Preliminary Molecular and Biological Characterization of Mourilyan Virus (MoV): A New Bunya-Related Virus of Penaeid Prawns

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ABSTRACT

Mourilyan virus (MoV) is a newly identified virus that infects penaeid prawns. Spherical to ovoid enveloped particles (85 x 100 nm dia.) possess bunyavirus-like morphology. RT-nested PCR testing has indicated that natural MoV infections occur commonly in black tiger (*Penaeus monodon*) and Kuruma (*Penaeus japonicus*) prawns from the wild or farmed commercially in Queensland. In each species, low-level MoV infections can be detected by *in situ* hybridisation (ISH) in vacuolated 'spheroid bodies' within the lymphoid organ. In heavily infected prawns, MoV is detected throughout the lymphoid organ and in connective tissues of other organs. In some *P. japonicus*, MoV has been identified in midgut and nerve tissues displaying histopathology consistent with gut-and-nerve syndrome. However, MoV infection has not been consistently observed within these abnormal tissues and additional studies are required to determine the relevance of MoV to this syndrome. Preliminary data suggests that the MoV genome comprises 4 segments of (-) sense single-stranded RNA. BLAST searches using open reading frames (ORFs) encoded in 3 segments identified distant relationships to the L (RNA-dependent RNA polymerase), M (G1/G2 glycoprotein) and S (N nucleoprotein) proteins of Uukuniemi virus and other viruses within the genus *Phlebovirus* of the *Bunyaviridae*. In phleboviruses, the S RNA segment also contains a small non-structural protein (NSs) gene that is encoded in ambisense. In the MoV S1 RNA there is no ambisense coding strategy but a somewhat larger protein not obviously related to NSs is encoded in the small (S2) fourth RNA segment. Evidence of elevated levels of viral infection associated with disease episodes suggests that MoV may be a significant pathogen of farmed prawns in Queensland and elsewhere in the Asian region.

THE IDENTIFICATION OF MOURILYAN VIRUS IN AUSTRALIAN FARMED PRAWNS

Mourilyan virus (MoV) was first identified in diseased Penaeus monodon collected from a farm near the township of Mourilyan in northern Queensland in 1996. These prawns were also infected with high levels of gill-associated virus (GAV) (Spann *et al*., 1997). Three MoV clones were identified in a cDNA library randomly amplified from a > 22 kbp dsRNA purified from lymphoid organ total RNA of P. monodon injected with an inoculum derived from these diseased prawns (Spann *et al*., 1997; Cowley *et al*., 2000). Clones in this cDNA

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library were used to determine a large proportion of the sequence of 26.2 kb (+) ssRNA genome of GAV (Cowley *et al*., 2000; Cowley and Walker, 2002). We assume that the MoV cDNAs must have been derived from viral RNA species that co-purified with the large GAV dsRNA. This is supported by evidence described below for co-infection of *P. monodon* with these two viruses.

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GenBank Acc. M17417: UUKV M-RNA segment, membrane glycoprotein G1/G2
Score = 77.0 bits (188), Expect = 1e-13
Identities = 50/175 (28%), Positives = 88/175 (49%), Gaps = 5/175 (2%)

MoV4.1   8  LPYKGNGFTILSGKSGIVAHSTSTALLSLNIQLGKLIVKKEKARYKCHAQFVKLTGCYSC  67
            LP      T    ++ + A     A  ++ I+L    ++      KC  +F+ L+GCY+C
UUKV   805  LPSTVGSVTFWPTETSVEAAIPDLASATMLIRLDGYTIQFRSDSNKCSPRFLSLSGCYNC 864

MoV4.1  68  MSGATLTILVGSTSDSSEAVLNCPDVNYTTIV-VANKVEESVTSTLHLTQSMIDMKCEVV 126
             +GA L +   +   ++  +L CP + YTT   V N +E+S+  T+HL  S ++ KC
UUKV   865  EAGAKLELEHVTDFGTALGILECPSLGYTTYYEVKNTLEKSI-RTMHLNGSHVEAKCYFR 923

MoV4.1 127  CPSSRTYLDVTGSLLYI--PDVDPETRTMSVDVHQNGASFTLNPLGSYKSAILYA      179
            CP+S + L + G L+Y+   D+    +T+S  +     S   +P G +K++ L A
UUKV   924  CPNSESQLTIRGELIYLFNDDIRHHNQTLSPGLSPKSGS-GWDPFGWFKASWLRA      977

Score =  130 bits (326), Expect = 1e-29
Identities = 66/221 (29%), Positives = 110/221 (48%), Gaps = 4/221 (1%)

