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Molecular Analysis (PCR by isothermal isolation) and Fresh Analysis to Detect the Hepatobacter penaei (NHP) Bacteria in Two Shrimp Farms in the State of Falcón, Venezuela

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ABSTRACT

Background: *Necrotizing Hepatopancreatitis (NHP) is a bacterial disease caused by Gram-negative bacteria (Hepatobacter penaei). This rickettsia affects shrimp farmed in several countries around the world. To prevent the outbreak of this disease, shrimp farms establish sampling programs to review, from a health point of view, the cultured organisms, carrying out specific analyses with the support of quality control or pathology laboratories.*

Objectives: *The objective of this research is to correlate molecular analysis (PCR by isothermal isolation) and fresh analysis to detect the bacteria H. penaei (NHP) in two shrimp farms in the state of Falcón, Venezuela.*

Methods: *These shrimp farms (categorized as F1 and F2) are located on the western coast of Falcón state (Gulf of Venezuela) where an NHP outbreak occurred. Samples were taken from all ponds (from both farms), capturing the animals with a two-meter cast net, and immediately taking them to the laboratory to perform fresh analysis and molecular analysis.*

Results: *In F1 a Relative Frequency (RF) for NHP of 0.02 was obtained and a RF: 0.05 (in F2) with fresh analysis. In the case of the molecular analysis, an RF: 0.06 for NHP was obtained in F1, and an RF: 0.25 for NHP for F2. When calculating the correlation, an R: 0.5654 was obtained in F1, showing a medium relationship, and an R2: 0.3197, relating both analysis techniques by 31.97%. In F2, R: 0.3973 and R2: 0.1578 were obtained, determining little relationship between both analyses for this shrimp farm.*

Conclusion: *the results of the relative frequency (RF) and correlation (R and R2) between molecular and fresh analysis techniques in shrimp farms show a moderate relationship in the first farm (F1) and a weak relationship in the second farm (F2).*

Keywords

Necrotizing Hepatopancreatitis, Shrimp, PCR, Fresh analysis, NHP.

Introduction

Recently, the intensification of the aquaculture industry has generated risks in production, mainly in the field of aquaculture health, product quality, and environmental impact. From this

perspective, some organizations such as FAO hope to generate the search for new strategies that promote the efficient use of natural resources aimed at aquaculture, in order to promote sustainable aquaculture through the optimization of production processes and the integration of animal and plant crops [1].

Diseases in shrimp have always caused significant impacts on the economic area for this agro-industrial sector, with some examples being: loss in the quality of produced animals, problems in the production cycle, increased feed conversion factor, loss of income and jobs for workers, among others [2].

Necrotizing Hepatopancreatitis (NHP) is a bacterial disease caused by gram-negative bacteria (*Hepatobacter penaei*). This bacteria affects farmed shrimp in several countries [3]. This disease is severe and caused by intracellular rickettsia, which infects and divides (binary fission) in the host cytoplasm of the epithelial cells of the hepatopancreas tubules. This disease (NHP) was known as "granulomatous hepatopancreas disease" since granulomas are formed, causing significant mortality in animals on many farms at the continental level. Such mortalities could be between 20% and 95% on farms of *Litopenaeus vannamei* (white shrimp). This pathology is also associated with physicochemical and environmental factors such as salinity and temperatures outside the range for extended periods [10]. However, necrotizing hepatopancreatitis is evolving and emerging. Hence, the OIE (World Organization for Animal Health) has it in its catalog, and this institution maintains constant *Vibrio parahaemolitycus* updating of diseases within the aquatic code. However, more research is needed on shrimp-related diseases [4].

It is important to mention that this type of bacteria (rickettsias) not only affects shrimp at the cytoplasmic level but has also been detected in other species of aquaculture and fishing interest (such as some bivalve mollusks), where they form intracytoplasmic vacuoles, which can cause tissue damage and massive mortalities in these species [5]. Similarly, other bacterioses cause damage to the hepatopancreas of shrimp, such as acute hepatopancreatic necrosis disease (AHNPD), which is caused by the bacteria, generating progressive dysfunction of this organ in crustaceans, as the epithelial cells of the hepatopancreatic tubules begin to degenerate, causing loss of lipid reserves, followed by cellular detachment from the tubular lumen, followed by an inflammatory response caused by the immune response mediated by hemocytes [6]. Sometimes, within shrimp farms, significant outbreaks of diseases can occur. These companies, to reduce such epidemic outbreaks, establish sampling programs to review, from a sanitary point of view, the cultured organisms, performing specific analyses with the support of quality control or pathology laboratories. This activity is of great importance since the absence of frequent evaluations can generate disease outbreaks in the pools or ponds [7]. From this argumentative framework, fresh analysis presents some advantages that have allowed its general application in most companies in the shrimp industry since it can be executed with minimal laboratory equipment (optical microscope, stereoscopic magnifying glass, dissection equipment, basic glass material, among others) and low investment in resources. However, these clinical tests can be established on farms, seeking to reduce the proliferation of diseases [8].

