JOURNAL OF
GENERAL VIROLOGY

RESEARCH ARTICLE
Owens, *Journal of General Virology* 2023;104:001878
DOI 10.1099/jgv.0.001878

MICROBIOLOGY SOCIETY

OPEN ACCESS

Abolishment of spawner-isolated mortality virus and where the remaining science leads

Leigh Owens*

Abstract

In the late 1980s, there was histological and electron microscopy evidence for a parvovirus-like virus in Australian prawns. The data were consistent with infectious hypodermal and haematopoietic necrosis virus (IHHNV). However, these cases did not fit the then current paradigms of the known viruses and sequencing did not find any meaningful sequence homology. The virus was named spawner-isolated mortality virus (SMV; GenBank AF499102.1) in order to allow publication of the information about its occurrence to inform the scientific and aquacultural communities. This virus was present in the early years of mid-crop mortality syndrome (1993–1995). However, as time passed, nucleotide and protein databases have expanded and sequence investigation tools have become more cost effective. The sequence of the entity known as SMV is now shown to be of *Carnobacterium divergens* (CP016843.1). Therefore, the publications with regard to SMV have been assessed and a recommendation to abolish the name with the still valid science transferred to IHHNV and *C. divergens*.

INTRODUCTION

At scientific conferences conducted by NSW Fisheries, Port Stephens, Salamander Bay, Australia, in ~1987 and by IFREMER in Tahiti 1989 [1], histological evidence was presented for the presence of infectious hypodermal and haematopoietic necrosis virus (now named penstylhamaparvovirus [2]; herein named IHHNV as this is the common name known to most virologists and aquaculturalists) to be present in Australia infecting prawns. This conclusion was based partially on the pathognomonic eosinophilic Cowdrey type A inclusion and the rarer basophilic inclusion found in the lymphoid organ of prawns. This was an unacceptable view due to biosecurity concerns and the evidence was derided by Australian senior scientists particularly at the Port Stephens meeting.

Efforts to get electron microscopy images of the associated virions were pursued, resulting in the finding of parvovirus-like arrays (18–20 nm) in the lymphoid organ of juvenile *Penaeus monodon* [3]. The peer-reviewers of that paper [3] would not accept the Australian strain of IHHNV for these lesions or electron micrographs as these cases did not meet the flawed OIE (Office International des Epizooties) definition of IHHNV due to the age/size difference, juveniles in the Australian cases vs post-larvae in the OIE definition. The name lymphoidal parvovirus was coined to allow the publication to proceed, but the strong implication in the paper was that it was IHHNV (see text and Table 1 of Owens *et al.* [3]). Later in 1991, an IHHNV epizootic occurred in hybrid *Penaeus monodon* crossed with *Penaeus esculentus* prawns in a research facility [4] and the peer-reviewers allowed the use of the name IHHNV in this publication. The evidence included positive histopathology, electron microscopy and ELISA titres all consistent with IHHNV. Thus it was undeniable that IHHNV was present in Australia. Later, this was confirmed by almost complete genome sequencing [5] (GenBank Accession KM593908.1).

Later in the same research facility, 300 wild-caught, mature spawners of *P. monodon* starting dying at an unprecedented rate, so much so that the original experiment was abandoned [6]. Surviving prawns (five of 110 prawns in the worst three tanks 19 weeks after stocking) were frozen for later examination. Cell-free extract was infectious as were fed carcasses with almost 100% mortality at the termination of the infection experiments. Histopathology was inconclusive but electron microscopy of midgut cells showed cytoplasmic ~20 nm virions spilling out of nuclear pores into the cytoplasm. Again the implication was that IHHNV was involved given that it was the same research facility, geographical location (prawns from northern Queensland), the time sequence following

Received 19 June 2023; Accepted 25 July 2023; Published 03 August 2023
Author affiliations: [1]College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville 4811, Australia.
*Correspondence: Leigh Owens, leigh.owens@jcu.edu.au
Keywords: spawner-isolated mortality virus; IHHNV; *Carnobacterium divergens*.
Abbreviations: GAV, gill-associated virus; IHHNV, infectious hypodermal and haematopoietic necrosis virus; LDI, lightner double inclusion; MCMS, mid-crop mortality syndrome; SMV, spawner-isolated mortality virus; TEM, transmission electron microscopy; WzSV8, Wenzhou shrimp virus 8.
001878 © 2023 Crown Copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution License. This article was made open access via a Publish and Read agreement between the Microbiology Society and the corresponding author's institution.

