Standardization of *In Situ* Hybridization Techniques and Electron Microscopy for the Diagnosis of Aquatic Organism Diseases

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Received:- 06 February 2024/ Revised:- 13 February 2024/ Accepted:- 21 February 2024/ Published: 29-02-2024

Abstract— *Numerous etiological agents cause acute, chronic, granulomatous, systemic, or focal diseases in animals. In aquaculture, especially in fish, reptiles, amphibians, and crustaceans, pathogenic microorganisms have led to significant production losses due to the mortality of infected animals or the deteriorating condition of diseased ones, rendering commercialization unfeasible and resulting in economic losses. Furthermore, some of these pathogens affecting aquatic animals are zoonotic, impacting public health. Therefore, with the aim of aiding in the rapid and efficient diagnosis of diseases in aquatic organisms, the standardization of diagnostic techniques began in 2009 at the Interinstitutional Laboratory for Aquaculture Health - LISA (Instituto Biológico/São Paulo/Brazil). These techniques include the in situ hybridization (ISH) under light or photonic microscopy and negative staining (rapid preparation) immunoelectron microscopy (IEM), immunocytochemistry with colloidal gold particles (IMCG), and embedding of fragments in resin for transmission electron microscopy. Adaptations to the initially developed protocols for mammals were made, such as the removal of melanin from melanomacrophages. Due to the high presence of melanomacrophages with brownish melanin granules in the organs of ectodermic animals (frogs and fish), it was necessary to remove this melanin to facilitate the visualization of the diaminobenzidine (DAB) chromogen without interfering with the in situ hybridization technique, in which specific nucleotide sequences were identified in histological sections. This modification prevented false-positive results. These standardized techniques aided in the accurate and efficient diagnosis of pathologies such as White Spot Disease in shrimp, the occurrence of Mycobacterium spp. and Francisella spp. in fish, and chytridiomycosis in frogs.*

Keywords— In situ hybridization, Electron microscopy, diseases, aquaculture.

I. INTRODUCTION

1.1 *In Situ* Hybridization:

This technique was first described in 1969 (Gal, 1969), but it only garnered the interest of the medical community in the 1980s. The appearance of cells and their architectural arrangement within a morphologically complex tissue represents only a fraction of the information within a histological section. These tissues contain all cellular proteins and express genes that will determine the biological behavior of the cell, and even provide clues about the origin and pathogenesis of diseases and their varying degrees (Murakami, 2001).

From this perspective, Jin & Lloyd (2001) assert that DNA or RNA analysis techniques will become increasingly commonplace, shedding light on unresolved questions. The undeniable benefits arising from the integration of this more refined technology with conventional Pathology are contributing to a remarkable increase in our knowledge of certain diseases.

The primary allure of the *in situ* hybridization reaction lies in its ability to precisely locate a specific gene or its transcripts within paraffin-embedded or frozen tissue. While PCR can detect mRNA or DNA in tissue extracts, it does not allow us to observe the distribution of transcripts or DNA within a specific population of cells or in areas of adult or developing tissue (Young, 1989).

1.2 Nucleic Acid Probes:

In *in situ* hybridization, probes are utilized to locate specific nucleic acid sequences at a subcellular level. Biotin is commonly employed in probes for non-radioactive detection. Biotin can be visualized through numerous methods, utilizing either avidin or streptavidin, both of which exhibit high affinity for this amino acid. Standard biotin detection employs enzymatic conjugates of streptavidin, producing a precipitation product that signals chromogenic enzyme substrates. Following the initial binding of biotinylated probes with streptavidin-peroxidase, peroxidase catalyzes the oxidation of biotinyl-tyramide, and this reaction deposits a significant amount of biotin at the hybridization site. This free biotin captures more streptavidin-peroxidase, amplifying the cycle further until the reaction reaches saturation. This signal is ultimately revealed using the chromogenic indicator diaminobenzidine (DAB), which is oxidized by peroxidase, yielding a dark brown precipitate at the hybridization site (Braissant and Wahli, 1998).

A probe is a known segment of DNA or RNA obtained through molecular cloning or chemical synthesis, which is complementary to a target sequence of interest and contains a label that enables selective visualization. DNA probes function similarly to antibodies used in immunocytochemistry in that they bind to a target and carry a signal. However, DNA hybridization probes offer certain advantages over immunodiagnostics because DNA is much more stable and easily preserved than most proteins. RNA is readily degraded by ribonucleases. The optimal probe size is around 200-500 base pairs for improved tissue penetration (although larger probes can be designed). The optimal hybridization temperature typically ranges from 15 to 25°C below the melting temperature (Warford and Lauder, 1991).

1.3 Application:

This technique can primarily be used to detect RNA or DNA from microorganisms and differentiate productive from non-productive infections. It provides morphological information and allows for the observation of gene expression (mRNA), especially when protein expression is low or when it is rapidly exported from the cell, making it challenging to detect through Immunohistochemistry. Additionally, it also verifies the possibility of post-transcriptional control mechanisms (for example, the hybridization of viral mRNA in the liver has helped in understanding the complexity of hepatitis B infection) (McNicol and Fraquharson, 1997).

1.4 Electron Microscopy Negative Staining Technique:

The negative staining technique involves a quick and easy preparation, providing results within minutes, making it the most productive approach in electron microscopy in terms of the number of samples. Particles from a suspension are adsorbed onto the surface of a specimen support, stabilized, and typically contrasted with drops of heavy metals. With this approach, particles can be visualized down to subnanometer sizes and categorized based on their morphology. The original term "negative staining" was introduced by Brenner and Horne in 1959.

