Wenzhou virus 8 (WZV8) diagnosis by unique inclusions in shrimp hepatopancreatic E-cells and a molecular detection method

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Background

To assist shrimp pathologists worldwide we are providing here photomicrographs of unique basophilic inclusions that are produced by Wenzhou shrimp virus 8 (WZV8) (Li et al. 2015) that was discovered in 2015 by wide screening of marine animals for RNA viruses using high throughput sequencing (GenBank record KX883984.1). A more recent publication from China (Liu et al. 2021) also gives the full sequence under GenBank record OK662577 that is highly similar to WZV8 (97% coverage and 95.4% sequence identity) but under the newly proposed name *Penaeus vannamei* picornavirus (*Pv*PV). Although that paper contained no histological analysis, it did include an electron micrograph of a cytoplasmic viral inclusion within a vacuole of an unspecified hepatopancreatic epithelial cell type (Liu et al., 2021).

Using the sequence of KX883984.1, we designed PCR primers and *in situ* hybridization probes for detection of WZV8. Subsequent ISH assays with shrimp RT-PCR positive for WZV8 samples allowed us to identify unique inclusions described herein as linked to WZV8 in hematoxylin and eosin (H&E) stained tissues. In some of the specimens positive for WZV8 with ISH assays, positive ISH reactions were also seen in normal nuclei in the central region of the HP and in the subcuticular epithelium and underlying connective tissue (especially in the stomach) indicating that these tissues are of no use for histological diagnosis of WZV8 infection because of their normal appearance with H&E staining.

Going back over our previous histological reports and archived slides, we have found the unique WZV8 inclusions in E-cells of normal shrimp samples from several shrimp farming countries in Austral-Asia since at least 2008. More recently we have obtained samples of *P. vannamei* from the Americas that also show these inclusions. We have noticed these inclusions for many years as unique basophilic, cytoplasmic inclusions of unknown origin that occur mostly in E-cells of the tubule epithelia of the hepatopancreas (HP) of both diseased and normal, cultivated *P. monodon* and *P. vannamei*. In diseased samples, mortality

was ascribed to bacteria or known lethal viruses. As a result, the additional presence of these inclusions of unknown origin and their relatively common presence also in shrimp with no signs of disease resulted in their relative neglect while efforts were focused on more urgent problems.

We urge shrimp pathologists to review their records and archived and current specimens for the presence of the unique WZV8 E-cell inclusions described herein. Hopefully, this will result in data that will provide a global view of the current prevalence and impact of WZV8-like infections.

Characteristics of WZV8 inclusions

With hematoxylin and eosin (H&E) staining, the cytoplasmic, deeply basophilic WZV8 inclusions are circular in section and are contained within a cytoplasmic vacuole (**Figs. 1, 2**). They should not be confused with metaphase plates (**Fig. 3**) commonly seen in E-cells where there is a high rate of cell division. Although the deeply basophilic, circular inclusions are dominant, there are also variations in their appearance. For example, they are sometimes associated with a nearby or attached, eosinophilic, circular inclusion, usually of smaller size (**Fig. 4**). Sometimes similar inclusions are also seen in differentiated HP tubule epithelial cells and an example photomicrograph of WZV8 inclusions in R-cells is shown in **Fig. 5**. In addition, the double inclusions are sometimes separated by an unstained space from a surrounding basophilic to magenta colored "surround" of variable thickness that is also separated from the host cell cytoplasm by an unstained space.

The dual inclusion sets consisting of a basophilic and eosinophilic partner may be common but the eosinophilic partners are mostly small in comparison to the larger basophilic inclusion such that the probability of them appearing together in 4-micron tissue sections would be small. Thus, it is difficult to determine whether this unique pairing occurs regularly or is of low occurrence. More work is needed to determine the relationship among these variable inclusions and how they develop. Hopefully, transmission electron microscopy (TEM) will help to achieve this goal.

Photomicrographs of semi-thin sections of WZV8 inclusions in E-cells are also shown in **Fig. 6** stained with toluidine blue. The dual-inclusion cells are so distinctive in their nature by both H&E staining and in semi-think sections that their occurrence, together with their common location in E-cells may be considered pathognomonic for WZV8 infection. We propose calling these unique inclusions "Lightner double inclusions" (LDI) to honor recently deceased Prof. Donald V. Lightner to whom we are greatly indebted for his monumental contributions in the field of shrimp pathology.

We originally speculated that these inclusions might be developmental stages of the microsporidian *Enterocytozoon hepatopenaei* (EHP), but they were subsequently found to be negative for EHP using a specific *in situ* hybridization (ISH) probe. In addition, we discovered by histological analysis and ISH assays that EHP does not infect E-cells (Flegel, 2012; Chaijarasphong et al., 2020).

Upon the discovery and reporting of the WZV8 genome sequence (GenBank accession number KX883984.1) as a novel virus in the order Picornavirales, we were able to design PCR primers and *in situ* hybridization probes for its detection. Subsequent ISH assays with shrimp RT-PCR positive for WZV8 samples allowed us to identify the inclusions described herein as linked to WZV8. A review of our archived slides has revealed WZV8 inclusions in

normal shrimp samples from several shrimp farming countries in Austral-Asia since at least 2008. More recently we have seen such inclusions in *P. vannamei* from two countries in the Americas.