Table 1. The effect of the withdrawal of information on SMV/LPV contained in published manuscripts

The red colour means the conclusions based on that data should be discarded. Orange means the mortality profiles, histopathology and TEM of viruses should be transferred to other viruses. Blue means the data pertain to *Carnobacterium*. Green means the data are unaffected as most of the sequencing data are correctly about IHHNV and hepanhamaparvovirus (HPV).

Publication	Synopsis/topic	Consequences to data
Owens *et al.* 1991	Lymphoidal parvovirus	Data transferred to IHHNV
Fraser & Owens 1996	Spawner-isolated mortality virus coined	Data transferred to IHHNV
Owens *et al.* 1998	SMV involvement in MCMS	*In situ* hybridization incorrect
Owens & McElnea 2000	SMV in crayfish	Limited data to IHHNV
		Discard the paper: except for the description of stress-related deaths
Owens *et al.* 2003	SMV in hatcheries and growout	Data to *Carnobacterium* probiotics
Owens 2013	Nuclear location signals in parvoviruses	95% paper unaffected: SMV removed
Reports		
Anderson & Owens 2001	Mid-crop mortality syndrome	~30% incorrect, data to IHHNV and GAV
Owens & Cullen 2004	Diagnostic improvement for SMV	PCR-ELISA; discard the report: except for assessing the accuracy of detecting
		Carnobacterium by PCR

closely on the accepted IHHNV infection and the size of the virions, but again the peer reviewers would not allow publication of the name IHHNV without the pathognomonic eosinophilic Cowdrey type A inclusions. Therefore the name spawner-isolated mortality virus (SMV; GenBank AF499102.1) was coined to allow publication and the release of this information.

Epizootics on prawn farms that received juveniles from the research facility started to occur in late 1993/early 1994 and, by 1995, an industry-wide problem existed that was characterized by mortality that appeared after 110–120 days of grow-out. This was termed mid-crop mortality syndrome (MCMS) [7]. This allowed further studies on SMV with the production of digoxygenin, *in situ* hybridization gene probes, of 1800 and 400 bp [8], partial sequencing, PCRs with amplicons of 260 and 207 bp [9], and a PCR-ELISA [10].

As time progressed, data banks increased their sequence libraries (NCBI) and molecular tools, and cost-effective availability of bioinformatics software has led to the point where previous conclusions can be meaningfully challenged. This paper will present evidence for the removal of the entities spawner-isolated mortality virus and, as a corollary, lymphoidal parvovirus, and recommends the dismissal of some of the related science and allocation of the remaining robust, unaffected science to its correct micro-organism.

RESULTS AND DISCUSSION

Re-examination of sequence data for SMV

In the production of the SMV *in situ* gene probe, DNA was extracted from $CsCl_2$ gradients, double stranded, ligated to EcoR1, cut by the low-frequency cutter BAMH1 restriction enzyme, cloned into pGEM7zf(+) and transformed into *Eschericia coli* JM109 [8]. Importantly, *E. coli* clones carrying prawn DNA were eliminated via dot blot hybridization against *P. monodon* DNA and discarded. Chosen clones were partially sequenced and compared against sequences available at the time in NCBI using BLASTn against all sequences and then viral sequences. Notably, in 1995, PCR primer-sized sequences (27 bp) from the end of the chosen clone screened against viruses hit with the moth virus *Galleria mellonella* densovirus (GenBank L32896; 100% identity; -/+ TTTA TCATAAGCTTCGTCGTGTTCTTT) at *P*=0.0013 and other mammalian and insect parvoviruses at non-significant levels. Even today (May 2023) with the massive increase in the GenBank database, using BLASTn, only the leading 15% of BE6 clone hits with *Carnobacterium divergens* (CP016843.1) were with 94% identity (e-125) (Fig. 1).

