2009). A DAS-ELISA detection method for the epizootic haematopoietic necrosis virus (EHNV), which is a member of the *Ranavirus* similar to STIV, has also been described (Zupanovic et al., 1998).

In this study, a DAS-ELISA for the detection of STIV was developed, including four monoclonal antibodies against STIV. This is the first report of the development of an mAbs against STIV. The 2F9 mAb, whose titer is the highest among all the samples, was selected for development of DAS-ELISA.

To obtain detection limits, serial dilution of known copies of virus standards is always required. However, the quantitation of a virus cannot be obtained by simply purifying STIV from tissue cultures or infected cells, since the purified viral proteins may contain host proteins which will not indicate the actual amount of SITV copies. As it is difficult to quantify standard samples by weight or copies in detection limit tests, the $TCID_{50}$ of virus stock solutions is measured to quantify the serial dilution. The detection limit of this ELISA was 10^3 PFU/ml. This result shows that the DAS-ELISA was sensitive to detecting very low concentrations of the STIV virus. Results from different test viruses showed that the DAS-ELISA had high specificity for STIV and had no cross-reaction with other types of virus.

Tissue culture is the “gold standard” for iridovirus detection (Chen et al., 1999). As CO cell line is not only sensitive with STIV, the results of tissue culture must be identified by other detection methods. PCR detection method is the most common method for the detection of STIV at present. In this study, the correlation rate between DAS-ELISA and PCR was 98.4%. The number of positive samples detected by DAS-ELISA was two more compared to that determined by the tissue culture and PCR method. It is possible that the STIV gene concentration was lower than the sensitivity of tissue culture or PCR, and two false negatives may have been detected. The DAS-ELISA method could also detect antigens of STIV. In other words, it could detect live viruses in samples. In contrast, PCR could only detect gene fragments. These results clearly suggest that the DAS-ELISA has high specificity and sensitivity, and produces more reliable results than PCR.

In summary, the DAS-ELISA method can detect STIV efficiently. It appears to be a better method for monitoring STIV in entry-exit inspections and quarantines.

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