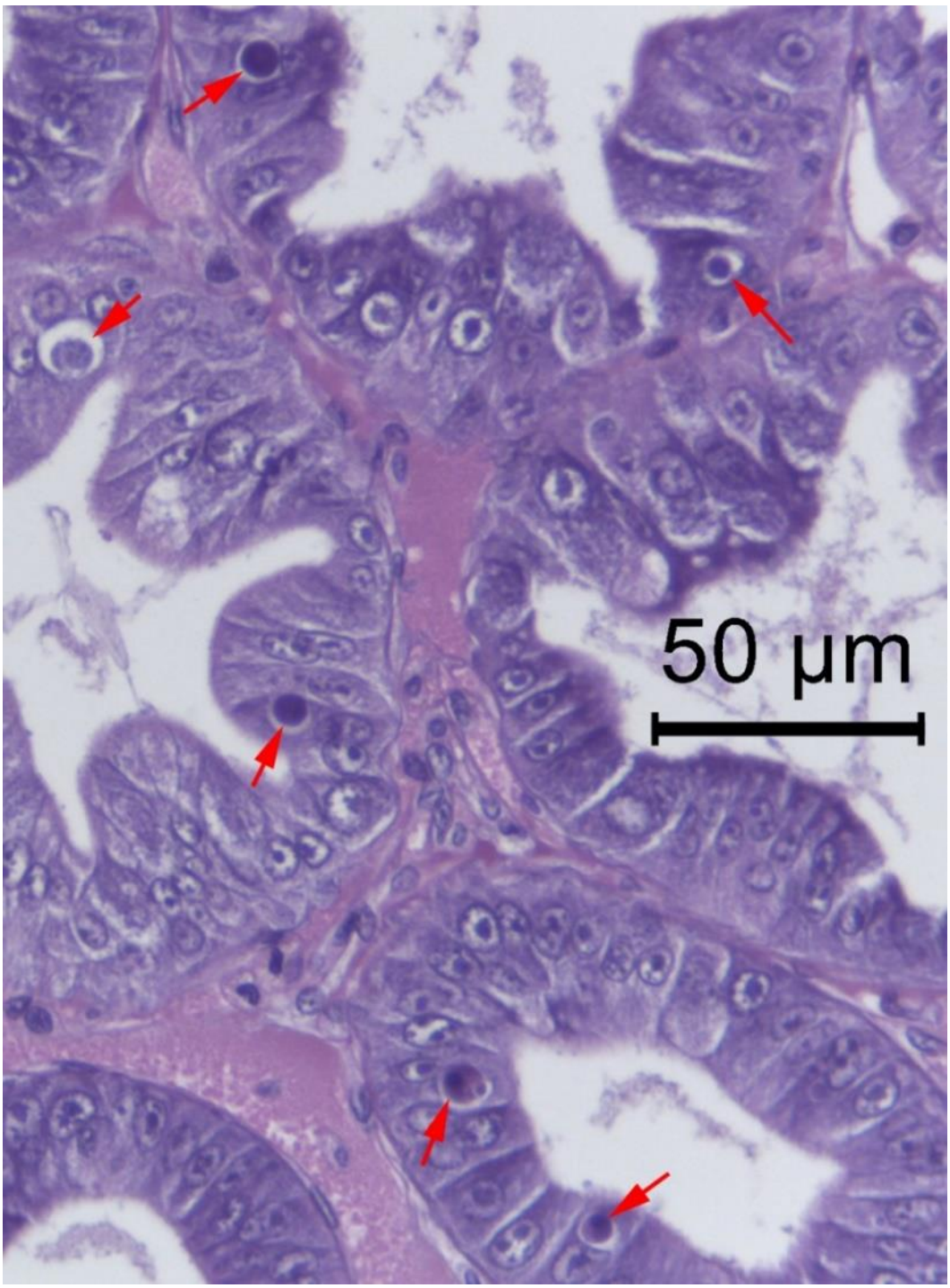

Figure 1. Photomicrograph taken using a 40x objective showing E-cells of the shrimp hepatopancreas containing WZV8 cytoplasmic inclusions within vacuoles (red arrows). They have variable morphology and staining properties. The dominant ones are deeply basophilic and mostly perfectly circular. The lightly

basophilic inclusion on the far left may be an early developmental stage, while the bottom two are more complex and show some eosinophilic staining in addition to a central, circular, densely basophilic inclusion.