Due to its ease of use and comparatively high yield, negative staining is often employed for quality assurance, such as testing virus cultures. Various types of samples can be easily transferred into a suspension without disrupting the structure of viral particles. Efficiency in terms of preparation speed is a critical factor in transmission electron microscopy diagnostics, making it a frontline method in this field (Curry et al., 2006). Furthermore, the open view of electron microscopy provides direct information about all nanoparticles present in a sample. Virus particles are identified based on morphological parameters such as size, shape, surface structure, and peculiarities. Considering that the morphology of a virus is relatively stable throughout evolution, diagnosis is easily achieved even if nucleic acids have undergone significant mutation, making identification through other methods more challenging. Therefore, diagnostic electron microscopy is valuable for identifying viruses in emerging infectious diseases or suspected bioterrorism cases. In veterinary medicine, diagnostic electron microscopy plays an even more crucial role because other diagnostic tools are often unavailable (Laue, 2010).

To combine structural information with molecular data, negative staining can be paired with immunolabeling. This immunonegative staining can enhance specificity in diagnostic electron microscopy or provide insights into the molecular topology of viruses (Biel and Gelderblom, 1999; Biel and Madeley, 2001).

1.5 Electron Immunomicroscopy:

Electron immunomicroscopy (IEM) is employed when the number of viral particles in a sample is very low, when virions are pleomorphic and difficult to identify due to the absence of typical viral morphology such as defined symmetry, shape, spikes, particle size, or capsomer number and arrangement or when samples are very electron-dense, and the aggregates generated by the technique can aid in identification (Lavazza et al., 2015). It allows for virus identification through specific antigen-antibody reactions, relying on the morphological characteristics. It is also used to serotype morphologically similar (but antigenically distinct) particles (Katz Kohn, 1984; Fields et al., 1996).

Various variations of the method, such as immune agglutination or direct immunoelectron microscopy (DIEM) (Anderson et al., 1973) or immune aggregation electron microscopy (IAEM) (Lavazza et al., 2015), solid-phase immunoelectron microscopy (SPIEM) (Derrick, 1973) have been employed. Hyperimmune sera, monoclonal antibodies, or convalescent sera can be used in performing the technique (Hazelton et al., 2003; Lavazza et al., 2015). SPIEM has been used to detect most viruses causing gastroenteritis, such as bovine rotavirus, swine rotavirus, equine rotavirus, canine parvovirus, and bovine viral diarrhea virus (BVDV).

IAEM has been used to detect porcine rotavirus (PoRV), porcine torovirus (PoToV), and porcine epidemic diarrhea virus (PEDV) in pigs with enteritis using convalescent serum (Lavazza et al., 2015).

1.6 Immunogold Colloidal Particle Labeling in Negative Staining Technique :

In this technique, the antigen-antibody reaction is enhanced by labeling the antigen with colloidal gold particles associated with protein A, using specific antibodies for the type and genus. This method also allows for the detection and identification of virus-induced antigen structures and their location in infected cells, serotyping viral strains (Kjeldsberg, 1986), and determining antigenic variants in isolated strains (Patterson & Oxford, 1986).

This technique has been used to label porcine epidemic gastroenteritis virus (TGEV) particles in feces and small intestine fragments of infected pigs (Martins et al., 2013), type A rotavirus and coronavirus in fecal samples from diarrheic calves and winter dysentery in cattle (Kooijman et al., 2016), the simultaneous presence of coronavirus and rotavirus in the feces of calves with diarrhea (Catroxo et al., 2007), and BVDV in the feces of cattle with diarrhea (Catroxo et al., 2007).

1.7 Resin Fragment Inclusion Technique:

The resin embedding technique, followed by ultrathin sectioning, is especially important for revealing fine details of the ultrastructure of all types of cells and tissues (Martins et al., 2013). In an infectious process, it allows the observation of infection pathogenesis and the identification of the agent (Fields et al., 1996). Ultrathin sections have the advantage of allowing the observation of virus-cell interaction, revealing the site of viral replication and maturation within host cells, which is pertinent information for the identification of unknown viruses (Fong, 1989). The overall ultrastructural details not only determine infection but also the course of disease in populations (Catroxo & Martins, 2015). The resin embedding technique has allowed the study of various ultrastructural aspects of the intracellular behavior of TGEV in intestinal fragments of infected pigs (Martins et al., 2013) and parvovirus in intestinal fragments of neonatal dogs with diarrhea (Catroxo & Martins, 2015). This technique also enables the study of the effectiveness of vaccines based on non-infectious virus-like particles (RVLPs) produced *in situ* (Meier et al., 2017).

In this work, we aimed to review the standardizations and protocols of *in situ* hybridization techniques in optical or photonic microscopy and immunoelectron microscopy (IEM), immunocytochemistry with immunogold colloid particle labeling (IMCG), and resin fragment embedding in transmission electron microscopy to enhance the efficiency of diagnosing diseases in aquatic organisms.

II. MATERIAL AND METHODS

The material and methods follow a chronological order as the techniques were developed and adapted. The first disease to be studied was White Spot Syndrome Virus in 2009, with the diagnostic techniques of *In Situ* Hybridization and Electron Microscopy being described sequentially.

White Spot Syndrome Virus (WSSV) Hipólito et al. (2012) published an article on the use of ISH and EM in the diagnosis of White Spot Syndrome Virus in shrimp.

Shrimp farming stands out as one of the most successful and economically attractive agro-industrial segments. This rapid advancement of marine shrimp farming is associated with, among other factors, the introduction of the exotic species known as Pacific white shrimp (*Litopenaeus vannamei*).

