Detection of IHHN virus in shrimp tissue by digital color correlation

Josué Alvarez-Borrego a,*, María Cristina Chávez-Sánchez b,1

a Departamento de Óptica, División de Física Aplicada, Centro de Investigación Científica y de Educación Superior de Ensenada, Km. 107 Carretera Tijuana-Ensenada, Ensenada, B.C., C.P. 22860, Mexico

b Centro de Investigación en Alimentación y Desarrollo, A.C., Unidad Mazatlán en Acuicultura y Manejo Ambiental, Sábalos Cerritos S/N, Apdo. Postal 711, Estero del Yugo, Mazatlán, Sinaloa, C.P. 82010, Mexico

Received 20 January 2000; received in revised form 10 July 2000; accepted 9 August 2000

Abstract

Shrimp culture has suffered from disease outbreaks that significantly affect the economy and restrain aquaculture production in many countries around the world. For research and diagnosis of shrimp diseases, histopathology is a powerful tool to identify pathogens and to understand the cellular, tissue and organ changes that result as a consequence of disease. One problem of the technique is that it requires considerable time, although shrimp producers demand results in the shortest possible time in order to take preventive or control measures. The introduction of techniques, such as processing of digital images in histopathology, could significantly reduce the time for precise diagnosis. Here, we present an attempt to implement this technique with infectious hypodermal and hematopoietic necrosis virus (IHHNV) because it presents difficulties for diagnosis by conventional histological techniques. In order to determine the viral histopathology that could be identified with the digital color correlation in different tissues, some very clear and conventional Cowdry type A inclusion bodies (CAIs) were selected. It was demonstrated that color digital correlation can be used to detect the virus IHHN in shrimp tissues. More researches are needed to be done, which consider several aspects, like rotation, scale, color, natural morphology of the virus and the use of different kinds of filters to determine whether the technique can be applied to other viruses. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Histopathology; Virus; Shrimp; Digital color correlation

* Corresponding author. Tel.: +52-61-74-50-50, 74-50-51; fax: +52-61-75-05053.

E-mail addresses: josue@cicese.mx (J. Alvarez-Borrego), marcris@servidor.unam.mx (M.C. Chávez-Sánchez).

1 Tel.: +52-69-88-01-57, 88-01-58; fax: +52-69-88-01-59.

0044-8486/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.

PII: S0044-8486(00)00504-4
1. Introduction

Shrimp culture has suffered from disease outbreaks that significantly affect the economy and restrain aquaculture production in many countries around the world. The causes are complex and are attributed to viruses, bacteria, fungi, pesticides and environmental degradation (Gjedrem and Fimland, 1995). It is widely recognized that improved regulatory and technical measures are necessary to prevent the introduction and spread of diseases. This is necessary to protect the aquaculture industry and the aquatic environment. Uniform practices and procedures designed to study the diseases in each country have been developed in Asia, Europe, Canada and the United States. Therefore, many countries are now involved in determining which diseases are present in wild and cultured aquatic organisms in order to facilitate the development of national and international codes of practice on health certification.

For diagnosis and research on infectious and non-infectious diseases, shrimp pathologists use different techniques. Recently, accurate and precise biological and molecular techniques based on DNA have been developed to diagnose viral and bacterial diseases, by procedures such as dot–blot, in situ hybridization, ELISA, and PCR (Lightner, 1996). In spite of these new techniques, histology is still one the techniques commonly used for research and diagnosis. With this technique, it is possible to observe the pathological changes in the cells, tissues and organs, and it also allows the identification of new pathogens, which are sometimes difficult to recognize using other techniques.

The basic method involves a series of steps to obtain a slide containing a layer of tissue, 1–5 \( \mu \)m thick, stained with hematoxilin–eosin for analysis under the microscope (Bell and Lightner, 1988; Lightner, 1996). However, this useful technique has some drawbacks: (a) getting the histological slide requires time consumption through all the process of fixation, dehydration, embedding, sectioning and staining, (b) analyzing one single histological slide requires short time, but when it is necessary to analyze many of them for diagnosis or research in a population, it takes a longer time because it is necessary to “sweep” carefully each section under the microscope and search for any kind of histopathologies and/or pathogens. In aquaculture, for example, to obtain a precise diagnosis of an identified pathogen or disease in a farm, it is necessary to analyze many slides from a large sample, delaying the results to the farmers for at least a week, and (c) for viral diseases, it is usually not possible to diagnose when the infection is in its early stages.