MoV48    4  IQLKCEKQSLYYVPRAVGKCTSVSHCSTVSGCSAKACLEFKANGSLPEWTSE---MDHYG  60
            I+L+C ++ LY+VPR   +C     C  +  C  +AC EFK N   PEW  E   M   G
UUKV   576  IKLECVRRDLYWVPRVTHRCIGTRRCHLMGACKGEACSEFKINDYSPEWGHEEELMAQLG 635

MoV48   61  WSRCQGIPGCAANGCFYCDDGCLWWREYFTNPKSEVYEIIRCPTWVFEVDLEVVLSNITT 120
            WS C    G A   CF     C + R+ F++   + + I  C  W + +++ V  ++  +
UUKV   636  WSYCVEQCGGALCQCFNMRPSCFYLRKTFSHLSQDAFNIYECSEWSYRINVLVSTNSTHS 695

MoV48  121  HVVLSPGATKAVGSVRLSLEALSAPPEPILGDCFYQLGPVTRLGP-CNERGSLSPGKVGE 179
            ++ L  G   ++    +SL ++S PP     +CF +    T+    CN R   + G++GE
UUKV   696  NLTLKLGVPDSIPHGLISLSSVSQPPAIAYSECFGEDLHGTKFHTVCNRRTDYTLGRIGE 755

MoV48  180  LQCPSKESARRADASCFANEAMVRTTVSSAGVSCHFSIVDP                    220
            +QCP+K  A      C ++++++ + V    V C  SI+DP
UUKV   756  IQCPTKADALAVSKRCISSDSIIFSKVHKDSVDCQSSIIDP                    796


GenBank Acc. D10759: UUKV L-RNA segment, L RNA polymerase
Score = 58.2 bits (139), Expect = 8e-08
Identities = 45/180 (25%), Positives = 87/180 (48%), Gaps = 10/180 (5%)

MoV1.2  14  TQVFYGIIVTNELKVCSNLPLTEEQVNELVFRCRMAIDIQAELGLLGVSFHEEDDEMSQT  73
            + + +G++V +E  V +NL L +++V+EL FR  +A  +  E+    +    +D++  +
UUKV   159  SSILFGVVVVSETTVVTNLNLNQQEVDELCFRFLVARAVHLEMTTKMIIPEYDDEDEDKR 218

MoV1.2  74  SAEVKTILRNIPLSFHFDDKY---VTREVYEN-SFGAPDSEYLKTMISTLMTDGRKNDLA 129
            S EVK    ++   ++  +      +R ++ N +   PD EYL    + ++ D  K   A
UUKV   219  SREVKAAFHSVQPDWNVTEANFAPFSRRMFSNFAQMEPDKEYL----AHIILDSLKQAQA 274

MoV1.2 130  TGAGMNFCDEEKKWEGPSITKNLNECLSSIKKYERDLME-GERQHSSTKAIVQLPAWVAK 188
               G ++ +E    E   + +N  E L+ +K +ERD     +R   S K+ V  P  + K
UUKV   275  DLDGNHYLNESLT-EQARLDRNREESLNMVKDFERDFNNAAQRSAWSHKSTVPFPGVIPK 333
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Figure 1. BLAST matches with ORFs in cDNA clones pMoV4.1, pMoV48 and pMoV1.2 with the membrane glycoprotein G2 and L RNA polymerase protein of UUKV. Cysteine (C) residues in MoV and UUKV G2 glycoprotein alignments are highlighted in bold.

MoV cDNA Sequence Homologies

BLAST searches (http://www.genome.ad.jp) using open reading frames (ORFs) encoded in the 3 MoV cDNA clones identified distant (25 to 29% amino acid identity) but significant ($8e^{-08}$ to $1e^{-29}$) relationships to the G2 and L proteins of Uukuniemi virus (UUKV) (Fig.1), a tick-borne phlebovirus infecting birds (Rönnholm and Pettersson, 1987; Elliott *et al*., 1992). Slightly lower but significant levels of similarity were evident with the G2 and L proteins of other phleboviruses infecting mammals and transmitted either by mosquitos or phlebotomine flies (Accardi *et al*. 1993; Collett *et al*. 1985; Gro *et al*. 1997; Ihara *et al*. 1985; Muller *et al*. 1991). Particularly striking was the conservation of Cys residues between ORFs encoded in pMoV4.2 and pMoV48 and the UUKV G2 glycoprotein. This suggests a high degree of structural integrity has been preserved in this envelope surface glycoprotein despite the significant evolutionary divergence evident between these virus and their hosts.