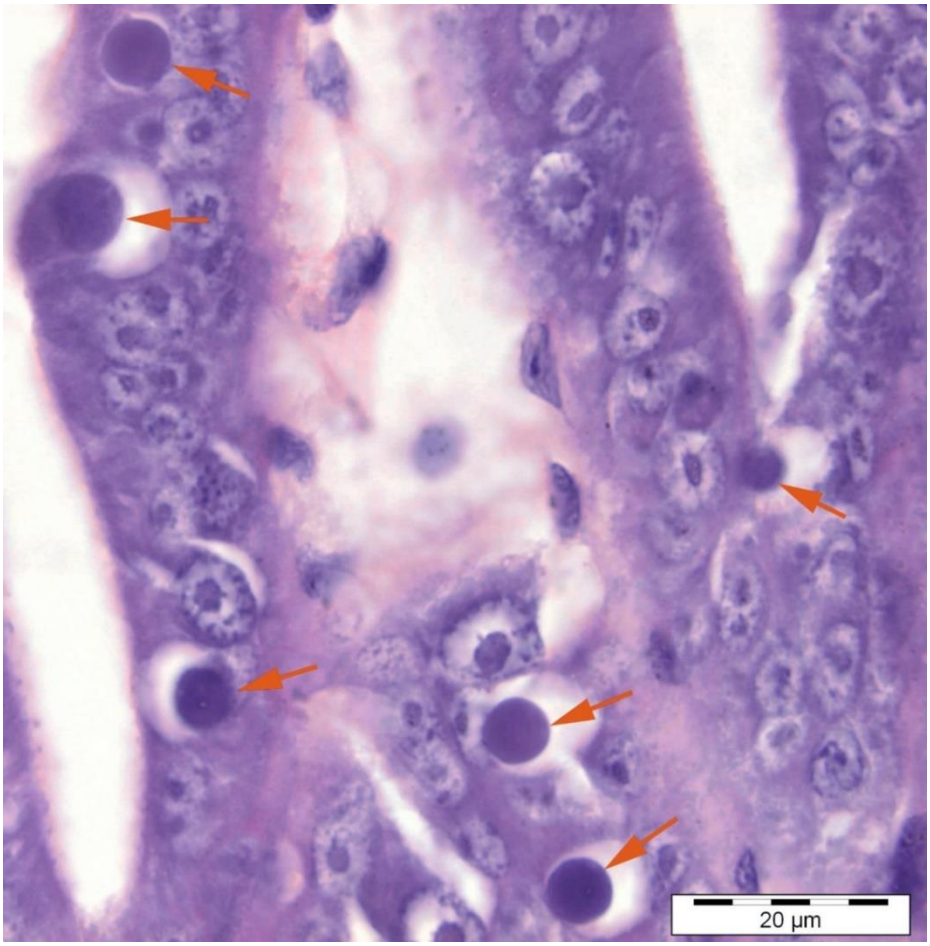

Figure 2. Example photomicrographs of the most common, circular, lightly to deeply basophilic, cytoplasmic inclusions of WZV8 within vacuoles in E-cells.

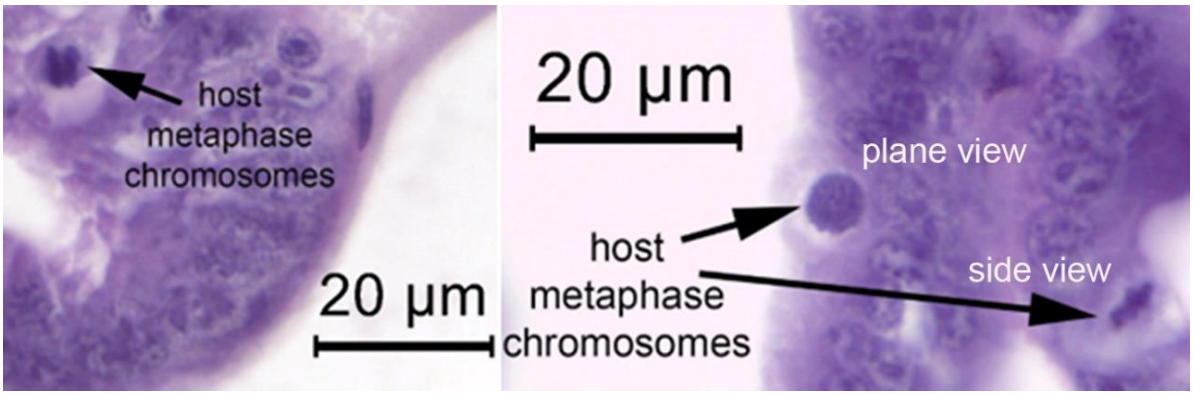

Figure 3. Example photomicrographs of metaphase chromosomes in E-cells that may sometimes resemble WZV8 inclusions when the tissue section passes through the plane of the metaphase plate rather than the side. One must be careful not to confuse these with WZV8 inclusions.

