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Confirming validity measures of visual assessment of PaV1 infection in Caribbean spiny lobsters

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ABSTRACT: *Panulirus argus* virus 1 (PaV1) affects wild populations of Caribbean spiny lobsters. PaV1 can be lethal but shows predilection for juvenile lobsters. Because *P. argus* is one of the most valuable fisheries around the wider Caribbean region, monitoring disease prevalence in local populations is desirable. Diseased lobsters are easily recognized by their milky hemolymph, but this sign only becomes evident in advanced stages of infection. Other methods have been developed to detect PaV1, but are less practical for long-term monitoring of patterns of infection in populations. A previous study estimated the validity measures (sensitivity and specificity) of detection of PaV1 infection by observed clinical signs against endpoint PCR assays, using a representative sample of lobsters comprising mainly subadults and adults from a commercial fishing area. In the present study, these validity measures were estimated in a similar manner for a different population comprising mainly juveniles from a protected nursery area. We obtained virtually the same sensitivity and specificity values (0.48 and 1, respectively) for observed clinical signs as in the previous study (0.51 and 1, respectively), confirming the validity of applying a simple 2× correction factor to monitor the patterns of PaV1 infection over time based on more easily conducted visual assessments of a representative sample of the population.

KEY WORDS: *Panulirus argus* virus 1 · Sensitivity · Specificity · Diagnostic tests · Clinical signs · PCR · Crustacean

1. INTRODUCTION

The Caribbean spiny lobster *Panulirus argus* (Latreille, 1804) (Crustacea: Decapoda: Achelata: Palinuridae) is a valuable fishing resource for 26 countries in the wider Caribbean region (FAO 2018). After a protracted pelagic larval phase, postlarvae of *P. argus* settle in seagrass habitats and marine vegetation of reef lagoons and shallow bays. The small juvenile lobsters (~6–50 mm carapace length, CL) remain in these habitats for several months, until the large juveniles, or subadults (~50–80 mm CL), migrate to coral reefs, which are inhabited by adults (~80–200 mm CL). *P. argus* is affected by the pathogenic virus *Panulirus argus* virus 1 (PaV1). Since its discovery in Florida (USA) in 1999 (Shields & Behringer 2004), PaV1 has been detected in many other Caribbean countries (Moss et al. 2013). PaV1, which can be lethal, shows predilection for juvenile lobsters. Macroscopic (clinical) signs of PaV1 infection are a milky hemolymph that fails to clot and a reddish discoloration over the carapace (Shields & Behringer 2004, Lozano-Álvarez et al. 2008). However, these signs do not become evident until the infection is in a rather advanced stage (Cruz Quintana et al. 2011). Therefore, an unknown proportion of individuals in a given population could be subclinically infected at any given time. To understand the potential effects of PaV1 on populations of *P. argus*, it would be desirable to monitor prevalence levels in local lobster populations over time.

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Because only lobsters in advanced stages of PaV1 infection exhibit clinical signs, visual assessments only estimate apparent ('clinical') prevalence of infection. Therefore, validity measures (sensitivity and specificity) of visual assessment as a diagnostic test should be compared against results of a 'gold standard,' i.e. a test with a much better performance to diagnose the disease (Altman & Bland 1994, Lalkhen & McCluskey 2008). By using the endpoint PCR assay developed by Montgomery-Fullerton et al. (2007) as a gold standard, Huchin-Mian et al. (2013) evaluated the sensitivity at which PaV1 infection was detected by observed clinical signs, and the specificity of these clinical signs. The sensitivity of a test measures the proportion of truly diseased animals that the test correctly identifies as diseased. The specificity measures the proportion of non-diseased animals that the test correctly identifies as non-diseased (Cameron 2002). Sensitivity of visual assessment was 0.51 (0.43–0.59, 95% CI), indicating that for each clinically infected lobster there was another lobster subclinically infected. Specificity was 1 (0.99–1.0), indicating that in *P. argus*, the milky hemolymph was a specific sign for PaV1 infection. Based on these values, a simple 2× correction factor could suffice to accurately estimate true prevalence of PaV1 based on visual assessment of lobsters (Huchin-Mian et al. 2013).

