Urgent announcement on usefulness of the lymphoid organ (LO) as an additional prime target for diagnosis of decapod iridescent virus 1 (DIV1) in diseased *P. vannamei*

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Summary

We carried out laboratory injection challenges that employed extracts prepared from shrimp naturally infected with decapod iridovirus 1 (DIV1). We found that diseased shrimp from the injection trials showed pathognomonic lesions for DIV1 in the hematopoietic tissue that matched those reported for DIV1 in *P. vannamei* from China (Qiu et al. 2017. Scientific Reports. 7). In addition, we also found distinctive lesions in the lymphoid organ that could be used as an additional indicator in confirming diagnosis of DIV1 disease. Also, the lesions from shrimp challenged with the 10x dilution were more severe than those from 100x dilution, and for some shrimp in the 100x dilution, the lesions were very clear in the LO but absent in the HPT. Altogether, the results suggested that histology of the HPT and LO could be used together to help in the diagnosis of DIV1 in conjunction with PCR, amplicon sequencing and *in situ* hybridization (ISH) analysis. This is particularly important in confirming the presence of virulent isolates of DIV1 in new geographical locations.

Brief methodology

We received frozen samples of shrimp purportedly infected with DIV1 from an undisclosed source. These were processed to prepare extracts for shrimp injection in a biosecure, quarantine facility. Presence of DIV1 was confirmed by PCR testing using a published method (Qiu et al. 2017. Scientific Reports. 7) and by an in-house method based on a second widely-separated region of the published DIV1 genome (Qiu et al. 2018. Arch. Virol. 163). After confirmation of the presence of DIV1 by PCR, extracts were prepared for injection into shrimp in the biosecure laboratory at 10x and 100x dilution. The batch of shrimp for the challenge tests consisted of juveniles of *P. vannamei* provided by a local supplier and tested by PCR for absence of DIV1. After injection challenge, moribund or control shrimp were removed at

intervals and separated into halves, one for PCR analysis and the other for histological analysis plus ISH using standard methods with adjacent tissue sections of the cephalothorax tissue.

RESULTS

Pathognomonic DIV1 lesions

Photomicrographs of HPT are shown here at low and high magnification from moribund shrimp obtained from the challenge tests that gave strong positive PCR results for DIV1. These can be compared to the published photomicrograph (Qiu et al. 2017. Scientific Reports. 7) from similarly challenged *P. vannamei* in China that show lightly-basophilic, cytoplasmic inclusions in cells of the HPT reported to be characteristic of disease caused by DIV1 (**Fig. 1B**). Since such inclusions have not been previously reported from shrimp, they may be considered pathognomonic for DIV1 pathology. The HPT tissue also showed some deeply basophilic inclusions of various sizes that were of little diagnostic value since they could not be distinguished from pyknotic and karyorrhectic nuclei.

Shrimp from our laboratory challenge samples also showed lightly-basophilic cytoplasmic inclusions in the cytoplasm of cells in the hematopoietic tissue (HPT) of moribund shrimp (example **Fig. 1C**, white arrows), similar to those reported from natural and laboratory infections of DIV1 in China. These were accompanied by densely-basophilic inclusions that could not be distinguished from pyknotic or karyorrhectic nuclei in the same tissue sections (black arrows). In these studied samples, the structural organization of the HPT was also abnormal (i.e., nuclei more scattered, less densely stained and sometimes vacant).

Figure 1. Example photomicrographs of normal HPT tissue and HPT showing lightly-basophilic cytoplasmic inclusions (black arrows) pathognomonic for DIV1 infection. (A) Normal HPT tissue from an unchallenged shrimp specimen. (B) Copy of photomicrograph in the Chinese report (Qiu et al. 2017. Sci. Rep. 7) showing the lightly basophilic cytoplasmic inclusions (black arrows) pathognomonic for DIV1 infection contrasted with deeply basophilic inclusions (white arrows) in the HPT tissue; (C) HPT from a moribund shrimp from our challenge test also showing lightly basophilic cytoplasmic inclusions (white arrows) similar to those reported in DIV1-infected shrimp from China. Note also the common occurrence of relatively vacant nuclei (asterisks).

