

Monoclonal antibodies against extra small virus show that it co-localizes with *Macrobrachium rosenbergii* nodavirus

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ABSTRACT: The capsid protein (CP) gene of extra small virus (XSV) expressed in *Escherichia coli* as a 42 kDa glutathione S-transferase (GST)-fusion protein (GST-XCP) or a 20 kDa His₆-fusion protein (His₆-XCP) were purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), combined, and used to immunize Swiss mice to produce monoclonal antibodies (MAbs). Using dot blot, Western blot, and immunohistochemistry (IHC) methods, 4 MAbs specific to the XSV CP detected XSV in the freshwater prawn *Macrobrachium rosenbergii* without cross-reaction to host proteins or to proteins of *Macrobrachium rosenbergii* nodavirus (MrNV) or 5 of the most pathogenic viruses of penaeid shrimp. In dot blots, the combined MAbs could detect down to ~10 to 20 fmol μl^{-1} of purified GST-XCP protein, which was somewhat more sensitive compared to any single MAb. Used in conjunction with an MrNV-specific MAb, white tail disease (WTD) was diagnosed more effectively. However, the sensitivity at which the combined 4 MAbs detected XSV CP was 1000-fold lower than XSV RNA detected by RT-PCR. IHC analysis of *M. rosenbergii* tissue sections using the MAbs showed XSV infection to co-localize at variable loads with MrNV infection in heart and muscle cells as well as cells of connective tissues in the hepatopancreas. Since XSV histopathology remained prominent in tissues of some prawns in which MAb reactivity for MrNV was low compared to MAb reactivity for XSV, XSV might play some role in WTD severity.

KEY WORDS: Immunohistochemistry · *Macrobrachium rosenbergii* nodavirus · MrNV · Monoclonal antibody · Capsid protein · Western blot · Extra small virus · XSV

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INTRODUCTION

Outbreaks of white tail disease (WTD) in hatchery-reared postlarvae (PL) of the giant river prawn *Macrobrachium rosenbergii* have occurred in many parts of the world including the West Indies (Arcier et al. 1999), Taiwan (Tung et al. 1999, Wang et al.

2008), China (Qian et al. 2003), India (Sahul Hameed et al. 2004), Thailand (Yoganandhan et al. 2006), and Australia (Owens et al. 2009). WTD is caused by *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV) (Arcier et al. 1999, Qian et al. 2003), is characterized by whitened abdominal muscle, and can cause almost complete

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losses of PL in hatcheries and nursery ponds within 5 d of gross signs first appearing (Arcier et al. 1999). In muscle tissue affected by WTD, MrNV is always found in conjunction with XSV (Qian et al. 2003). MrNV is a small (27 nm diam.) non-enveloped icosahedral virus with a genome comprising 2 single-stranded RNAs 2.9 kb and 1.3 kb in length and a capsid comprised of a single 43 kDa protein (Bonami et al. 2005). The XSV genome is a single-stranded RNA 796 nucleotides in length that encodes a 16–17 kDa capsid protein (CP). XSV is considered to be a satellite virus dependent on the MrNV RNA-dependent RNA polymerase for genome replication (Sri Widada & Bonami 2004, Bonami et al. 2005).

Dot blot and *in situ* hybridization (Sri Widada et al. 2003, Hsieh et al. 2006, Wang et al. 2008), RT-PCR (Sri Widada et al. 2003, Sahul Hameed et al. 2004), multiplexed RT-PCR (Yoganandhan et al. 2005, Tripathy et al. 2006, Owens et al. 2009, Senapin et al. 2010, 2012), real-time RT-PCR (Zhang et al. 2006), and reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Pillai et al. 2006, Haridas et al. 2010, Puthawibool et al. 2010) have been developed to detect MrNV and/or XSV RNA. While highly specific and sensitive, all of these molecular detection methods are quite complicated and expensive and thus not practical for pond-side detection of these viruses. Sandwich ELISA and other protein detection methods have also been developed using either polyclonal antibodies (Romestand & Bonami 2003, Sahul Hameed et al. 2011) or monoclonal antibodies (MAbs) (Qian et al. 2006, Wangman et al. 2012) prepared to either native or recombinant MrNV proteins. Here we describe the preparation of MAbs to the XSV CP expressed in bacteria as 2 types of recombinant-fusion proteins and their use in various immunodetection methods to detect XSV in association with MrNV in *Macrobrachium rosenbergii* affected by WTD.