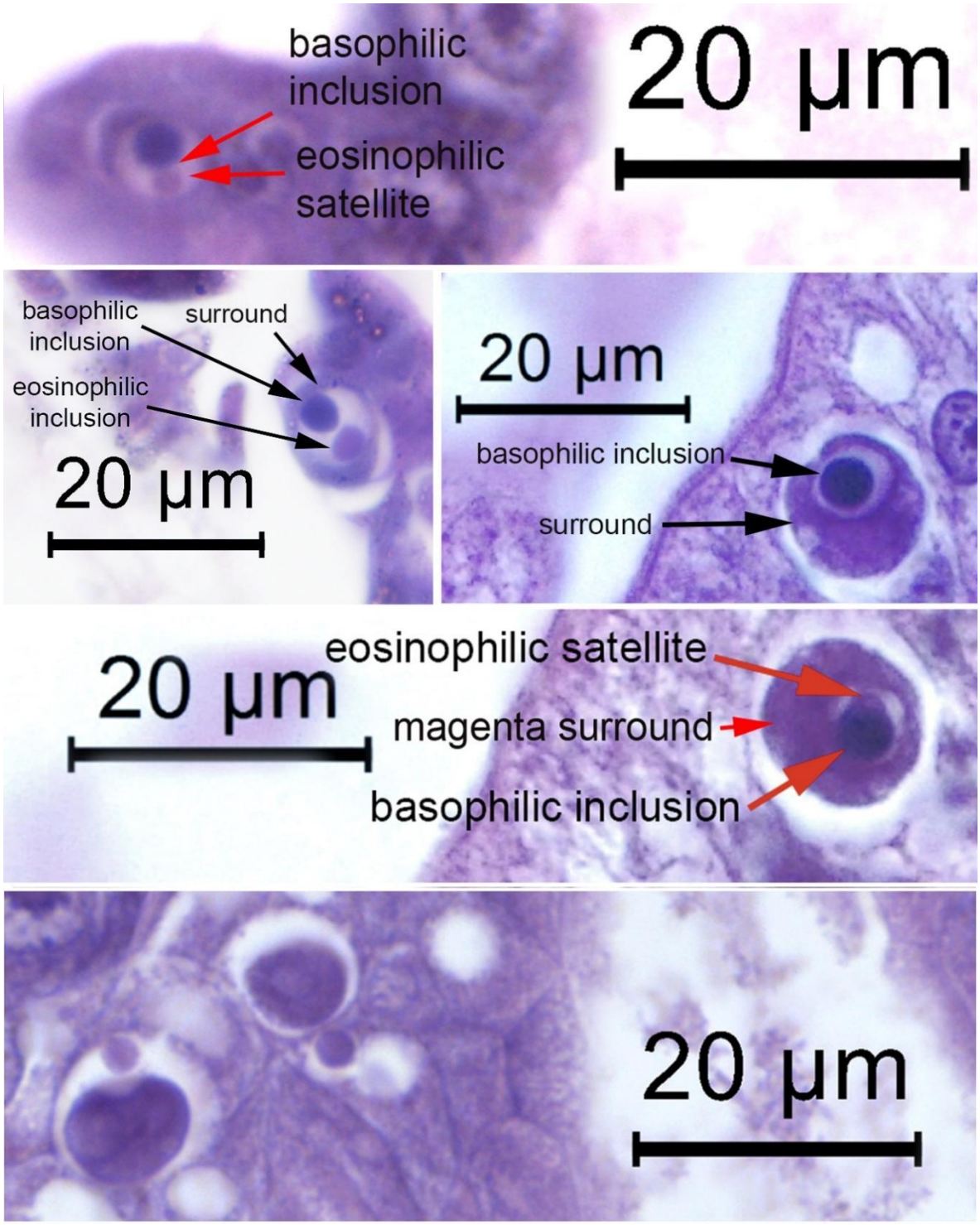

Figure 4. Variations in basophilic WZV8 inclusions that are sometimes accompanied by usually smaller, circular, eosinophilic satellite inclusions. This is a highly distinctive combination, such that "densely basophilic, circular inclusions accompanied by a closely associated satellite, eosinophilic inclusion within an E-cell vacuole" may be considered pathognomonic for WZV8.

