Hepatitis A virus and environmental quality indicators in aquatic ecosystems for oyster farming in the Northeast of the State of Pará, Brazil

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Abstract - *Research into the occurrence of microbiological contaminants, including hepatitis A virus (HAV), in river waters intended for oyster farming is of extreme importance for public health. This study aimed to detect the occurrence of HAV in the aquatic environment for ostreiculture in northeastern Pará, Brazil, and correlate with microbiological, physico-chemical and climatological variables. The HAV research was based on the method of water concentration by filtration membrane adsorption-elution and in the organic flocculation method with skim milk, followed by Nested-PCR. Quantification of coliforms, Enterococci and heterotrophic bacteria was performed. The physico-chemical variables were measured with multiparametric probe and spectrophotometry. Positive samples were purified and submitted to sequencing. From March 2017 to December 2018, 203 samples of river water were collected and analyzed in the municipalities of Augusto Corrêa, Curuçá, Salinópolis and São Caetano de Odivelas. In 10.8% of the analyzed samples the HAV RNA was detected, in all localities the HAV was classified in genotype IB. There was no significant difference between the concentration methods of the water samples. The only physicochemical variable that most influenced HAV detection was dissolved oxygen. Regarding the bacterial indicators, the highest statistical significance occurred with thermotolerant coliforms and Escherichia coli. The detection of HAV in the four municipalities studied shows that the virus is circulating in the aquatic environment and, therefore, in the community. In this context, more effective hygienic-sanitary measures are necessary in these communities dedicated to oyster farming.*

Keywords— Hepatitis A Virus, Quality Indicators, Water and Ostreiculture.

I. INTRODUCTION

The hepatitis A virus (HAV) belongs to the order of *Picornavirales*, family *Picornaviridae*, genus *Hepatovirus*, species *Hepatovirus A* [1]. It is an icosahedral symmetry virus, not enveloped, with a diameter of 27 to 32 nanometers. Classified in six genotypes, according to the phylogenetic analysis of the complete VP1 protein sequence. Genotypes I, II, and III are found in humans, being subdivided into subgenotypes IA and IB; IIA and IIB; IIIA and IIIB [2].

HAV is transmitted enterally by the ingestion of contaminated food and water, causing Hepatitis A, a self-limiting, infectious, symptomatic or asymptomatic disease of benign evolution, with the occurrence of fulminant cases. HAV infection is prevalent throughout the world, but with different epidemiology according to age of exposure and immunization [3].

With increasing pollution of the aquatic system, microorganisms that cause water-borne diseases can occur in oysters due to contamination by human waste or chemical pollution [4]. The quality of seawater where oysters are grown, as well as the animal itself, is of extreme importance for public health, since food and / or water contaminated by pathogens are the main causes of the occurrence of gastrointestinal diseases in Brazil [5]. Due to the various contaminants and forms of contamination of the aquatic environment, it is necessary to use quality micro-organisms that ensure the absence of other pathogens. The most commonly used indicators are coliform bacteria, total coliforms, thermotolerant bacteria, and Escherichia coli. However, depending on the environment studied, heterotrophic bacteria and Enterococci can be used to evaluate water quality [6].

One of the most widely used methods in environmental virology is based on the adsorption of virus particles to the filter media by loading interaction and subsequent elution of the virus by a pH adjusted solution [7]. Another method of viral

concentration is organic flocculation, where an alkaline proteinaceous solution containing glycine with meat extracts or skimmed milk is used, promoting the recovery of virus particles adsorbed on the flakes [8].

After the concentration processes, molecular tests are carried out by PCR techniques, which has allowed advances in the detection of enteric viruses. However, the assay is susceptible to inhibitors found in aquatic environments [9]. The higher the level of water pollution, the lower the detection efficiency of the viral genome, the presence of particulate matter or suspended solids in water [10].