GENOME ORGANISATION OF MOV

A 5'-RACE technique (Cowley *et al*. 2000) was used to generate clones that were used to determine the nucleotide sequence to the 5' end of the putative (+) sense RNA transcribed from the MoV M RNA segment. Sequence analysis of multiple clones revealed that the terminal 10 nucleotide (nt) sequence of the MoV M mRNA was conserved with the consensus inverted terminal repeat sequence preserved at the ends of the 3 (-) genomic RNA segments (L, M, and S) of UUKV and the other phleboviruses. Based on this data, RT-PCR using a primer designed to anchor to the terminal 10 nt region of the M mRNA was used as a starting point to identifying the genome organisation and sequence of MoV (Fig 2.).

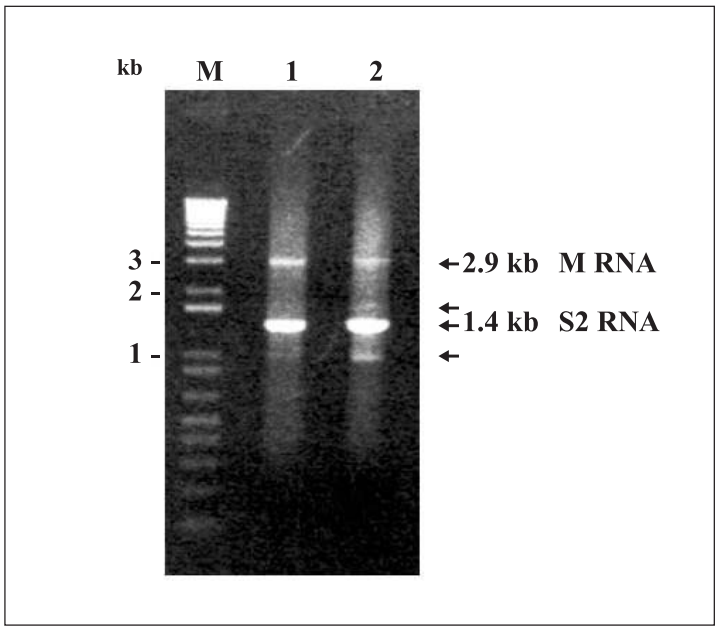

Figure 2. RT-PCR products obtained using the anchored terminal MoV primer and lymphoid organ total RNA from 2 different sources of MoV-infected *P. monodon* (lanes 1 and 2).

The single-primer RT-PCR generated 2 primary DNA products of ~2.9 kb and ~1.4 kb and minor products of ~1.6 kb and ~1.0 kb. Cloning and sequence analysis of the PCR products revealed that the 2.9 kb DNA comprised the complete MoV M RNA segment and that the 1.4 kb DNA comprised one (designated S2) of two small MoV RNA segments. Individual clones of the other small MoV RNA segment (designated S1) were found to vary in length from 1.1 and 1.55 kb, suggesting that they might be derived from DNA intermediate in size between the two minor PCR products. The heterogeneity detected in the five S1 RNA segment clones was due to the presence of a variable-length (157 - 635 nt) imperfect dinucleotide (ie. GA in the (+) sense RNA) repeat. The repeat sequence initiated immediately downstream of a 246 amino acid (aa) ORF and continued to a position 99 nt upstream of the 3'-end of the S1 RNA. BLAST searches of the S1 ORF indicated that it was homologous to the nucleocapsid (N) protein of UUKV and the other phleboviruses. The complete sequence of the long (6.3 kb) MoV L RNA segment was obtained using RT-PCR products amplified using the anchored terminal primer in combination with overlapping L segment-specific primers designed to sequences in clone pMoV1.2.

Overall, the data obtained for the MoV RT-PCR clones and PCR products generated using the anchored terminal primer indicated that the MoV genome comprises 4 RNA segments. We have designated these as L, M and S1 and S2. A schematic representation showing the length of the 4 RNA segments (combined length using the longest S1 RNA segment = 12227 nt), depicted as (+) sense RNA for convenience, the relative position of the encoded ORFs and the lengths of the 5' and 3' untranslated regions is shown in Fig. 3. The length and deduced molecular mass of the ORF encoded in each segment, together with putative functional designations of each protein, are also indicated in Fig. 3.

