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Misuse of PCR assay for diagnosis of mollusc protistan infections

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ABSTRACT: Polymerase chain reaction (PCR) assays are useful tools for pathogen surveillance, but they are only proxy indications of pathogen presence in that they detect a DNA sequence. To be useful for detection of actual infections, PCR assays must be thoroughly tested for sensitivity and specificity, and ultimately validated against a technique, typically histology, which allows visualization of the parasite in host tissues. There is growing use of PCR assays for pathogen surveillance, but too often the assumption is made that a positive PCR result verifies an infection in a tested host. This assumption is valid only if the assay has been properly validated for the geographic area and for the hosts examined. Researchers should interpret unvalidated PCR assay results with caution, and editors and reviewers should insist that robust validations support all assertions that PCR results confirm infections.

KEY WORDS: PCR assay · Validation · Histology · *In situ* hybridization · *Marteilia* · *Haplosporidium*

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Polymerase chain reaction (PCR) assays are extremely useful tools for pathogen surveillance in aquatic animal pathology. It is important to remember, however, that PCR-based techniques detect DNA sequences (Hiney 2001); these sequences may or may not be present in viable pathogen cells, and such cells may or may not be from an established infection. PCR-based diagnostic methods are based on the accuracy of the assumption that the positive signal is from the target species. Positive PCR assays may indicate the presence of an infectious agent, but the technique does not verify the existence of an infection. In addition, false-positives can be caused by poor specificity of PCR primers and resulting cross-reaction with DNA of non-target organisms (Claydon et al. 2004).

Conversely, negative PCR assays do not confirm the absence of either targeted pathogen cells or infections. False-negatives can result from sampling error due to relatively small proportional abundances of pathogen DNA (especially from hosts with localized or very light infections) among the typically small DNA samples that are analyzed (10 to 50 ng). Likewise, DNA sequence variations among different pathogen genetic strains may inhibit PCR primer hybridization to target DNA templates. Finally, inhibitors may be present in the sample that affect DNA polymerases, whose efficient activities are critical to PCR assay performances (Hiney 2001, Reece & Burreson 2004).

The focus here is exclusively on errors, misinterpretations, and unjustified consequences of unvalidated assumptions that PCR amplifications of parasite target DNA sequences verify infections. Infection occurs after a host is exposed to a parasite, after the parasite overcomes external host barriers to invasion, and after the parasite evades internal host defenses to become established in target cells, tissues or organs. Because of the possible misuse or misinterpretation of positive findings from PCR assays alone, the OIE Aquatic Animal Health Standards Commission recently revised the definition of infection from 'the presence of a disease

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agent in a host' to 'the presence of a multiplying or otherwise developing or latent disease agent in a host' (OIE 2007, p. 9). PCR assays alone cannot determine that a parasite has become established in a host. To be interpreted appropriately, PCR-based analyses for determination of protistan pathogen infections must be validated against an established technique, typically histology, which allows visualization of the infective agent.

As defined by Hiney (2001), validation is an investigation of the extent to which a technique can be legitimately used for a particular purpose. Validation determines whether the technique detects the species, whether it detects all strains and life history stages of that species (inclusivity), and whether there is a cross-reaction with any non-target species (exclusivity) (Reece & Burreson 2004). The latter is particularly problematic because it is impossible to test any assay against all other organisms, so false positives from unknown cross-reactions are always a possibility. This becomes especially important if an assay is used in a new host, or new geographic area for which it has not been validated. Sequencing of amplification products can verify that the DNA is from the target organism, but it does not confirm that there is an actual infection.

Validation against histology determines the extent to which the PCR assay detects an actual infection. Validation should be accomplished by field trials in which the same sample is tested by both PCR-based methods and histology. When using PCR assays to detect parasites in hosts or geographic regions where validation of the assay has not been accomplished, infections must be verified by histology. Of course, it is well documented through many validation studies that molecular diagnostic techniques are more sensitive than histology when infections are of low intensity, especially if the parasite is small. In these circumstances, *in situ* hybridization (ISH) assays may increase the sensitivity of histology by facilitating visualization, and also provide assurance (when the ISH assay has been tested for specificity) that the organism observed is the target organism.

When using PCR assays to search for parasite DNA in potential intermediate hosts, it is critical to confirm infections with ISH assays. In such circumstances, the morphology of parasite life cycle stages in an intermediate host may be different from their morphology in the definitive host, so routine histology may be ambiguous or misleading. ISH assays verify both the identity of the parasite and confirm that the intermediate host is actually infected (Audemard et al. 2002).