MATERIALS AND METHODS

Virus preparation

The presence of MrNV and XSV in 2 batches of *Macrobrachium rosenbergii* PL showing gross signs of WTD and obtained from hatcheries in Chachoengsao Province, Thailand, was verified by RT-PCR (Senapin et al. 2012). Some PL from each batch were homogenized in 0.15 M phosphate-buffered saline (PBS; pH 7.2) at approximately 1:10 (v:v) and cen-

trifuged at $3000 \times g$ for 30 min, and aliquots of the supernatant were stored at -70°C . The remaining PL from each batch were fixed in Davidson's fixative and processed for histology. Uninfected *M. rosenbergii* collected from elsewhere were used as negative controls in various tests.

RNA extraction

WTD-affected *Macrobrachium rosenbergii* were homogenized in lysis buffer (50 mM Tris-HCl, pH 9, 100 mM EDTA, 50 mM NaCl, 2% sodium dodecyl sulfate [SDS]), and RNA was extracted from 200 μl homogenate using a High Pure viral nucleic acid kit (Roche Molecular Biochemicals) using the protocol described by the manufacturer.

XSV CP gene expression constructs

Random-primed cDNA was synthesized from RNA of XSV-infected prawns using the Superscript 1-step RT-PCR system (Invitrogen) and amplified by PCR using the primers XSVF70 (5'-CGG GAT CCT CTA ATC TCA TTC CTT AC-3') and XSVR (5'-CGT CGA CTT ACT GTT CGG AGT CCC AAT ATG-3') containing 5'-terminal restriction endonuclease sites (underlined) and designed to amplify a portion of the XSV CP gene devoid of its N-terminal hydrophobic transmembrane region. The thermal cycling conditions used were 50°C for 30 min, 95°C for 5 min, and 4°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 15 min. The 471 bp PCR product was purified and digested with *Bam*HI and *Sal*I, cloned into these sites in the pGEX-6P-1 expression vector (Amersham Bioscience), and transformed into *Escherichia coli* strain BL21. The continuity of the glutathione S-transferase (GST)-fusion protein (GST-XCP) open reading frame in selected recombinant plasmids was verified by DNA sequencing. A full-length XSV CP gene coding sequence was amplified similarly using the primers XSV-F (5'-GGA ATT CCA TAT GAA TAA GCG CAT TAA TAA-3') and XSV-R (5'-CGC GGA TCC TTA CTG TTC GGA GTC CCA ATA-3I), and the 538 bp amplicon was digested with *Nde*I and *Bam*HI and cloned into these sites in pET15b (Novagen) vector, and the continuity of His₆-XCP fusion protein open reading frame of selected recombinant plasmids was verified by DNA sequencing.

XCP fusion protein expression and purification

Escherichia coli strain BL21 transformed with either plasmid pGEX-6P-1-XCP or pET15b-XCP was cultured in Luria-Bertani (LB) broth to the exponential phase before recombinant protein expression was induced using 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). After 4 h additional growth, bacteria were collected by centrifugation at $3000 \times g$ for 20 min. Bacterial pellets were suspended in buffer (100 mM NaH_2PO_4 , 10 mM Tris-HCl, and 8 M urea, pH 8) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated until the lysate cleared. Proteins in the lysate were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% gel. After treatment of the gel in 0.3 M KCl, the GST-XCP and His₆-XCP recombinant fusion proteins were excised, placed in dialysis bags, eluted with a Transblot apparatus (BioRad) at 70 V for 6 h, dialyzed, and concentrated using a Savant centrifuge vacuum concentrator. The concentration of the purified recombinant protein solutions was determined by Bradford assay (Bradford 1976) and following dilution to 0.5 mg ml⁻¹, small aliquots of each dilution were stored at -70°C.

Mouse immunization and MAb production

Purified GST-XCP and His₆-XCP proteins mixed 1:1 were emulsified in Freund's complete adjuvant (FCA) at a 1:1 ratio and injected into the intraperitoneal cavity of 4 Swiss mice at 0.05 mg protein per mouse. Mice were injected similarly 3 more times at 2 wk intervals using the same proteins emulsified in Freund's incomplete adjuvant (FIA). One week after the final injection, mice were bled to collect antisera that were tested for reactivity to the GST and to the GST-XCP and His₆-XCP fusion proteins by SDS-PAGE and Western blotting. The mouse with the highest antibody levels was subsequently boosted again 3 d before hybridoma production. All procedures using mice adhered to regulations described in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes, The National Research Council Thailand. The P3X myeloma cell fusion protocol used to produce MAbs was that developed by Köhler & Milstein (1976) modified as described by Mosmann et al. (1979). Cell fusion products were plated onto thirty 96-well microculture plates and positive cul-

tured fluids were identified initially by dot blot using GST as well as the GST-XCP:His₆-XCP protein mixture and subsequently by Western blotting and immunohistochemistry (IHC). Cell clones were selected and recloned from immunoreactive cultures using the limiting dilution method and stored in liquid nitrogen.