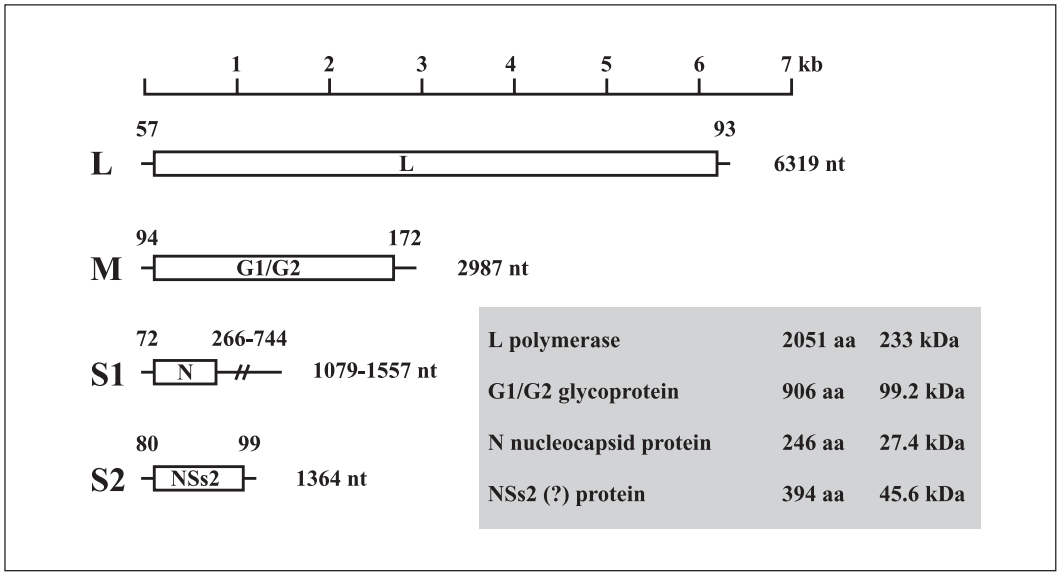

Figure 3. Genome organisation and putative protein coding strategy of MoV.

Northern blotting using a DNA probe specific to each MoV RNA indicated that all 4 RNA segments are expressed in MoV-infected prawn tissue (data not shown). Interestingly, the probe to the MoV S1 RNA detected a discrete ~1.5 kb band that was no more smeared than bands detected by the probes to the MoV L, M and S2 RNA segments. We are currently trying to determine whether variability in the length of the GA repeat within the S1 RNA segment occurs in vivo or whether the heterogeneity in size identified among the S1 cDNA clones was due to an amplification artefact of the random RT-PCR prompted by the long repeat sequence.

The genome organisation of MoV differs from that of viruses formally classified within the Bunyaviridae in that it comprises 4 rather than 3 segments of RNA. However, we have yet to demonstrate unequivocally that (-) sense RNA segments are packaged within MoV virions. BLAST searches and Clustal W multiple sequence alignments using complete L polymerase, G1/G2 glycoprotein and N protein sequences confirmed preliminary data obtained using the partial MoV4.1, MoV48 and MoV1.2 coding regions and indicates that MoV is evolutionarily most closely related to UUKV (data not shown). However, unlike UUKV and the other phleboviruses (Giorgi, 1996), MoV appears not to use an ambisense coding strategy in the S1 RNA segment for expression of a non-structural NSs protein. The MoV S1 RNA contains a GA (in the + sense) or CU (in the - sense) repeat in this region. BLAST searches using the protein sequence encoded in MoV S2 RNA segment failed to identify any proteins with significant homology in the combined databases. However, the 250-316 aa NSs protein of phleboviruses, which is encoded in the S RNA in ambisense with the N protein, display far greater sequence divergence than the phlebovirus virion proteins. In fact, BLAST searches using the UUKV NSs protein do not detect any similarity with the cognate protein of the phleboviruses transmitted by mosquitos or phlebotomine flies, or any other proteins in the combined databases. Based on lack of sequence similarity among the phlebovirus NSs proteins and the absence of an NSs ORF in the MoV S1 RNA, it is tempting to speculate that the 394 aa protein (NSs2) encoded in the MoV S2 RNA may correspond to the NSs protein of phleboviruses.

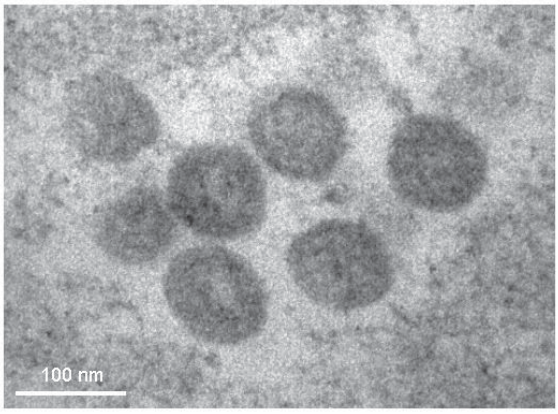

Figure 4. Transmission electron micrograph showing putative spherical to ovoid shaped enveloped MoV particles in thin sections of a cell within the lymphoid organ of a *P. japonicus*. (bar = 100 nm)