However, risk analyses of cultivated shrimp highlight that diseases caused by viruses are a highly limiting factor in the industry. Monitoring the health status of cultivated shrimp is important not only from a bioecological perspective but also from an economic-financial standpoint.

White Spot Disease, or White Spot Syndrome (WSS), is caused by the virus of the same name (WSSV), belonging to the Whispovirus genus. This virus has a wide range of hosts, has been detected in all growth phases, and can be transmitted horizontally or vertically. At the end of 2004, the first cases of this disease in Brazil were reported in the southern region, causing alarm in the shrimp farming sector due to its severity, leading to 100% mortality on some farms. Due to limited knowledge of this disease, its occurrence disrupted the entire production sector (aquaculture and fisheries), leading to restrictions on the movement and trade of any aquatic species.

For the detection of WSSV, techniques such as *in situ* hybridization and Nested-PCR are used. The main objective of this project was to detect the presence of White Spot Virus in shrimp destined for the State of São Paulo using methods recommended by the World Organization for Animal Health (WOAH). The project aimed to implement protocols for histopathology, *in situ* hybridization, Nested-PCR, and Transmission Electron Microscopy and train technicians from the Department of Agriculture in these techniques. To achieve this, samples of WSSV from clinically affected shrimp (*L. vannamei*) from farms in the State of Santa Catarina were collected. The target organs for diagnosis were the gills, digestive tract, pleopods, and hemolymph. Through training and the implementation of these techniques in Department of Agriculture laboratories, the goal was to fill a gap in the detection of diseases in aquatic organisms, especially viral diseases that are subject to mandatory reporting, which is currently observed in the State of São Paulo.

2.1 *In Situ* Hybridization Technique:

For in situ hybridization, a commercial kit for *In Situ* Hybridization (ISH) was used, along with biotinylated probes in the diagnosis of WSSV. The biotinylated probe was constructed in the same sequence as the PCR, with sequences 146F1 (5'-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3') and 146R1 (5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3'). (FIG 1, 2, and 3).

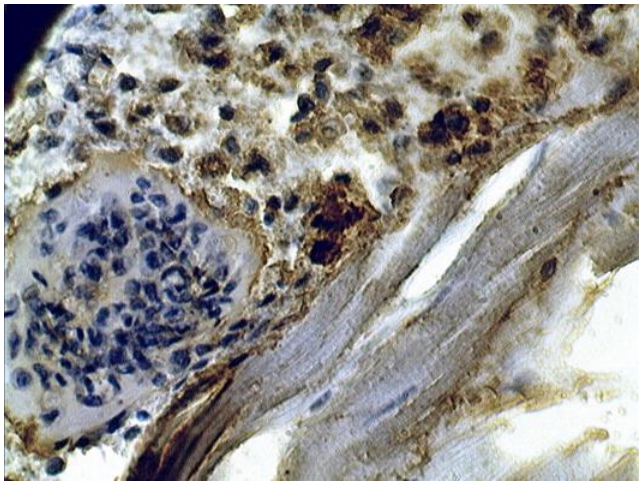


FIGURE 1: Photomicrograph in brown, positive result (white arrow), negative result (black arrow) Heart and hemocytes x 630.

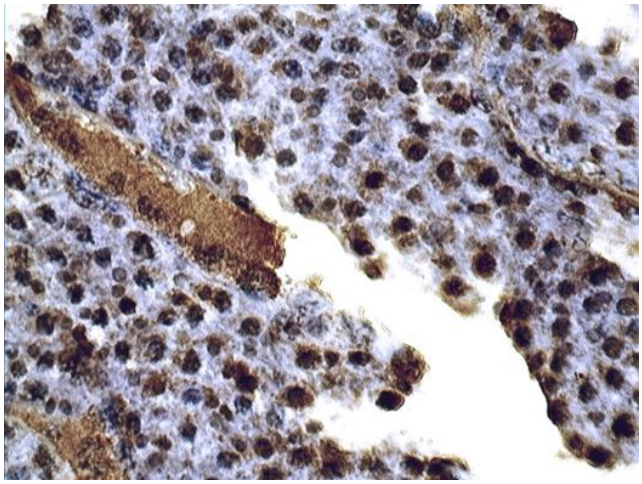


FIGURE 2: Photomicrograph in brown, positive result (white arrow), negative result (black arrow) Hepatopancreas x 630.

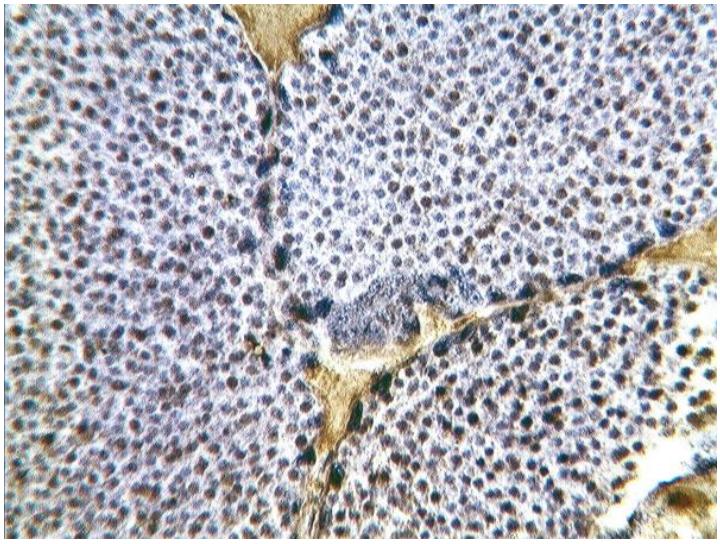


FIGURE 3: Photomicrograph in brown, positive result (white arrow), negative result (black arrow) Testicle x 400.