Dot blot method

Lysates of *Escherichia coli* strain BL21 expressing GST, GST-XCP, or His₆-XCP, and homogenates of uninfected or WTD-affected prawn tissue were applied to nitrocellulose membranes (1 μ l spot⁻¹), baked at 60°C for 10 min, and incubated in hybridoma-conditioned culture medium diluted 1:20 in 1% blocking solution (1% nonfat dry milk, 0.1% Triton X-100 in PBS) for 4 h. After extensive washing in 0.5% blocking solution (4 \times 15 min), membranes were incubated in horseradish peroxidase-labeled goat anti-mouse gamma immunoglobulin heavy and light chain-specific antibody (GAM-HRP, Bio-Rad) diluted 1:1500 for 3 h. Membranes were then washed in blocking solution for 4 \times 15 min before being incubated for 5 min in a substrate solution (0.03% diaminobenzidine [DAB], 0.006% H₂O₂, and 0.05% CoCl₂ in PBS) for color development (Sithigorngul et al. 2002). To determine the detection limit of the XSV MAbs in dot blots, serial dilutions of purified GST-XCP protein in PBS were spotted onto nitrocellulose membranes and detected as described in this section.

Western blot method

Lysates used in dot blots were also separated by SDS-PAGE in a 15% gel electrophoresed at 60 V for 3 h as described by Laemmli (1970). Proteins in one gel section were stained using Coomassie Brilliant Blue R-250, and proteins in the other section were transferred onto a nitrocellulose membrane using a Transblot apparatus (BioRad). Membranes were incubated in 1% blocking solution for 10 min before adding MAbs or mouse anti-XCP protein antiserum (preabsorbed with *Escherichia coli* lysate containing GST) for 3 h. After extensive washing in 0.5% blocking solution, membranes were incubated with GAM-HRP dilution 1:1500 for 4 h, and washed extensively again before being incubated in a substrate solution (0.03% DAB, 0.006% H₂O₂, 0.05% CoCl₂ in PBS) for color development.

IHC method

WTD-affected *Macrobrachium rosenbergii* PL fixed in Davidson's fixative solution for 24 h were processed into paraffin and serial tissue sections (8 μ m thick) and processed for indirect immunoperoxidase staining. Briefly, each section was covered for 12 h with each MAb diluted to 1:100 and then with GAM-HRP diluted to 1:1000 in 10% calf serum in PBS. Color was developed by incubating sections in 0.03% DAB and 0.006% H₂O₂ in PBS. Sections were counterstained with hematoxylin and eosin Y (H&E), dehydrated in graded series of ethanol concentration, cleared in xylene, and mounted in Permount (Sithigorngul et al. 2002). Positive reactions appeared as brown coloration against pink cytoplasm and purple nuclei. To examine if XSV co-localized with MrNV, serial histological sections of 18 WTD-affected PL processed were detected using the XSV-1 MAbs (the present study) in combination with MAb MrNV-12 (Wangman et al. 2012).

Determining MAb class and subclass

Classes and subclasses of the mouse immunoglobulins produced by the hybridomas were determined by sandwich ELISA using Mouse MonoAb ID Kit (Zymed).

MAb cross-reactivity testing

Histological tissue sections of penaeid shrimp infected with *Penaeus monodon* densovirus (PmDENV), *Penaeus monodon* nucleopolyhedrovirus (PemoNPV), Taura syndrome virus (TSV), infectious myonecrosis virus (IMNV), white spot syndrome virus (WSSV), and yellow head virus (YHV) were examined by IHC using the XSV-specific MAbs. Results were compared to IHC tests performed using MAbs specific to PmDENV (Rukpratanporn et al. 2005), TSV (Longyant et al. 2008), IMNV (Kunanopparat et al. 2011), WSSV (Chaivisuthangkura et al. 2004), YHV (Sithigorngul et al. 2002), and PemoNPV (Boonsanongchokying et al. 2006).