MOV PARTICLE MORPHOLOGY

As the molecular data indicated that MoV was most closely related to bunyaviruses, we sought to find electron microscopic evidence of cytoplasmic particles with bunyavirus-like morphology in *P. monodon* and *P. japonicus* from eastern Queensland that, by RT-PCR analysis, were shown to be infected with MoV. Spherical to ovoid (~100 x 85 nm) particles of variable electron density were identified in both species and an example of particles observed within the lymphoid organ of a MoV-infected *P. japonicus* is shown in Fig. 4. The particles appeared to be enveloped (sometimes with a more electron dense membrane-like margin) and possessed a fuzzy surface layer. In *P. monodon* gill cells, these spherical particles were often found in association with rod-shaped virions of GAV (data not shown), which is highly prevalent in the population of *P. monodon* from northeastern Queensland. The MoV virions display some morphological similarity to the 50-90 nm spherical particles identified recently in the eye of Australian *P. monodon* (Smith, 2000) and to spherical enveloped virus particles coexisting with a haemocytic rod-shaped virus identified in experimental hybrids of Australian *P. esculentus* and *P. monodon* (Owens, 1993). In regard to other crustacean viruses, MoV is structurally similar to crab haemocytopenic virus (CHV) that has been identified in haemocytes of the European shore crab and reported to possess bunyavirus-like morphology (Bang, 1971; Hoover and Bang, 1976). With regard to viruses of terrestrial organisms, the particle morphology of MoV is essentially indistinguishable from UUKV (Saikku and von Bonsdorff, 1968; Von Bonsdorff *et al*., 1970) and other bunyaviruses (Murphy *et al*., 1973).

TISSUE DISTRIBUTION OF MOV

In situ hybridisation (ISH) using a digoxigenin-labelled DNA probe prepared by random priming from the excised cDNA insert in clone pMoV4.1 was used to examine the tissue distribution of MoV in healthy and diseased *P. monodon*. MoV tissue distribution was also determined in *P. japonicus* collected either from farm ponds or from tanks at a research facility in which farmed stocks were being over wintered and suffering idiopathic mortalities. In overtly healthy farmed prawns of either species, MoV ISH signal was primarily restricted to spheroid bodies within lymphoid organ (Fig. 5).

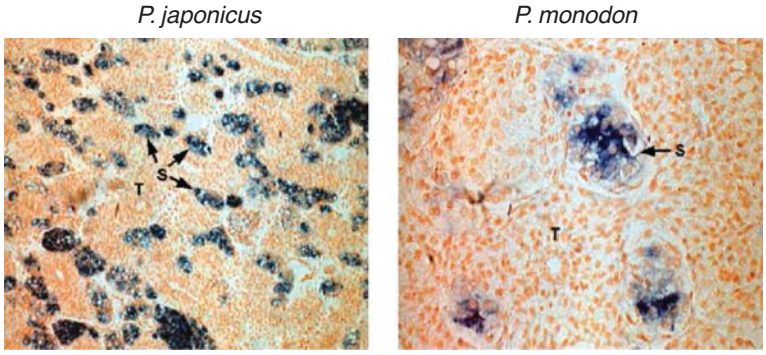

Figure 5. MoV ISH signal in the lymphoid organ of healthy *P. japonicus* and *P. monodon* (S = spheroid, T = normal tubule).

More extensive tissue distribution of MoV was found in the last survivor of a group of adult *P. japonicus* being over wintered in tanks at a research facility. Animals in this group suffered progressive idiopathic mortalities and all died within 3 months of being moved into tanks. The lymphoid organ of these prawns displayed extensive spheroid formations, which were highly vacuolated, and MoV ISH signal was obvious throughout both spheroid and normal tubule tissues (Fig. 6). MoV distribution throughout the lymphoid organ was also seen in moribund *P. monodon* either from farms disease episodes or generated by injection of an inoculum derived from diseased, farmed *P. monodon* containing high levels of both MoV and GAV (Fig. 6). Vacuolisation within spheroids, however, was more evident in *P. japonicus* than in *P. monodon*. Conversely, structural degeneration associated with spheroid disintegration and a loss of normal tubules was more evident in the lymphoid organ in moribund *P. monodon* than in *P. japonicus*, most probably as a result of the co-existence of GAV (Spann *et al*., 1997).

