Chapter 1
Context concerning PCR utilization in water molecular microbiological diagnosis

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Water is an indispensable resource for life with various uses for humans, ranging from direct consumption, food preparation, personal and domestic hygiene, agriculture, recreation, transportation and energy production; thus it is essential that this resource ensure the well-being and health of human beings, because today developing countries water service problems, are not solely restricted to the lack of drinking water, but also to its quality. At the global level, there are high morbidity rates due to water related diseases, such as gastroenteritis, diarrhea and infectious hepatitis; around 25 million people die every year in the world because of water pollution; with diarrheal diseases being the most prevalent and the third infant death leading cause (1).

Pathogenic viruses and bacteria present in infected persons feces may eventually have contact with water sources supplies and cause epidemic outbreaks, since many of these microorganisms have shown a high resis-

tance to pH, temperature and humidity extreme conditions and disinfection and water purification treatments which has contributed to its persistence in the aquatic environment (2).

Fecal borne water contamination measure is carried out mainly through indicator microorganisms enumeration, whose presence may indicate that the sample was exposed or has pathogenic microorganisms such as *Vibrio cholerae*, *Salmonella* spp, *Legionella pneumophila*, *Escherichia coli* O157: H7, *Listeria monocytogenes*, among others (3). These microorganisms diagnosis in water is based on the use of traditional methods, however, in about half of the cases of waterborne diseases, the causative agent is not identified because many of these detection methods are not enough sensitive and fast; this causes late results for decision making to solve the problem associated with the water resource (4,5).

Others problems facing the waterborne pathogens detection is the lack of a method applicable to all of them, as well as being technically difficult and laborious because of pathogens low concentration in most water samples, requiring concentrating large water volumes for its determination, which makes it difficult to detect microorganisms in the aquatic environment (6).

However, with molecular biology techniques development and application, such as Polymerase Chain Reaction (PCR) and Real-Time Polymerase Chain Reaction (RT-PCR), it has been found that most of waterborne diseases have been caused by not detected bacteria when traditional methods were used. The PCR is a highly sensitive, fast and specific method that improves results reliability; however, its implementation in waterborne pathogens diagnosis is confronted with certain problems such as the high microorganisms dilution, chemical contaminants presence

and organic compounds present in environmental water samples, which leads to the need of efficient bacterial methods concentration and reaction inhibitors purification strategies, therefore, it is important to standardize the RT-PCR method for its implementation (7).

PCR technique standardization process for its use on waterborne microorganisms' identification starts with the evaluation of bacterial concentration method, which allows the determination of the most appropriate technique according to test methods needs; the most utilized techniques for this purpose are centrifugation and filtration. Recovery verification using these techniques is done by the addition of microorganism known concentrations; then the added samples are subjected to the concentration processes and the recovery capacity is evaluated by culturing the obtained concentrate and comparing it with the amount of inoculated bacteria.

When selecting filtration as concentration method, it is needed to evaluate bacterial cells elution method from the filters. The elution process is performed by submerging the filter into different types of buffer and subsequently exerting friction forces necessary for cells separation, through vortexing, agitation, and grinding. Once the cells are released, DNA extraction is carried out using standardized protocols or the use of commercial kits.

In the case of PCR technique application to pathogens detection such as *Salmonella* spp. It is required as in the classic microbiology method, a pre-enrichment phase after the bacterial concentration process. Pre-enrichment phase objectives are to increase the probabilities of detecting the pathogen when it is in a low number, bacterial cells that are under stress activation and also allows determining in a certain way cellular viability, for molecular methods case.

One aspect to be taken into account in relation to molecular methods is that they detect microorganism DNA and not viable cells. Thus, its application in microbiological analyzes is limited to determining the microorganism DNA, indicating that the pathogen was present at some point in the sample but cannot be guaranteed that the microorganism is alive; however, when sample pre-enrichment is performed, if viable cells are present, they increase in number and are reflected in the decrease on the Ct value in the real-time PCR (7).

The pre-enrichment stage is necessary for pathogens such *Salmonella* spp. detection in both water and food, this is generally done with enriched media such as peptone water buffered during 18 to 24 hours at 37°C; however, its use is applicable in the diagnosis where it is not necessary the pathogens enumeration and it is usually utilized in qualitative analysis. From the pre-enrichment culture the DNA extraction is performed either by a conventional methodology through organic extraction or by the use of commercial kits, which allows to obtain DNA of sufficient quantity and quality to be amplified by the PCR.

Some commercial real-time PCR kits for microorganism determination do not specify the range or maximum Ct value to define a positive sample, it corresponds to the kit user to define this aspect based on his experience and in function of its behavior once it has been evaluated with matrices inoculated with target microorganism. Based on the above, it is possible to define a Ct value, which indicates *Salmonella* spp. presence in a given sample, performing an assay where samples are inoculated with a specific amount of bacteria and its behavior is analyzed in relation to the obtained Ct values. It must be taken into account that this does not fully guarantee that the detected cells are alive in the sample, but the fact of having low Ct

values indicates a greater number of bacterial cells and therefore cellular multiplication processes, unless there were a high amount of not viable cells in the sample.

The real-time PCR variant also allows quantitative data to be produced, which would be very useful considering that in the water samples microbiological evaluation this type of information is also required to show water state and evolution of environmental and health quality. This evaluation uses contamination indicators, such as the bacterium *Escherichia coli*, which indicates the possible presence of other enteric bacteria, some of which are pathogenic to the human, and thus determining the microbial load in relation to the number of *Escherichia coli* present in a sample constitutes a key element to determine the water quality.

Real-time PCR can yield quantitative data by relative quantification using standard curves that relate obtained Ct values with a gene copies number or with different CFU amounts of a bacterium. The latter case is of great use in microbiology by allowing results reporting in units comparable to microbiological standards. Therefore, it is proposed to develop standard curves from *Escherichia coli* inoculum dilutions, and then perform the DNA extraction and amplified it by real-time PCR.

The objective of this project was to standardize the PCR technique in real time for the pathogenic microorganism detection, selecting as models *Salmonella* spp. due to its importance in public health and *Escherichia coli* for microorganism enumeration indicating water sanitary quality. The matrices used for the standardization process were drinking water because of their impact at the public health level and in order to project this technique implementation in the monitoring of drinking water quality in the region, and the other matrix was sea water due to its complexity and in order to

use it for monitoring beaches environmental quality. The purpose of this book is to make the information presented extrapolable to other public health importance microorganisms such as *Listeria monocytogenes*, *Vibrio cholerae*, *Legionella pneumophila*, *Escherichia coli* O157: H7, etc.

This research results presentation is based on the standardization of DNA direct extraction method from water for *Escherichia coli* enumeration in drinking water and sea water by qPCR, evaluating different strategies for bacterial concentration and elution, followed by the development of an *E. coli* standard curve for this bacterium quantification in sea water and drinking water samples. On the other hand, for the standardization of the *Salmonella* spp detection method, DNA extraction from a pre-enrichment culture was evaluated and the Ct value was defined to determine if a sample is positive for *Salmonella* spp. The methodology and results are presented so that the techniques can be applied in microbiological diagnostic laboratories, in research processes and academic practices.

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