Sensitivity and specificity of a test are considered as fixed characteristics of a particular test (Lalkhen & McCluskey 2008); however, they can vary between different populations due to a number of factors, such as the stage of disease or the presence of other microorganisms (Cameron 2002). Therefore, validation of a test requires reassessment of its performance characteristics for each unique population of animals to which it is applied (Jacobson 1998). The aim of the present study was to obtain an independent estimate of sensitivity and specificity of clinical signs of PaV1 in order to test the validity and potential general applicability of the correction factor proposed by Huchin-Mian et al. (2013). Those authors sampled 1397 lobsters over a size range of 12.8–168.0 mm CL, in a large bay sustaining an important local lobster fishery. In contrast, we sampled hundreds of juvenile *P. argus* in a reef lagoon within a protected area.

2. MATERIALS AND METHODS

2.1. Study area

Our study was conducted at the Puerto Morelos Reef National Park, a marine protected area located on the Caribbean coast of Mexico, centered at 20° 52′ N, 86° 53′ W. An extended fringing coral reef, interrupted by several channels, runs slightly diagonally from the coast, at a distance of ~500–2000 m. A shallow reef lagoon (<5 m in depth) extends between the shoreline and the coral reef. The bottom of the reef lagoon is mostly calcareous sand covered by extensive seagrass meadows, providing adequate settlement habitat for postlarvae of *Panulirus argus*. Fishing for lobsters in the reef lagoon and back-reef zone has been banned since 1998. Experimental 'casitas' (artificial shelters that can harbor multiple lobsters), scaled to house juvenile lobsters, are occasionally used in the reef lagoon exclusively for research purposes (Briones-Fourzán et al. 2007). PaV1 was first recorded in Puerto Morelos in 2001 (Huchin-Mian et al. 2008), and samples for the present study were obtained in 2014–2015.

2.2. Biological samples

Lobsters were extracted from casitas and natural shelters by skin- and SCUBA-divers using hand nets. Each lobster was measured with Vernier calipers (carapace length, CL, in mm) and carefully examined for clinical signs of PaV1 infection, i.e. milky hemolymph (clearly visible through the translucent membrane between the cephalothorax and the abdomen), sometimes accompanied by a reddish discoloration over the clear marks of the carapace (Lozano-Álvarez et al. 2008). Using a sterile 1 ml tuberculin syringe (30-gauge 0.5-inch needle), we extracted ~200 µl of hemolymph from the basis of 1 of the fifth pereopods after swabbing the surface with 70% ethanol. The hemolymph was fixed in 95% ethanol (ratio of 1:3, v/v) and transported in a cooler with refrigerant gels to the laboratory, where samples were stored at –20°C until assayed.

2.3. PCR assays

DNA was extracted from hemolymph using aseptic techniques to avoid sample cross-contamination. DNA was extracted following a salt precipitation protocol similar to the one described by Aljanabi & Martínez (1997). DNA precipitation was achieved after adding 200 µl of 3 M sodium acetate, pH 5.2, instead of NaCl. The integrity of DNA was characterized by electrophoresis in a 1% agarose gel. DNA of PaV1 was amplified by PCR in a 25 µl

reaction containing 1 µl of the extracted DNA, 0.33 µM of each 45aF and 543aR primer (Montgomery-Fullerton et al. 2007), 2.5 mM of $MgCl_2$ (Promega), 0.6× reaction buffer (Promega), 0.4 mM of dNTPs mixture (Promega), and 0.75 U *Taq* DNA polymerase (Promega). The thermal cycling conditions were 1 cycle of 94°C for 10 min, followed by 30 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 1 min, followed by 72°C for 10 min. The presence of the expected 499 bp PaV1 amplicon was determined by resolving 5 µl of the PCR product and 3 µl of loading buffer in a 2% agarose gel containing 0.1% ethidium bromide, and DNA visualization was done using UV illumination (MiniBis Pro®). Ultrapure water and hemocyte DNA extracted from lobsters heavily infected with PaV1 were used as negative and positive controls, respectively (Huchin-Mian et al. 2013).