Comparison of dot blot and RT-PCR for detecting XSV

Tissue homogenates from WTD-affected prawns serially diluted in homogenates from an unaffected prawn were processed to detect XSV by dot blot and RT-PCR. In dot blots, XSV MAbs and MAb combinations as well as an MrNV MAb (Wangman et al. 2012) were tested, and the lowest dilution of homogenate showing color development was taken as the end point. Dot blots were also performed using an *Escherichia coli* lysate containing GST-XCP diluted similarly. Total RNA isolated from the same prawn homogenate and serially diluted in total RNA isolated from an unaffected prawn was tested by RT-PCR using primers XSVF70 and XSVR as described in 'XSV CP gene expression constructs' above.

RESULTS

XSV CP gene expression

The recombinant 42 kDa GST-XCP and 20 kDa His₆-XCP fusion proteins expressed in *Escherichia coli* were resolved by SDS-PAGE (Fig. 1). Recombinant protein bands excised from the gel and eluted from gel pieces were also analyzed to demonstrate their effective recovery, purity, and integrity (Fig. 1).

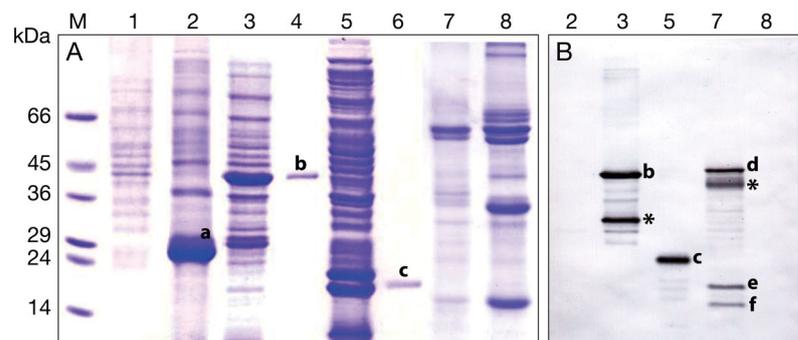


Fig. 1. *Escherichia coli* and *Macrobrachium rosenbergii*. SDS-PAGE and Western blotting. Lysates of *E. coli* strain BL21 without plasmid (1) or containing plasmids pGEX-6P-1 (2), pGEX-6P-1-XCP (3), or pET15b-XCP (5), and samples of purified GST-XCP (4), purified His₆-XCP (6) or homogenates from white tail disease-affected prawns (7) or uninfected postlarvae (8) were electrophoresed in 15% polyacrylamide gel. (A) Part of the gel was stained with Coomassie Brilliant Blue, and (B) another part of the gel was transferred to nitrocellulose membrane and treated with monoclonal antibodies XSV-1 + MrNV-12. M: standard marker proteins; a: glutathione S-transferase (GST) (26 kDa); b: GST-XCP (43 kDa); c: His₆-XCP (20 kDa); d: natural *Macrobrachium rosenbergii* nodavirus (MrNV) capsid protein (42 kDa); e and f: natural extra small virus (XSV) capsid proteins (17 and 16 kDa, respectively); * : degradation products or partial synthesis proteins

MAB production

After multiple injections of 4 mice with a 1:1 mixture of the gel-purified GST-XCP and His₆-XCP proteins, antiserum from all mice was found to react with GST-XCP, His₆-XCP, and XSV protein from homogenates of WTD-affected prawns. However, antiserum from only 1 mouse was found to be strongly immunoreactive based on its ability to detect GST-XCP, His₆-XCP, and double bands of native XSV CP when diluted 1:10 000. This mouse was thus selected as the spleen donor to produce hybridomas. In total, ~1600 micro-culture wells containing hybridoma cells were obtained, with culture fluid from ~20 wells binding GST-XCP and His₆-XCP in initially screens. After further screening of culture fluid from clones derived from these wells by dot blot and Western blot, and by IHC with tissue sections from WTD-affected prawns, 4 MABs with high specificity for XSV CP were selected from established hybridoma cell lines. MABs were determined to be from the immunoglobulin G (IgG) subclasses IgG1, IgG2a, or IgG2b (Table 1).

