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Research Article

Preparation of Polyclonal Antiserum to the Recombinant TiLV-S8 Protein and Its Application in the Detection of Naturally Tilapia Lake Virus (TiLV) Infected Tilapia

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ABSTRACT

Tilapia lake virus (TiLV) is classified as a negative-sense, single-stranded RNA virus in the family Amnoonviridae. It is an enveloped virus with 10 genomic RNA segments, each coding for a protein. TiLV causes disease in tilapia, and outbreaks can lead to significant economic losses for the tilapia aquaculture industry. In this study, the gene encoding the segment 8 protein of TiLV was cloned into the expression vector pET15-b and then transformed into *Escherichia coli* strain BL21. After induction, the recombinant TiLV-S8 protein (rTiLV-S8), with a molecular mass of 20 kDa, was expressed, purified, and used to immunize mice. The mouse antiserum against rTiLV-S8 protein demonstrated specific immunoreactivity for the viral protein, approximately 19 kDa in TiLV-infected fish tissues, as determined by Western blotting. According to the results of the dot blotting assay, the antiserum was about 80 times less sensitive than one-step RT-PCR in detecting TiLV in homogenates of infected fish samples and showed no cross-reaction with uninfected fish tissues, other common fish viruses, or prevalent bacterial species found in aquatic animals. Furthermore, this polyclonal antiserum could be employed to identify TiLV-infected fish in the field using dot blotting assay, and the results can be confirmed by immunohistochemistry.

Keywords: Nile tilapia, Tilapia Lake virus-TiLV, Polyclonal antiserum, Immunohistochemistry, Dot blotting

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Introduction

Tilapia, scientifically known as *Oreochromis niloticus,* is an important commercial fish with a high potential for aquaculture production in countries like Thailand, China, Indonesia, Egypt, and the Philippines. The major epidemic that causes severe damage to tilapia aquaculture is tilapia lake virus disease (TiLVD), caused by the tilapia lake virus (TiLV). TiLV outbreaks have been reported in various tilapia farming regions across Asia, America, and Africa, including Ecuador, Israel, Colombia, Thailand, Egypt, Uganda, Malaysia, Indonesia, India, China, the Philippines, Peru, and Mexico. These outbreaks have led to substantial economic losses [1-5].

TiLV is a negative-sense, single-stranded RNA virus that was initially classified as an Orthomyxo-like virus [6, 7]. However, it was later reclassified into the family Amnoonviridae, genus *Tilapinevirus*, and species *Tilapia tilapinevirus*. TiLV has 10 genomic segments containing approximately 10,323 nucleotide bases, each containing open reading frames (ORFs) that may encode 14 different proteins [3, 7].

TiLVD is typically characterized by skin lesions, skin darkening, abdominal distension, scale protrusion, and exophthalmos in moribund fish [4, 6]. Several detection techniques have been established to identify TiLV, and they are supported by clinical symptoms, cell culture, histopathology, and transmission electron microscopy [6, 8-10]. Molecular-based methods such as *in situ* hybridization (ISH) have been utilized to identify the viral genomic RNA of TiLV infection in fish tissues using digoxigenin (DIG)-labeled oligonucleotides [3, 11]. However, the detection sensitivity of this method is still relatively low, and it is also time-consuming and technically demanding. Subsequently, a technique for detecting TiLV infection using a PCR primer set has been developed for reverse transcriptase PCR (RT-PCR) assays [6]. A more sensitive nested RT-PCR, using two pairs of primers specific to the RNA of TiLV, has been published and is suitable for detecting TiLV in clinical cases [12]. A semi-nested RT-PCR is even more sensitive than nested RT-PCR, capable of detecting the virus at 7.5 viral copies/reaction [9]. A real-time SYBR green assay with an analytical sensitivity of 2 copies of plasmid [8] and a *Taq*Man probe-based RT-qPCR assay targeting genomic segment 3 of TiLV are also employed [13]. Furthermore, RT-LAMP has also been developed to improve sensitivity and detect early viral infections. This reduces the examination time and has a higher sensitivity than traditional diagnostic tests [14, 15].

Although molecular techniques can be used quickly and efficiently to detect TiLV-infected fish, they require well-trained personnel with biomolecular skills and expensive equipment, making them impractical for on-farm testing by farmers. Therefore, immunodiagnostic assays can be developed to identify viral antigens in fish tissues, such as immunohistochemistry (IHC) [11], anti-tilapia lake virus IgM in sera, or TiLV antigen in tissue homogenates and mucus samples, tested by indirect enzyme-linked immunosorbent assay (indirect ELISA) [16, 17]. These serological methods are simple, inexpensive, sensitive, and highly specific, making them ideal for the frequent monitoring of fish cultivation [11, 16].

In this research, we aimed to generate a polyclonal antiserum specific to the TiLV segment 8 protein, as a previous study reported that this segment is highly antigenic and can produce high titers of specific antibodies [16]. We developed an immunoblot assay to detect TiLV-infected fish using antibodies against a recombinant TiLV-S8 fusion protein (His-TiLV-S8). This method is highly specific, easy, convenient, and can detect many suspected samples simultaneously.

Materials and methods

Ethics approval and consent to participate

All animal experiments were authorized by the Srinakharinwirot University Animal Care and Use Committee, Thailand, with approval code COA/AE-002-2566.

Fish sample collection

Naturally TiLV-infected Nile tilapia, *O. niloticus*, in the moribund stage with clinical symptoms [18] and healthy fish were acquired from a fish farm in Phetchaburi Province, Thailand. The fish had a length and body weight of 7.0-8.0 cm and 8.0 ± 9.0 g, respectively. Fish samples were kept at -70°C for future use. The gills of each fish were dissected and confirmed for TiLV infection by RT-PCR, as described below [6].