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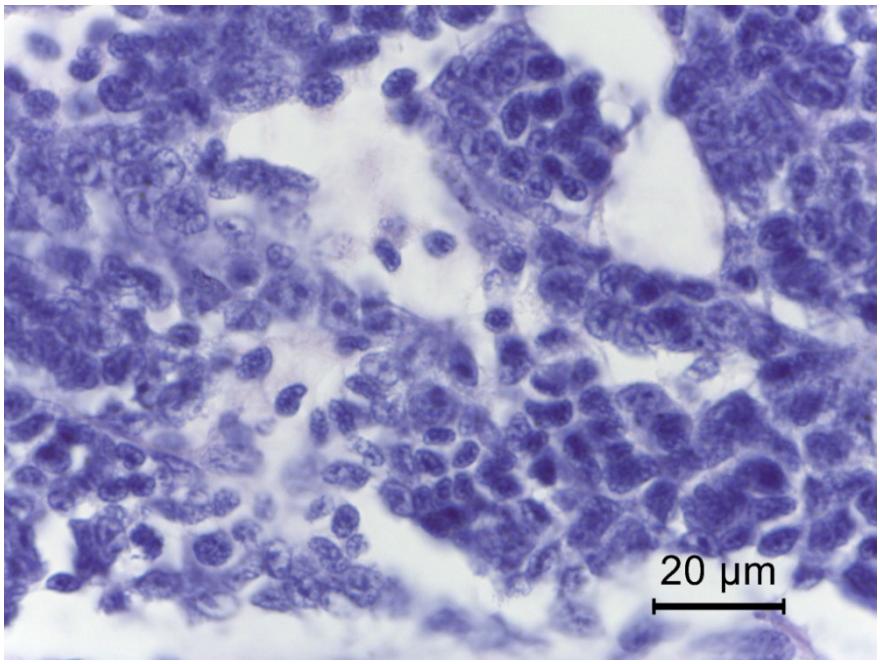

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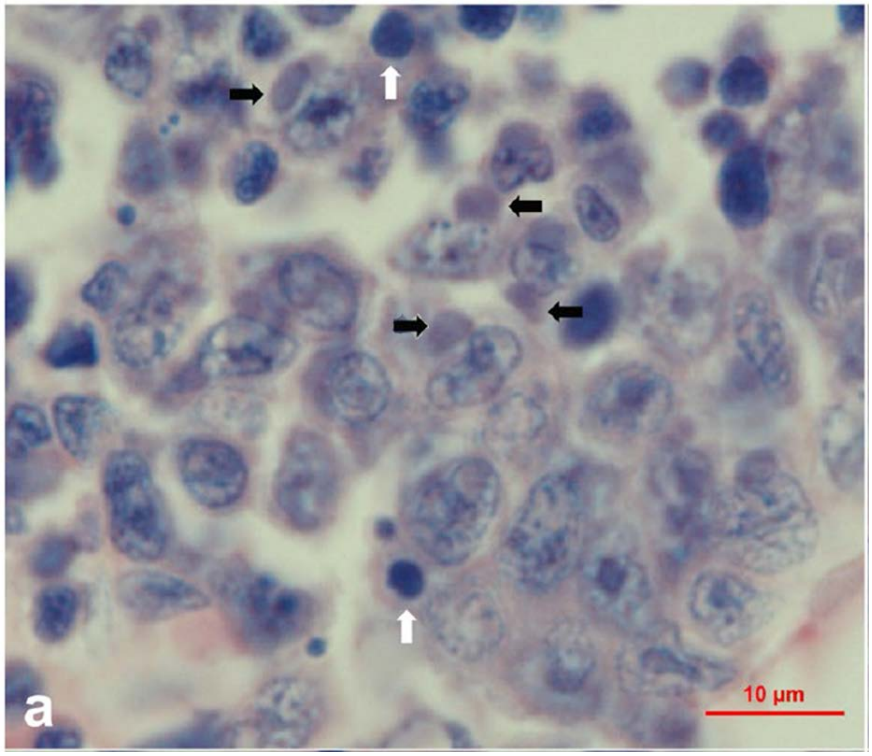