On the other hand, the appearance of the Polymerase Chain Reaction (PCR) analysis was a high point in molecular diagnosis, and in biology and medicine in general. Its creator, Kary Mullis, was recognized in 1993 with the Nobel Prize in Chemistry. The process that allows the amplification of specific DNA sequences was initially described by Kjell Kleppey and the 1968 Nobel laureate Har Gobind Khorana [9].

The Polymerase Chain Reaction (PCR), with its high specificity, is a powerful tool for detecting pathogens in plants, animals, or humans. PCR reactions are normally carried out in a thermocycler that provides the required temperatures for the different stages of the reaction. Since most of the cycle time is spent increasing the temperature to bring the solutions to the appropriate temperature, reducing the reaction time would accelerate the entire process. PCR allows the amplification of specific nucleic acid fragments, extending widely in the field of pathogen detection, mutation detection in genes, and clinical diagnosis [10,11].

The nucleic acid isothermal amplification technique consists of the amplification and detection of very specific DNA or RNA fragments under constant temperature conditions, using two pairs of specific primers, with the help of an easy-to-use analyzer that ensures the conditions of the PCR [12]. This technique has the advantages of strong specificity, and sensitivity, concerning its analyzer equipment, which is very basic, providing the possibility of rapid reading, even in the field since many of these analyzers are portable. This technique is also known as "LAMP" (Loop-mediated isothermal amplification), is similar to conventional PCR in the use of primers to anchor to specific nucleic acid sequences and a polymerase to amplify the sequence between the primers. However, LAMP does not require heating and cooling cycles, as happens with conventional PCR. Instead, LAMP is performed at a constant temperature, making it more efficient, and obtaining faster results compared to traditional PCR [13].

Another advantage of using molecular techniques is that they allow the study of bacteria (in this case, *H. penaei*) without performing any type of isolation, thus avoiding biases that arise with methods based on bacterial cultures. This has been a determining factor for molecular detection techniques to become, on various occasions, the primary choice for the diagnosis of infectious pathologies, leaving behind bacterial cultures (and other less precise techniques) as reference means [9]. The objective of this research is to correlate the molecular analysis (PCR by isothermal isolation) and the fresh analysis to detect the *Hepatobacter penaei* (NHP) bacteria in two shrimp farms in the state of Falcón, Venezuela. In these mentioned farms, an outbreak of NHP occurred in 2021, so fresh analysis and iiPCR (by isothermal isolation) were used for its detection.

Materials and Methods

Study Area

This research was carried out on the western coast of the state of Falcón (Venezuela), specifically in the coastal zone of the Mauroa municipality, which is part of the Gulf of Venezuela, in the west of the country. Several shrimp farms where the white shrimp *Litopenaeus vannamei* is cultivated are located in this area. This trial was carried out on two farms (which we will name F1 and F2). Shrimp farm 1 (F1) is located in the border region between the states of Zulia and Falcón, near the town of San Félix (Mauroa municipality of Falcón state) at coordinates 10º58´19" N 71º14´38" W. It is worth noting that this farm has 50 operational pools. Shrimp farm 2 (F2) is located near the town of Casigua, at coordinates 11º04´36" N 71º01´52" W. It is important to note that F2 has 60 operational pools or ponds.

Capture and Diagnosis of Litopenaeus Vannamei

The shrimp were sampled using a 2-meter diameter cast net, making random throws at different points in the pools. The animals are stored in plastic containers and immediately transported to the laboratory for PCR and Pathobiology analysis. Likewise, attention is paid to the characteristic clinical signs of possible hepatopancreas damage caused by the *Hepatobacter penaei* bacteria, the promoter rickettsia of NHP [14].