Pool gill fish (3-4 samples) from TiLV-infected fish or healthy fish were homogenized in 0.3 M PBS (pH 7.2) at a ratio of 0.2 g/mL, followed by centrifugation at 4,000 × g at 4°C for 20 min. Aliquots of the 0.5 mL supernatant were kept at -20°C until use for immunoassay testing of antibody specificity and sensitivity.

RT-PCR verification of TiLV-infected fish

TiLV infection was verified by RT-PCR using TiLV gene-specific primers. Total RNA was isolated from gill tissue using the High Pure Viral Nucleic Acid Kit (Roche™), following protocols from the manufacturer. The RT-PCR amplification consisted of 1.0 µL of RNA template, 0.25 µM of a pair of TiLV-specific primers (Nested ext-1: TATGCAGTACTTTCCCTGCC and Nested ext-2: TTGCTCTGAGCAAGAGTACC), 2X reaction mix buffer, and 0.5 µL of SuperScript™ III One-Step RT-PCR/Platinum *Taq* (Invitrogen™). Reactions included cDNA synthesis at 50°C for 30 min before denaturation at 94°C for 2 min. This was followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, elongation at 68°C for 30 s, and additional elongation at 68°C for 5 min. The amplification product, which had a size of 491 bp, was separated on a 1.2% agarose gel electrophoresis.

Expression of recombinant His-TiLV-S8 protein

The 525-base pair TiLV genome segment 8 (TiLV-S8) was synthesized using the nucleotide sequence reported in the GenBank database (accession number MN687772.1) and cloned into an expression vector (pET-15b) by GenScript Biotech Corporation (New Jersey, U.S.A.). ORF validity by DNA sequencing, and the plasmid pET15-b containing the TiLV-S8 gene was transformed into *E. coli* BL21 [19]. The bacteria were grown in LB broth containing ampicillin (100 µg/mL) and grown at 37°C with shaking at 225 rpm until the OD_{600} reach 0.6. To induce expression of the recombinant protein (His-TiLV-S8 or rTiLV-S8), isopropyl-β-D-thiogalactopyranoside (0.5 mM) was added and incubated for an additional 4 h with shaking at 225 rpm. After centrifuging at 4,000 x g for 20 min, the pellet was resuspended in a binding buffer containing 0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole at pH 7.9, and 1 mM phenylmethylsulfonyl fluoride. It was then sonicated and centrifuged to collect the soluble protein fraction,

which was confirmed by SDS-PAGE with Coomassie blue R250 staining. The solubility of recombinant protein expression was purified using an affinity chromatography method with the Ni-NTA Purification Kit (Novagen®), following protocols from the manufacturer. Briefly, a 10 mL sample of the total soluble protein was applied to the column and washed with washing buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl, pH 7.9). The rTiLV-S8 protein was then eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and dialyzed against PBS. SDS-PAGE was used to confirm the purity of the purified rTiLV-S8 protein, and its concentration was measured using the Bradford protein assay [20].

Immunization and preparation of polyclonal antiserum (anti-rTiLV-S8 antiserum)

Three six-week-old female Swiss mice were maintained in the experimental animal facility and injected intraperitoneally with 50 µg of purified rTiLV-S8 protein mixed with complete Freund's adjuvant (1:1 ratio) at the first dose. The same protein concentration was then combined with incomplete Freund's adjuvant and injected three more times as a booster at 14-day intervals. Seven day after the fourth injection, mouse blood was obtained from the orbital sinus. Following centrifugation at 6,000 x g at 4°C for 20 min, the serum was specifically tested against lysates of *E. coli* BL21 or *E. coli* BL21 with expressing His-TiLV-S8 protein and gill homogenates from TiLV-infected or uninfected tilapia using Western blotting and dot blotting assays. The mouse antisera that demonstrated the best immune reactivity was further used for the detection of naturally TiLV-infected tilapia.

Specificity testing

SDS-PAGE and Western blot analysis

Lysates of *E. coli* BL21 and *E. coli* BL21 expressing His-TiLV-S8 protein, as well as gill homogenates from TiLV-infected and uninfected tilapia samples, were separated on 15% SDS-PAGE gels and electrophoresed for 2.5 h at 70 V, according to the method described by Laemmli [21]. Protein profiles in the gel were stained with 0.1% Coomassie Brilliant Blue R250. For Western blot analysis, after separation on the gel, the protein was electroblotted to 0.45 µm pore size nitrocellulose membrane using a Blotting apparatus. The membrane was blocked for 10 min with 5% blocking solution (5% nonfat dry milk and 0.1% Triton X-100 in PBS, pH 7.2), followed by incubation with mouse anti-rTiLV-S8 antiserum diluted 1:5,000 in 1% blocking solution for 1 h at RT. After washing in 0.1% blocking solution, the membrane was probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig (HRP-GAM; Bio-Rad) diluted 1:3,000 in 1% blocking solution for 1 h at RT. To reveal the immunoreactive protein band, the membrane was washed extensively in PBS, then immersed for 2 min in a chromogenic substrate solution comprised of 0.006% H_2O_2, 0.03% 3,3′-diaminobenzidine (DAB), and 0.05% $CoCl_2$ in PBS, and finally washed in distilled water to stop the reaction.

To reduce the non-specific binding of antibodies to non-target proteins, mouse polyclonal antiserum was preabsorbed with lysate of *E. coli* BL21 at a 1:10 ratio for 1 h at 4°C before being used in all experiments in this study.

Specificity and cross-reactivity testing

Dot blotting

The specificity and cross-reactivity of the mouse polyclonal antiserum were examined by dot blotting assay. Lysates of *E. coli* BL21, *E. coli* BL21 expressing His-TiLV-S8 protein, gill homogenates from uninfected or TiLV-infected tilapia, and other common fish viruses [22, 23], including tissue homogenates from Asian sea bass (*Lates calcarifer*) infected with red spotted grouper nervous necrosis virus (RGNNV) or infectious spleen and kidney necrosis virus (ISKNV) or scale drop disease virus (SDDV), and heat-killed suspensions (10^8 CFU/mL) of prevalent bacterial species found in aquatic animals [24] as summarized in Table 1, were tested.