2.2 Transmission Electron Microscopy:

Another one of these additional tests involved the utilization of transmission electron microscopy. In this instance, the employed technique encompassed transmission electron microscopy in conjunction with immunoelectron microscopy (IEM) and immunocytochemistry, such as immunolabeling with colloidal gold particles (IMCG). The samples, fragments of hepatopancreas and pleopods, originated from marine shrimp, L. vannamei, cultivated intensively within Brazilian shrimp farms. In IEM, the fragments were processed through grids brought into contact with specific monoclonal antibodies. Following washes with PBS drops, the grids were incubated in viral suspension drops. Subsequently, the grids were contrasted using 2% ammonium molybdate at pH 5.0. In IMCG, for negative staining, screens were exposed to viral suspension and specific monoclonal antibodies. After successive PBS drop washes, the screens were incubated with protein A in conjunction with 10 nm colloidal gold particles. The grids were then contrasted with 2% ammonium molybdate at pH 5.0. We employed a Philips EM 208 transmission electron microscope. The results revealed a positive reaction for both techniques. In transmission electron microscopy for IEM, the presence of virus-antibody aggregates was observed. In IMCG, the antigen-antibody reaction was enhanced by the colloidal gold particles. Viral particles displayed oval to bacilliform or ellipsoid shapes, with measurements ranging from 230-290 nm in length and 80-160 nm in diameter, exhibiting projections within the nucleus. It is noteworthy that this is the first study employing these techniques in samples of marine shrimp, *L. vannamei*, in Brazil and it has demonstrated its viability as a complementary tool for diagnosing the presence of the virus (Figures 4, 5, 6, 7).

2.3 Negative Staining, Immunoelectron Microscopy, and Immunolabeling with Colloidal Gold Particle Techniques:

For negative staining, fragments of hepatopancreas and carapace were suspended in 0.1M phosphate buffer at pH 7.0. These suspensions were placed on metal grids, previously coated with collodion film and stabilized with carbon. Subsequently, the screens were drained with filter paper and negatively contrasted with 2% ammonium molybdate at pH 5.0.

In the immunoelectron microscopy (IEM) technique, copper grids, prepared in the same manner, were incubated for 15 minutes in 40 µL of viral suspension (antigen), sensitized with primary polyclonal anti-WSSV antibody against VP 664 protein (Abcam®), diluted at 1:200, and washed with 40 drops of PBS buffer. They were then incubated for an additional 10 minutes with WSSV viral suspension, washed successively with distilled water, and negatively contrasted with ammonium molybdate under the same conditions (Hayat and Miller 1990).

For the immunolabeling with colloidal gold particle technique (IMOC), copper grids, also previously prepared, were incubated for 30 minutes in 40 µL drops of viral suspension, sensitized with the same antibody diluted at 1:80, and washed with PBS buffer. Subsequently, they were incubated for 30 minutes with secondary antibody (protein A conjugated with 10nm diameter

colloidal gold particles - Electron Microscopy Sciences®) diluted at 1:20 in 0.5% PBS and also negatively stained using ammonium molybdate (Knutton 1995).

The samples were examined via transmission electron microscopy using a Philips EM 208 microscope. As a control for the technique, every 10 incubated samples, one was treated as described previously, replacing the antibody with distilled water.

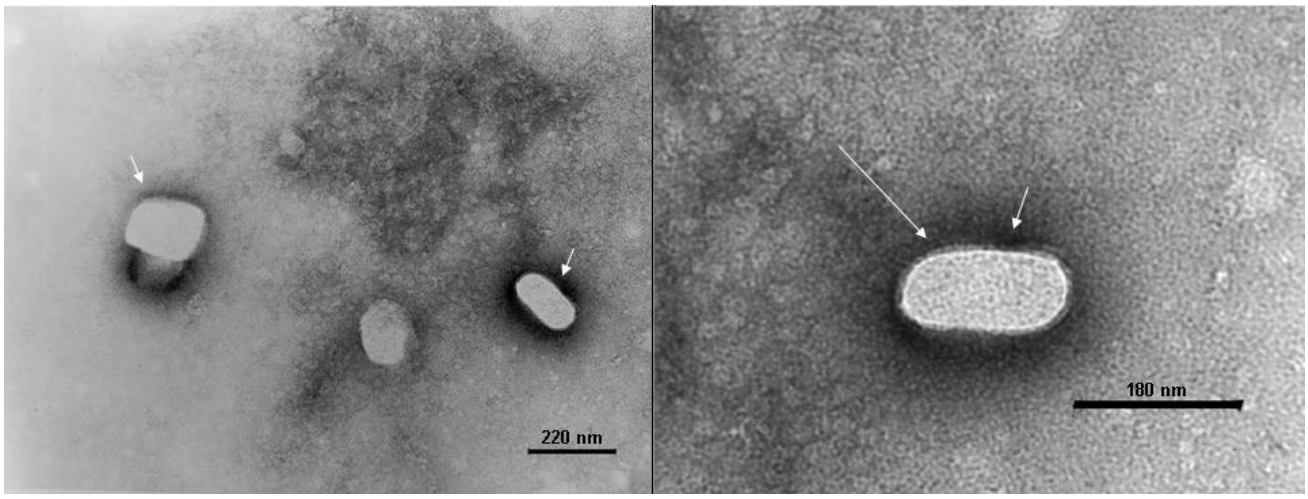


FIGURE 4: Electrophotomicrograph of WSSV viral suspension from L. vannamei hepatopancreas. Particles with oval and bacilliform shapes (large arrow) and an outer envelope (small arrow).