2.4. Estimating sensitivity and specificity of visual assessment

Sensitivity was estimated as the number of lobsters with clinical signs divided by the total number of PCR-positive lobsters. Specificity was estimated as the number of lobsters without clinical signs divided by total number of PCR-negative lobsters (Pestal et al. 2003, Huchin-Mian et al. 2013). As sensitivity and specificity are proportions, we estimated their 95% confidence interval (CI) using Wilson's score method with continuity correction (Newcombe 1998).

3. RESULTS

In total, we sampled 452 lobsters over a size range of 10.7–95.6 mm CL. However, only 12 lobsters measured >80 mm CL, and the mean size (± SD) was 38.2 ± 16.0 mm CL; therefore, the sample consisted mostly of juvenile lobsters. Of the total lobsters, 65 (14.4%) exhibited clinical signs of PaV1 infection (Table 1). However, 136 lobsters (30.1%) tested positive for PaV1 by PCR (Table 1). Therefore, the sensitivity at which PaV1 infection was detected by visual assessment was 65/136 = 0.48 (95% CI: 0.39–0.56). This result indicates that the diagnosis based on clinical signs detected about one half (48%) of the lobsters infected with PaV1, i.e. that for each clinically infected lobster there was another subclinically infected lobster. In contrast, no clinical signs were evident in any of the 316 lobsters that tested negative to PaV1 by PCR (Table 1), indicating that the specificity of the visual assessment was 316/316 = 1 (95% CI: 0.98–1.0). That is, the milky hemolymph is a clinical sign specific for the PaV1 disease in the population of *Panulirus argus* in the Puerto Morelos reef lagoon. These validity measures of visual assessment of PaV1 were virtually identical to those obtained by Huchin-Mian et al. (2013) (Fig. 1).

Table 1. Relation between results of visual assessment of clinical signs of *Panulirus argus* virus 1 infection and end point PCR (reference test)

Visual assessment	Endpoint PCR (reference test) Negative	Positive	Total
Negative	316 (true negatives)	71 (false negatives)	387
Positive	0 (false positives)	65 (true positives)	65
Total	316	136	452

4. DISCUSSION

Our results confirm the values of sensitivity and specificity of visual assessment of clinical signs of PaV1 previously estimated by Huchin-Mian et al. (2013) in a different population of *Panulirus argus*. The sensitivity value was very similar between our study (0.48) and the study of Huchin-Mian et al.

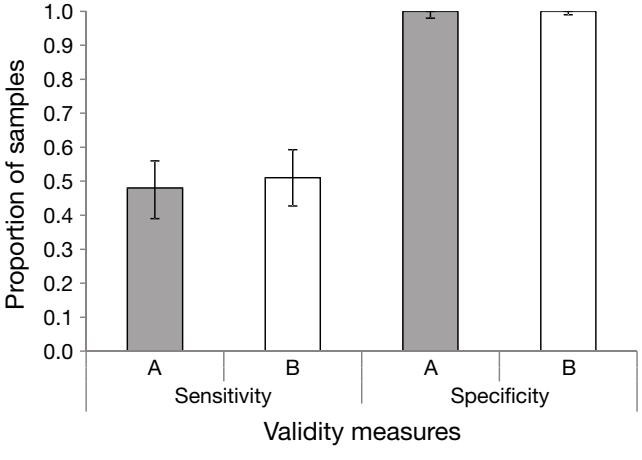

Fig. 1. Comparison of validity measures (sensitivity and specificity) of visual assessment of clinical signs of *Panulirus argus* virus 1 in *Panulirus argus* (tested against endpoint PCR) obtained in the present study (A, gray columns; n = 452, size range = 10.7–95.6 mm carapace length [CL]) and by Huchin-Mian et al. (2013) (B, white columns; n = 1397, size range = 12.8–168.0 mm CL). Error bars are 95% confidence intervals