The investigation of the contamination of water in relation to enteric viruses using molecular techniques may be impaired due to the large volume of water in the environment in relation to the low concentration of viruses in these environments, and also by the possible presence of inhibitors of enzymatic reactions that can be found in these samples that compromise the detection of viral genomes [9].

This study aimed to detect the occurrence of HAV in the aquatic environment for ostreiculture in northeastern Pará, Brazil, and correlate with microbiological, physico-chemical and climatological variables.

II. MATERIAL AND METHOD

2.1. Description of the study

This is a cross-sectional field research that was carried out in a descriptive way, with a quantitative analysis approach developed from March 2017 to December 2018.

2.2. Study area

The samples were collected in four different rivers for ostreiculture, in the municipalities of Salinópolis, Augusto Corrêa, Curuçá and São Caetano de Odivelas. The municipalities belong to the Meso-region of the Northeast of the State of Pará, Brazil.

2.3. Sampling

The water samples were collected in three distinct points (mouth, oyster and spring) of a river in each municipality studied. In São Caetano de Odivelas, an extra point was added due to the occurrence of a point of launch of a rainwater gallery near the ostrich. A total of 203 water samples were collected: 48 samples in Salinópolis, 45 in Augusto Corrêa, 48 in Curuçá and 62 in São Caetano de Odivelas.

2.4. Collection and storage of samples

At each collection point the samples were stored in previously sterilized plastic polypropylene bottles and kept at ± 4 ° C until the time of analysis, which did not exceed 24 hours in any opportunity. Two liters of water were collected for each method of concentration of the virus particles, one liter for the physico-chemical analyzes and 500 mL for quantification of the microbiological indicators of water contamination. All methodology complied with the recommendations of ISO 5667-14, Standard Methods for the Examination of Water and Wastewater [11] and CETESB [12].

2.5. Chemical physical analysis

The pH, Temperature, Electrical Conductivity (CE), Total Dissolved Solids (STD), Salinity and Dissolved Oxygen (OD) parameters were analyzed by potentiometry in a previously calibrated Professional Plus YSI® multiparameter probe. Turbidity was determined by spectrophotometry on the HACH® DR3900 equipment. The analytical methods employed for the determination of the physicochemical parameters obeyed the procedures and recommendations described in the Standard Methods for Examination of Water and Wastewater [11] and Procedures Manual HACH-Spectrophotometer DR-2800.

2.6. Quantification of microbiological indicators

The most probable number (MPN/100mL) of total coliforms, thermotolerant, Escherichia coli, Enterococcus and heterotrophic bacteria was determined using the COLLILERT 18 / QUANTI-TRAY®, ENTEROLERT / QUANTI-TRAY® and SimPlate TM for chromogenic substrate method HPC Unit Dose from IDEXX Laboratories, Inc. © from IDEXX Laboratories, Inc., following the manufacturer's recommendations and the Standard Methods for the Examination of Water and Wastewater [11].

2.7. Concentration of viral particles

2.7.1 Method of organic flocculation with skimmed milk

One liter of the sample was acidified with hydrochloric acid (1N HCl) for pH 3.5 adjustment. 100 mL of 0.01% w / v pre-flocculated skim milk solution was added to the acidified sample. The samples were kept under stirring for eight hours, followed by resting for an additional eight hours for sedimentation of the flocculated material. The supernatant was carefully removed and the final volume containing the pellet centrifuged at 7000 x G for 30 minutes at 12ºC. After removal of the centrifuged solution supernatant, the pellet was resuspended in 8 mL of 0.2 M phosphate buffer pH 7.5 (1: 2 0.2 M Na2 HPO4 and 0.2 M NaH2 PO4). Once dissolved, phosphate buffer was added to a final volume of 10 mL. The concentrate was stored at -20 ° C [13].