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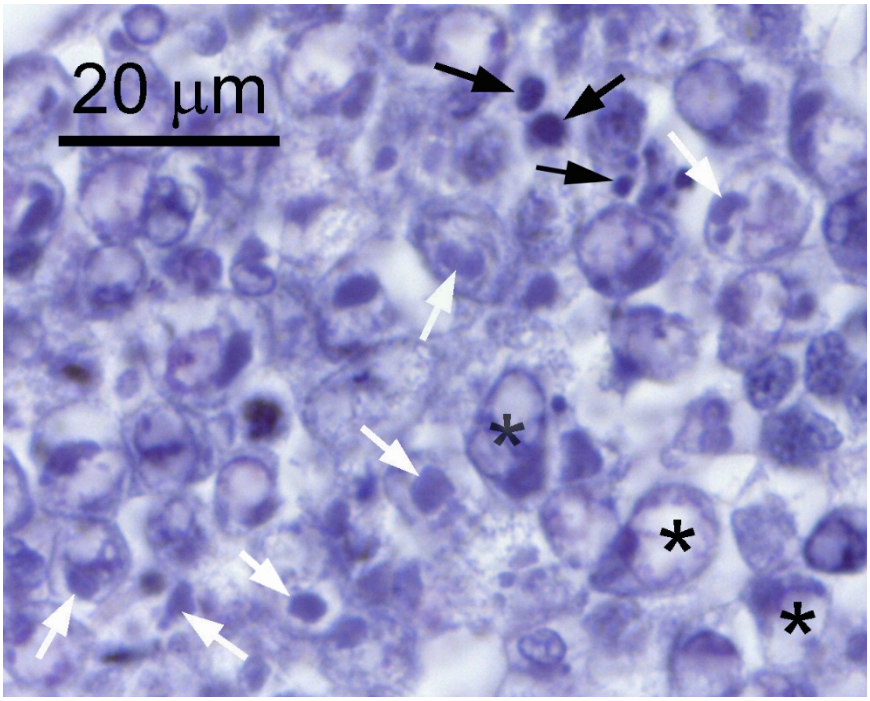

Unique lymphoid organ (LO) lesions accompanying the HPT histopathology described above

Although not described in any of the Chinese publications on DIV1 (Xu et al. 2016. Dis Aquat Org. 120, 17-26; Qiu et al. 2017. Scientific Reports. 7), we found that all the moribund shrimp that showed the HPT pathology described above also showed unique pathology of the LO tissue (**Fig. 2**) whenever it was present in the cephalothorax sections. This consisted of disorganization of the LO-tubule matrix accompanied by abnormal morphology of the nuclei

and the presence of karyorrhectic and pyknotic nuclei that could not be distinguished from basophilic cytoplasmic inclusions. This LO pathology somewhat resembles that of yellow head virus (YHV) and is therefore not distinctive enough for specific diagnosis of DIV1 infection. In some samples, lymphoid organ spheroids were also present or in the process or being formed and they showed similar cytoplasmic inclusions. Although the LO pathology is not distinctive enough in itself to indicate DIV1 infection, we believe it can be used to support a presumptive diagnosis of DIV1 disease when combined with the features of the pathognomonic lesions in the HPT. In addition, in the shrimp challenged with the 100x dilution of DIV1, some specimens showed no HPT lesions and were negative for DIV1 by ISH when the LO did show a clear but less severe similar pathology to that in Fig. 2b & 2c and a positive ISH reaction, suggesting that the LO may serve as an early indicator of the possibility of DIV1 infection especially when supported by PCR and ISH results (not shown).

Figure 2. Photomicrographs of normal LO tissue and abnormal LO also present in moribund shrimp from DIV1 challenge tests. (A) Normal LO tissue. (B) Low magnification of abnormal LO tissue from a farmed moribund shrimp showing a disorganized tubule matrix containing many pyknotic and karyorrhectic nuclei. (C) High magnification of LO tissue shown in B with the asterisk (*) at the same tissue position in both photomicrographs.