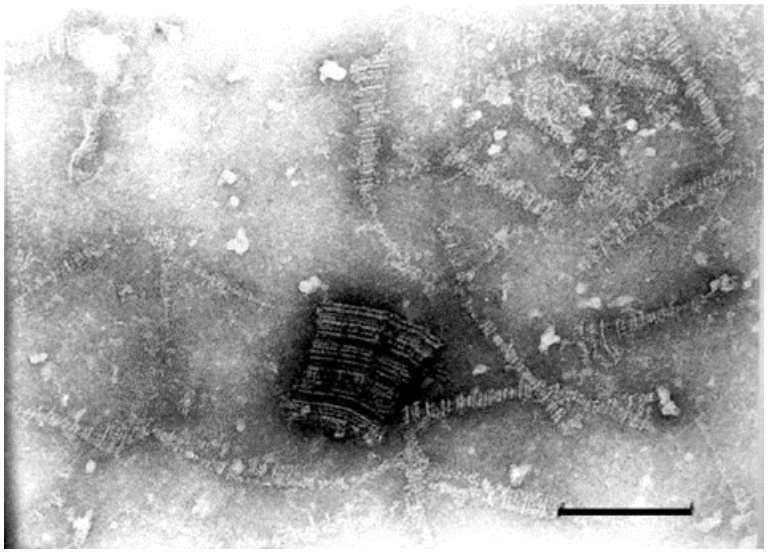


FIGURE 5: Electrophotomicrograph of WSSV viral suspension from L. vannamei carapace, showing WSSV nucleocapsids with streaks. Negative staining technique.

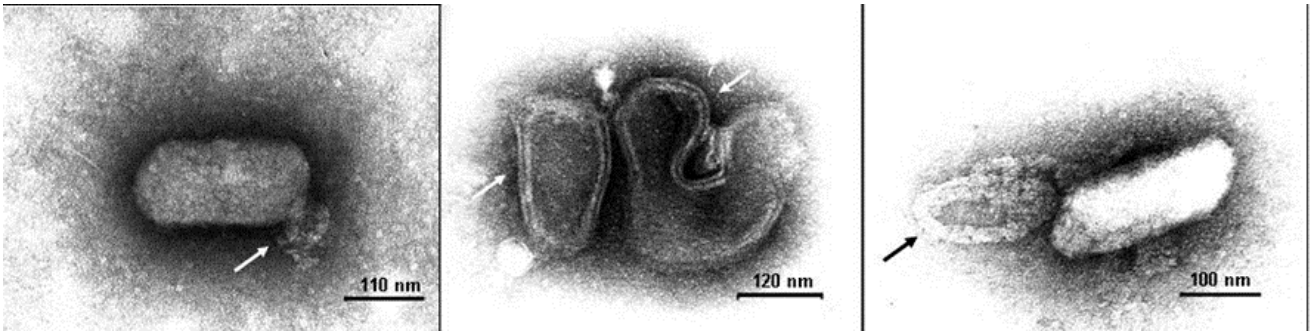


FIGURE 6: Negative staining of WSSV viral suspension showing ring -like particles attached to the virion (arrows).

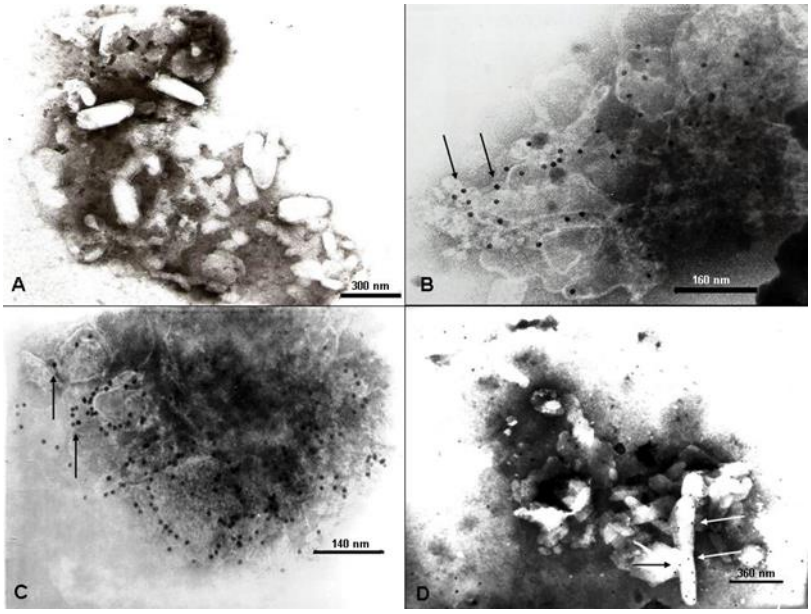


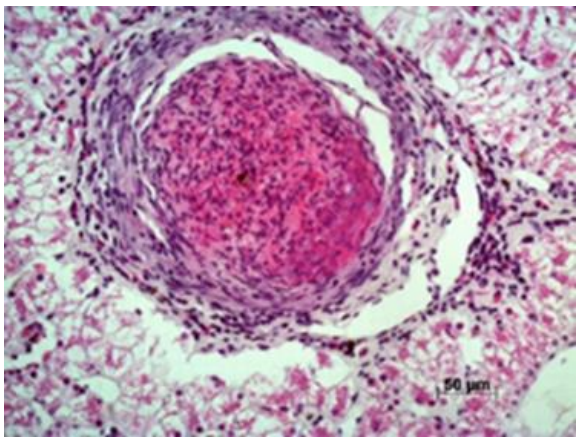
FIGURE 7: Immunoelectron microscopy and immunolabeling with colloidal gold particles. A - WSSV particles aggregated to the immune complex. B, C, D - Strong antigen-antibody interaction evidenced by gold particles on the virion (Arrows).