Diagnosis through Fresh Analysis

The fresh analysis is based on the direct observation and analysis of tissue samples or affected areas of the shrimp, through direct observation under the microscope, to establish a possible diagnosis [15,16]. To perform the fresh analysis, tubular deformation, atrophy, and/or constriction of the hepatopancreatic tubules with detachment and cellular hypertrophy must be observed in the sampled organisms. The observation of these signs is made using optical microscopy, using 10x and 40x objectives [17]. The analyzed sample (for each pool or pond) corresponds to 10 individuals, to determine the number of sick animals.

Diagnosis through Molecular Analysis (iiPCR)

In 2021, an outbreak of NHP occurred in two farms of white shrimp *L. vannamei* (previously mentioned), so samples (animals) were collected from all the ponds of F1 and F2, for a total of 110 pools to evaluate. The POCKIT™ nucleic acid analyzer was used, which combines highly specific iiPCR technology with a short response time (approximately 1 hour). The specific primers (NHPB IQ Plus Kit) used for this assay were supplied by IQ Plus, which can detect NHP disease in one hour, simplifying the extraction procedure and facilitating the assay.

DNA Extraction Procedure

Samples of hepatopancreas (between 10% to 25%) from F1 and F2 were used for this study. These samples were subjected to digestion (with solutions 1 and 2 provided by IQ Plus). After centrifugation, the supernatant was placed in column-type tubes, which were centrifuged again. The supernatant was discarded, and the column tube was transferred to a new tube. Then, 200 µL of solution 3 (IQ Plus) was added to obtain the DNA extract. It is worth noting that for this methodology, primers are designed to identify eight target regions, which gives the procedure high specificity [18]. For the reaction procedure, 50 µL of premix buffer was added to specific premix tubes for the detection of *Hepatobacter penaei* DNA (NHPB IQ Plus). Then, an inoculation loop (IQ Plus) was used to inoculate the DNA extract, which was then immersed in the premix tube. Next, 50 µL of premix solution was transferred to tube R (IQ Plus), which was immediately capped and centrifuged for 10 seconds. Tube R was then placed in the nucleic acid analyzer, 520 nm + 550 nm was selected, and "run" was pressed on the POCKIT™ equipment [19].

Results and Discussion

Fresh Analysis (Pathology)

Through the fresh analysis (pathology) test, deformation of the hepatopancreatic tubules was detected in animals from pool 17 (Farm 1) and pools 1, 30, and 31 of Farm 2 (Figure 1).

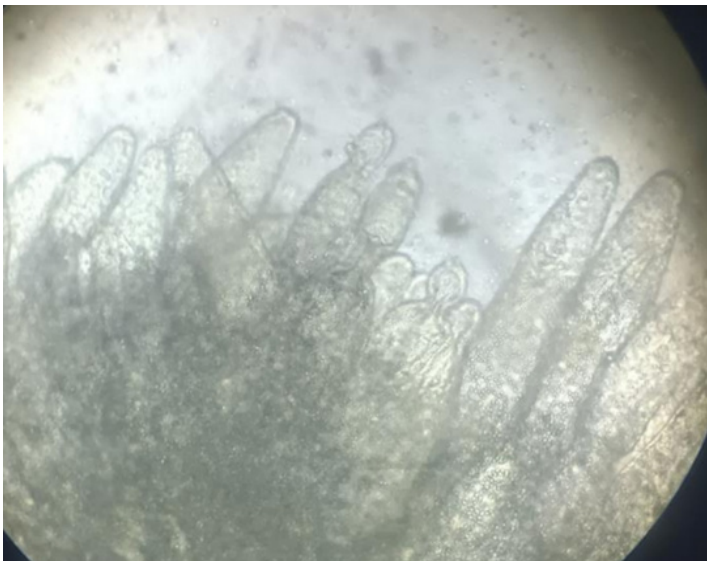


Figure 1: Deformed hepatopancreatic tubules, in the center of the image, observed through simple optical microscopy in shrimp from F1 using the fresh analysis (pathology) test.