A study of nuclear location signals in crustacean parvoviruses [11] showed that the sequence of SMV derived from clone BE6 was likely to be reversed and in the second (reversed) reading frame in the clone. Nevertheless, the sequences submitted to NCBI should still have found homologous sequence with BLASTn. When this new information was taken into account, a protein to protein BLASTp from translated nucleotide 1885 to nucleotide 782 (reversed, reading frame 2 of GenBank AF499102.1), 100% of this portion of the clone hits with a domain containing protein *Carnobacterium divergens* (WP_109841005.1) with 98.64% identity (e-0.0) (Fig. 2). This includes the area for the PCR primers for the diagnostic SMV PCR.

Carnobacterium divergens strain TMW 2.1579 chromosome, complete genome

Sequence ID: CP016843.1 Length: 2666320 Number of Matches: 1

Range 1: 170059 to 170361 GenBank Graphics ▼ Next Match ▲ Previous

Score	Expect	Identities	Gaps	Strand
464 bits(514)	3e-125	286/303(94%)	3/303(0%)	Plus/Plus

```
Query  42      AGTAGGGATGCTTTTAGCGAAGAATCCCGTTTAGCAATCACAAACAGGACACAAACTACT  101
               |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  170059  AGTAGGGATGCTTTTAGCGAAGAATCCCGTTTAGCAATCACAAACAGGACTCAAACTACT  170118

Query  102     GATTTTTCAGTGTTCAAAGCCCTTCTAAAGTAATAAAATTGTAATGACTGAATGGAACAT  161
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  170119  GATTTTTCAGTGTTCAAAGCCCTTCTAAAGTAATAAAATTGTAATGACTGAATGGAACAT  170178

Query  162     TGGCGTTGTTTGGTTAGCTTTAGTTGTTTGAGTCATCATCATTGTATTCTCCTTTTCGTT  221
               ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  170179  TGGCGTTTTTTGGTTAGCTTTAGTTGTTTGAGTCATCATCATTGTATTCTCCTTTTCGTT  170238

Query  222     TGGGTTCTTCattta--ttttaatttaattttaatctaattcttatttactaatctaatt  279
               |||||||| ||||||  |||||||||| ||||||||||||||||||||||||||||||||
Sbjct  170239  TGGGTTCTCCATTTATTTTTTAATTTATTTTTAATCTAATTCTTATTTACTAATCTAATT  170298

Query  280     ctaatCAAGAGAAATGTCAAGCATTAttttttg-tttttAGACTATTATTTAATaaaaaa  338
               ||||||    |  |||||||| ||||||||||| ||| ||||||||| |||||||||| |
Sbjct  170299  CTAATCTCATGGGATGTCAAGTATTATTTTTTGATTTCTAGACTATTTTTTAATAAAATA  170358

Query  339     aCA  341
               |||
Sbjct  170359  ACA  170361
```

Fig. 1. The nucleotide alignment of SMV with NCBI GenBank database for *Carnobacterium divergens* (CP016843.1).

Furthermore, after an area of stop codons (sequencing errors?), the sequence for *C. divergens* continues from nucleotide 752 until another stop codon at nucleotide 440 (data not shown). After this stop codon, the next 28 amino acids hit non-significantly with a number of bacteria including *Pseudomonas vividiflava*. This almost abuts to the end of the nucleotide sequence in reading frame 1 of *C. divergens* in Fig. 1. Altogether, >95% of the SMV cloned sequence is bacterial, mostly by far *C. divergens*.

In addition, within the BE6 clone of SMV, there are five areas of nucleotide homology with moths – area 1 (465–509 bp): *Crambus* moth; area 2 (reversed 682–734 bp): *Ecliptoptera silaceata*; area 3 (773–814 bp): *Charanyca ferruginea*; area 4 (979–1028 bp): *Melanargia galathea*; and area 5 (reversed 1097–1152 bp): *Marasmarcha* moth. This homology probably allowed the PCR-produced, dioxygenin-labelled (DIG) SMV gene probes to partially specifically bind in many places to the Pancrustacean genome particularly when DNA was abundant as in replicating cells. Parvoviruses require rapidly dividing cells in S-phase to replicate, congruent with where the SMV gene probe would bind, hence supporting an incorrect conclusion. The tissues where the SMV probe bound were often the gut and the endocuticle, both areas of DNA replication, microbial and physical assault, and repair.