2.4 Chytridiomycosis (*Batrachochytrium dendrobatidis*):

Martins et al. (2020) described the melanomacrophage bleaching technique, which was proposed due to the significant presence of melanomacrophages in the organs of ectodermal animals such as amphibians and fish. These animals exhibit brownish melanin granules in various tissues, and to facilitate the observation of organ fragments under direct light microscopy during the use of biotinylated probes, it was decided to remove this pigment.

This bleaching facilitated the visualization of the DAB (diaminobenzidine) chromogen without interfering with the in situ hybridization technique, in which specific nucleotide sequences were identified in histological sections, and it prevented false-positive results (Figures 8 and 9). The bleaching process is gradual oxidation by hydrogen peroxide (KORYTWSKI & Sarna, 1990).

Fragments of both healthy and diseased animals were fixed in 10% formalin, dehydrated in increasing alcohol grades, cleared in xylene, and embedded in paraffin. Histological sections of 4.5 microns in thickness were affixed to silanized slides. These slides used for bleaching were immersed in 10% hydrogen peroxide (H2O2) in 0.2 mol/L Tris-HCl buffer at pH 7.4 for 24 hours at room temperature. During this process, the material was kept in the dark. Following this procedure, the normal staining protocol for IHC and ISH was followed.



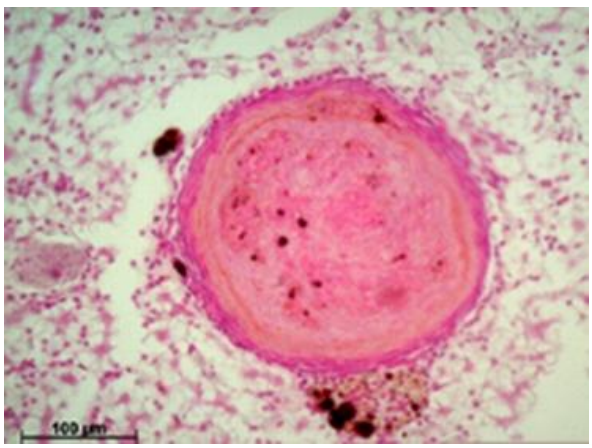


FIGURE 8 AND 9: Photomicrographs of granulomas in tilapia organs with bleaching treatment (left) and without treatment, thus containing melanin in the granuloma and melanomacrophages (right).

2.5 *In Situ* Hybridization Technique:

Schloegel et al. (2010) detected the non-hyphal fungus *Batrachochytrium dendrobatidis* in captive frogs in Brazil, affecting amphibians (Urodela and Anura) at all stages of their life, developing exclusively in the outer keratinized layer of the epidermis. Chytridiomycosis is an emerging disease and considered the primary cause of the global decline of these animals.

Subsequently, in line with this study, Hipolito et al. (2013) employed *in situ* hybridization and resin embedding techniques in electron microscopy as diagnostic tools to analyze keratinized tissues of larval forms of *L. catesbeianus* suspected of chytridiomycosis. The material utilized comprised parts of the tadpole mouth with partial discoloration of typically dark-colored denticle structures. *In Situ* Hybridization (ISH) is a molecular biology technique that detects the presence of nucleic acids in lesions using specific probes, which are complementary nucleotide sequences marked with biotin, binding to the fungus DNA in the infected tissue. The presence of bound biotin is amplified by the streptavidin-peroxidase enzyme complex and the chromogenic indicator DAB (diaminobenzidine). The "Gen-Point® DAKO Amplification System" kit and DAKO® S2450 hybridizer were employed. Hybridization was performed on slides containing sections of tadpole mouth tissues from bullfrog tadpoles. The biotinylated probe used consisted of Primer 1 (Forward Primer): ITS1-3 Bd: 29 bases [Biotin-5'-CCT-TGA-TAT-AAT-ACA-GTG-TGC-CAT-ATG-TC-3'] and Primer 2 (Reverse Primer): 5.8S Bd: 22 bases [Biotin-5'-AGC-CAA-GAG-ATC-CGT-TGT-CAA-A-3']. A positive result for hybridization is indicated by a dark brown precipitate, resembling structures akin to zoosporangia in this coloration (Figure 10).

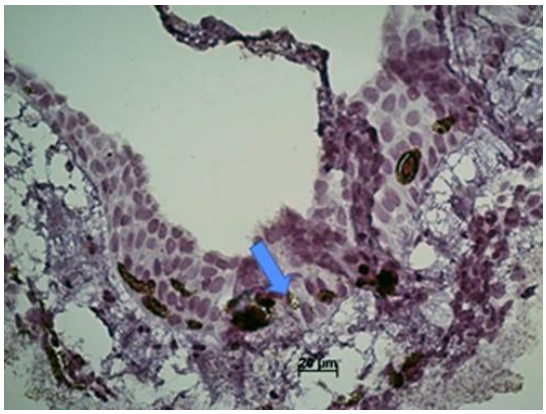


FIGURE 10: Photomicrograph of histological section of the mouth of a bullfrog tadpole, Lithobates catesbeianus, using the *In Situ* Hybridization (ISH) technique, demonstrating a vacuolar structure resembling sporangia, containing spores, in brown coloration (arrow).