For the dot blotting assay, 1 µL of each sample protein was spotted onto a 0.45 µm pore size nitrocellulose membrane, air dried at RT for 5 min, and blocked for 10 min with 5% blocking solution. After blocking, the membrane was probed with anti-rTiLV-S8 antiserum at a dilution of 1:5,000 in 1% blocking solution for 1 h at RT. The membrane was then washed with 0.1% blocking solution and probed with 1:3,000 diluted HRP-GAM in 1% blocking solution for 1 h at RT. After the final washing, the membrane was immersed in the substrate solution and then washed in distilled water to stop the reaction, as described in the Western blot analysis section. A positive result was indicated by a clear dark spot on a nitrocellulose membrane obtained in triplicate.

Table 1 List of bacteria used in this study for specificity and cross-reactivity testing [24].

No.	Bacteria	Institute	Source
1	*Flavobacterium columnare* 1301 (FC1)	CPF	*Oreochromis niloticus*
2	*Flavobacterium columnare* AT (FC2)	CPF	*O. niloticus*
3	*Chryseobacterium massiliae* 1205 (CM)	CUVET	*O. niloticus*
4	*Chryseobacterium taichungense* 1217 (CT)	CUVET	*O. niloticus*
5	*Chryseobacterium indologenes* 1219 (CI)	CUVET	*O. niloticus*
6	*Flectobacillus roseus* 1207 (FR)	CUVET	*O. niloticus*
7	*Aeromonas hydrophila* AE1 (AH)	CPF	Fish
8	*Aeromonas veronii* (AV)	CENTEX	Fish
9	*Streptococcus agalactiae* (SA)	CENTEX	Fish
10	*Vibrio vulnificus* 4907000 (VV)	DBSWU	Shrimp
11	*Vibrio harveyi* H1 (VH)	CPF	Shrimp
12	*Vibrio parahaemolyticus* VPV (VP)	VMARC	Aquatic animal

CPF = Charoen Pokphand Foods Public Co. Ltd

CUVET = Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University

CENTEX = CENTEX Shrimp, Faculty of Science, Mahidol University

DBSWU = Department of Biology, Faculty of Science, Srinakharinwirot University

VMARC = Veterinary Medical Aquatic Research Center, Chulalongkorn University

Sensitivity testing

To determine the sensitivity of the dot blotting assay, 2-fold serial dilutions of lysates of *E. coli* BL21 expressing His-TiLV-S8 protein and gill homogenates from TiLV-infected fish in PBS were spotted onto nitrocellulose membranes in 1 µL. The membrane was allowed to air dry at RT for 10 min and processed for dot blotting assay using the mouse anti-rTiLV-S8 antiserum as described earlier. To assess the relative sensitivity of the dot blotting and RT-PCR assays, viral nucleic acid was isolated from the same TiLV-infected fish sample and serially 10-fold diluted with nucleic acid from an uninfected fish. Each diluted sample was then used as a template for one-step RT-PCR as described above. The two assays were compared by determining the lowest sample dilution yielding a clear positive result in both, with measurements performed in triplicate.

Detection of TiLV from naturally infected tilapia

To detect TiLV in field specimens, 12 naturally TiLV-infected Nile tilapia and 12 healthy tilapias from an aquaculture farm in Phetchaburi Province were used. The gill tissues from each fish were homogenized in PBS at a ratio of 0.2 g/mL and divided into equal parts. One part was directly applied (1 µL/spot) to each square of nitrocellulose membrane and processed for dot blotting assay using the anti-rTiLV-S8 antiserum obtained in this study. The other part of the sample was subjected to RNA isolation using an RNA extraction kit and processed for one-step RT-PCR as described in the previous section, and the results of each assay were obtained in triplicate.

To confirm the TiLV-infected fish, the remaining gill tissue from each fish was preserved separately in Bouin's fixative solution for 24 h and processed to immunohistochemical detection. The samples were then rinsed thoroughly in tap water, dehydrated in a series of alcohols (50%-95% ethanol and butanol), and embedded in paraffin wax. Serial tissue sections (8-µm thick) were prepared and stained using indirect immunoperoxidase staining, as described by Longyant et al. [25]. In brief, tissue sections were probed with mouse anti-rTiLV-S8 antiserum diluted 1:2,000 in 10% fetal bovine serum in PBS and incubated at 4°C for 6 h in a humid chamber. After washing the slides with PBS, the sections were incubated with HRP-GAM diluted 1:1,000 in 10% fetal bovine serum solution at 37°C for 3 h at RT, followed by another wash with PBS. The immunoperoxidase activity was visualized by incubating the sections within a chromogenic substrate solution (0.006% H_2O_2, 0.03% DAB in PBS) for 5 min, followed by washes with distilled water. Tissue sections were stained with either eosin Y alone, which facilitated the identification of brown immunoreactivity, or hematoxylin and eosin Y (H&E). The sections were then dehydrated in an ethanol series, cleared in xylene, and mounted in Permount. Positive reactions appeared as brown coloration against the pink cytoplasm and purple nuclei.

Results

Recombinant His-TiLV-S8 protein expression

After induction, the expression of the recombinant His-tagged TiLV-S8 fusion protein (rTiLV-S8) was visualized as a band with the expected molecular mass of 20 kDa on a Coomassie blue-stained gel (Figure 1, lane 3). The rTiLV-S8 protein was purified using affinity chromatography, yielding a highly pure fusion protein (Figure 1, lane 4). This purified rTiLV-S8 protein was then used for immunization at a concentration of 1 mg/mL.