(2013) (0.51) despite broad differences in sample size, size range of lobsters, and average apparent prevalence in the sampling locations (5.4% in Bahía de la Ascensión: Candia-Zulbarán et al. 2012, 14.4% in Puerto Morelos reef lagoon: R. Candia-Zulbarán unpubl. data). Also importantly, no false positives were detected in the PCR assays in either study, resulting in a 100% diagnostic specificity for PaV1 of the clinical sign represented by milky hemolymph. In other decapods, different pathogens can cause the hemolymph to turn milky, e.g. rickettsia-like bacteria in *Panulirus homarus* and *P. ornatus* grown in sea cages in Vietnam (Nunan et al. 2010), the RV-CM virus in *Carcinus maenas*, the CoBV virus in *Chionoecetes opilio*, the Bi-facies virus in *Callinectes sapidus* (reviewed by Bateman & Stentiford 2017), and the dinoflagellate *Hematodinium* spp. in *Nephrops norvegicus* and numerous species of brachyuran and anomuran crabs (reviewed by Morado 2011). However, to our knowledge, those pathogens have not been reported to affect *P. argus*.

In addition to PCR (Montgomery-Fullerton et al. 2007), current methods to detect PaV1 include histology (Shields & Behringer 2004), fluorescence *in situ* hybridization (FISH) (Li et al. 2006), and quantitative PCR (qPCR) (Clark et al. 2018). Histology can detect active infections in individual lobsters but requires sacrificing the animals. The remaining methods are valuable to diagnose subclinically infected individuals, but PCR is less expensive than qPCR and can detect the DNA of pathogens much faster than FISH (e.g. Pestal et al. 2003). The qPCR assay, which had not yet been developed when the current work was conducted, could become the new gold standard, as its limit of detection is 10 copies of plasmid DNA (Clark et al. 2018) compared to ~37 copies of DNA of the endpoint PCR assay (= 0.02 fg PaV1 per µl of DNA; Moss et al. 2012). However, using qPCR would probably not substantially improve our estimation of the sensitivity of the visual assessment, because if the test used as the gold standard (e.g. endpoint PCR) is much better than the test being evaluated (e.g. visual assessment), then there is little difference between the relative sensitivity and specificity and the true sensitivity and specificity (Cameron 2002). Although PCR cannot determine if individuals testing positive will develop the disease or are only carriers (Montgomery-Fullerton et al. 2007), it is useful to evaluate how broadly distributed a particular pathogen is in asymptomatic host populations (Peinado-Guevara & López-Meyer 2006).

A first important step for managing disease is to develop a working knowledge of the diseases and compromised health states present in a given area (Cameron 2002). Management of disease emergencies (i.e. when diseases cause significant ecological, economic, or social impacts) requires improved surveillance, which in turn requires fast, accurate diagnoses (Groner et al. 2016). PCR techniques are becoming less costly, but their use for long-term monitoring of marine diseases is still far from becoming routine, especially in developing countries. Visual assessment is easier to implement over the long run, but it clearly underestimates PaV1 prevalence. We used PCR as a gold standard against which sensitivity and specificity of observation of clinical signs was assessed. Because sensitivity and specificity of a diagnostic test are independent of prevalence (Lalkhen & McCluskey 2008), if the specificity of a given test is 1 (i.e. 100% compared to the gold standard), then the sensitivity value can be used as a correction factor for prevalence (Pestal et al. 2003). Thus, our results confirm the contention of Huchin-Mian et al. (2013) that, provided a representative sample of individuals in a population of *P. argus* is examined, then the true prevalence of PaV1 in that population could be estimated by applying a 2× factor to the apparent prevalence. This could greatly simplify efforts to monitor changes in prevalence of PaV1 in local populations of *P. argus*.

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