Amongst others, the diagnostic SMV PCR primers BLAST hit non-significantly with the springtail *Dicyrtomina minuta* at 100% coverage and 100% identity and *Carnobacterium* sp. 17-4 at 95% coverage and 100% identity within that coverage for the forward primer (SMVfor TAGCTATTTTTTGGTCGTCTG). The reverse primer hit with the moths *Eilema depressum* and *Pyrausta nigrata* at 83% coverage and 100% identity [SMVrev GCCGCAATTTACCAGTGTTTGAAG (reverse/complemented)]. The highest bacterial hit was the marine bacterium *Pseudalkalibacillus hwajinpoensis* at 79% coverage and 100% identity. Notably, whilst the PCR primers have some probability of amplifying *Carnobacterium* or other Gram-positive pseudalkalibacilli, the entire PCR DNA product is not recognized as *Carnobacterium* in BLASTn global searches, or those searches limited to only bacteria with only *Enterobacter* sp. E76 hitting significantly (e-0.03) at 23% coverage and 84% identity within that coverage.

In summary, the mortality, histopathology and virology including transmission electron microscopy (TEM) ascribed to SMV including the early years of what became MCMS (1993 to March 1995) should be attributed to the Australian isolates

DUF6382 domain-containing protein [Carnobacterium divergens]

Sequence ID: WP_109841005.1 Length: 641 Number of Matches: 1

See 5 more title(s) See all Identical Proteins(IPG)

Range 1: 159 to 526 GenPept Graphics ▼ Next Match ▲ Prev

Score	Expect	Method	Identities	Positives	Gaps
727 bits(1876)	0.0	Compositional matrix adjust.	363/368(99%)	365/368(99%)	0/368(0%)

```
Query  1    EIELENDIEDLIQYLNESYFSVEGFGKMLYRQSRNLPVFEEENRKQVAKKPLSNIKKTTN  60
            EIELEND+EDLIQYLNESYFSVEGFGKMLYRQSRNLPVFEEENR QVAKKPLSNIKKTTN
Sbjct  159  EIELENDVEDLIQYLNESYFSVEGFGKMLYRQSRNLPVFEEENRIQVAKKPLSNIKKTTN  218

Query  61   STQEAIESPLKKFFNQIGMVHKHNDTSKKEVVTMSDDQKIAKEKLKKDSIVLVVLGFLTS  120
            STQE IESPLKKFFNQIGMVHKHNDTSKKEVVTMSDDQKIAKEKLKKDSIVLVVLGFLTS
Sbjct  219  STQEPIESPLKKFFNQIGMVHKHNDTSKKEVVTMSDDQKIAKEKLKKDSIVLVVLGFLTS  278

Query  121  FVIYQLVPDPFVTASVAFLIGTLVLNYVHKKRTAFYQEFPELVNKNQKKTDPLTVENELA  180
            FVIYQLVPDPFVTA+VAFLIGTLVLNYVHKKRTAFYQEFPELVNKNQK TDPLTVENELA
Sbjct  279  FVIYQLVPDPFVTAAVAFLIGTLVLNYVHKKRTAFYQEFPELVNKNQKITDPLTVENELA  338

Query  181  VRKIKRNKIAVVAIALLAIVAIQQLIQDVALRGCLILLVLATAFFYIRKLTRQSAFPEVE  240
            VRKIKRNKIAVVAIALLAIVAIQQLIQDVALRGCLILLVLATAFFYIRKLTRQSAFPEVE
Sbjct  339  VRKIKRNKIAVVAIALLAIVAIQQLIQDVALRGCLILLVLATAFFYIRKLTRQSAFPEVE  398

Query  241  AHKTGKSFMKNKHVSTSEPKKKLADLLNPELPDSNEQTNETISTQVENKIDEKEHIVEAK  300
            AHKTGKSFMKNKHVSTSEPKKKLADLLNPELPDSNEQTNETISTQVENKIDEKEHIVEAK
Sbjct  399  AHKTGKSFMKNKHVSTSEPKKKLADLLNPELPDSNEQTNETISTQVENKIDEKEHIVEAK  458

Query  301  TEKVTLSLDEHEQLREKLREELKAEITAKIQNEIEQERLEKQRNTEEKVGTPKKIQTDSK  360
            TEKVTLSLDEHEQLREKLREELKAEITAKIQNEIEQERLEKQRNTEEKVGTPKKIQTDSK
Sbjct  459  TEKVTLSLDEHEQLREKLREELKAEITAKIQNEIEQERLEKQRNTEEKVGTPKKIQTDSK  518

Query  361  IETTISTI  368
            IETTISTI
Sbjct  519  IETTISTI  526
```