MAB specificity

None of the 4 XSV CP-specific MABs reacted with *Escherichia coli* proteins, *E. coli*-expressed GST, or tissue homogenates from uninfected prawns in either dot blots (Fig. 2) or Western blots (Fig. 1). All 4 MABs bound to GST-XCP and His₆-XCP and to double bands (16 kDa and 17 kDa) on native XSV CP present in tissue homogenates from WTD-affected prawns (Fig. 1B). In comparison to these 4 MABs, a MAB specific to the MrNV CP (MrNV-12; Wangman et al. 2012) reacted more strongly with tissue homogenates from WTD-affected prawns in both dot blots and Western blots (Figs. 1B & 2). By IHC, all 4 XSV CP MABs reacted with muscle tissue and with loose connective tissues in the hepatopancreas and

Table 1. Characterization of monoclonal antibodies (MABs) specific to extra small virus (XSV). IgG: immunoglobulin G; IHC: immunohistochemistry; +++: strong immunoreactivity

MAB (isotype)	Sensitivity (fmol μl^{-1})	Western blot	IHC	Cross-reactivity
XSV-1 (IgG2b)	10	+++	+++	None
XSV-8 (IgG2a)	20	+++	+++	None
XSV-10 (IgG1)	20	+++	+++	None
XSV-17 (IgG2b)	20	+++	+++	None

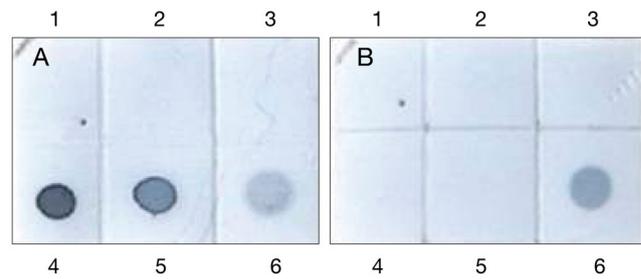


Fig. 2. *Escherichia coli* and *Macrobrachium rosenbergii*. Dot blot analysis of monoclonal antibodies (MABs). Lysates of *E. coli* strain BL21 without plasmid (1) or containing plasmids pGEX-6P-1 (2), pET15b-XCP (4), or pGEX-6P-1-XCP (5), and homogenates from uninfected (3) or white tail disease-affected (6) postlarval prawns were spotted (1 μl spot⁻¹) onto a nitrocellulose membrane and treated with MABs (A) XSV-1 or (B) MrNV-12

in the heart of WTD-affected prawns (Fig. 3). None of the MABs exhibited cross-reactivity to tissues from uninfected *Macrobrachium rosenbergii* prawns or penaeid shrimp infected with IMNV, WSSV, YHV, TSV, PmDENV, or PemoNPV (data not shown).

Co-localization of XSV and MrNV

IHC of consecutive tissue sections from 18 WTD-affected prawns using XSV-specific MAB XSV-1 and the MrNV-specific MAB MrNV-12 showed that 3 reacted with XSV-1 and all 18 reacted with MrNV-12. In some tissues of the 3 XSV-infected prawns, immunoreactions were equally intense for MAB XSV-1 and MAB MrNV-12, while in other tissues, XSV-1 reactions were either more or less intense than MrNV-12 reactions (Fig. 3). In all cases, XSV-1 and MrNV-12 reactions co-localized.

Test sensitivity

To test the detection sensitivity of XSV-specific MABs, serial dilutions of GST-XCP protein were detected to a limit of 0.5 $\mu\text{g ml}^{-1}$ with MAB XSV-1 and a limit of 1.0 $\mu\text{g ml}^{-1}$ with MABs XSV-8, XSV-10, and XSV-17, equivalent to 10–20 fmol μl^{-1} or 0.5–1 ng protein μl^{-1} . However, in dot blots using tissue homogenates of WTD-affected prawns, the detection sensitivity of MAB XSV-1 was between 2- and 8-fold higher than any of the other 3 XSV-specific MABs (Fig. 4A–D). Detection sensitivity was increased only slightly when MABs XSV-8, XSV-10, and XSV-17 were combined (Fig. 4E) but resulted in

a 2-fold increase when MAb XSV-1 was also included (Fig. 4F) compared to XSV-1 alone (Fig. 4A). The detection sensitivity of the combined XSV MABs was comparable to MAb MrNV-12 (Fig. 4H), and when combined with the 4 XSV MABs, its detection sensitivity increased 2-fold (Fig. 4G).

To compare the sensitivity of the MAb dot blot method with RT-PCR, RNA extracted from the same prawn homogenates used in dot blots was amplified. As shown in Fig. 5, it was still just possible to detect a 471 bp RT-PCR product with 10^{-6} diluted RNA, thus making this method on the order of 1000-fold more sensitive than detection of a XSV protein by dot blot using all 4 XSV MABs combined (Fig. 4F).