2.7.2 Adsorption-elution method on filter membranes

Two liters of the samples were acidified with hydrochloric acid (6N HCl) to adjust pH 5, when necessary. Subsequently, after concentrating on 0.45 μm and 142 mm diameter pore HA membranes (MILLIPORE), after the sample was passed through the membrane, 350 mL of sulfuric acid (5 mM H2SO4 pH 3.0) was filtered for elimination of cations. Elution was performed with 15 mL of 1 mM NaOH solution pH 10.5 for 10 minutes over orbital shaking. The eluate was neutralized with 50 ul H2SO4 (50mM) and 50μL TE 100x (pH 8). The concentrate was stored at -20 ° C [14].

2.8. Ultrafiltration

Ultrafiltration was performed by means of a membrane using a vacuum system. The samples concentrated by the adsorption-elution method were reconcentrated in Amicon Ultra-15 (MILLIPORE) device at 5000 RPM for 15 minutes at 4 ° C, obtaining a final volume of 2 mL, stored at -20 ° C until extraction of the nucleic acid.

2.9. Molecular analysis

2.9.1 Viral RNA Extraction

RNA extraction was performed with commercial QIAamp Viral RNA kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's recommendations.

2.9.2 Preparation of Complementary DNA (cDNA)

After extraction of the nucleic acid, the cDNA was prepared from 9.5 μL of the extracted RNA, with a random primer Pd (N) 6 (Invitrogen) and reverse transcription using Super Script III (Invitrogen), with a cycling of 25 ° C for 5 minutes, 50 ° C for 60 minutes and 70 ° C for minutes.

2.9.3 Nested RT-PCR

For detection of HAV by the Nested RT-PCR technique, after reverse transcription, the VP1/2A junction region of the HAV genome was amplified (409 bp). The sequences of the external primers used were F6-CTATTCAGATTGCAAATTAYAAT and F7-AAYTTCATYATTTCATGCTCCT (Y = C or T), and internal primers F8-TATTTGTCTGTYACAGAACAATCAG and F9-AGGRGGTGGAAGYACTTCATTTGA (R = A or G, Y = C or T) [15].

2.9.4 Sequencing

Sequencing of the positive samples was performed from the 2nd round product of Nested-PCR, purified with the commercial BigDye XTerminator Purification Kit (Applied Biosystems) according to the manufacturer's instructions. For the reaction, the BigDye Terminator Cycle Sequencing kit was used. 3.1 (Applied Biosystems) and the 3130xl Genetic Analyzer (Applied Biosystems) following the manufacturer's recommendations. Briefly, two μL of the purified genetic material was used in the preparation of the sequencing reactions, which is composed of 250ng of each of the primers (F8 and F9), in separate reactions, together with the reaction buffer and BigDye, totalizing a volume end of 20 μL. The reactions were subjected to 25 cycles where the steps of denaturation occurred at 96 ° C, hybridization at 50 ° C and extension at 60 ° C. After the cycling of the sequencing reaction, the DNA was precipitated (125 mM EDTA, ethanol) to remove excess reagents that might interfere with obtaining the data during electrophoresis.

2.9.5 Sequence analysis and genotyping

The sequences were edited and aligned in the Geneious version 8.1.3 program and compared to prototype sequences of the HAV genotypes, available from the National Center for Biotechnology Information (NCBI) database. The comparative analysis was performed with BLAST (BlastN) to compare the nucleotide sequence to be analyzed against available sequences in the NCBI database. To evaluate the existence of recombination between the sequences, the phi-test of the program Splits tree version 4.13.1 was applied.

2.9.6 Phylogenetic analysis

The alignment was manually inspected in the Geneious program version 8.1.3 and corrected insertion and deletion regions shared by more than one strain. In order to evaluate the genetic distance and the choice of the nucleotide substitution model, MrModeltest was used to select the best evolutionary model for the phylogenetic tree generation process, where the Neighbor-Joining method was selected.

The evolutionary distances were calculated using the Tamura-Nei method. The rate variation between the sites was modeled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each pair of sequences. There were a total of 225 positions in the final dataset. The visualization of the phylogenetic tree was performed in the Mega 6.06 program.