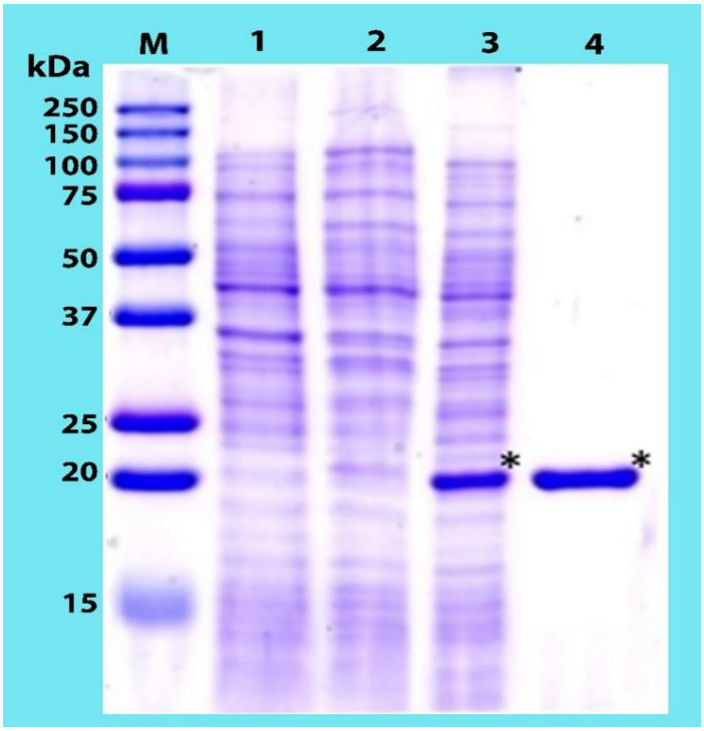

Figure 1 SDS-PAGE analysis for recombinant His-TiLV-S8 proteins expression and purity. 1) Lysate of *E. coli* strain BL21, 2) lysate of *E. coli* strain BL21 containing pET15b plasmid, 3) lysate of *E. coli* strain BL21 containing pET15b-TiLV-S8 plasmid, and 4) purified recombinant His-TiLV-S8 protein were separated by 15% SDS-PAGE. The proteins were then stained with Coomassie brilliant blue R250. M = Standard marker proteins; * = Recombinant His-TiLV-S8 protein of molecular mass about 20 kDa.

Specificity and cross-reactivity of the polyclonal antiserum

The immunoreactivity of the anti-rTiLV-S8 antiserum, raised in mice, was evaluated and confirmed for specificity and cross-reactivity using immunoblot assays. All mice exhibited almost identical immunoreactive bands in Western blot analysis. The results revealed that the mouse antisera (from mouse No. 1-3) specifically recognized the rTiLV-S8 protein at approximately 20 kDa in *E. coli* BL21 lysate expressing His-TiLV-S8 protein. Interestingly, they bound to the smear protein band approximately at 45 kDa. This band was expected to be an aggregated form of the rTiLV-S8 protein, given that high protein

concentrations during overexpression in *E. coli* BL21 can promote misfolding and aggregation due to improper protein-protein interactions [26], while a faint protein band at 17 kDa was observed, indicating the degradation of this protein (Figure 2B to 2D, Lane 2). Additionally, the antisera also bound to the S8 protein of TiLV at approximately 19 kDa in TiLV-infected fish homogenate (Figure 2B to 2D, Lane 3) but did not recognize any proteins in the homogenate from uninfected fish (Figure 2B to 2D, Lane 4). However, the antisera also cross-reacted with some protein bands in *E. coli* BL21 lysate and *E. coli* BL21 lysates expressing the His-TiLV-S8 protein (Figure 2B to 2D, Lanes 1 and 2). This non-specific antibody binding can be effectively eliminated by preabsorbing with lysate of *E. coli* BL21 before use. After the pooled polyclonal antiserum from all mice was preabsorbed and tested, non-target protein bands were not observed (Figure 2E, Lanes 1 and 2). Similar results were observed in the dot blot assay. The antiserum exhibited strong immunoreactivity specific to a lysate of *E. coli* expressing His-TiLV-S8 protein, TiLV-infected fish homogenate, and purified rTiLV-S8 protein, but showed no cross-reactivity with uninfected fish homogenate, or tissue homogenates from other virally infected fish (RGNNV, SDDV, and ISKNV), or eleven prevalent bacterial species found in aquatic animals (Figure 3).