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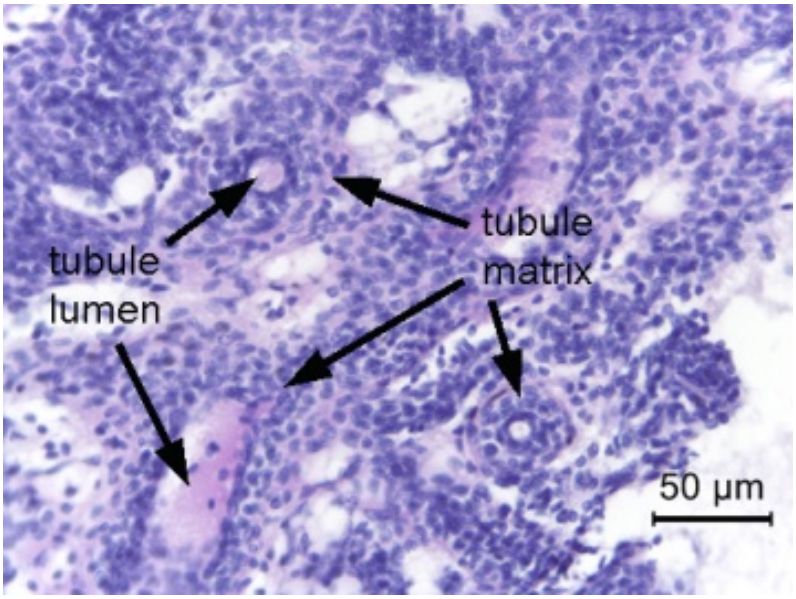

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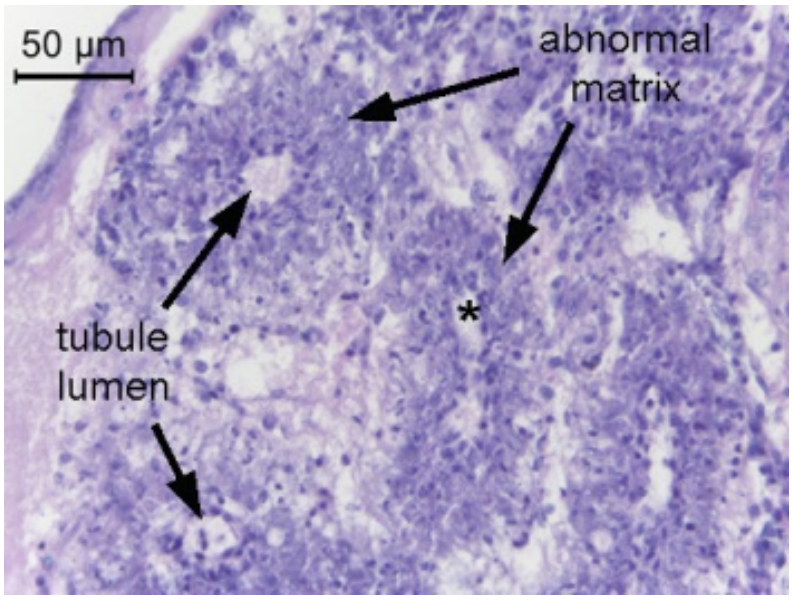