DISCUSSION

Here, 4 MABs generated from 2 forms of purified recombinant XSV CP expressed in *Escherichia coli* were assessed for their ability to detect XSV in *Macrobrachium rosenbergii* PL prawns affected by WTD. The MABs, and particularly all 4 MABs combined, were effective at detecting XSV using dot blot and Western blot methods as well as in histological sections of tissues by IHC. While antibody-based ELISA and Western blot methods have been reported to detect MrNV (Romestand & Bonami 2003, Qian et al. 2006, Sahul Hameed et al. 2011) or XSV (Sahul Hameed et al. 2011), this is the first report of XSV

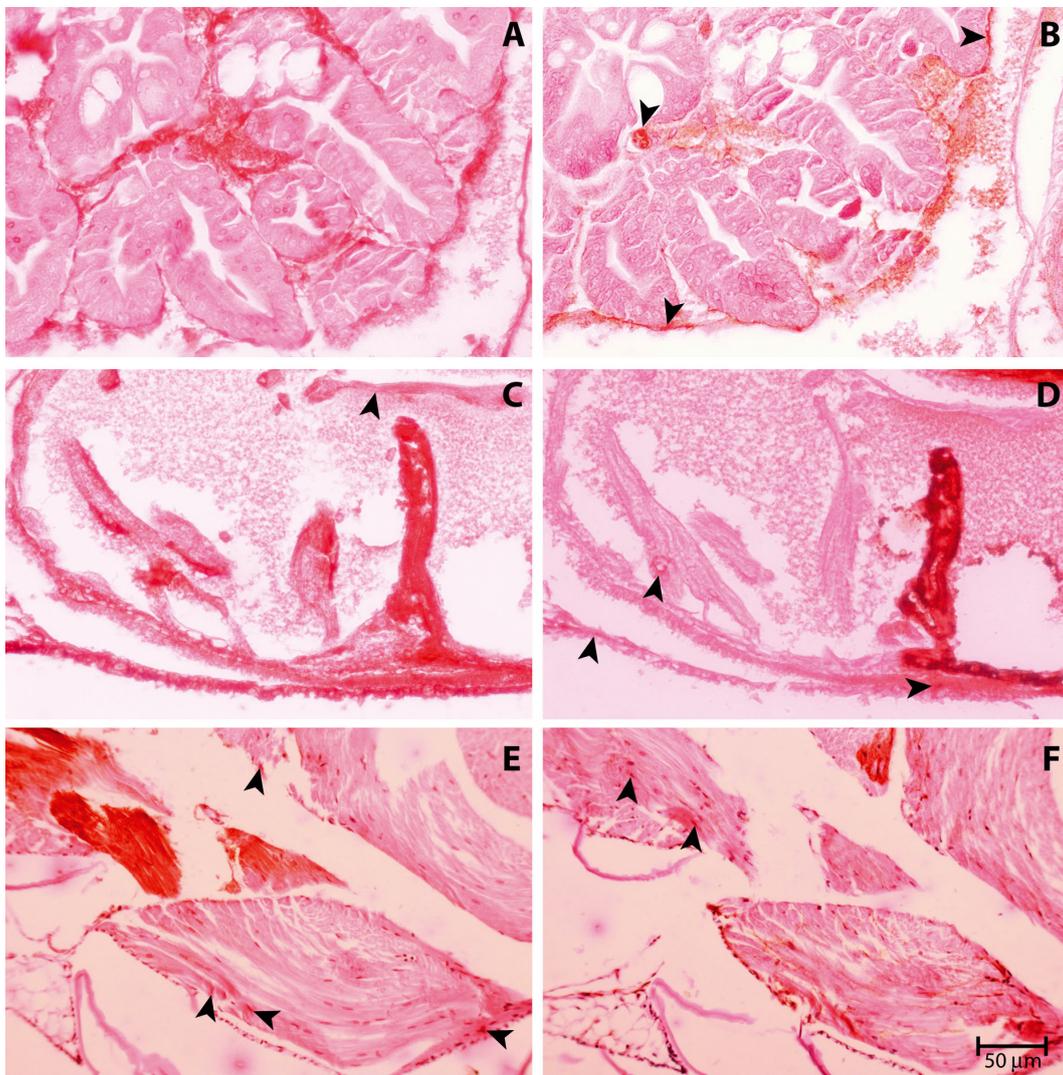


Fig. 3. *Macrobrachium rosenbergii*. Immunohistochemical analysis. Tissue sections from white tail disease-affected postlarvae were treated with (A,C,E) monoclonal antibodies XSV-1 or (B,D,F) MrNV-12 and counterstained with H&E. Strong immunoreactivity was exhibited in (A) intertubular connective of hepatopancreas, (B) heart, and (C) striated muscle. Lightly immunoreactive areas are indicated (black arrowheads). Scale bar = 50 μ m for all panels