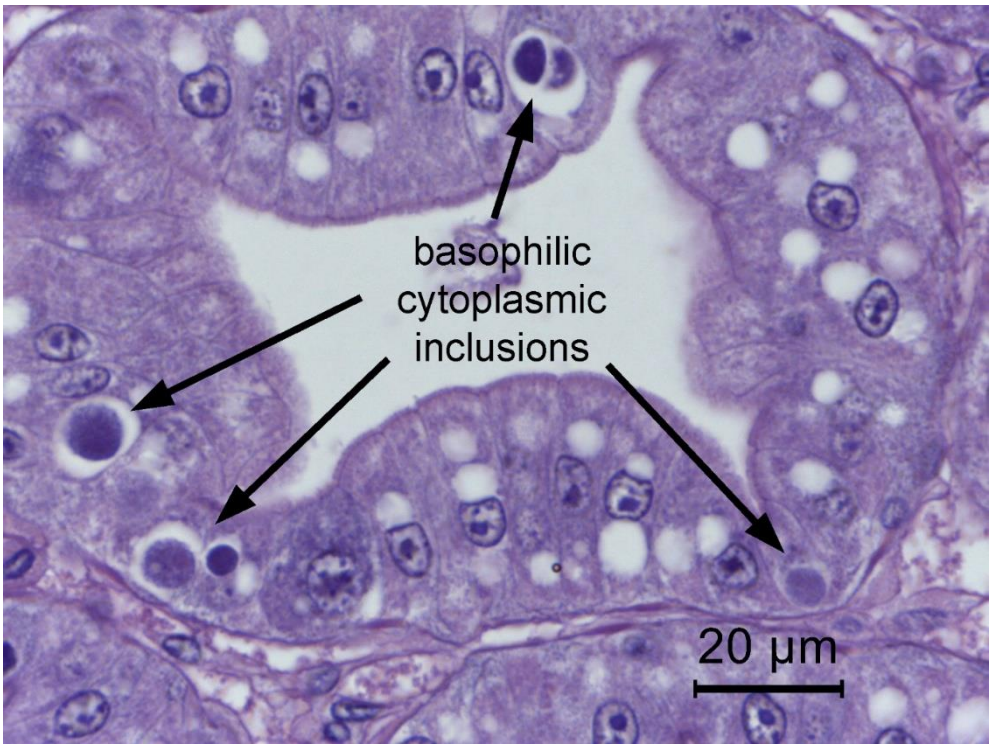

Figure 5. Photomicrograph of WZV8 basophilic inclusions in differentiated cells (R-cells) of the shrimp hepatopancreas.

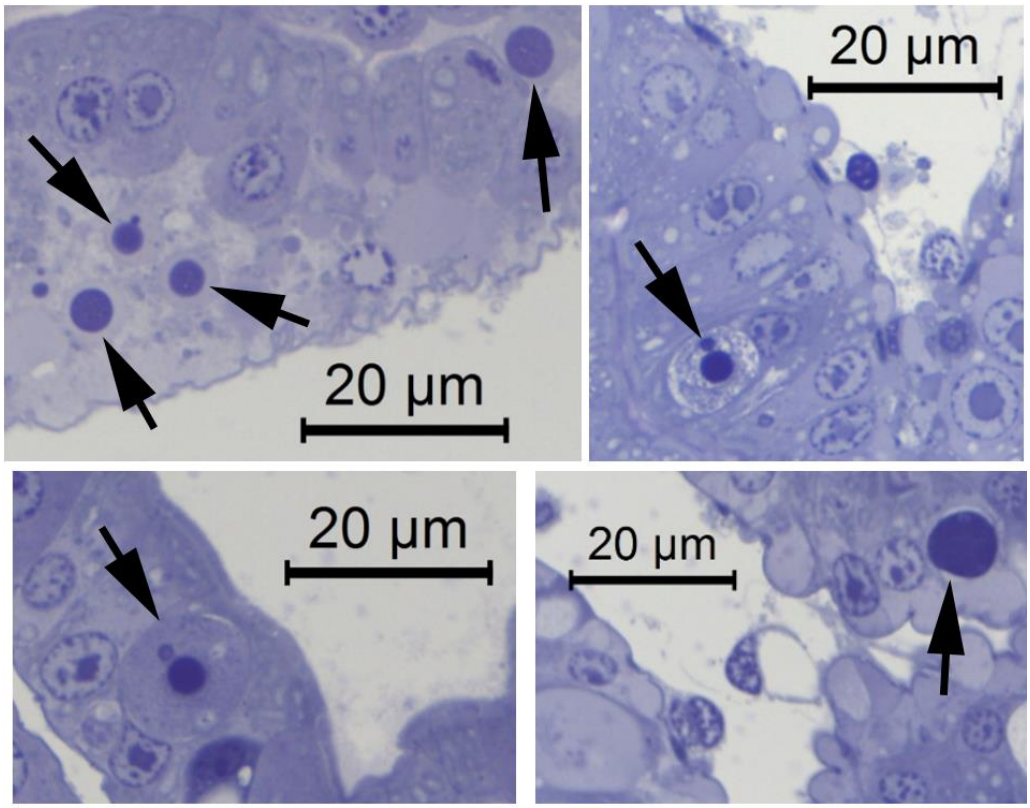

Figure 6. Photomicrographs of semi-thin sections of shrimp HP tissue showing variation in WZV8 inclusions (dark blue and circular) in E-cells of the shrimp hepatopancreas. Some show a smaller, adjacent satellite inclusion or other vacuolar contents.

RT-PCR detection of WZV8

We also provide here details for a nested RT-PCR detection method for WZV8 based on the original sequence from Li et. al (2015) (GenBank record KX883984.1). A more recent publication from China (Liu et al. 2021) also gives the full sequence of PvPV under GenBank record OK662577. The forward primer and reverse primers for the first RT-PCR and nested PCR were designed from the putative RNA-dependent RNA polymerase (RdRp) gene of GenBank record KX883984.1. This was done before release of the PvPV sequence at GenBank in 2022. We have been using this RT-PCR method successfully to screen shrimp RNA samples from global clients. We are willing to provide (for free) a plasmid containing the target for this PCR method to anyone who requests it. This will be provided as a dried plasmid on filter paper that will be sent by ordinary airmail and can be eluted to transform *E. coli*. This will provide a perpetual supply of the plasmid for use as a positive control in RT-PCR tests and as a target to make a WZV8 specific DNA probe for ISH by PCR. The primer sequences are shown in **Table 1**.

Table 1. Primers used for a nested RT-PCR amplification in this study.