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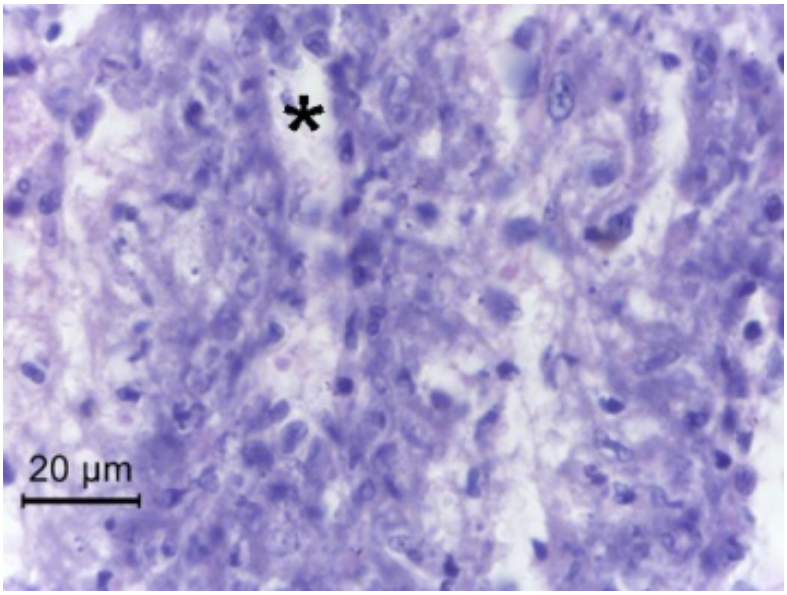

ISH results confirming the presence of DIV1-DNA in HPT and LO tissues of moribund shrimp from laboratory challenge tests

ISH tests were carried out with tissue sections adjacent to those stained with H&E using two different DNA hybridization probes. One was based on the amplicon obtained using the Chinese PCR method with the ATPase gene of DIV1 as the target, as described above. The other was based on the amplicon obtained from the in-house PCR method with the MCP gene of DIV1 as the target (NCBI accession no. KY681039.1). All of the moribund shrimp samples from the DIV1 challenge tests gave strong positive ISH reactions in the abnormal HPT (**Fig. 3**) and LO (**Fig. 4**) tissues described above. In the case of the HPT, examination of the positive ISH cells at high magnification with a light microscope (100x objective) revealed that the positive signals originated from cytoplasmic inclusions, often adjacent to nuclei of the host HPT cells. These positions corresponded to the positions of the lightly basophilic inclusions described as characteristic of DIV1 infection in the Chinese publications and confirmed the presence of DIV1 in our specimens.

Figure 3. Example photomicrographs of ISH test results for DIV1 in the HPT of a moribund shrimp specimen from the challenge tests. (A) H&E-stained section showing lightly-basophilic cytoplasmic inclusions characteristic of DIV1 histopathology (black arrows). (B) Adjacent tissue section showing a negative ISH reaction with the no-probe control. (C) Adjacent tissue section showing positive ISH reactions in cytoplasmic locations (white arrows).