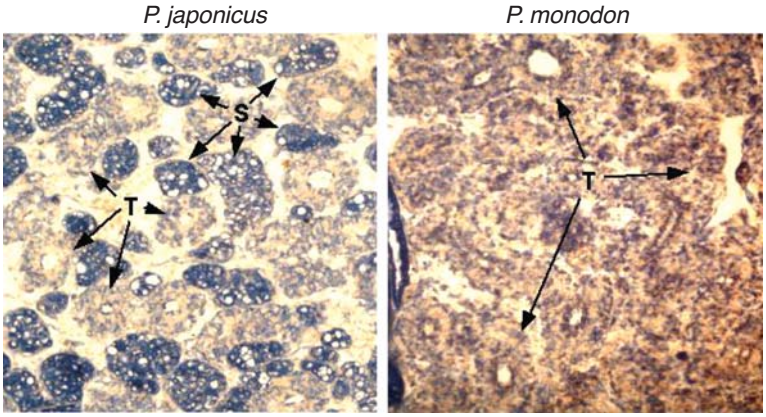

Figure 6. MoV ISH signal in the lymphoid organ of a *P. japonicus* from a group suffering idiopathic mortalities and a moribund *P. monodon* containing high levels of both MoV and GAV. S = spheroids, T = normal tubules

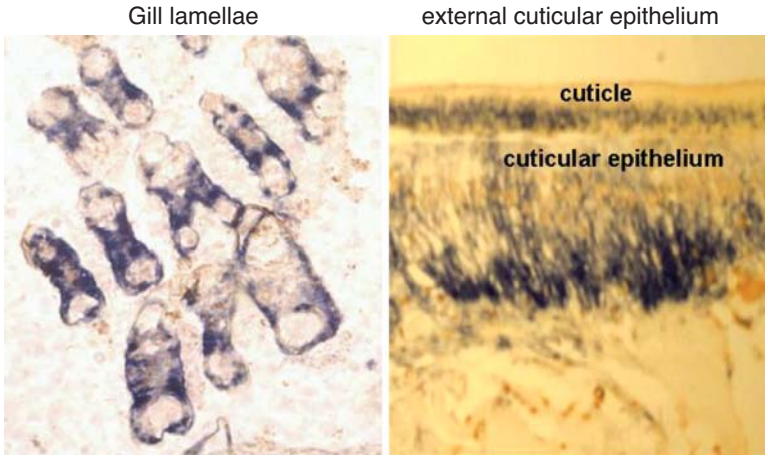

Figure 7. MoV ISH signal in the gill lamellae and the external cuticular epithelium of a *P. japonicus* heavily infected with MoV.

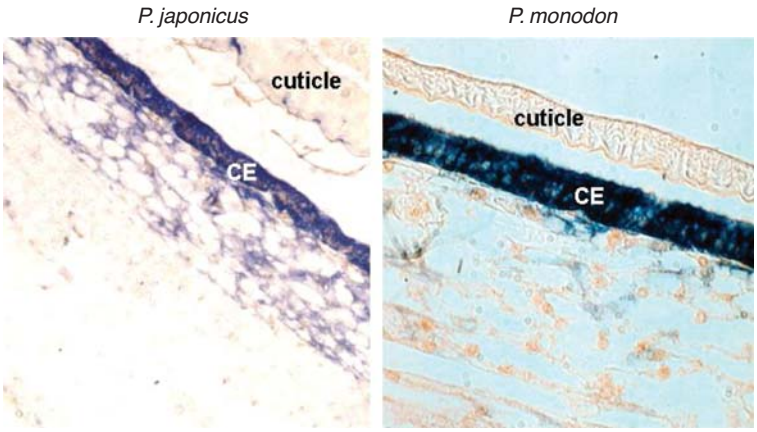

Figure 8. MoV ISH signal in the foregut cuticular epithelium of *P. japonicus* and *P. monodon* heavily infected with MoV (CE = cuticular epithelium).

In heavily infected *P. japonicus*, MoV ISH signal was also detected in all tissues of mesodermal and ectodermal origin. These included the connectives of all major organs, haematopoietic tissues, gills, epicardium, heart, cuticular epithelia, the epithelia of the foregut, midgut and antennal gland, the ventral and peripheral nerves, the segmental nerve ganglia and the epineurium lining of these nerves. Micrographs displaying examples of ISH signal within gill lamellae, external cuticular epithelium and the cuticular epithelial lining of foregut are shown in Figs. 7 and 8. The tissue distribution of MoV in moribund *P. monodon* that contained high levels of MoV and GAV was not significantly different to that seen in *P. japonicus* and was remarkably similar to that identified for GAV and YHV using ISH (Spann *et al*., 2003; Tang and Lightner, 1999; Tang *et al*., 2002). In different moribund *P. monodon* that have been examined, tissues of mesodermal and ectodermal origin displayed variable ISH signal intensity that is likely to reflect the infection stage in the particular prawn. A micrograph showing the presence of MoV in the midgut cuticular epithelium of a moribund *P. monodon* is shown in Fig. 8.