Method	Primer name	Sequence (5'-3')	Amplicon size (bp)
First RT-PCR	WZV8-482F	ATGCCTCTGGAAAGCGATAC	482
	WZV8-482R	GGTGTTAGATCGCTCCTTCTTC	
Nested PCR	WZV8-168F	GAAAGCGATACTCCTACGACAG	168
	WZV8-168R	TCTTGAGTTTGAGGAAGGTGAG	

As shown in **Fig. 7**, the Outer Forward Primer for the first-step RT-PCR of this method has a one-base mismatch at the final base when aligned with the GenBank record of PvPV, while the Inner Forward Primer has 3 mismatches. Thus, this RT-PCR method and plasmid control target can only be considered as a temporary emergency method that will need to be modified as full sequences of more isolates of WZV8 accumulate and are aligned in search of a more universal target sequence.

The first step RT-PCR reaction is performed in 12.5 µl mixture consisting of 1X Reaction Mix (Invitrogen, USA), 0.4 µM each of 482F and 482R primers, 0.5 µl of SuperScript III RT/Platinum Taq Mix (Invitrogen, USA) and 100 ng of RNA template. The RT-PCR protocol begins with 50°C for 30 min followed by 94°C for 2 min and then by 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 68°C for 45 sec plus a final extension at 68°C for 5 min.

For the nested PCR step, the 12.5 µl mixture contains 1X OneTaq Hot Start Master Mix (NEB, USA), 0.2 µM of each 168F and 168R primer, and l µl of the product solution from the first RT-PCR step. The nested PCR protocol is 94°C for 5 min, followed by 25 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 30 sec plus a final extension for 5 min at 72 ºC. The amplicons yielded are 482 bp and 168 bp, respectively.

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TGCCAAGAGCGTTTTCGTCTCCATTGGGGACGACATCATCTTCGCAGATTTGGCCACGAGGAGAATTCTG 3850
AAGAATTCTAATCTCTCTCCAGTCCAAATTTACACCTCTAGTGACAAGAAACAAGTCTACGTCAAGGATG
                                                  Forward outer primer  ATG

CCTCTGGAAAGCGATATACCTATGACAGTGAGAAGTTCATCACTCGCGTGACCACCACCCTTCAAGACGT
CCTCTGGAAAGCGATAC (one mismatch)
      GAAAGCGATACTCCTACGACAGC Forward inner primer (three mismatches)

CAGCTACTGGCAGGGCCTCCCGCTAGAAGCCATCATCTACCTCATGAAGAATCATCAAGTCATCATCGAA
GAGCACTTTCCCCTCACCTTCCTCAAACTCAAGAAGAAGGCCCGCAGAGATAGAAACATGCAGATCGTCC
            CTCACCTTCCTCAAACTCAAGA Reverse inner primer (no mismatch)

AACAGATCACTAACTCCAAGGCCTTCCAGGTTGGGTTCCCCATCGCTGTGATCGGAGTTGCCGCTACAGC
AATCTTCGGGATCGCGAAACTCTCCTCCAAGAAAGAAAAGTCCCCAAAGAAACCCTCCCCACCTGACAGC
GACAGCTCTTCAAGCGAGTCCGAATCAGACGAAGAACAATCTGCCCCTCAGAAGTCTGAGAAGTCTCAGA
CGCGAGTTGCTAGGAAATCCAAGCAATCCGCACCCGAGAAGAAGGAGCGATCTAACACCAAAGTCCTCCG
                Reverse outer primer GAAGAAGGAGCGATCTAACACC (no mismatch)

CAAGCAGAAACAGTCTGAGCCTGAGCACAACCCCAAGTCTTCAACTAAGGTTGCCAGGAARAAGCCCCAA
TCAGAACCCCAAAAATCTGACAAGTCCTCCACCAAAGTTGCTCGAAAGTTGATGCAGACCATCCTCGAAG 4550
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Figure 7. A portion of GenBank record OK662577.1 of the *Penaeus vannamei* picornavirus (PvPV)-sequence (nucleotide no. 3,781 to 4,550). The primers for our RT-PCR method (red text) designed from GenBank record KX883984.1 were aligned with the PvPV sequences. There is one mismatch with the final base of the Forward Outer Primer and 3 mis-matches with the Forward Inner Primer, while the 2 reverse primers have no mismatches.

Given only 1-base mismatch at the 3' end of the Forward-outer primer with the matching region of the PvPV genome and no mismatch with the Reverse-outer primer, it is likely that this method could be modified slightly as a temporary semi-nested RT-PCR method using an RNA extract from PvPV-infected shrimp. Specifically, the first RT-PCR step would use the same protocol as that described above with a product also of 482 bp. However, for the second PCR step, the same Forward-outer primer as in the first-step RT-PCR would be used to replace the Forward-inner primer together with the Reverse-inner primer (no mismatch). This would give a PCR product 9 bp longer than that for WZV8 in Table 1 (i.e., 177 bp instead of 168). This is because the forward sequence of the template from the first-step reaction will have replaced the original PvPV target while the reverse primer is already identical to the target in the original PvPV sequence.

A photograph of an example gel of PCR products is shown in **Fig. 8**. The sensitivity of the method is 20 copies per reaction vial using the purified WZV8 amplicons as the template.