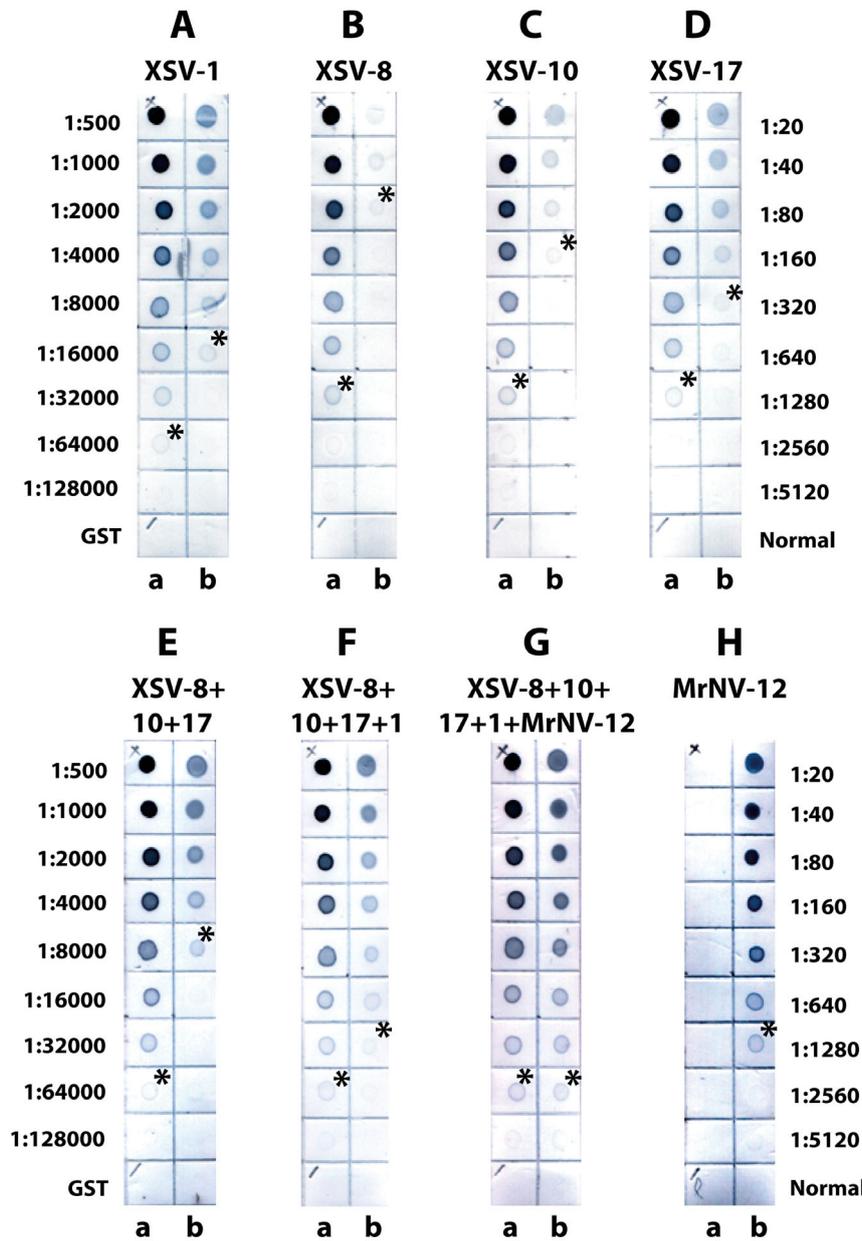


Fig. 4. *Escherichia coli* and *Macrobrachium rosenbergii*. Sensitivity of extra small virus (XSV) detection by dot blotting. Lysate of *E. coli* strain BL21 containing the GST-XCP fusion protein (a) or homogenate from white tail disease-affected postlarval (PL) prawns (b) were serially diluted with phosphate-buffered saline (PBS) and spotted ($1 \mu\text{l spot}^{-1}$) onto each square of nitrocellulose membranes. The last square of each column was spotted with lysates of *E. coli* strain BL21 containing glutathione S-transferase (GST) or homogenate from uninfected PLs (Normal). Each nitrocellulose membrane was treated with a single monoclonal antibody (MAB) or a combination of MABs specific to XCP (XSV capsid protein) and *M. rosenbergii* nodavirus (MrNV): (A) XSV-1, (B) XSV-8, (C) XSV-10, (D) XSV-17, (E) XSV-8 + XSV-10 + XSV-17, (F) XSV-1 + XSV-8 + XSV-10 + XSV-17, (G) XSV-1 + XSV-8 + XSV-10 + XSV-17 + MrNV-12, and (H) MrNV-12. (*) Lowest limit of detection for the methods. Numbers along left panel are dilution factors of lysate containing GST-XCP and those along right panel are dilution factors of prawn homogenate

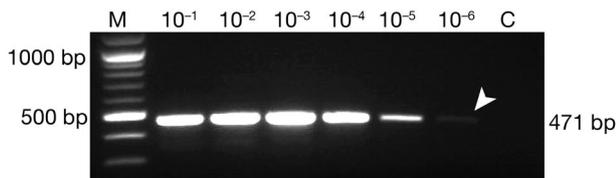


Fig. 5. *Macrobrachium rosenbergii*. RT-PCR analysis. The same sample of homogenate from white tail disease-affected postlarval prawns used for dot blotting was diluted from 10^{-1} to 10^{-6} and processed for RT-PCR. Lowest limit of detection for the methods is indicated (white arrowhead). M: DNA markers; C: negative control using distilled water

MABs and their use in conjunction with an MrNV-specific MAB to show clearly that this virus and presumed satellite virus co-localize to the same cells within affected muscle tissue.

Dot blots of tissue homogenates from 18 WTD-affected *Macrobrachium rosenbergii* showed XSV to be present in only 3, and in these, while XSV was always found associated with MrNV, the detection intensity of XSV varied upward and downward in comparison to MrNV at different sites of infection and pathology within individuals. The low prevalence of XSV detected among the 18 WTD-affected