Infectious hypodermal and hematopoietic necrosis (IHHN) is a viral disease which was first recognized in 1981, in Hawaii, in imported shrimp stocks of the blue shrimp *Penaeus stylirostris*, from culture facilities in South and Central America, but not from Mexico (Lightner, 1983). However, in 1987 and the subsequent 3 years, this virus was introduced and spread with imported stocks of penaeid shrimp in western Mexico that bordered the Gulf of California (Lightner et al., 1992). Since then, this disease has spread throughout the country, causing losses in cultured and wild shrimp.

To diagnose this viral disease histologically, it is necessary to demonstrate the presence of the prominent Cowdry type A, eosinophilic, intranuclear inclusion bodies surrounded by marginated chromatin in hypertrophied nuclei of cells in tissues of ectodermal (gills, epidermis, hypodermal epithelium of fore and hindgut, nerve cord and
nerve ganglia) and mesodermal origin (hematopoietic organs, antennal gland, lymphoid organ, connective tissue and striated muscle) (Lightner et al., 1983). Classical Cowdry type A inclusion bodies (CAIs) are not easy to identify, due to the different organs where they occurred and the difference in size, tones of purple (light to very dark basophilic), shapes and size of the classic white ring that is also characteristic of these inclusions. These features depend on the stage of the virus, type of tissue infected and quality of the staining. Thus, it is necessary to develop skill and experience to recognize these inclusions.

The introduction of techniques, such as digital processing of images directly from the microscope in the diagnosis or research of infectious diseases, could significantly reduce the time taken to obtain precise results. However, in this paper, we used transparencies in order to test this new method, but the general idea is to use images directly from the microscope. We selected IHHN because of the difficulty presented in diagnosing this viral disease through conventional histological techniques.

2. Materials and methods

Experimental shrimps were obtained from a farm in the state of Sinaloa, Mexico and transported live to the laboratory to be fixed in Davidson’s solution according to Lightner (1996). After 24 h, the fixative was discarded and the shrimps were preserved in 50% alcohol until processed by the conventional histological techniques, as suggested by Lightner (1996). For this study, and in order to determine if the inclusion bodies could be identified with the digital color correlation in different tissues, some very clear and conventional Cowdry type A inclusion bodies (CAIs) were selected from the cuticular epithelium, nerve cord, gills and connective tissue under the microscope (Olympus BX60). The selected IHHNV inclusion bodies were microphotographed with an Olympus camera PM-CB30. All the photographs were taken as 35 mm transparencies with the 60 objective.

An HP Photosmart color digital scanner was used to obtain the matrix data from the transparencies, and defined the function \( f(x, y) \) for every pixel of coordinates \( x \) and \( y \) on the image. In general, a polychromatic object presents different shape and amplitude distributions \( A_\lambda(x, y) \) when illuminated with different wavelengths \( \lambda \). However, two different objects may present similar amplitude distribution when they are illuminated with a determined wavelength \( \lambda_0 \). So, in an optical pattern recognition process using a correlator illuminated with a wavelength \( \lambda_0 \), these objects will give very similar amplitude correlation distributions, and some false alarms will appear. To avoid this problem, it is necessary to use the information about the dependence of the object amplitude distributions on the wavelength (Campos et al., 1991).

Most natural colors can be obtained as a combination of three colors (called primaries) if they are well selected. Each of them has to be on the red (R), green (G), and blue (B) regions of the visible spectrum. When the object is transparent, the amplitude transmittance obtained by illumination with one of these primaries is called the red, green and blue components of the object.