The deformation of the hepatopancreas tubules is one of the clinical signs to evaluate for the possible presence of the *H. penaei* bacteria. However, these signs are not decisive; it is feasible to say that a fresh analysis sample is positive (by determination under the optical microscope) when there is atrophy of the hepatopancreas, reduction of epithelial lipid vacuoles, melanized tubules, hemocytic infiltration, multifocal necrosis and tubular strangulation (Figure 2). In turn, it is feasible to observe an important bacterial population, intracellularly and/or extracellularly in the hepatopancreatic tubules [20]. From this perspective [21], also establishes that bacterial diseases that affect the hepatopancreas in shrimp (including NHP) can generate many types of histopathological lesions, with a variable impact. The reports that have been generated, through fresh analysis, must be

taken into account, in the analysis of hepatopancreatic tubules, degraded tubules, empty tubules, and atrophied tubules.

Fresh Analysis on Farm 1

In the F1 shrimp farm, sampling was carried out in fifty pools (between fattening and pre-farming pools). With the fresh analysis, only a Relative Frequency (RF) of 0.02 was observed (in sick pools); and in healthy pools, an RF of 0.98

Considering that the Relative Frequency (RF) would be the proportion of individuals (within a pool or pond) that presented NHP symptoms, about the total population. In this particular case of F1, the fresh analysis indicates that only 2% of the sampled pools or ponds in this shrimp farm presented the clinical characteristics of the disease (Figure 2a).

Fresh Analysis on Farm 2

The F2 shrimp farm consists of 60 pools or ponds (between fattening and pre-breeding pools). In this case (shrimp farm 2), an RF of sick pools or ponds with an NHP of 0.05 was observed, and from healthy ponds, an RF of 0.95 (Figure 2b), indicating that the disease is rare.

PCR analysis at Farm 1

Employing PCR analysis through isothermal isolation, an RF of 0.06 was obtained from sick ponds and an FR of 0.94 from pools that were negative for the presence of genetic material from the *H. penaei* bacteria.

Comparing the results obtained with the fresh analysis, it can be observed that the number of positive cases increased using molecular techniques (PCR), however, according to the results obtained, NHP disease was uncommon in F1 (Figure 3a).

PCR analysis at Farm 2

Using the isothermal isolation PCR technique (in F2), an RF of 0.25 was obtained from sick pools, and an RF of 0.75 from healthy pools. In this case, greater detection of the *H. penaei* bacteria was evident using molecular techniques (iiPCR) compared to the fresh analysis, yielding only 0.05 in F2 (Figure 3b). It is evident that the fresh analysis showed limitations in terms of specificity and sensitivity to detect NHP, however, it can work to provide preliminary data, helping to generate a first alert and initiate decision-making to generate corrective actions on the farms, with low costs. It is important to mention that health monitoring of this type (routine) could also make an early detection of any disease outbreak, however, it is advisable to limit biases (supporting fresh analysis, with molecular techniques, for example) and carry out the corrective actions necessary to avoid significant economic losses in shrimp production [22].

Comparison between Molecular Analysis (PCR) and Fresh Analysis (Pathology) in the Study Farms

When comparing the number of positive cases for NHP for each type of analysis (in shrimp farm 1), we can notice that the highest report of positive cases appears for the PCR analysis.

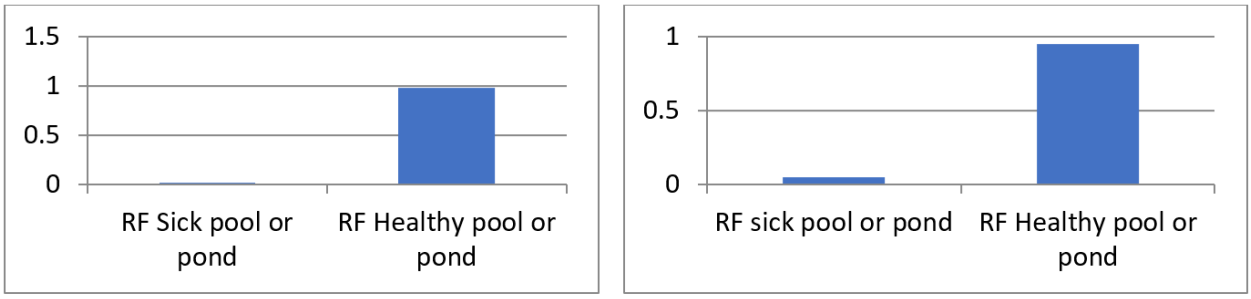
The positive cases for fresh analysis were observed in pond 17; and with PCR they were in pools 17, 18, and 34 (Figure 4a). Regarding the Correlation Coefficient (R), a value of 0.5654 was obtained, indicating a certain dependence on the variables (X and Y), that is, the two analysis procedures (fresh analysis and PCR) were moderately related to shrimp farm 1 (Figure 4b).