prawns examined here is consistent with reports of RT-PCR testing which have found that, generally, XSV-positive individuals are also positive for MrNV but MrNV-positive prawns are not always infected with XSV at levels detectable by PCR (Tripathy et al. 2006, Yoganandhan et al. 2006, Bonami & Sri Widada 2011). However, in unusual cases when XSV was detected in the absence of MrNV, it has been speculated that this might be due to MrNV existing at very low levels below that detectable by the method used (Senapin et al. 2010). Indeed, the IHC data obtained in the present study indicate that some tissues rich in XSV can be infected at only low loads with MrNV, suggesting that little MrNV replication might be needed to support extensive XSV replication. Moreover, tissues in which infection loads of XSV were apparently high when MrNV loads were low showed typical WTD histopathology. It is, therefore, possible that XSV might contribute to disease severity rather than being a benign companion virus.

Application of the XSV MAbs and an MrNV MAb to IHC showed the viruses to co-localize in striated muscle and in connective tissues or organs and appendages. XSV was infrequently detected in gill or heart muscle, but when detected in the heart, the IHC signal was distributed homogeneously with the MrNV IHC signal, similarly to striated muscle cells. This tissue infection pattern differs from viruses primarily causing muscle pathology in *Penaeus vannamei* shrimp, such as *Penaeus vannamei* nodavirus (PvNV) (Tang et al. 2007) and IMNV (Tang et al. 2005, Kunanopparat et al. 2011), which in heart tissue display an IHC signal limited to fixed hemocytes.

The quantities of XSV obtained in pooled samples in the present study were much lower than those obtained for MrNV due its less frequent infection, as seen by an approximately 2-fold lower detection limit for XSV than for MrNV by dot blotting (Fig. 4A,H) and Western blotting (Fig. 2). However, the detection of XSV via MAbs would still be useful, since a combination of MAbs specific to XSV and MrNV resulted in increasing detection sensitivity (Fig. 4G). In the case of co-infection of XSV with light infection of MrNV, WTD was detected and confirmed when using MAbs specific to MrNV and XSV together (Fig. 3). As for other viruses (W. Sithigorngul et al. 2006, 2007, P. Sithigorngul et al. 2011), MAbs targeted to different epitopes on the XSV CP in combination with the MAb generated recently (Wangman et al. 2012) to the MrNV CP should allow immunochromatographic strip tests to be developed for the rapid pond-side diagnosis of WTD.

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