Fig. 2. The first listed amino acid alignment (reversed, second reading frame) of SMV with GenBank database for *Carnobacterium divergens* (WP_109841005.1).

of IHHNV. It should be noted that late in MCMS (March 1995 onwards), gill-associated virus (GAV) started to overwhelm the picture of the MCMS epizootics.

The *in situ* gene probe data for SMV are completely incorrect and are probably DNA–DNA partial hybridization with arthropod DNA when there are large quantities of DNA in the cells that show DIG signal. These data should be dismissed. The PCR-generated data are probably showing the presence of *Carnobacterium* spp. in the samples. As a member of the lactobacilli, *Carnobacterium* have been used in mixes as commercial probiotics in aquaculture for decades and are registered for use in human food products. In aquaculture, lactobaccilli are often used to fortify (improve nutritional value of) rotifers and *Artemia* before feeding them to larval prawns. Hence, it is highly likely that the data generated from PCR studies on SMV were largely tracking *Carnobacterium* through the aquaculture systems.

Consequences for publications related to errors and SMV removal

Although a major error in interpretation has occurred, much information is still useful. An attempt has been made to evaluate the major published literature on SMV to understand how much value remains (Table 1). There are two publications that are almost totally incorrect [10, 12] but they still have some useful information. Owens and McElnea [12] describe the early years of stress-related deaths in crayfish which ultimately led to the studies and discovery of the viruses *Cherax iflavirus* and *Cherax bunyavirus* [13, 14]. Owens and Cullen [10] demonstrated the robustness (accuracy, specificity and sensitivity) of a PCR that now could be used to detect *Carnobacterium*.

CONCLUSIONS

This abolishmant of SMV, whilst unfortunate, still has some valuable lessons to teach. First, whilst the peer review system is the best we have, it is still responsible for mistakes, particularly if you need to go against a current paradigm that is in vogue, as was in this case. We had evidence for IHHNV in Australia that did not meet the flawed OIE definition. To make our information available to others, the authors coined politically acceptable names for the virus we had found. Second, linking different assays to the same entity can be difficult as assumptions have to be made. In this case, the virus seen with TEM and the lesions seen with histopathology had to be linked with *in situ* DNA–DNA hybridization which could not be conducted on the same histological slide, just on thin sections cut further into the same blocks. At the time we were concerned that we had no independent confirmation test, but we assumed (incorrectly) we had the same entity in all sections. *In situ* hybridization is becoming less popular as PCRs with confirmation steps (sequencing, probes, nesting and melt curves) allow greater surety of results. Third, even though genomic databases are extensive, they are only as good as the information that is supplied to them and there is a lot of information and undiscovered entities that have not been entered into even the best databases.

Recently, Srisala *et al.* [15] have published on Wenzhou shrimp virus 8 (WzSV8) detected by PCR, histopathology and *in situ* hybridization. The pathognomonic Lightner double inclusions (LDIs) for WzSV8 are identical to those depicted within the lymphoidal parvovirus paper of Owens *et al.* [3] suggesting WzSV8 was present in Australia by 1990. WzSV8 presence in Australia as been confirmed by sequence information [15]. The eosinophilic inclusion of the double inclusion is probably the RNA of the displaced nucleolus. Nevertheless, the *in situ* hybridization images of Srisala *et al.* [15] do not correspond well to the purported histopathological changes or the semi-thin sections. Furthermore, the *in situ* probe binds to inclusions in vacuoles which could easily be intranuclear with the nuclear membrane being interpreted as a vacuole membrane. The probe may be binding to areas with large amounts of DNA as occurred in our case. Indeed, the semi-thin sections are even more consistent with a condensed, basophilic intranuclear inclusion with a displaced eosinophilic nucleolus (the LDI). The number of RNA viruses that produce intranuclear inclusions can be counted on one hand. To protect Srisala *et al.* [15] from the same mistake that we made, we would urge those authors to re-examine their *in situ* hybridization results and be satisfied with their conclusions.