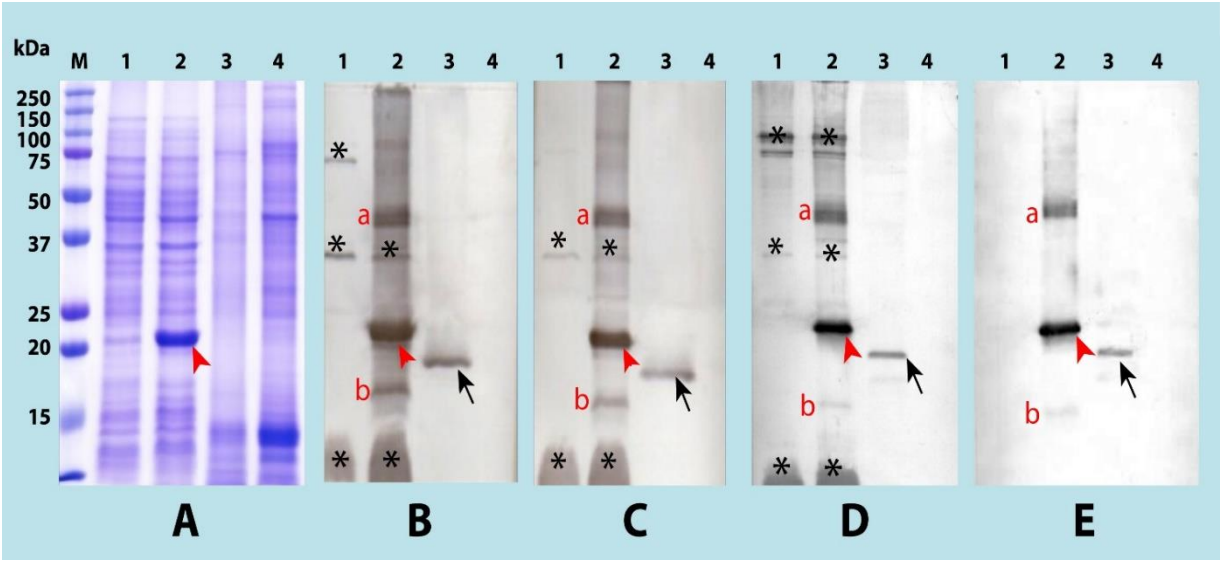

Figure 2 Specificity testing of anti-rTiLV-S8 antiserum by SDS-PAGE (A) and Western blotting (B - E). 1) lysate of *E. coli* BL21, 2) lysate of *E. coli* BL21 containing pET15b-TiLV-S8, gill homogenate of 3) TiLV-infected, or 4) uninfected fish were electrophoresed by 15% SDS-PAGE and stained with Coomassie brilliant blue R250 (A). Then, the protein was transferred onto nitrocellulose membranes and probed with mouse antiserum No. 1 (B), 2 (C), and 3 (D) or with pooled mouse antiserum preabsorbed with lysate of *E. coli* BL21 (E). M = standard protein marker; Red arrowhead = TiLV immunoreactive bands of the rTiLV-S8 protein (20 kDa); Black arrow = TiLV-S8 protein (19 kDa); * = protein bands were cross-reacted by antiserum. a = aggregated protein form of rTiLV-S8 (45 kDa); b = degraded product of rTiLV-S8 (17 kDa)

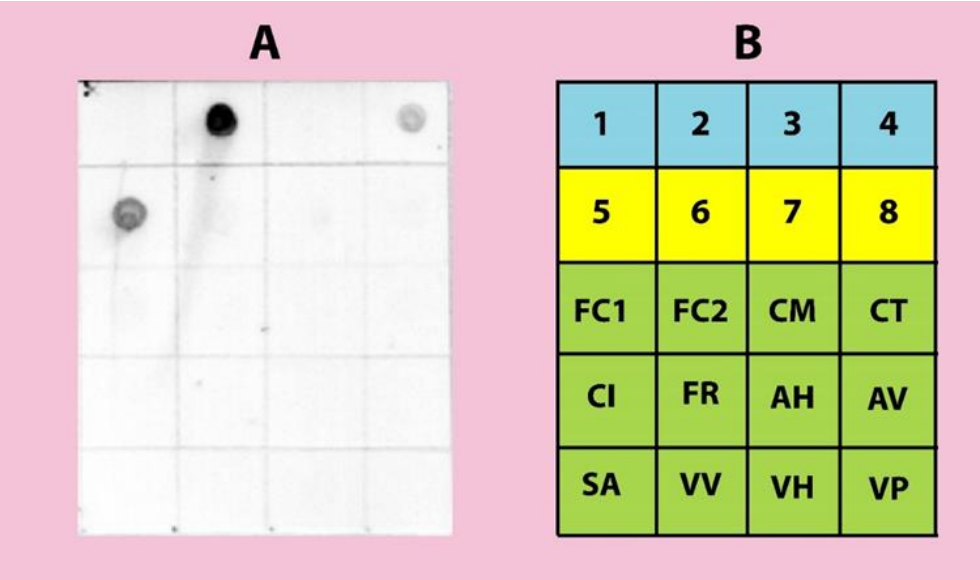

Figure 3 Specificity and cross-reactivity testing of anti-rTiLV-S8 antiserum determined by dot blotting. Different lysate proteins of virus-infected fish, the tissue homogenates from uninfected or virus-infected fish, and heat-inactivated bacteria (10^8 CFU/mL) were spotted (1 μL/spot) onto each square of a nitrocellulose membrane (A) and probed with mouse anti-rTiLV-S8 antiserum. A list of lysate proteins and bacteria is summarized in diagram (B) as follows: 1) Lysate of *E. coli* BL21, 2) lysate of *E. coli* BL21 containing His-TiLV-S8, gill homogenate from 3) uninfected, or 4) TiLV-infected Tilapia, 5) purified rTiLV-S8 protein, tissue homogenate from different virus-infected fish including 6)RGNNV, 7) SDDV, 8) ISKNV, and heat-killed bacteria: *Flavobacterium columnare* 1301 (FC1), *F. columnare* AT (FC2), *Chryseobacterium massiliae* 1205 (CM), *C. taichungense* 1217 (CT), *C. indologenes* 1219 (CI), *Flectobacillus roseus* 1207 (FR), *Aeromonas hydrophila* AE1 (AH), *A. veronii* (AV), *Streptococcus agalactiae* (SA), *Vibrio vulnificus* 4907000 (VV), *V. harveyi* H1 (VH), and *V. parahaemolyticus* VPV (VP)

Sensitivity of polyclonal antiserum

The detection sensitivity limits of the mouse anti-rTiLV-S8 antiserum were determined by dot blot assay. The antiserum could bind to the lysate of *E. coli* expressing His-TiLV-S8 protein and TiLV-infected fish tissue homogenate at ratios of approximately 1:1,600 and 1:128 dilutions, respectively (Figure 4A). In comparison with TiLV detection by the RT-PCR method, the same TiLV-infected fish sample was used for nucleic acid extraction. The expected PCR product of 491 bp was still observable at a dilution of 10^{-4} dilution (Figure 4B). Therefore, the dot blot method by using this antiserum was approximately 80-fold less sensitive than one-step RT-PCR for TiLV-infected fish tissue homogenate detection.