2.6 Transmission Electron Microscopy:

2.6.1 Resin Embedding Technique Followed by Positive Contrast of Ultra-Thin Sections:

Samples of tadpole mouth fragments were processed following standard resin embedding procedures based on Gonzalez-Santander's methods (1969). When employing this technique, freshly collected samples are fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer at pH 7.0, post-fixed in 2% osmium tetroxide in 0.2M phosphate buffer at pH 7.0, stained with 0.5% uranyl acetate, dehydrated in an increasing acetone series (50 to 100%), and embedded in Spurr resin. After ultrasectioning of the blocks, the ultrathin sections obtained are positively stained through a sequential treatment with uranyl acetate (Watson, 1958) and lead citrate (Reinolds, 1963) before being observed under the Philips EM 208 transmission electron microscope (Figure 11).

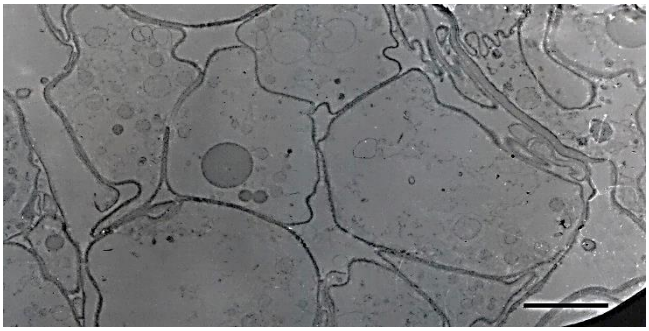


FIGURE 11: Transmission electron micrograph of ultrathin section of the mouth of a tadpole infected with the fungus *Lithobates catesbeianus*.

Martins et al. conducted an analysis in 2021 on various diagnostic techniques for *Mycobacterium* spp. and *Francisella* spp. in captive or free-ranging fish. This study focused on granulomatous diseases caused by these bacteria, which have significant implications for Animal Health, Public Health, and the Agribusiness sector. In this context, the occurrence of circulating samples of these bacteria was determined using fragments of organs obtained from passive and active collections in the State of São Paulo, employing the following techniques: *In Situ* Hybridization (ISH), Immunohistochemistry (IHC), Optical Microscopy (OM) (H&E and Ziehl-Neelsen or Fite-Faraco staining), and Transmission Electron Microscopy (TEM) with negative staining.

Mycobacterium spp. is a pathogen capable of causing severe and costly diseases in various invertebrates and vertebrates, including humans (tuberculosis, leprosy, Buruli ulcer), livestock (bovine tuberculosis), and ectothermic animals (reptiles, amphibians, and fish). In recent years, there has been increased interest in aquaculture due to a decline in fishing activities, which has favored the development of diseases such as mycobacterioses (Whipps, Watral, Kent, 2003).

More recently, the bacterium belonging to the *Francisella* spp. genus, highly virulent for numerous animal species, has been found in marine and freshwater fish, amphibians, reptiles, and even mollusks. It has been associated with massive mortalities of tilapia in commercial farms in Taiwan, Hawaii, and Costa Rica (Mauel et al., 2007; Soto et al., 2009), leading to declines in production ranging from 5% to 80%, with an average of 50%. In 2005, this bacterial disease, initially mistaken for a disease caused by bacteria of the Piscirickettsia genus (commonly responsible for septicemia in salmonids), decimated the tilapia stocks of one of the major producers and exporters of fresh fillets to the United States, Aqua Corporation, in Costa Rica.

2.7 *In Situ* Hybridization Technique For *in situ* hybridization, we utilized the "Gen-Point® DAKO Amplification System" kit and DAKO® S2450 hybridizer, along with the following probes:

2.7.1 *Francisella spp.* (Hsieh et al., 2007). Individual primer sequences:

- FLB16S180f: 5'-GCG-GATTAA-AGG-TGG-CCT-TTG-C-3' (forward primer)
- FLB16S465r: 5'-CCT-GCA-AGC-TAT-TAA-CTC-ACAGG-3' (reverse primer)

2.7.2 *Mycobacterium spp.* (Talaat et al., 1997). Primers specifically amplified:

- 924-bp fragments, T-39 (5′-GCGAACGGGTGAGTAACACG-3′) and T-13 (5′-TGCACACAGGCCACAAGGGA-3′). Or Partially degenerate primers (Zerihun et al., 2011):
- Myco-rtf (5¢-GGTGGACRTCATCYTGAACA-3¢) and Myco-rtr (5¢-TCCARRATCTGGCCGATGT-3¢).
- Amplifying a 63bp fragment and containing the TaqMan probe: Myco-rtpr (5¢-CACGGTGTGTGCCGCGTCGTATG-3¢) (Figures 13, 14, 15).

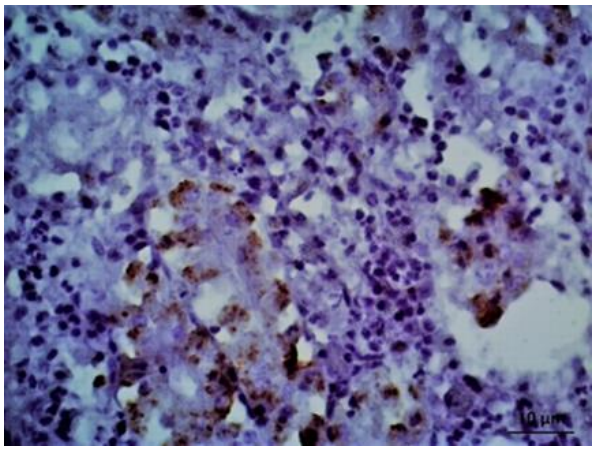


FIGURE 13: Photomicrograph of kidney and liver positive for Mycobacterium spp. using the *In Situ* Hybridization (ISH) technique at 400x magnification