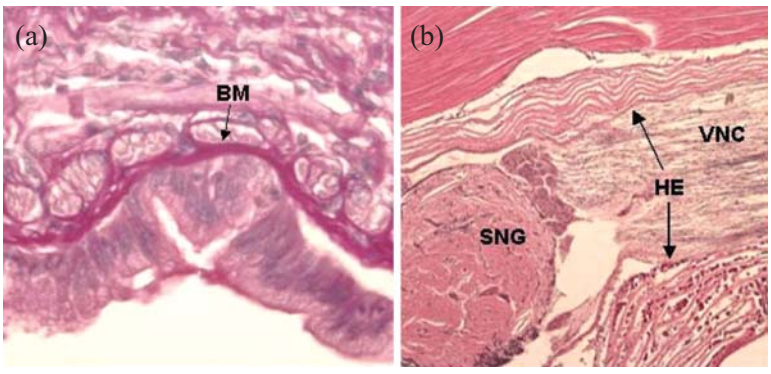

Figure 9. (a) PAS stain highlighting thickening of the basement membrane (BM) of the midgut. (b) H&E stain highlighting hyperplasia of the epineurium (HE) lining of the ventral nerve cord (VNC) and segmental nerve ganglion (SNG) in a *P. japonicus.*

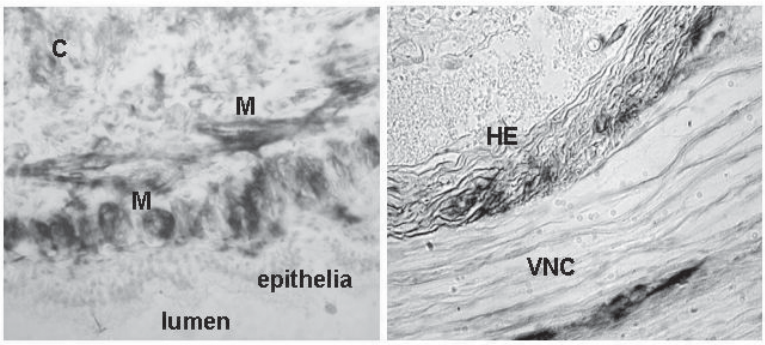

Figure 10. (a) MoV ISH signal in the underlying muscle (M) and associated connectives (C) of the midgut. (b) MoV ISH signal in hyperplastic epineurium (HE) and in ventral nerve cord (VNC) of a heavily infected *P. japonicus*.

Histopathology consistent with gut-and-nerve syndrome (GNS) (Lightner *et al*., 1984), namely hyperplasia of the epineurium and thickening of the midgut basement membrane, has been observed to variable extents in farmed *P. japonicus* representing the progeny of wild broodstock captured from Mackay harbour in northern Queensland (Fig. 9). In these animals, MoV ISH signal has been detected in the layered (hyperplastic) epineurium, ventral nerve cord, segmented nerve ganglia and underlying muscle and associated connectives of the midgut (Fig. 10). However, ISH signal was not consistently identified in tissues displaying characteristic GNS histopathology (data not shown). There is a previous report of a reo-like virus (double shelled; 61 nm diameter) in the hepatopancreas of *P. japonicus* with GNS (Lightner *et al*., 1985; Tsing and Bonami, 1987). However, the limited data currently available on this reo-like virus and MoV are insufficient to clearly associate either virus with GNS.