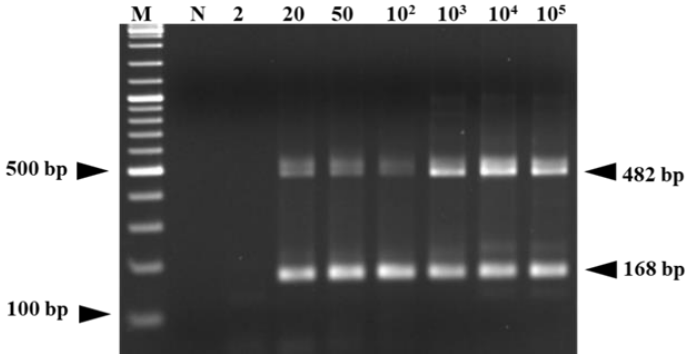

Figure 8. Sensitivity testing for the WZV8-PCR method. Agarose gel electrophoresis analyses the WZV8 amplicons using serially dilutions of the purified PCR products of WZV8 at concentrations from 2 up to 2×10^5 copies/reaction.

Amplicon sequence comparisons

A Clustal Omega alignment of the consensus sequences of RT-PCR amplicons from our current samples (WZV8-AQHT, origin not specified) and excluding primer sequences is shown in **Fig. 8**. The % identity among the three RdRp sequences is significant (**Table 2**), even for the two GenBank sequences from China. This indicates relatively high variability in existing types that may possibly differ in virulence for shrimp. As shrimp pathologists, we wish this information to be distributed as widely and quickly as possible to encourage cooperation and exchange of information on WZV8-like viruses.

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WZV8-AQHT       ACCTACGACAGCGAGACGTTCATCACTCGTGTGACCACCACTCTTCAAGACGTCAGCTAC 60
KX883984.1      TCCTACGACAGCGAGAAGTTCATCACTCGCGTGACCACCACCCTTCAAGACGTCAGCTAC 60
OK662577.1      ACCTATGACAGTGAGAAGTTCATCACTCGCGTGACCACCACCCTTCAAGACGTCAGCTAC 60
                 **** ***** ********** ********** **********

WZV8-AQHT       TGGCAGGGTCTCCCGCTAGAAGCCATCATCTACCTCATGAAGAATCATCAAGTCATCATC 120
KX883984.1      TGGCAGGGCCTCCCGCTAGAAGCCATCATCTACCTCATGAAGAATCATCAAGTCATCATC 120
OK662577.1      TGGCAGGGCCTCCCGCTAGAAGCCATCATCTACCTCATGAAGAATCATCAAGTCATCATC 120
                ******** ************************************************

WZV8-AQHT       GAAGAGCACTTTCCCCTCACCTTCCTTAAGCTCAAAAAGAAGGCCCGTCGAGATAGAAAC 180
KX883984.1      GAAGAGCACTTCCCCCTCACCTTCCTCAAACTCAAGAAGAAGGCCCGCCGAGATAGAAAC 180
OK662577.1      GAAGAGCACTTTCCCCTCACCTTCCTCAAACTCAAGAAGAAGGCCCGCAGAGATAGAAAC 180
                *********** ************** ** ***** ***********  ***********

WZV8-AQHT       ATGCAGATCGTCCAACAGATCACAAACTCCGACGCCTTCAAGATTGGGTTCCCCATCGCT 240
KX883984.1      ATGCAGATCGTCCAACAGATCACTAACTCCAAGGCCTTCCAGGTTGGGTTCCCCATCGCT 240
OK662577.1      ATGCAGATCGTCCAACAGATCACTAACTCCAAGGCCTTCCAGGTTGGGTTCCCCATCGCT 240
                ********************** ****** * ****** ** ****************

WZV8-AQHT       GTGATCGGAGTTGCTGCTACAGCAATCTTCGGGATCGCGAAACTCTCCTCCAAGAAAGTA 300
KX883984.1      GTGATCGGAGTTGCTGCTACAGCAATCTTCGGGATCGCGAAACTCTCCTCCAAGAAAGAA 300
OK662577.1      GTGATCGGAGTTGCCGCTACAGCAATCTTCGGGATCGCGAAACTCTCCTCCAAGAAAGAA 300
                ************** ***************************************** *

XZV8-AQHT       AAGTCCTCTAAGACACCCTCCCATCAAGACAGTGACAGCTCTTCAAGCGAGTCCGAA--- 357
KX883984.1      AAGTCCCCAAAGAAACCCTCCCGACCTGACAGCGACAGCTCTTCAAGCGAGTCCGAATCA 360
OK662577.1      AAGTCCCCAAAGAAACCCTCCCCACCTGACAGCGACAGCTCTTCAAGCGAGTCCGAATCA 360
                ****** * **** ********  *  ***** ************************

WZV8-AQHT       GACGAAGAACAATCCGCTCCCCAGAAGTCTGAGAAATCTCAGACGCGAGTTGCAAGAAAA 417
KX883984.1      GACGAAGAACAATCTGCCCCTCAGAAGTCTGAGAAGTCTCAGACGCGAGTTGCTAGGAAA 420
OK662577.1      GACGAAGAACAATCTGCCCCTCAGAAGTCTGAGAAGTCTCAGACGCGAGTTGCTAGGAAA 420
                ************** ** ** ************** ***************** ** ***