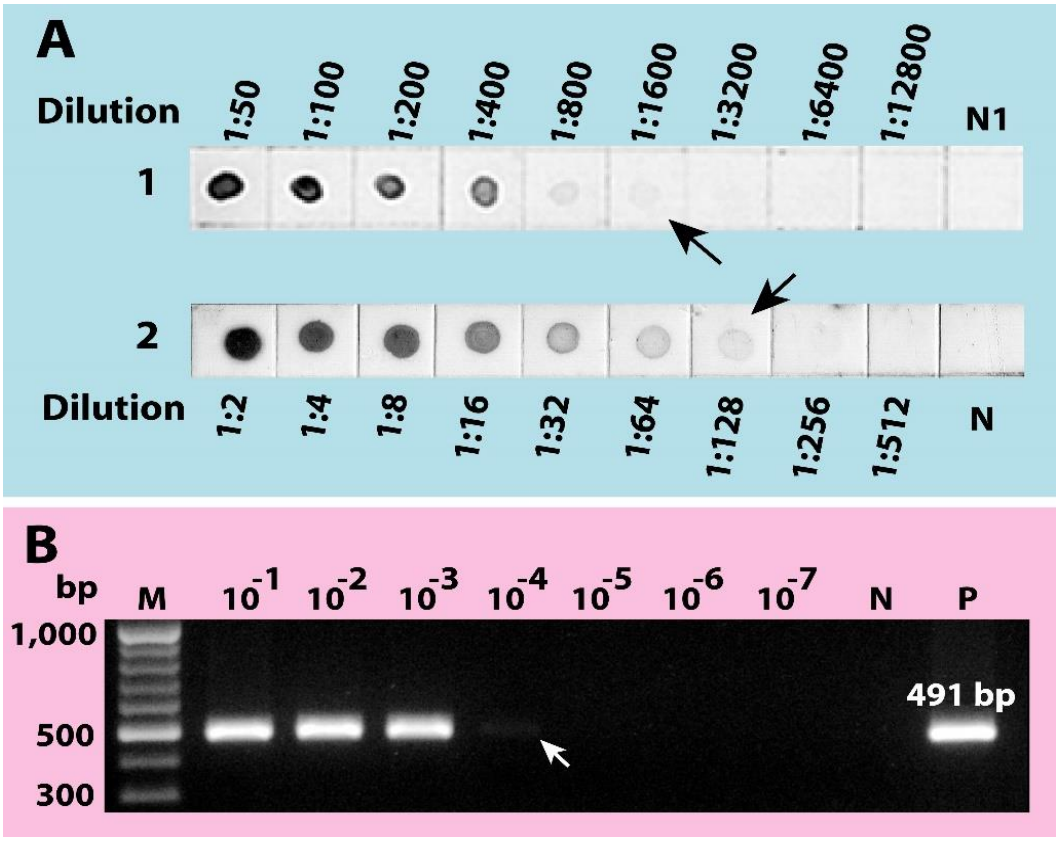

Figure 4 Comparative sensitivity of TiLV detection between (A) dot blotting analysis and (B) RT-PCR. In the dot blot, two-fold serial dilutions of lysates of 1) *E. coli* BL21 with His-TiLV-S8 (dilutions 1:50-1:12,800) and 2) gill homogenate extracts from TiLV-infected fish (dilutions 1:2-1:512) were spotted onto each square of a nitrocellulose membrane (1 µL/spot) and probed with anti-rTiLV-S8 antiserum (preabsorbed with expressed lysate of *E. coli* BL21). For the RT-PCR assay, the same gill TiLV-infected fish homogenate at each dilution was utilized for DNA extraction. M = DNA molecular weight markers; Arrow = the lowest detection limit; Negative control (N1 = lysate of *E. coli* BL21 at dilution 1:20; N = gill homogenate extracts from normal fish at dilution 1:5); Positive control (P = tissue homogenate of TiLV-infected fish) showed a 491 bp band.

Comparison of naturally TiLV-infected tilapia detection by dot blotting and RT-PCR

Dot blotting and RT-PCR assays were compared for the detection of naturally TiLV-infected tilapia in field specimens. Both assays demonstrated positive results in all 12 infected fish samples and gave negative results for all 12 uninfected fish samples (Figure 5A and 5B). Although some fish (No. 2 and 10) showed slightly positive results in the dot blotting assay, it aligns with the findings from the RT-PCR results.

Immunohistochemical analysis confirmed alignment with the results of the dot blotting and RT-PCR assays, with all TiLV-infected fish samples demonstrating positive immunoreactivity. The antiserum exhibited specific binding to antigens in TiLV-infected cells, generating a brown chromogenic reaction

within the gill arch and gill filaments of infected tissue (Figure 6A and 6B column I). These immunoreactivity results indicated that sample No. 2 had a low level of TiLV infection (Figure 6A), while sample No. 9 exhibited a high level of infection (Figure 6B). No positive results were observed in the control sample (No. 14, Figure 6C) and all uninfected fish samples. Furthermore, histopathological examination by staining with hematoxylin and eosin revealed syncytial cell formation (Figure 6A and 6B column II).