Some recent publications highlight problems associated with over-interpretation of PCR-based diagnostic results for protistan pathogens that were not properly validated for the geographical region or hosts investigated. Based on PCR analyses alone, Ulrich et al. (2007) reported the presence of *Haplosporidium nelsoni* infections (causative agent of MSX disease) in *Crassostrea virginica* and several other oyster species in the Gulf of Mexico; these results indicated both geographic and host range expansions for *H. nelsoni*. The PCR assay they used was developed by Stokes et al. (1995). This assay has been tested for specificity with other haplosporidians and local pathogens to the extent possible, and has been validated for detection of *H. nelsoni* in *C. virginica* in Chesapeake Bay. The validation consisted of monthly comparisons of PCR results and histology in 25 oysters through the May–December infection period of *H. nelsoni* (N. Stokes unpubl. data). The assay has not been validated for the Gulf of Mexico. Ulrich et al. (2007) report that '30 of 41 oysters were positive for MSX' (p. 196). They also make the following statements: '…despite the wide distribution of *H. nelsoni* **infections**' (Abstract); '…we are confident that these **infections** represent…' (p. 197); 'Given the … prevalence of **infections** in the Gulf of Mexico…' (p. 198); 'We also identified *H. nelsoni* **infections** in oysters originating from … Venezuela.' (p. 198, all emphases mine). Ulrich et al. (2007) acknowledge that they did not confirm infection intensities by histology, but in reality they did not even confirm actual infections. Nevertheless, they repeatedly assert the presence of infections, as shown above. Clearly these authors have made an interesting finding worthy of additional research, but they have not documented infections by *H. nelsoni* in *C. virginica* in the Gulf of Mexico. In contrast, thousands of *C. virginica* oysters from over 50 sites in the Gulf of Mexico that were collected during all seasons of the year for over 20 yr have been examined histologically by competent pathologists as part of the National Status and Trends Program Mussel Watch Project (available at: ccma.nos.noaa.gov/stressors/pollution/nsandt/), with no infection by *H. nelsoni* ever observed.

Other recent examples of misuse of PCR assay results are those of Carrasco et al. (2007a,b), who investigated the life cycles of *Marteilia* spp. (documented pathogens of oysters and mussels in Europe). These authors used an unvalidated (sensu Hiney 2001) PCR assay (López-Flores et al. 2004) to detect *Marteilia* spp. DNA in zooplankton populations containing potential intermediate hosts. No histology was performed on the samples and reported results are based on PCR assays alone. Carrasco et al. (2007a) state that: 'Identification of zooplankton species **found infected** allowed two new *Marteilia hosts* to be proposed.' (Abstract, emphasis mine). Throughout the Discussion section of the paper the authors mention 'infected' samples. They state that: 'The *Marteilia* parasite was present in different groups of copepoda species and

showed a lack of specificity. Therefore, other copepod species could also probably have been **infected**…' (p. 69, emphasis mine). Finally, the authors conclude that there needs to be '…a more detailed study on the role of the newly identified **hosts** in the life cycle of the *Marteilia* parasite.' (p. 69, emphasis mine).

In a subsequent paper, Carrasco et al. (2007b) use the same unvalidated PCR assay to detect the presence of *Marteilia refringens* DNA in zooplankton samples. In their Table 1, they report the results of the PCR assay as positive or negative, which is appropriate. However, in the legend of both their Tables 1 and 2 they use the phrases '…zooplankton samples **found to be infected** by *Marteilia refringens*…' or '…zooplankton samples **detected to be infected** by *Marteilia refringens*…' (emphases mine). Furthermore, the following statement appears in the Results section: 'Therefore, ecologically diverse marine crustacean groups, such as calanoida, cyclopoida and harpacticoida copepods, and decapod crustacean larvae **were parasitized** *by M. refringens*…' (p. 1547, emphasis mine). Finally, in the Discussion section it is stated that: 'Copepod species more frequently **found infected** in the present study were *A. discaudata* and *Oithona* sp.' and 'A number of zooplankton species **were found to be infected** by the parasite using nested PCR.' (p. 1549, emphases mine). Other authors, to their credit, did not report *Marteilia* sp. infections in copepods until they were verified by an ISH assay (Audemard et al. 2002). The interpretations of Carrasco et al. (2007a,b) are especially troubling because *M. refringens* is an OIE-reportable pathogen of molluscs and the presence of infections can have potential economic consequences. Therefore, for internationally reportable protistan pathogens, researchers have a special obligation to verify infections with a technique that allows visualization of the parasite.

In the studies mentioned above, PCR assay results were interesting and certainly emphasize the need for additional study, but in no case was infection of a host by a parasite confirmed, though repeatedly asserted. I urge researchers of protistan parasites to test their PCR assays for specificity and to validate them against histology to empirically determine the limitations for legitimate inferences on infection status that may be concluded from PCR results. Only then is the assumption that a positive PCR result confirms an infection valid. PCR-only results from unvalidated assays that are not confirmed by histology should be interpreted with caution. Likewise, editors and reviewers of manuscripts reporting PCR diagnostic results must insist that robust validations support all assertions that PCR results confirm infections among tested hosts.

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