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Production of polyclonal and monoclonal antibodies against gill-associated virus and the development of an ELISA

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Abstract

Gill-associated virus (GAV) is the type species of the genus *Okavirus* in the Roniviridae of the Nidovirales and has been associated with mortalities in farmed *Penaeus monodon* (black tiger prawn). Polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) specific to GAV were produced following immunisation with protein subunits of purified GAV isolated from the gill tissues of *P. monodon* into either a six week old chicken (PAbs) or into a six week old Balb/c mouse for the production of the MAbs. Western blot analysis indicated that the PAbs reacted with 63, 110 and 170 kDa proteins, while the MAbs reacted to the 20 kDa protein of GAV. The MAbs were further investigated to ensure specificity by immunohistochemistry and elution of affinity purified GAV protein from the MAbs. An enzyme linked immunosorbent assay was then developed using the prepared PAbs and MAbs for the detection of GAV.

Keywords: Gill-associated virus; Yellowhead virus; ELISA; Detection method; Antibodies

1. Introduction

Gill-associated virus (GAV) of *Penaeus monodon* and by inference, yellowhead virus (YHV) is the type species of the genus *Okavirus* in the Roniviridae of the Nidovirales (Mayo, 2002) and has been associated with mortalities in farmed *P. monodon* (black tiger prawn) (Chantanachookin et al., 1993; Spann et al., 1997). The two viruses are closely related, sharing 95.8% amino acid sequence and 85.1% nucleotide identity (Cowley et al., 1999). The level of sequence divergence between YHV and GAV is much greater than individual isolates of GAV, which share 98.5% identity at the nucleotide level, indicating that YHV and GAV represent distinct genetic lineages and that they are geographical topotypes (Cowley et al., 1999). Nadala et al. (1997) identified four structural proteins (170, 135, 67 and 22 kDa) from purified YHV, and by inference GAV and determined that the 135 kDa protein was glycosylated.

Attempts to prevent and control GAV/YHV associated disease have relied primarily on diagnosing and removing infected prawns from the farm population. Reverse transcription nested polymerase chain reaction (RT-nPCR) has been developed for the detection of both GAV and YHV (Wongteerasupaya et al., 1997; Cowley et al., 1999, 2004) and more recently, a real time PCR has been developed for the two viruses (Dhar et al., 2002; Vega et al., 2004). Despite these PCR techniques being highly sensitive for the detection of the viruses, there is a practical limitation to their widespread application. These limitations include the requirement for specialised equipment, expensive molecular reagents and well-trained

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personnel. Consequently, these high costs result in the assay being non-viable for the majority of prawn farmers or small support laboratories that screen for viruses.

Immunological techniques are widely used for the diagnosis of many viral diseases. They are highly sensitive and accurate for specific viruses and results can be quickly obtained at a relatively low cost, making them ideal for on-farm screening of disease and pathogen load, when used correctly. Monoclonal antibodies to YHV have been previously reported (Sithigorngul et al., 2000, 2002, Soowannayan et al., 2003). However, the assay (Western blot, dot blot analysis and immunohistochemistry) could not be easily applied for field examinations.

The present work describes the first development of PAbs and specific MAbs against GAV and their use in the detection of GAV by capture ELISA.

2. Materials and methods

2.1. Purification of gill-associated virus

Methods used to purify GAV were adapted from Wongteerasupaya et al. (1995) and Nadala et al. (1997). Fifty grams of *P. monodon* gill tissue was homogenised in 500 ml (1 in 10 w/v) of TNE buffer (0.2 M NaCl, 0.02 M $NH_2C(CH_2OH)_3$, 0.02 M $C_{10}H_{14}N_2Na_2O_2{\cdot}2H_2O$, pH 7.4). This was then centrifuged at 5000 ×*g* for 10 min in an Eppendorf centrifuge (A-4-44 rotor). The supernatant was then centrifuged again at 5000 ×*g* for 30 min in an Eppendorf centrifuge (A-4-44 rotor). The supernatant was then filter sterilised through a 0.45 μm filter and centrifuged at 100 000 ×*g* for 1 h in a Beckman ultracentrifuge (Ti 70.1 rotor). The resulting pellet was resuspended in TNE buffer and overlayed onto a 25–55% sucrose gradient and centrifuged at 100 000 ×*g* for 20 h on a Beckman ultracentrifuge (SW40 rotor). After centrifugation, viral bands were visualised by top illumination and were removed using an 18 gauge needle and diluted 5 times in TNE before centrifugation at 100 000 ×*g* for 1 h. The pellet was then resuspended in 500 μl of phosphate buffered saline (PBS) (1.4 M NaCl, 0.014 M KH_2PO_4, 0.08 M Na_2HPO_4) and stored at −20 °C.

2.2. Polyacrylamide gel electrophoresis (PAGE)

One dimensional polyacrylamide gels of 7.5 cm length, using 0.75 mm spacers, with a 12% separating gel were prepared and run by the method of Pizzutto (1992), being a modification of the method of Laemmli (1970). A mini Protean II apparatus (Bio-Rad) was used to electrophorese the gels. SDS running buffer (0.1 M $NH_2C(CH_2OH)_3$, 1 M $C_2H_5NO_2$, 0.02 M $C_{12}H_{25}O_4SNa$, pH 8.3) was poured into the buffer tank containing the gels and 200 μl of sample was loaded into the two comb well. The sample consisted of 50% purified virus and 50% reducing buffer (0.06 M $NH_2C(CH_2OH)_3$, pH 6.8, 10% glycerol, 5% CH_6OS, 0.05% (w/v) bromophenol blue). Five microliters of broad range SDS-PAGE molecular weight standards (Bio-Rad) was used as markers. Electrophoresis was performed at 200 V until the tracking dye left the bottom of the gel. Protein bands were either visualised by staining with Coomassie blue or transferred for Western blotting.

2.3. Production of polyclonal antibodies in chickens

A multiple emulsion of PBS with presumptive GAV protein subunits 170, 110 and 63 kDa emulsion (excised from the polyacrylamide gel) was prepared in a 1:1 ratio. When completely homogenised, 1 ml of the mixture was injected into the breast muscle of three semi-adult chickens.

On days 14 and 28 after the initial injection, a booster dose was administered using the same technique as the original injection. On day 25, 3 ml of blood was removed from each chicken via the wing vein. The blood was allowed to clot at room temperature for 4 h. The serum was then removed and centrifuged at 1500 ×*g* for 10 min. The serum was then screened for polyclonal antibodies to the target antigen (method below) by indirect ELISA. On day 28, a further 10 ml of blood was removed from each chicken via the wing vein. The blood was then processed as before. The serum was subsequently stored in aliquots of 200 μl at −20 °C.

2.4. Western blots

2.4.1. Protein transfer

Protein samples were run using the SDS-PAGE technique. The procedure for wet electrophoretic transfer was adapted from Towbin et al. (1979). The gels were loaded into gel cassettes (Bio-Rad Laboratories) in direct contact with polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Australia). A cooling module was inserted into each chamber. The chambers were then filled with transfer buffer and the complete unit was placed in a water bath at a constant 4 °C. The blots were run at 50 V for 2.5 h. They were then removed from the cassettes and dried overnight at room temperature.

2.4.2. Immunostaining Western blots

Once the membranes (blots) were thoroughly dry, they were incubated with either MAbs or PAbs (primary antibody) for 1 h. The membrane was washed twice for 5 min each time using fresh PBS. The secondary antibody, either rabbit anti-chicken IgG (H+L)–HRP conjugate