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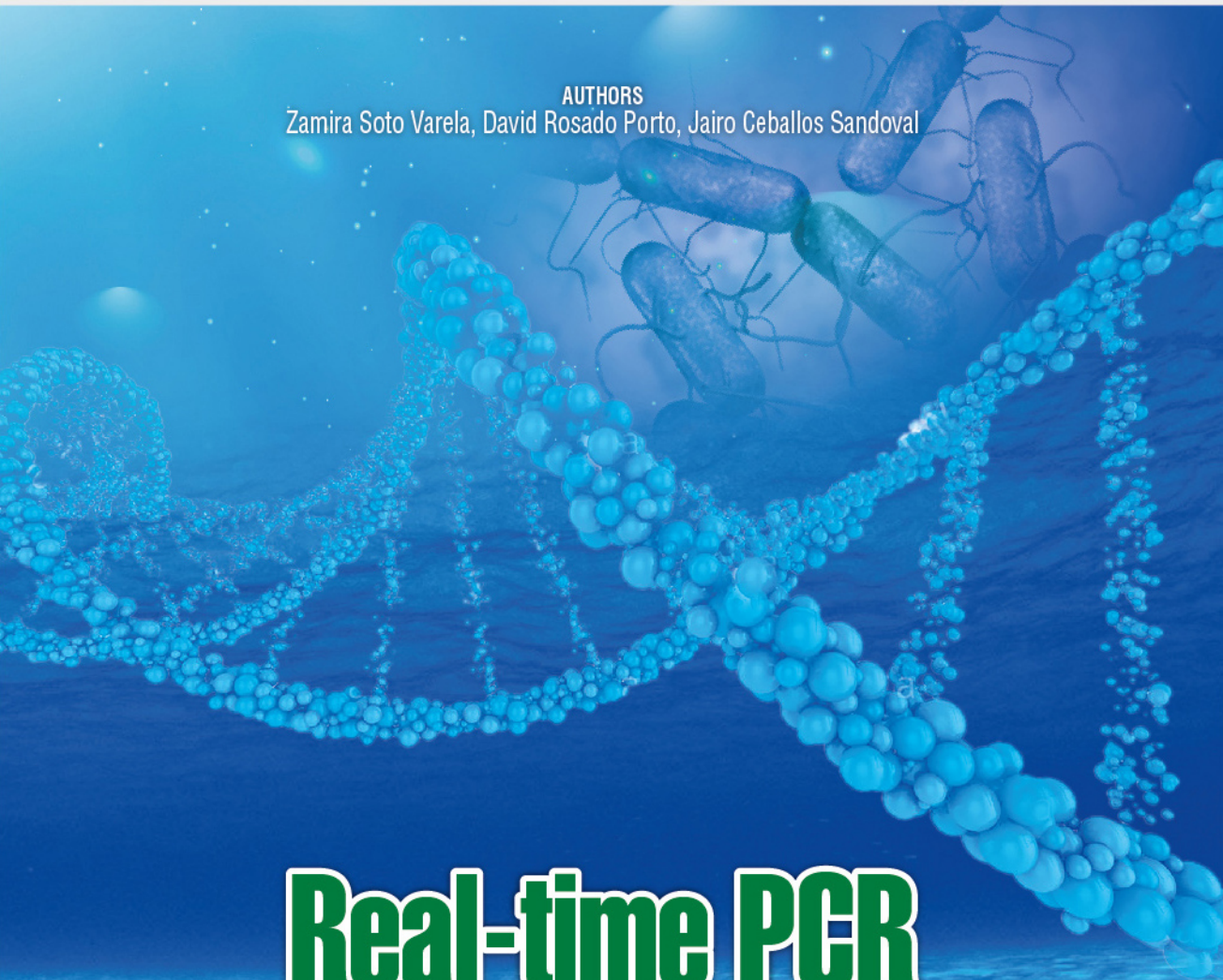

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applied to bacterial waterborne pathogens
detection and quantification

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Preface

Nowadays molecular methods use in the microbiological diagnosis is booming because they allow microorganisms detection in less time with greater sensitivity and specificity; within these, the top technique is the polymerase chain reaction (PCR), that allows to generate multiple copies of a target DNA fragment and by this way to be able to detect it. The PCR has many variants, each of one of them have specific applications, the most used variant is the real-time PCR, which as its name implies, enables the detection of each DNA copy generated at the time, allowing it to be applied in the quantification of gene copies and its expression with a greater sensitivity.

Although traditional microbiological methods continue to be the reference, the use of the PCR technique in various areas such as clinical, agricultural, veterinary, food and environment has been implemented to detect microorganisms of interest, many of them pathogenic to humans, plants and animals. In environment and health study fields, this methodology has been applied, mainly around water quality investigation, leaving aside the opportunity to use it as a routine method, once validated could be used in surveillance and water quality control, whether it be drinking water, recreational water, sea water, water used in agriculture, among others. The advantage offered by the use of real-time PCR is that it allows quantitative data to be released and thus to know the state and evolution of the microbiological water quality with a high sensitivity, since many waterborne pathogens are diluted in high volumes and therefore making difficult its detection.