Studies of the Progression of MoV Infection in Farmed *P. japonicus*

In the 2000-01 grow-out season, RT-nested PCR was used to follow the progression of MoV loads in *P. japonicus* in four ponds at a farm in southeast Queensland (Fig. 11). The PCR method, which utilised primers designed to sequences in clone pMoV4.1, will be described in detail elsewhere. Nested PCR detected MoV in the pools of 10 x PL10s in 2 of the 3 hatchery tanks. Postlarvae in the 3 tanks were mixed prior to seeding 4 ponds. At monthly intervals during grow-out, 10 prawns were sampled from each pond. Lymphoid organ tissue pooled from 5 prawns from each pond was used as a source of total RNA for PCR amplification. Nested (306 bp) PCR products were not uniformly detected until the prawns had been in ponds for 3 months, suggesting that MoV levels were very low and/or that only relatively few prawns were infected during the early months of grow out. At month 3, MoV levels in prawns from pond 4 were sufficiently high to allow detection of a primary (607 bp) PCR product. At month 4, prawns from 3 of the 4 ponds possessed MoV levels sufficient to generate a primary RT-PCR product and prawns from pond 4 were again most heavily infected. This farm, and 3 others in southeast Queensland, reported poor survival rates (as low as 20%) during the 2000-01 grow-out season and overall their average yield (2000 kg/ha) was well below that achieved in previous seasons. We are currently attempting to determine whether MoV was the etiological agent for the poor survival of *P. japonicus* farmed in Queensland during this season.

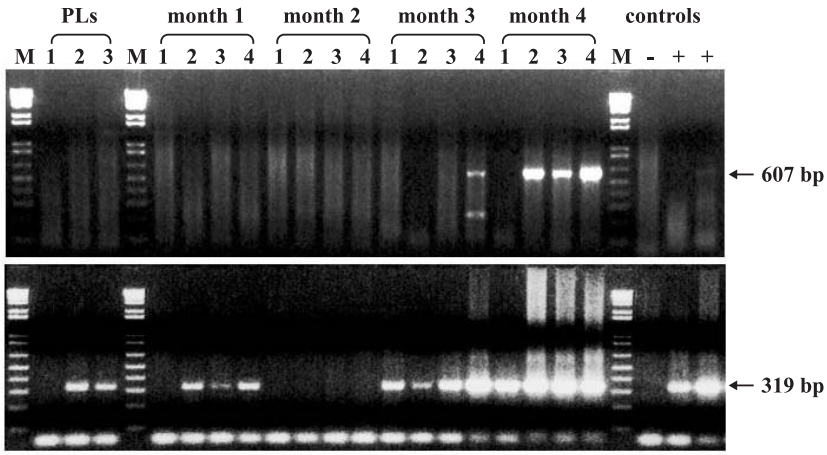

Figure 11. Detection of MoV by RT-nested PCR using total RNA isolated from pools of *P. japonicus* postlarvae (10 x PL10) or lymphoid organ tissue from prawns (pools of 5) collected at monthly intervals throughout the grow out in 4 ponds (1 to 4) at a farm in south-east Queensland. Primary RT-PCR products (607 bp, top panel) and nested PCR products (319 bp, bottom panel) amplified from the primary PCR were resolved in 2% agarose gels containing 0.5 μg/ml ethidium bromide. M = 1 kb PLUS ladder (Invitrogen) and MoV positive (+) and negative (-) RNA controls are indicated.

CONCLUSION

The genome sequence of MoV identified in *P. monodon* and *P. japonicus* from eastern Queensland, indicates that it is the first crustacean virus to be related genetically to viruses classified within the genus *Phlebovirus* of the *Bunyaviridae*. Of the phleboviruses, MoV is most closely related to UUKV, a tick-transmitted virus of birds. However, unlike the phleboviruses and all other viruses formally classified within the *Bunyaviridae*, the MoV genome comprises 4 rather than 3 segments of RNA. The additional small (S2) RNA encodes a protein unrelated to any proteins currently in the combined sequence databases. Moreover, unlike UUKV and the phleboviruses transmitted by mosquitos or phlebotomine flies, the MoV S1 RNA encodes a homolog of the phlebovirus N protein but does not encode an NSs protein homolog in ambisense. Rather, it contains an imperfect dinucleotide repeat in this region. Spherical to ovoid (~100 x 85 nm) particles presumed to represent MoV have been identified in both *P. monodon* and *P. japonicus*. The particles possess an envelope with a fuzzy outer surface and are morphologically indistinguishable from the virions of UUKV and other bunyaviruses. MoV in healthy *P. monodon* and *P. japonicus* was restricted mainly to spheroids in the lymphoid organ, implying that it can exist as a subclinical infection in both species. In moribund *P. monodon* co-infected with GAV, MoV can be present in significantly increased levels and be detected by ISH in all tissues of ectodermal and mesodermal origin. A similar widespread tissue distribution occurs in *P. japonicus* suffering idiopathic mortalities. To determine whether MoV is responsible for mortalities in the absence of GAV, we are currently attempting experimental infections in juvenile *P. japonicus*. Other work in progress aims to determine the prevalence of MoV in wild populations of *P. monodon* and *P. japonicus*, whether it is transmitted vertically and whether it is the cause of recent production losses in the *P. japonicus* farmed in southeast Queensland.

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