For the calculation of the Coefficient of Determination (R^2), a value of 0.3197 was obtained, which indicated that both techniques for diagnosing the disease are related in 31.97%, the remaining pools sampled (68.03%) were negative to NHP, using both methods. Making this similar comparison (in shrimp farm 2) the following graph is obtained:

On this occasion, an increase in the discovery of the *H. penaei* bacteria is noticeable, yielding better results with the molecular analysis (PCR). It can be seen (in Figure 5) that the positive pools (ponds) for fresh analysis were: 1, 30, and 31. For PCR analysis the detection was greater, with pools yielding positive results: 1, 8, 24, 30, 31, 34, 36, 37, 39, 42, 43, 46, 53, 54, and 55 (in blue in the graph – Figure 5a). Likewise, the number of "false negatives" is evident since most of the pools gave negative results for fresh analysis, and turned out to be positive for NHP with the molecular analysis.

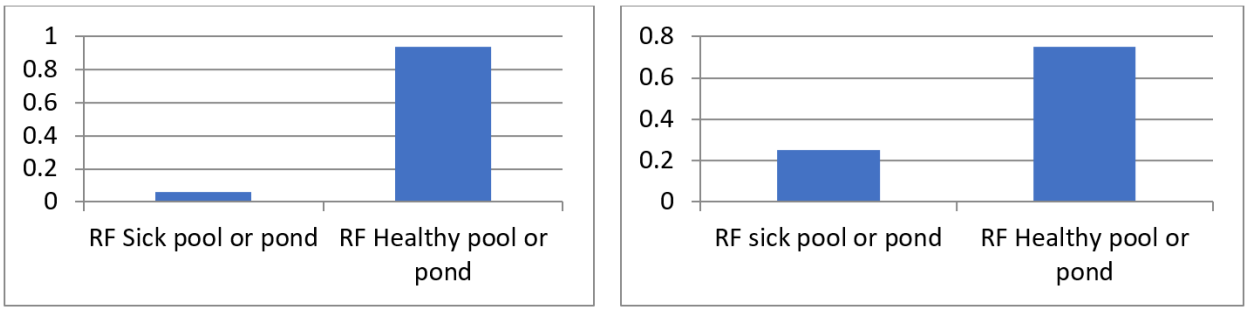
In medical sciences, a false negative could be defined as an error through which, when an analysis is carried out, its result does not detect any pathology or affection, however, a disease exists in a certain patient, or groups of patients. As in this case, two diagnostic processes were used, it is evident that the molecular clinical diagnosis techniques presented greater sensitivity than the Pathobiology analysis (fresh analysis), therefore, the PCR analysis did not present false negatives in this investigation [23,24]. Regarding the Correlation Coefficient (R), a value of 0.3973 was obtained, indicating little dependence on the variables (X and Y), that is, the two analysis procedures (fresh analysis and PCR) coincided equally way little related in shrimp farm 2 (Figure 5b). For the calculation of the Coefficient of Determination (R^2), a value of 0.1578 was obtained, which indicated that both techniques for diagnosing the disease are related only by 15.78%. We can establish, as a method with greater sensitivity, molecular techniques (PCR by isothermal isolation), from the hepatopancreas of animals suspected or with clinical signs of NHP. From this perspective, Vincent and Lotz [25], established histopathology and real-time PCR as methods with greater precision to determine, in an early manner, the infection caused by *H. penaei*. In what these authors obtained, they established that six days after infection, 33% of the shrimp used for analysis were positive for histopathological diagnosis.

It should be noted that F2 showed a higher frequency (RF: 0.25) of sick ponds, in contrast to F1, where the RF was lower (RF: 0.06). These results are consistent with the study by Pascal et al.