2.10. Statistical analysis

The G test was applied to analyze the correlation of independence of HAV detection with biotic and abiotic variables. For the analysis of variance between the biological indicators with biotic and abiotic variables, the Kruskal-Willis test was used. The Pearson test analyzed the correlation between biological indicators and abiotic variables. Fisher's exact test was used to analyze the relationship between the viral concentration methods used in the study.

III. RESULTS AND DISCUSSION

Oyster farming in the Northeast of the State of Pará is an income alternative carried out alongside or parallel to traditional fishing, during periods of detention or low fish production. Oyster farming makes it possible for the fishermen to take part in family life, serving as a complementary source of income. In the study period, there were no reports of hepatitis A outbreaks in the communities, however the occurrence of HAV in the study areas demonstrates a possible source of contamination of the cultivated molluscs, which may have a negative impact on public health, production and commercialization of these foods.

3.1 Occurrence of HAV

HAV-RNA was detected in 10.8% (22/203) of the analyzed water samples. All positive samples were confirmed by sequencing. HAV was detected in 11.1% (5/45) of Augusto Corrêa samples, 12.5% (6/48) of Curuçá, 10.4% of (5/48) Salinópolis and 9.7% (6 / 62) of São Caetano de Odivelas, this difference was not significant (p = 0.688 Test G).

As for the distribution of HAV positive results among the sampling points, a positivity of 30.4% (8/22) was observed among the samples collected at the source of the rivers, 31.8% (7/22) at the point of oyster culture, 31.8% (7/22) at the mouth and 4.5% (1/22) at the extra point, there being no statistical significance (p = 0.9835 Test G).

As for the concentration of water samples used in this study, HAV was detected in 4.4% (9/203) of the samples concentrated by the membrane adsorption-elution method and in 7.4% (15/203) of the samples. samples concentrated by organic flocculation with skim milk, this difference in the data was not statistically significant (p> 0.05 Fischer exact test). In only two instances were the samples positive by both methods. This fact calls for the use of at least two methods of water concentration for this type of study in order to obtain better results. In the case of salt water, Moresco (2011) emphasized the method of concentration by organic flocculation in relation to membrane filtration (adsorption-elution). Rigotto [16] detected HAV in 8.3% of the samples, using the adsorption-elution method, but using a pre-filter to reduce the presence of debris in the samples, demonstrating the need for adaptations according to the type of virus or matrix analyzed .

Of the 22 HAV positive samples, 30.4% (7/23) were concentrated by the membrane adsorption-elution method, 56.5% (13/23) only by the organic flocculation method with skim milk and 8.7 % (2/23) by both methods. The detection of HAV has also been reported by concentrating seawater samples by the polyethylene glycol (PEG) precipitation method [17]. The use of PEG may increase the recovery of some viral species, such as Poliovirus, but it may also be a potent inhibitor of PCR reactions [18].

In fact, the use of molecular biology tools to detect microorganisms in environmental samples may be limited due to the presence of polymerase chain reaction inhibitors, for example, humic acid may adsorb proteins or enzymes interfering in the sites of activity of the polymerase or chelate the cofactors of the enzymes, the cations of Ca2 + and Mg2 + [19]. It is also possible that marine bacterial enzymes can degrade the viral capsid, being a thermolabile biological factor active against enteric viruses, including HAV [19]. To decrease the action of possible inhibitors of the PCR reaction, the genetic material can be diluted 1:10, mainly in viral quantification reactions [18]. Rigotto [16] observed that the detection of HAV in six samples was only possible after dilution of the same. In the present study there was no analysis of diluted samples.

In two separate studies on seawater in Santa Catarina, Rigotto [16] detected HAV in 16% of the samples using Nested RT-PCR and Moresco [18] detected the HAV in 51.5% of seawater samples using the quantitative RT q-PCR method. This improved detection efficiency corroborates with a study by [15], in which HAV was detected in 23% of water samples by nested-RT-PCR and 92% by RT-qPCR, demonstrating a lower influence of enzymatic inhibitors on the samples in quantitative tests and the need for more a method of viral detection. As only the Nested RT-PCR was performed for the analysis of the samples from this study, it was not possible to compare the qualitative and quantitative results.