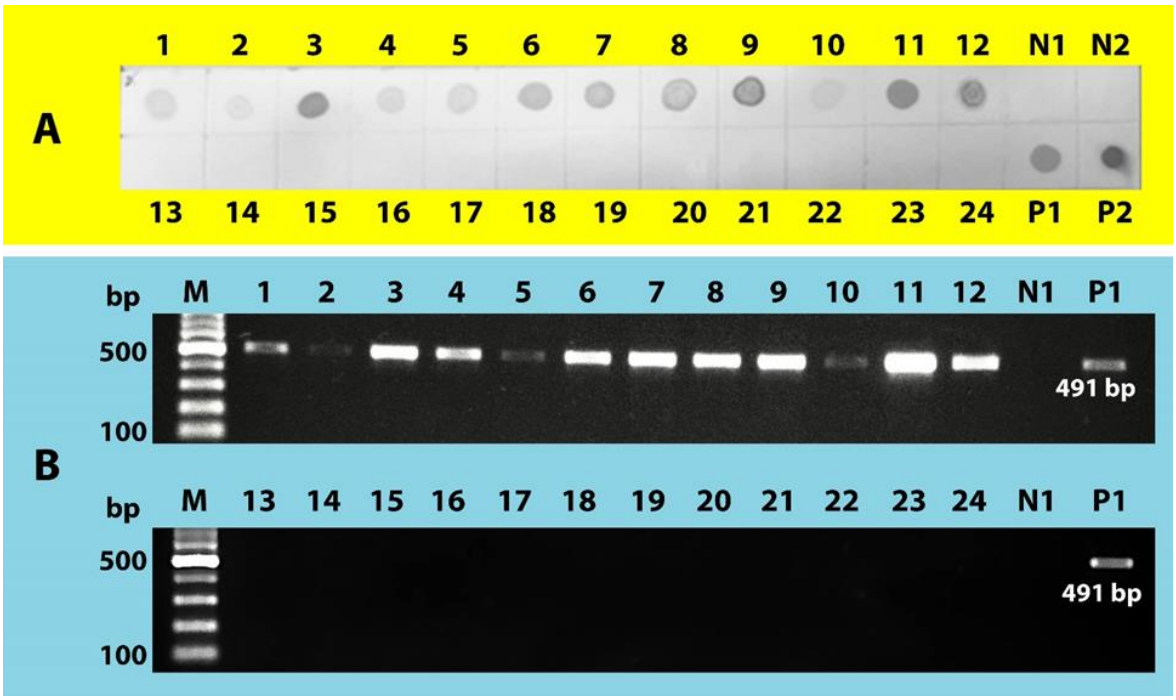

Figure 5 Comparison of TiLV-infected tilapia detection by dot blotting and RT-PCR analysis. Homogenates of gill extract from naturally TiLV-infected fish samples (Lanes 1–12) and normal fish samples (Lanes 13–24) were spotted on to nitrocellulose membrane and processed for (A) dot blotting using anti-rTiLV-S8 antiserum. The same tissue homogenate from each sample was used for DNA template preparation and processed for (B) RT-PCR analysis using TiLV Nested ext-1 and Nested ext-2 specific primers, revealing the presence of a positive band at 491 bp. Negative control (N1 = tissue homogenate of normal fish; N2 = lysate of *E. coli* BL21); Positive control (P1 = tissue homogenate of TiLV-infected fish; P2 = lysate of *E. coli* BL21 with His-TiLV-S8); M = DNA molecular weight markers.

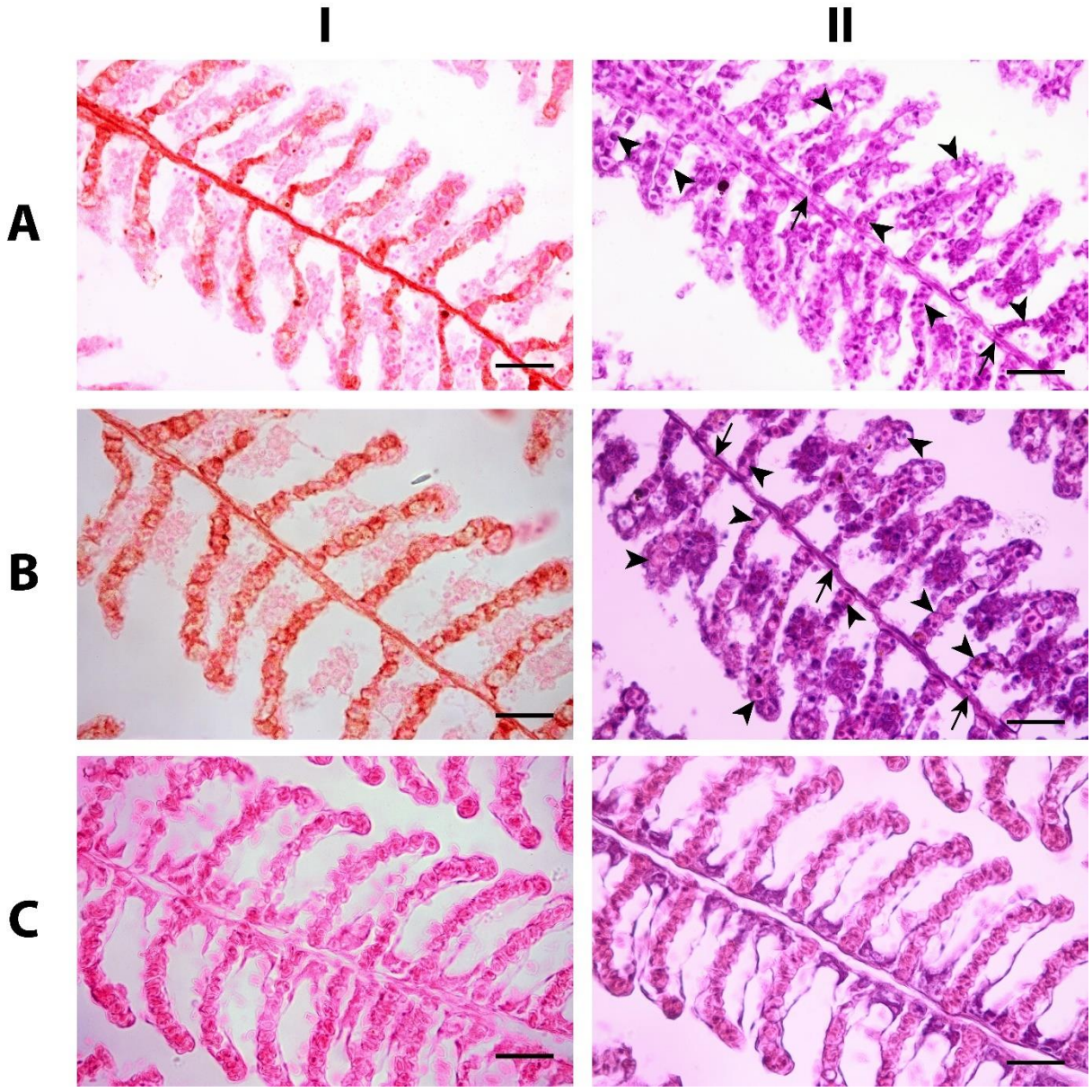

Figure 6 Gill tissue sections from naturally TiLV-infected tilapia with light infection (No. 2, row A) and heavy infection (No. 9, row B), as well as an uninfected fish (No. 14, row C) from Figure 5, were analyzed by immunohistochemistry. Sections in column I were treated only with anti-His-TiLV-S8 antiserum and counterstained with eosin, while sections in column II were not treated with the antiserum and stained with hematoxylin and eosin. Strong immunoreactivity (brown staining) was observed in the infected tissue (rows A and B, column I), primarily in the gill arch (arrows) and gill filaments (arrowheads). Scale bar=10 μm.