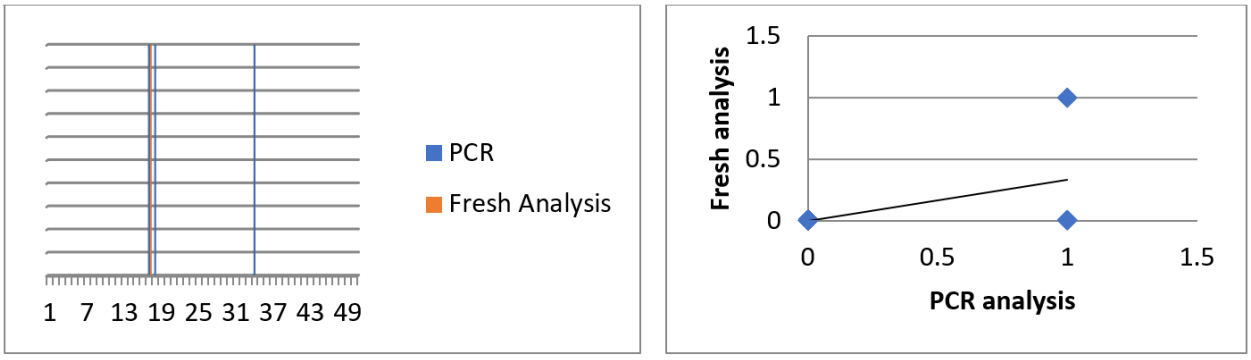
(a) **(b)**

Figure 2: (**a**) Description of Relative Frequency (RF) between sick pools and healthy pools detected with the fresh analysis in F1; (**b**) Relative Frequency (RF) between sick pools and healthy pools detected with the fresh analysis in F2.



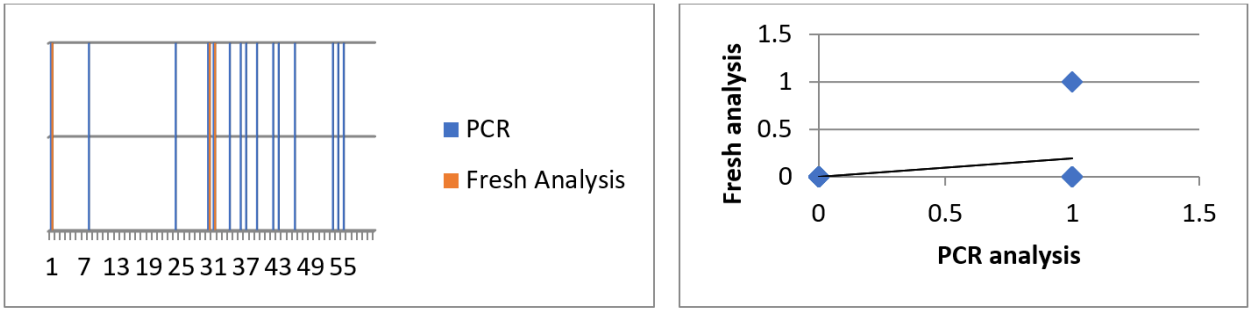
(a) **(b)**

Figure 3: (**a**) Relative Frequency (FR) between sick and healthy pools detected with PCR analysis in F1; (**b**) Relative Frequency (FR) between sick and healthy pools detected with PCR analysis in F2.



(a) **(b)**

Figure 4: (**a**) Positive cases (per pool or pond) of NHP; red for fresh analysis, blue for PCR in F1; (**b**) Correlation between molecular analysis (PCR) and fresh analysis for F1.



(a) **(b)**

Figure 5: (**a**) Positive cases (per pool) of NHP; red for fresh analysis, blue for PCR in F2; (**b**) Correlation between PCR analysis and fresh analysis for F2.

[26]; where they determined the prevalence of higher parasitic diseases in the same geographical area in which shrimp farm 2 (F2) is located, this could be related to the physicochemical and microbiological quality of the water in this farm.

The use of the Polymerase Chain Reaction technique by isothermal isolation (iiPCR) was a tool that presented great advantages, providing great versatility and analytical power when studying the samples from these farms. The significant amount of elements that this analysis technique has (reaction reagents, DNA polymerase, among others), made this method have a high specificity because it allowed the DNA fragments of *H. penaei* to be amplified (specifically) [27].

Conclusion

In F1, the Relative Frequency (FR) of NHP disease was lower compared to F2. Therefore, there were more reported cases of the disease diagnosed through molecular techniques (iiPCR). Comparing both techniques used (fresh analysis and PCR), the use of PCR demonstrated greater sensitivity and accuracy in detecting the presence of NHP disease. The "False negatives" increased considerably in shrimp farm 2 (F2).

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