From the 22 sequences of hepatitis A virus genome fragments obtained from the positive samples, it was possible to perform the phylogenetic analysis in 19 sequences, observing a nucleotide similarity of 98.4 to 100.0%. Samples were grouped with strains M14707 (wild-type), AY323047 (Rio de Janeiro) and AF268396 (Brazil), all genotype IB, with nucleotide identity varying from 98.2 to 100.0%. Phylogenetic analysis included complete genomic sequences which are available from GENBANK (Fig. 1).

Mendes [20] analyzed 144 samples from the Rodrigo de Freitas Lagoon, Rio de Janeiro, detected the HAV by RT q-PCR in 21.53% of the samples and only 3.47% by Nested RT-PCR, all of which were genotyped as genotype IB. The genotyped samples showed a small genetic difference between them, demo nstrating the circulation of different strains. In the State of Pará there are few studies that report the occurrence of HAV genotype in aquatic matrices, mainly in brackish or salt water. Santos [21] analyzed freshwater samples in Mosqueiro Island, Pará, and detected three samples of genotype IA and 11 of genotype IB, similar to IB isolates from Brazil.

In bivalve molluscs, IB and IB subgenotypes are the most commonly found in Brazil, France, China and Japan. Hepatitis A outbreaks involving IB genotype were associated with oyster ingestion in the Middle East, Egypt and Morocco [22]. In a study in Tunisia, the occurrence of genotype IA was observed both in clinical samples and in samples of oysters and culture water [23].