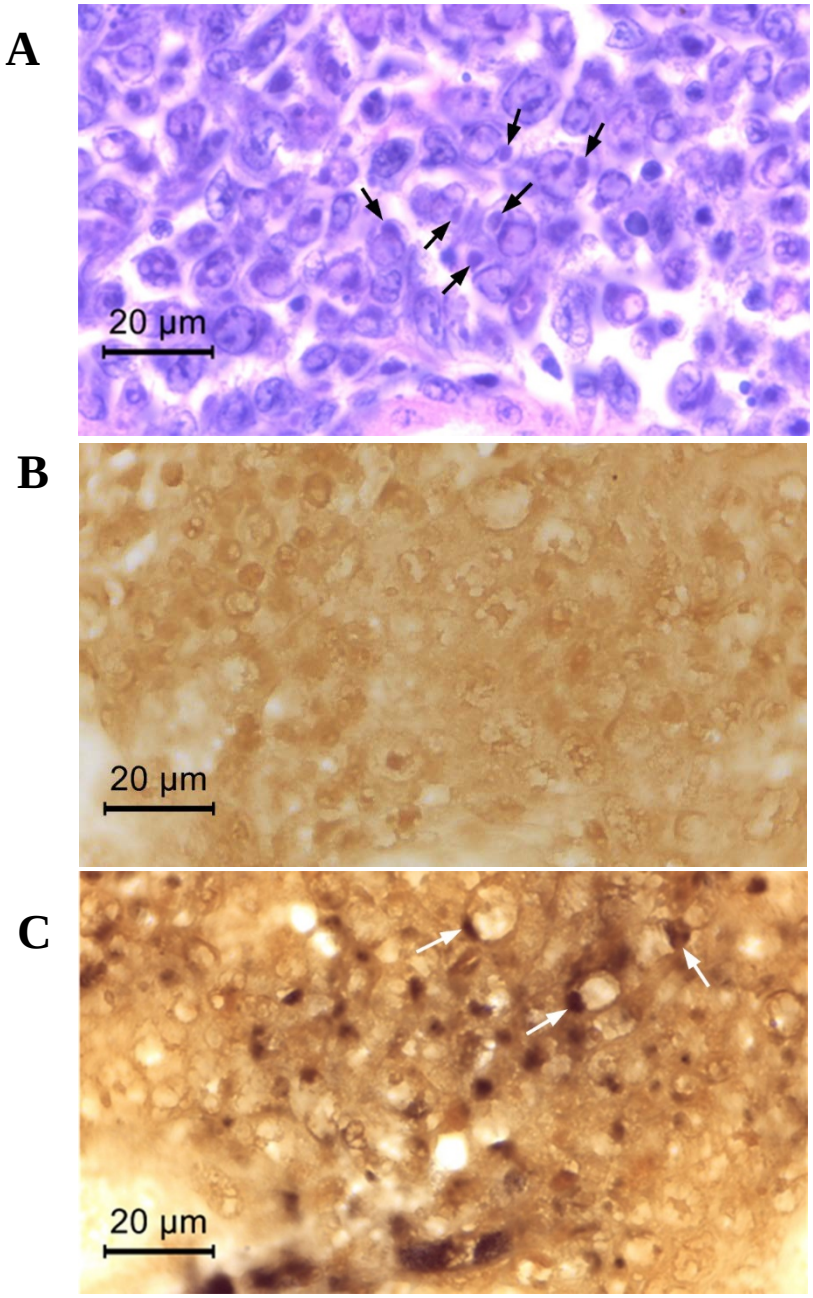

Figure 4. Low magnification photomicrographs showing positive ISH reactions for DIV1 in LO tissue, in a nearby muscle lesion and in connective tissue. (A) H&E stained tissue section showing an abnormal LO, an adjacent muscle lesion, connective tissue and a portion of the hepatopancreas (HP). (B) Adjacent tissue section showing a negative ISH reaction with the YHV probe control. (C) Adjacent tissue section showing positive *in situ* hybridization reactions in the LO, in the muscle lesion, in connective tissue, in the antennal gland and in interstitial spaces of the hepatopancreas (HP).