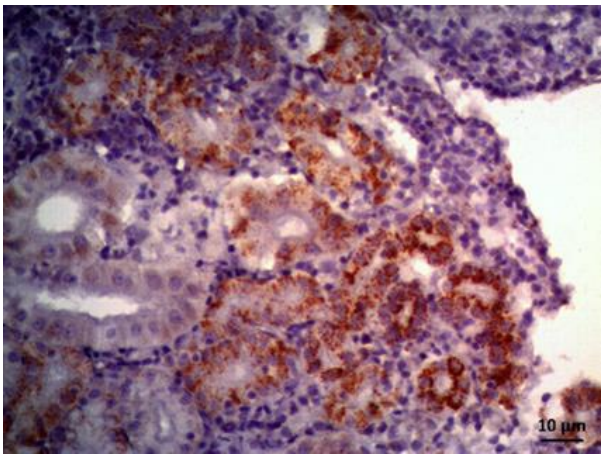


FIGURE 14: Photomicrograph of a kidney positive for Francisella spp. using the *In Situ* hybridization technique at 400x magnification.

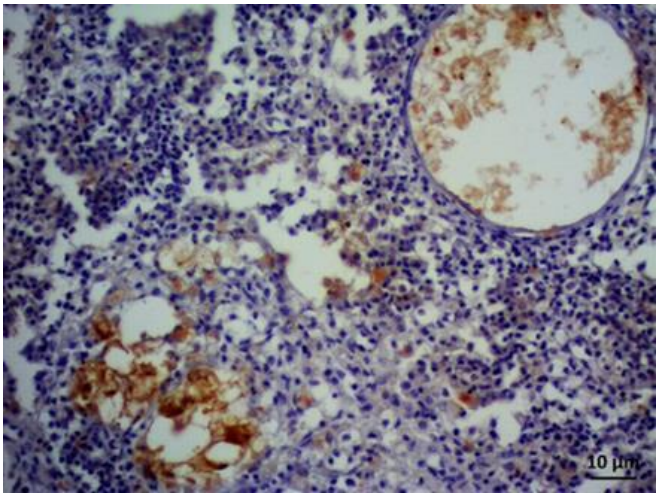


FIGURE 15: Photomicrograph of spleen positive for Francisella spp. using the *In Situ* Hybridization (ISH) technique at 400x magnification.

2.8 Negative Staining Technique

Fragments of fish organs were suspended in 0.1M phosphate buffer at pH 7.0. Drops of the obtained suspensions were placed in contact with copper mesh grids, previously coated with collodion film and stabilized with carbon. After draining with filter paper, the grids were negatively stained with 2% ammonium molybdate at pH 5.0 and examined under a Philips EM 208 transmission electron microscope (Figures 16, 17).

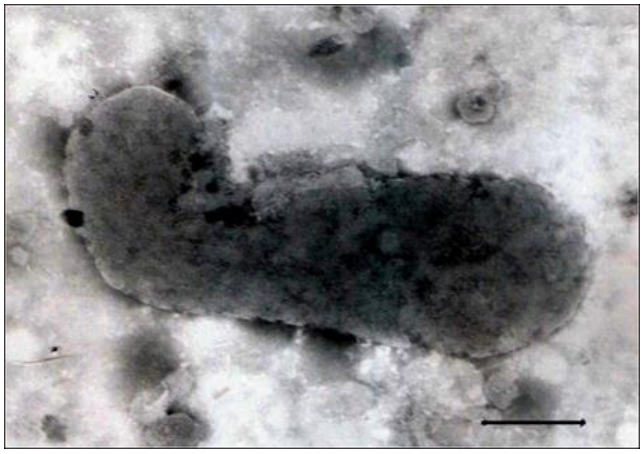


FIGURE 16: Electron micrograph of *Mycobacterium* spp., contrasted with ammonium molybdate using the negative staining technique. Scale bar: 100 nm.

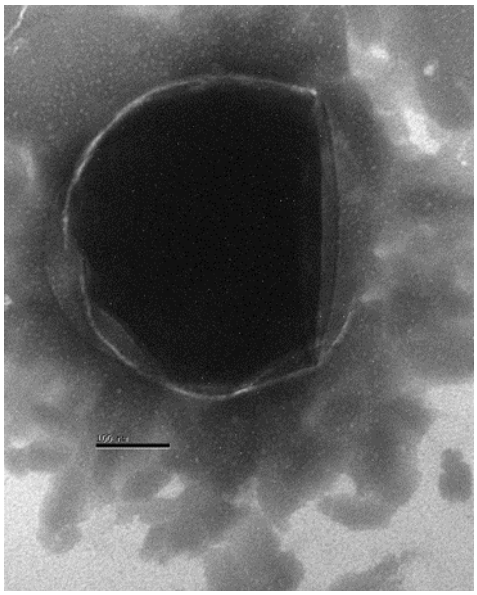


FIGURE 17: Electron micrograph of *Francisella* spp., contrasted with ammonium molybdate using the negative staining technique. Scale bar: 100 nm.

III. CONCLUSION

Considering that several etiological agents cause acute, chronic, granulomatous, systemic or focal diseases in aquaculture animals (fish, reptiles, amphibians and crustaceans), the standardization of histopathology and transmission electron microscopy techniques was essential to assist in the rapid and efficient diagnosis of such microorganisms, avoiding the mortality of infected animals, the unfeasibility of commercialization and economic losses to farms.

ACKNOWLEDGMENTS

This research was financially supported by FAPESP under grant numbers 09/14060-0, 2011/50009-9, and 2016/12930-0.

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