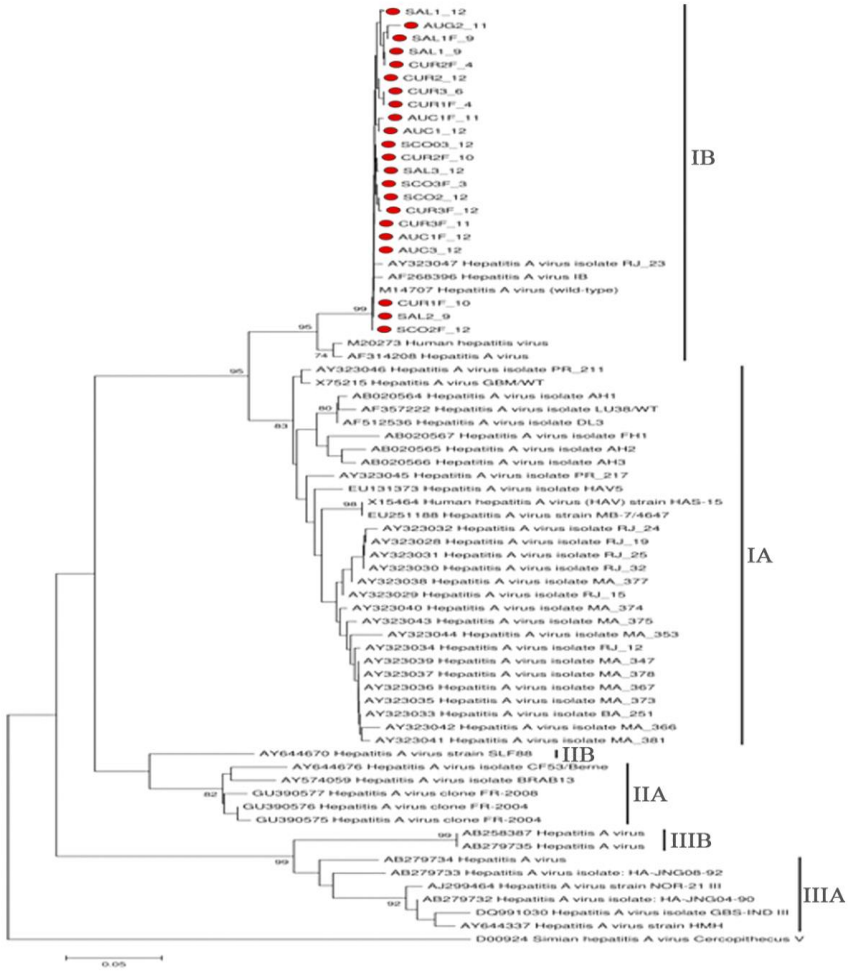

FIGURE. 1 - Neighbor-joining algorithm phyogenetic tree built on sequences of hav genetics studied, compared with sequences available in the data bank of the national center for biotechnology information (NCBI).

3.2 Climate variables

According to the website of the National Institute of Meteorology (INMET), the highest rainfall rates occurred in the months of March to July 2017 and from January to July 2018, indicating the rainy season in the study area. Regarding the seasonality of HAV occurrence, 4.0% (4/100) of the positive samples in the dry period and 17.5% (18/103) in the rainy season were observed, a highly significant factor (p = 0.0034 Test G). The higher the rainfall the greater the transport of solids to water, pathogenic organisms can remain attached to particles, using them as a substrate and form of protection and resistance [8].

Of the analyzed samples, 18.2% (37/203) were acidic (pH range between 5.28 and 6.99) and 81.2% (166/203) were basic (pH variation between 7.01 and 12 , 06). Catuxo et al. [24] observed values different from those obtained in the present study, relating them to the possible influence by leaching of organic acids to water. In quiescent and organic-rich waters, a great variation of organic and inorganic acids occurs and, therefore, they are often more acidic [25]. As for the occurrence of HAV in acidic or basic waters, 18.8% (4/22) occurred in acid medium and 81.2% (18/22) in basic medium, and although the highest number of HAV positive samples was found in basic water, this fact did not present statistical significance (p> 0.05 Fisher's exact test), as found by Hernandez-Morga et al. [26] who found statistical significance between pH and HAV RNA detection. HAV is more stable at acidic pH, even at pH close to 1, which would reproduce the pH of the human stomach [27]. The resistance of HAV to a more acidic medium is due to its extremely cohesive capsid [28].

3.3 Physico-chemical variables

Regarding the electrical conductivity, the parameter varied between 1.32 μS / cm and 111.7 μS / cm in the studied municipalities. A statistically significant difference was observed when EC values were compared between the municipalities and seasonality, especially in the rainy season (p <0.05 Kruskal-Wallis test). Generally, values above 100 μS / cm may indicate impacted environments [12], therefore, all the samples of this study would be within the acceptable limit. The highest values found for electrical conductivity occurred in the rainy season, which corroborates with the values found by Kiyatake [29], in a study carried out in São Caetano de Odivelas, possibly because of the smaller influence of the ocean along the estuary in this period.

The turbidity ranged from 1.0 to 247.5 nephelometric units (UNT), with an overall mean of 22.0 NTU. The relation of the turbidity with the municipality and with the period of highest rainfall index was statistically significant (p <0.05 Kruskal-Wallis test). It was also observed that, in the four studied rivers, the turbidity values were higher in the rainy season, a fact that can be justified by the loading of inorganic and organic material from the river bed by rainfall.

The analysis of the total dissolved solids data showed statistically significant relation with the municipalities studied and with respect to the occurrence of rain during the collection period (p <0.05 Kruskal-Wallis test). The STDs are directly related to rainfall, ie, the higher the rainfall, the higher the solids dilution in the water [30]. In the municipalities of Curuçá and São Caetano de Odivelas, the highest averages of STD were observed in the rainy season, different from the municipalities of Augusto Corrêa and Salinópolis. The fact that the rivers studied in Curuçá and São Caetano de Odivelas have less distance between their margins and lower depth corroborates a higher concentration of STD.