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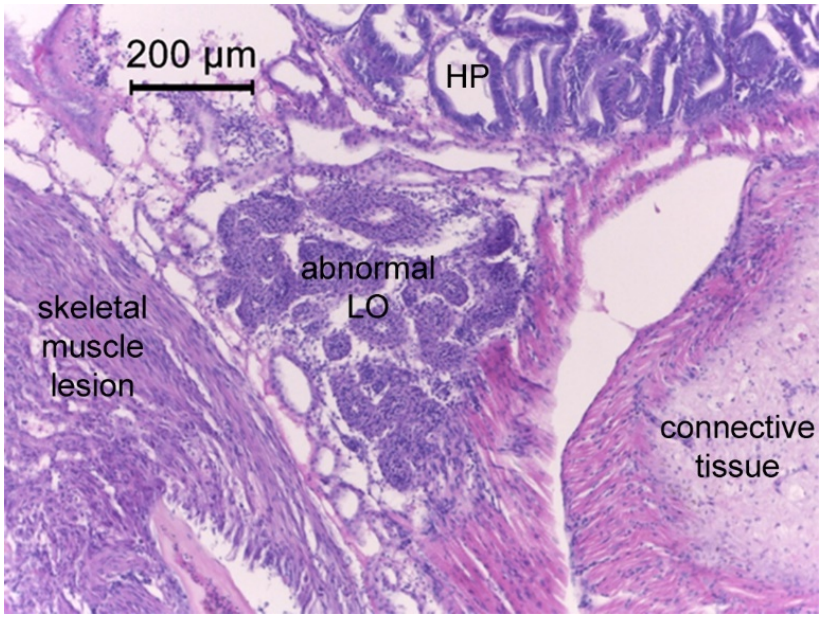
200 μm
HP
abnormal
LO
skeletal
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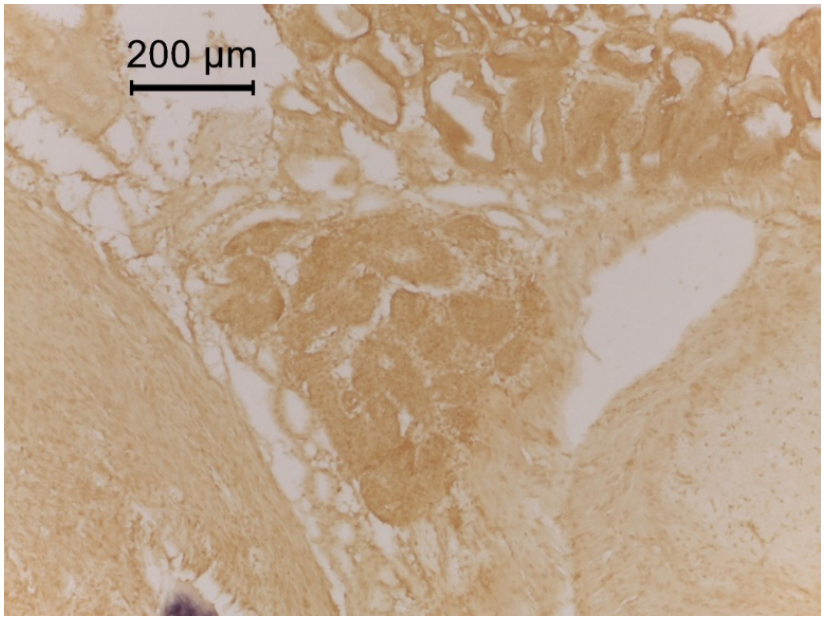
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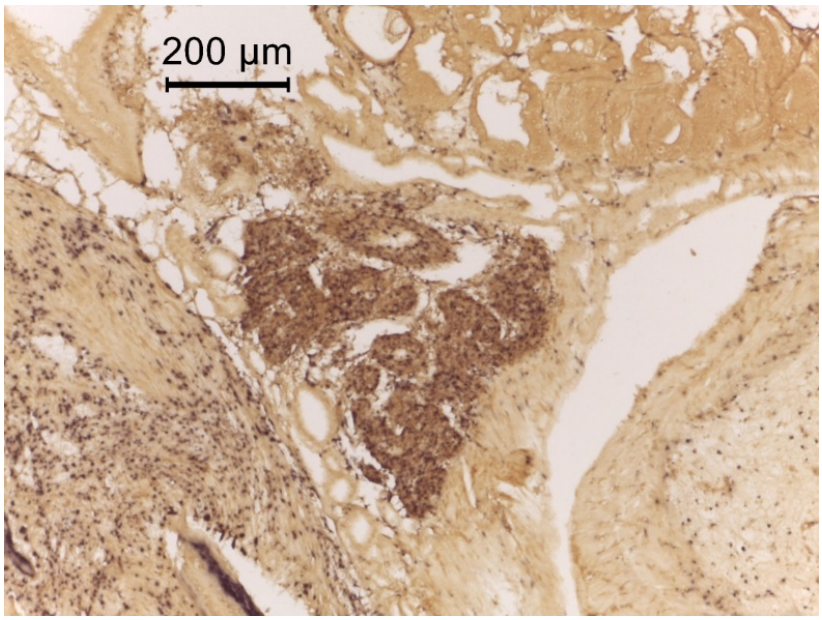
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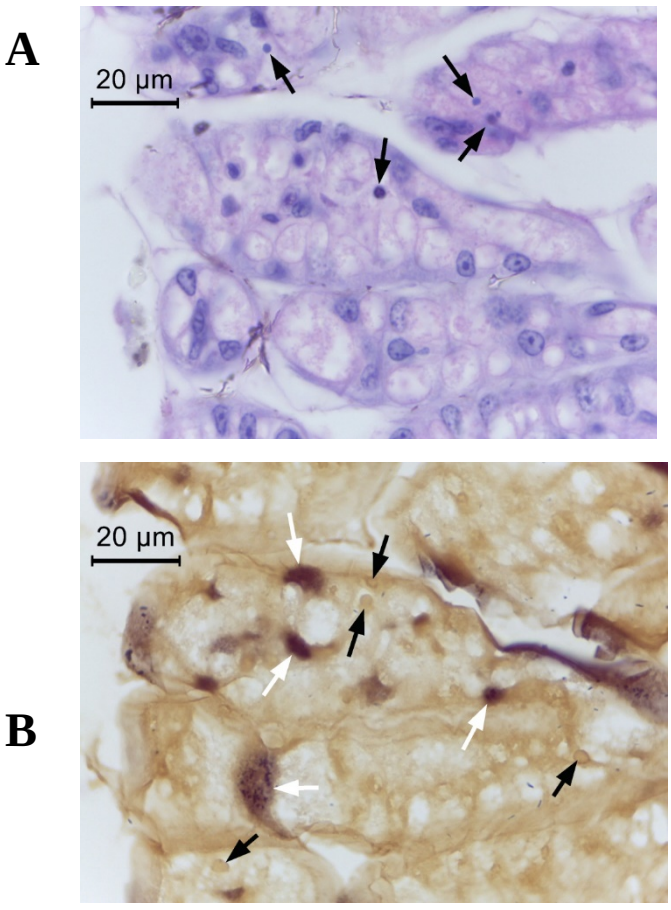
In addition to positive ISH signals for DIV1 in HPT and LO lesions in the moribund shrimp specimens described above, positive signals were also observed widely in other tissues. These included the gills (**Fig. 5**) and connective tissue throughout the cephalothorax and abdomen (e.g., Fig. 4). They were also found in connective tissue in interstitial spaces of the hepatopancreas (Fig. 4) and the anterior midgut cecum, around the ventral nerve cord and below the subcuticular epithelium and sometimes including it. Signals were also found in the antennal gland, heart muscle and skeletal muscle. The latter occasionally showed large, necrotic lesions that gave strong positive *in situ* hybridization reactions as seen with the muscle in Fig. 4. Thus, DIV1 causes systemic infections that include many tissues of ectodermal and mesodermal origin excluding the ventral nerve cord and ganglia and excluding tissues of endodermal origin (e.g., HP tubule epithelium, anterior midgut cecum epithelium, midgut epithelium). None of these tissues show unique features that would