Recognition of an object in a scene is achieved by decomposing the information in three monochromatic channels and by identifying the object independently in each
channel. Specifically, three correlations, \( C_x(x, y) \), were determined between the transparency to be analyzed, \( f_x(x, y) \), and the inclusion bodies to be detected, \( V_y(x, y) \). This was done by illumination with three wavelengths \( \lambda = R, G, B \) which covered the visible spectrum.

\[
C_x(x, y) = f_x(x, y) V_y(x, y)
\]

We referred to red, green and blue channels when the correlator was illuminated with the R, G, or B wavelengths, respectively. In each channel, the filter to be used was matched to the corresponding component of the target.

---

**Fig. 1.** Photomicrographs of the typical intranuclear Cowdry type A inclusion bodies (arrows) in: (a) and (b) cuticular epithelium and connective tissue (H&E 435.6X); (c) in the gill (H&E 435.6X); (d), (e), (f), (g) in mid-sagittal sections of the ventral nerve cord (H&E 732.6X); (h) transversal section of a nerve cord embedded in reserve cells and (H&E 435.6X); (i) in connective tissue (H&E 435.6X); (j), (k), (l) in mid-sagittal sections of the ventral nerve cord (H&E 435.6X).
Objects that have a determined component $A_1(x, y)$ that is similar to the same component of the target $V_1(x, y)$ will give a maximum of correlation in this channel ($\lambda_1$). Only the target will simultaneously give a correlation maximum in each channel. So, an object is detected as the target if it simultaneously produces a correlation peak in the three channels.

The filter used to detect the target $V_2(x, y)$ was a phase-only filter. Let $V_2(x, y)$ be the inclusion body to be recognized and let $V(u, v) = |V(u, v)|e^{i\phi(u, v)}$ be its Fourier transform (frequency information). The phase-only filter $V_{POF}(u, v)$ is defined as $V_{POF}(u, v) = e^{i\phi(u, v)}$, where $|V(u, v)|$ is equal to 1, and $u, v$ are the variables in the frequency domain.

Several authors (Yu and Chao 1983; Yu 1985; Yu and Javidi 1986; Yu and Zhou 1987) proposed color image recognition by matched filtering, taking into account the
shape and the spectral information of the object. This permits an improvement in discrimination sensitivity. In the last 10 years, several authors (Millán et al., 1989, 1992; Campos et al., 1991; Moreno et al., 1997) have obtained important results in this line. They have used some images like butterflies and letters of different colors.

3. Results and discussion

The selected CAIs were observed in the ventral cord of the nervous tissue, gills, the subcuticular epithelium and in connective tissue. Their digital results are presented for each one of the analyzed images (Fig. 1a–l). Well-defined correlation peaks can be observed exactly in the same place where the inclusion bodies are localized in the
images. Phase-only filters were used in the three channels (red, blue and green) in the digital color correlation. Fig. 1 shows, first, the results (RGB channels) after reducing the noise or background to less than 60% of the highest value. The green channel showed more noise than the other channels in five cases and in three cases in the blue channel. Nevertheless, correct identification of the viral inclusions was obtained by simultaneous correlated peak in the three channels. In these results, invariant correlations, like scale and rotation, have not been considered. In all cases, the viral inclusions

Fig. 2. Phase-only filter correlated with the phase-input image.
were well identified. In Fig. 1b, a fourth peak was present which was not considered to constitute detection of a viral inclusion. If we obtained the correlation of the phase-only filter with the phase of the input image (Fig. 1b in this case), we found that three peaks were well defined (Fig. 2).

As a first approximation, digital color correlation could be used to detect IHHN virus in shrimp tissue with a diagnosis time reduced to seconds.

Only a few transparencies with typical CAIs in some tissues were selected to demonstrate the possible use of digital processing. More information must be added to the system, including a wide range of variation in CAIs and to assure that the system will accurately diagnose this virus in less time than normal histological methods. This technique will also require adaptation to a microscope able to sweep the slides automatically and send the images directly to the computer to read the results. This work demonstrates the potential of the technique in diagnosing not only IHHNV but other viruses and pathogens which could be included in a similar digital program.

Acknowledgements

The authors wish to thank Dr. Don Lightner for reviewing the manuscript. The authors also wish to thank María Soledad Morales Covarrubias and Selene Abad Rosales for skilled technical assistance during this study in histological techniques. Thanks also to Valerie for the English review.

References