WZV8-AQHT       TCTAAGCAATCCGCACCCGA       437
KX883984.1      TCTAAGCAATCCGCACCCGA       440
OK662577.1      TCCAAGCAATCCGCACCCGA       440
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Figure 8. Clustal Omega alignment of the sequence of the 1st-step RT-PCR amplicon obtained using the method herein from a specimen of unspecified Asian origin (WZV8-AQHT) with the two GenBank records for WZV8 (KX883984.1) and PvPV (OK662577.1) from China. The gaps in the line of asterisks indicate regions of difference among the 3 sequences, while the grey background nucleotides indicate differences nucleotide between the three sequences. The % identities are shown in Table 2.

Table 2. Percent identity matrix among the three aligned sequences in Fig. 8 created by Clustal Omega 12.1. This compares with the much lower 95.4% sequence identity for 97% coverage from the BLASTn comparison of the whole WZV8 like sequences KX883984.1 and OK662577.1.

	WZV8-AQHT	OK662577.1	KX883984.1
WZV8-AQHT	100.00	92.45	93.14
OK662577.1	92.45	100.00	98.18
KX883984.1	93.14	98.18	100.00

Summary

The original publication describing WZV8 did not include information related to signs of disease, histopathology or pathogenicity (Li et al. 2015). Nor did the publication on PvPV (Liu et al. 2021), although the latter did state that the virus was isolated from moribund shrimp. However, to confirm virulence, isolation of a new virus from moribund shrimp must be accompanied by histopathological analysis for the presence of other known pathogens in the source specimens together with results from challenge tests employing the newly purified virus to show that it alone can produce the same pathology as seen in the original moribund shrimp. To date, there is no proof that WZV8-related viruses have caused disease, and we have many histological samples from normal shrimp dating back over a decade that show its distinctive LDI. Thus, the evidence in hand suggests that WZV8-like viruses have had little impact on production of cultivated *P. monodon* and *P. vannamei*.

In addition, we have carried out preliminary challenge tests (unpublished) using crude viral extracts injected into shrimp that have resulted in development of LDI in E-cells, but without any accompanying signs of disease for several weeks after injection. However, it is possible that other types of WZV8 may exist and that some may be lethal, or that WZV8 may contribute to mortality in combination with one or more other pathogens or under some environmental conditions. This is what we need to establish as quickly as we can. We hope that this rapid communication and offer of a free positive control plasmid and RT-PCR method will support this initiative and lead to the rapid acquisition of new information plus the development of a more universal RT-PCR method. At the same time, we should avoid the occurrence of a panic situation developing as a result of a rapid increase in reports of its presence simply because detection methods for it are now available. This is particularly relevant for RT-PCR detection without supporting evidence of a causal link to disease.

Recommendations to colleagues

In the current atmosphere of antagonism and threats from aquaculturists and governmental authorities towards scientists who study diseases of aquaculture animals, we have not given any indication here of the source of the samples we have studied. As research scientists, this is not our responsibility but the responsibility of the competent authorities of the countries from which the samples we study originate. In many cases, we receive samples from global clients. Like medical doctors, our work with clients is confidential. If the client is from a WTO member country, it is his or her legal obligation to inform the competent authority in their country if an unexpected disease outbreak has occurred. It is then the responsibility of the competent authority of the relevant country(ies), to inform OIE. It is now our policy to no longer reveal a country source of any material we use for research on the discovery of new and newly emerging organisms that may have pathogenic potential. In the case of WZV8, the majority of the shrimp samples we have examined were normal and showed no signs of

disease, so the clients had no obligation to report anything to their competent authority. It is not obligatory to report unusual histological anomalies that occur in normal shrimp if they are not associated with disease.

In retrospect, we regret our earlier action (Senapin et al., 2007) in which we published a paper on disease outbreaks caused by infectious myonecrosis virus (IMNV) in Indonesia based on samples we received from a client there. We regret that we did this without notifying or obtaining permission to do so from the government of Indonesia, and we apologize for our oversight on that occasion. Since then, we simply state that we have obtained samples from the Indo-Pacific region, the Americas etc. We strongly recommend that other research laboratories adopt the same approach. This will allow us and other scientists who adopt the same approach to isolate our work on new and newly discovered organisms free from political interference and to promote rapid scientific communications such as this to expedite a cooperative international, scientific response towards assessing threats and limiting the spread of potential pathogens.

It is unfortunate that scientists from some countries may not be able to easily join in this cooperation because it is illegal (punishable by fines and/or imprisonment in some countries) for them to even test for any pathogen that their government considers to be "exotic" to their country. This is despite the fact that it is hard to understand how a government knows whether a newly discovered pathogen is exotic to their country or not. Therefore, we recommend that researchers in such countries consult with their government officials before they apply any of the methods described in this communication lest they risk fines or imprisonment for doing so. On the other hand, we cannot see how anyone could be prevented from simply re-examining H&E-stained shrimp tissues for LDI. The question is, what should they do if they find them? It seems that silence would be their least risky choice.

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