be useful for diagnosis by standard H&E staining. Therefore, we recommend that the HPT and LO be the key tissues to examine while trying to diagnose a suspected case of disease caused by DIV1. Of these two, the LO can be examined using a 40x objective lens but the HPT may require a 100x objective lens to confirm the presence of lightly basophilic cytoplasmic inclusions.

Figure 5. Photomicrographs of gill tissue. (A) H&E stained section showing the presence of what appear to be pyknotic and karyorrhectic nuclei as the only prominent anomaly. (B) ISH hybridization assay result from an adjacent section to that shown in A with apparent pyknotic and karyorrhectic nuclei giving no reaction with the probe (black arrows), while the white arrows indicate larger, positive hybridization areas that do not correspond to similarly sized or shaped anomalies in the H&E stained section. This suggests that the DIV1 positive areas are not detectable by H&E staining and that the apparent pyknotic and karyorrhectic nuclei, in themselves, cannot be used for preliminary diagnosis of DIV1 infection.



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2. Qiu, L., Chen, M.-M., Wang, R.-Y., Wan, X.-Y., Li, C., Zhang, Q.-L., Dong, X., Yang, B., Xiang, J.-H., Huang, J., 2018. Complete genome sequence of shrimp hemocyte iridescent virus (SHIV) isolated from white leg shrimp, *Litopenaeus vannamei*. Arch. Virol. 163, 781-785.

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