Salinity is used as a parameter for water classification. Salt water has a high concentration of salt, the salinity is equal to or greater than 30 parts per million (ppm). Brackish water has salinity between 0.5 ppm and 30.5 ppm. Fresh water has a salinity of less than or equal to 0.5 ppm [31]. The salinity of the samples ranged from 0.09 to 42.08 ppm in the studied municipalities, 2.9% (6/203) of freshwater samples, 77.3% (157/203) of salt water and 19.8 % (40/203) of salt water. Statistical analysis showed that, in relation to the sampling point, the rivers of the four municipalities presented the highest concentration of salinity at the mouth and lower concentration at the source. (p <0.05 Kruskal-Wallis test). The concentration of salinity was inversely proportional to the values of rainfall, data also found by Kiyatake [29] in a study in waters in the same rivers, possibly due to the ions being more concentrated in the water. In a study carried out in a river in Santa Maria da Vitória, Bahia, Loss [32] verified that the farthest point of the mouth had a lower mean salinity due to the dilution of the salt water with the fresh water of the river, corroborating with the findings of this river study. The concentrations of salinity found in this study corroborate a study by Mok et al. [33] in water intended for oyster farming in Hansan Geojeman, Korea, which observed lower levels of salinity in the rainy season, even with the temperature occurring between 8.8 ºC and 25.5 ºC.

Dissolved oxygen (OD) ranged from 0.0 to 14.0 mg / L, with an average of 5.0 mg / L. In this study, 18.2% (37/203) of the samples were below 5 mg / L O2, limit established for DO by Resolution CONAMA 357/2005 [31], therefore unsuitable for oyster cultivation. Both the comparison between the municipalities and the collection points are not statistically significant (p <0.05 Kruskal-Wallis test).

The statistical analysis of this study demonstrated that the only physicochemical variable that presented statistically significant results when compared to the occurrence of HAV was OD ($p < 0.05$ test G), different from that found by Hernandez-Morga et al. [26], who observed that the variable that most possessed statistical significance with HAV detection was salinity.

3.4 Microbiological indicators of water quality

In the analysis of the occurrence of HAV in the study environment in relation to the bacterial indicators studied, there was statistical significance between the occurrence of HAV with thermotolerant coliforms and E. coli ($p < 0.05$ test G). The occurrence of HAV in samples with acceptable levels of microbiological indicators of water quality demonstrates the need to include virus research in the tests already performed in water quality analysis in order to improve epidemiological and sanitary surveillance.

In the analysis of variance of the mean concentrations of thermotolerant coliforms (CTT) and E. coli, obtained in the studied rivers, it was observed that there was no statistically significant difference between them, as well as when the collection points of the same river ($p > 0.05$ Kruskal-Wallis test). However, the analysis of concentrations of Total Coliforms (CT) and Enterococci was significantly positive in the source of the studied rivers ($p < 0.05$ Kruskal-Wallis test), the highest values were detected in the rivers. In a study carried out in Rio de Santa Maria da Vitória, Bahia, Loss (2012) [32] verified that the four collection points, including the one closest to residences, presented contamination by fecal residue, containing CT and Enterococci, probably by the release of debris from the sewage in the river water. In addition to sewage or faeces, organic plant materials, insects and reptiles may also be sources of Enterococci [34].

IV. CONCLUSION

The detection of HAV, genotype IB, in the four municipalities studied shows that the virus is circulating in the aquatic environment and, therefore, in the community. We suggest the use of more than one concentration method with a view to reducing false-negative results, since even though no significant difference was observed between the concentration methods of the water samples for HAV detection, in only two the results were concordant.

The results obtained in this research are important to evaluate the occurrence and concentration of microbiological and physicochemical contaminants in oyster farming areas in the Northeast of Para. In this context, it is necessary to make more hygienic-sanitary measures in the communities dedicated to the cultivation of this food so that it meets the minimum standards established in current legislation, aiming at the expansion of oyster farming in the region and greater sanitary security for consumers.

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