Funding information
This work received no specific grant from any funding agency apart from administration support (office, computer, library access) supplied by College of Public Health, Medical and Veterinary Sciences, James Cook University.

Conflicts of interest
The author declares he has no conflicts of interest.

References

1. Owens L, Hall-Mendelin S. *Recent Advances in Australian Penaeid Diseases and Pathology*. Advances in Tropical Aquaculture, Tahiti (1989). Actes de Colloques, 1990, pp. 103–112.
2. Pénzes JJ, Söderlund-Venermo M, Canuti M, Eis-Hübinger AM, Hughes J, *et al*. Reorganizing the family Parvoviridae: a revised taxonomy independent of the canonical approach based on host association. *Arch Virol* 2020;165:2133–2146.
3. Owens L, De Beer S, Smith J. Lymphoidal parvovirus-like particles in Australian penaeid prawns. *Dis Aquat Org* 1991;11:129–134.
4. Owens L, Anderson I, Kenway M, Trort L, Benzie J. Infectious hypodermal and haematopoietic necrosis virus (IHHNV) in a hybrid penaeid prawn from tropical Australia. *Dis Aquat Org* 1992;14:219–228.
5. Krabsetsve K, Cullen B, Owens L. *Investigation and Verification of Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) in Australian Penaeid Prawns*. Master of Science Thesis, James Cook University, unsubmitted, 2005.
6. Fraser C, Owens L. Spawner-isolated mortality virus from Australian Penaeus monodon. *Dis Aquat Org* 1996;27:141–148.
7. Anderson IG, Owens L. The diagnosis and prevention of the mid-crop mortality syndrome of pond-reared black tiger prawns (*Penaeus monodon*). In: *A final report for the Fisheries Research and Development Corporation Project 96/301 ISBN: 0 7345 0145 5*. 2001
8. Owens L, Haqshenas G, McElnea C, Coelen R. Putative spawner-isolated mortality virus associated with mid-crop mortality syndrome in farmed Penaeus monodon from northern Australia. *Dis Aquat Organ* 1998;34:177–185.
9. Owens L, McElnea C, Snape N, Harris L, Smith M. Prevalence and effect of spawner-isolated mortality virus on the hatchery phases of Penaeus monodon and P. merguiensis in Australia. *Dis Aquat Organ* 2003;53:101–106.
10. Owens L, Cullen B. *Development of Diagnostic Capability for Aquatic Animal Diseases of National Significance: Spawner-Isolated Mortality Virus*. James Cook University, 2004.
11. Owens L. Bioinformatical analysis of nuclear localisation sequences in penaeid densoviruses. *Mar Genomics* 2013;12:9–15.
12. Owens L, McElnea C. Natural infection of the redclaw crayfish Cherax quadricarinatus with presumptive spawner-isolated mortality virus. *Dis Aquat Organ* 2000;40:219–223.
13. Sakuna K, Elliman J, Owens L. Discovery of a novel *Picornavirales*, Chequa iflavirus, from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia. *Virus Res* 2017;238:148–155.
14. Sakuna K, Elliman J, Tzamouzaki A, Owens L. A novel virus (order Bunyavirales) from stressed redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Australia. *Virus Res* 2018;250:7–12.
15. Srisala J, Thaiue D, Saguanrut P, Taengchaiyaphum S, Flegel TW, *et al*. Wenzhou shrimp virus 8 (WzSV8) detection by unique inclusions in shrimp hepatopancreatic E-cells and by RT-PCR. *Aquaculture* 2023;572:739483.