Discussion

TiLV poses an extremely infectious threat to the global tilapia aquaculture industry. Natural outbreaks of TiLV in tilapia have resulted in mortality rates ranging from 20% to 90% in both farmed and wild fish [4, 6, 9, 27]. The presence of TiLV infection has been reported in tilapia at various developmental stages, including fertilized eggs, yolk sac larvae, fry, fingerlings, adults, and broodstock fish [11, 28, 29]. This widespread infection could have significant implications for food security and lead to substantial economic losses. Therefore, the need for simple, quick, and accurate diagnostic tools that do not require expensive laboratories is still necessary to reduce costs and effectively control the disease in tilapia farming.

In this research, we competently produced and purified recombinant TiLV-S8 (rTiLV-S8) protein, which can be used as an effective immunogen for mouse immunization. A previous study has demonstrated that the S8 protein from the genome-decoded segment 8 of TiLV is a highly potential antigenic protein capable of inducing high titers of specific antibodies, and it is used as a coating antigen for indirect ELISA. Since this S8 protein is abundant, identified by LC-MS/MS analysis as one of the four most prevalent proteins in purified TiLV [16], it implies increased exposure to the immune system, potentially triggering a robust antibody response – a crucial factor in achieving our goal of generating a highly immunogenic anti-TiLV antibody.

The anti-rTiLV-S8 polyclonal antisera were evaluated by Western blot for immunoreactivity. The specific immunogenicity against TiLV-antigens was observed in gill homogenates from infected fish at a 19 kDa band with a similar predicted molecular mass to those previously examined by mass spectrometry, which was later identified as TiLV segment 8 protein [3]. Therefore, the results of this study confirm that the recombinant TiLV-S8 fusion protein (rTiLV-S8) produced in *E. coli* is antigenically similar to the epitopes of the natural S8 protein of TiLV. After completely preabsorbing the antiserum with the lysate of *E. coli* BL21, the antiserum was assessed for specificity, cross-reactivity, and sensitivity tests. In dot blotting, the polyclonal antiserum showed high specific reactivity to rTiLV-S8 and TiLV in infected tissue homogenate, with no binding to uninfected fish tissues, other common fish viruses, or pathogenic bacteria in fish and aquatic animals.

In this study, the antiserum sensitivity limit, determined using a dot blot method for TiLV detection, showed lower sensitivity than RT-PCR. However, the level of antiserum sensitivity was comparable to that previously reported for MAbs specific to the expressed major capsid protein of ISKNV [23] or RGNNV [22]. The MAbs exhibited immunoreactivity staining in ISKNV or NNV-infected fish tissues, as shown by the immunohistochemistry (IHC) and can be utilized to identify naturally infected fish by dot blot assay. Therefore, the obtained antiserum could be helpful for the detection of TiLV-infected fish, similar to those available currently for the detection of TiLV antigens in fish tissues by IHC [11], the detection of specific antibodies against TiLV in sera, or the screening and detection of TiLV in fish tissue and mucus samples using indirect ELISA [16, 17]. In the investigation of field specimens of *O. niloticus* fish infected with TiLV, positive immunological activity against anti-rTiLV-S8 antiserum was observed. This was determined using dot blot testing, which yielded results consistent with RT-PCR analysis. Immunohistochemical

confirmation was visible as strong antigenic signals in the cytoplasm of the gill epithelium in TiLV-infected fish but not in uninfected fish tissues. This TiLV-IHC immunoreactivity occurred in a pattern similar to that previously observed in infected fish tissue using *in situ* hybridization [11].

The dot blot method offers several advantages over molecular-based techniques. It is a cost-effective approach that facilitates the simultaneous testing of a large number of samples. With this method, sample preparation does not require any special solutions or nucleic acid extraction processes. Tissue samples can be ground and directly spotted onto the nitrocellulose membrane. Additionally, it provides results within a relatively short timeframe of approximately 3 hours [24]. Consequently, the dot blot method, which utilizes the obtained antiserum, can be effectively employed for the preliminary screening or monitoring of TiLV infection.

Based on previous studies, most screens for TiLV-infected fish have relied on immunological methods that primarily include the generation of specific antiserum [11, 16, 17]. Hence, to enhance the efficacy of immunological techniques for detecting TiLV infection, it is necessary to pursue monoclonal antibody production. This involves acquiring highly sensitive, diverse, and specific antibodies for improving various immunoassays, such as an immunochromatographic strip test; it is user-friendly, simple, and provides immediate results without the need for additional equipment. Hence, it can be employed by farmers to investigate or detect this viral infection.

Conclusions

A polyclonal TiLV-specific antiserum was generated from mice immunized with rTiLV-S8 protein, whose specificity was confirmed by Western blot, dot blot, and immunohistochemistry. The antiserum showed no cross-reactivity with healthy fish tissues, other fish viruses, or bacterial pathogens. Although the sensitivity of the antiserum was lower than that of one-step RT-PCR, it can still be utilized to identify naturally TiLV-infected fish samples, as demonstrated in dot blotting. Therefore, this antiserum can be used to monitor and confirm TiLV infection in field fish samples.

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