Many microbiologists, analysts and laboratory technicians find it very difficult to implement these methodologies in their laboratories, believing that they are still novel methods and require a high level of expertise for their implementation, even though many commercial kits are available in the market. Consequently, this book has been written, from the results of the project "Implementation and standardization of qPCR technique for public health importance bacterial pathogenic microorganisms detection as water quality bioindicators", funded by the National Regalias Fund of Colombia. The main aim of the project was the implementation of real time PCR for the water microbiological diagnosis using as model microorganisms *Escherichia coli* and *Salmonella* spp. The former bacterium was used for the development of a quantitative type method and the latter for a qualitative type method.

The present book is categorized as a research project result book and is divided into six chapters, the first is a brief problem introduction and the justification; showing a panorama about the use of the PCR technique in the water microbiological diagnosis; the second chapter corresponds to the theoretical framework and the state of the art and it is exposed initially the theoretical basis of the processes involved in the standardization as the methods of bacterial concentration, DNA extraction and PCR in real time and finally a description of the PCR implementation in the microbiological diagnosis. The third chapter presents the developed methodology for *Salmonella* spp. and *Escherichia coli* techniques standardization. The fourth chapter presents the results obtained from the tests performed according to the described methodology. In the fifth chapter the discussion and conclusion of the results are carried out. Finally, an additional chapter is presented, which compiles the experiences of a guest author on the PCR standardization applied to microbiological diagnosis.

The methodology and results are organized in three sections: the first describes the standardization process of concentration and bacterial elution for *Escherichia coli* DNA extraction from drinking water and sea water samples. In the second, the processes for the elaboration of the standard curve for *Escherichia coli* quantification by real-time PCR and in the third the method standardization for *Salmonella* spp. detection by real-time PCR in drinking water and sea water. At this point it should be clear out that the selected matrices are justified by their impact on public health and environmental quality, being of direct consumption in the drinking water case and for recreational purposes and environmental impact in the sea water case.

The presentation of the book is done in a simple way so that the reader understands the developed methodology and the obtained results. Presented methods can be easily repeated in other laboratories or used to compare the obtained results in order to select the most appropriate strategies for the development of the real-time PCR technique. The readers might be students, teachers, researchers from exact and natural sciences and medicine fields, working on the topic of water microbiological quality or bacterial pathogens molecular diagnosis.

The authors' view is that this book can be very useful for those who work in the biological sciences field, but especially for those who wish to implement the PCR technique in their laboratory, either for research purposes or for the purposes of control and diagnosis. Thus, it is expected that molecular biology techniques, especially PCR, will be considered in a near future a useful tool for routine use in microbiological quality control.

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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Chapter 2

Theoretical framework and state of art on the implementation of a real time PCR for the *Escherichia coli* quantification and *Salmonella* spp detection

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Molecular methods such as polymerase chain reaction are considered an alternative technology in microbiological diagnosis that overcomes some current limitations of traditional culture methods, as they offer the possibility of reducing the test time, improving detection sensitivity and specificity and identify several pathogens at the same time. For PCR implementation it is necessary to take into account certain principles and theoretical bases of the processes that are carried out in the method development for water

microbiological control; starting from bacterial concentration methods, followed by the basis of bacterial DNA extraction and finally the basis of the PCR technique, specifically with the real-time variant.

BACTERIAL CONCENTRATION METHODS

In the microbiology laboratory it is very difficult to detect microorganisms that are in small numbers and besides being accompanied by a microflora and found in a complex matrix (8). Therefore, separation and concentration techniques have evolved to perform a specific detection of microorganism to be investigated, thereby avoiding false negative results (9). The general purpose of the separation and concentration process is to obtain the target cell in high number and separate them from the matrix components that negatively impact its detection, with the consequent reduction in the sample volume and heterogeneity (10,11). Concentration is an important step in improving the detection sensitivity of microbial contaminants, especially by molecular tools that use samples with very small volumes (12).

Concentration methods can be performed under two strategies, those based on non-specific approaches depending on physical and/or chemical principles and those based on specific approaches that are selective and based on ligands with target cell specificity (10). Non-specific approaches include centrifugation, filtration, ion exchange resins, and dielectrophoresis. On the other hand the immunomagnetic separation and flow cytometry stand out among the specific approaches.

Centrifugation is a separation method that uses the rotation around a fixed axis (10). The force generated during rotation drives the suspended particles in a liquid to settle, which depends on several parameters including cell diameter, particle density and solution, volume, angle and spin speed (10,11). Centrifugation has been classified according to the centrifugal force applied in low speed centrifugation (maximum 8000 x g), high speed

(8000 - 60,000 × g) and ultracentrifugation (maximum 700,000 x g) (11). Variables manipulation, such as solution density and particle size have allowed the creation of alternative centrifugation methods such as differential and density gradient centrifugation (11).

Differential centrifugation consists of a gradual increase in the centrifugation speed, sedimenting first the component of larger size and high density, followed by the small size and low density particles. Density gradient centrifugation is performed using a compound (sucrose, ficol and/or percol) that forms a density gradient in the tube in which the particles migrate differentially during centrifugation to the point where their density is in equilibrium with that of the solution, forming bands that can be removed for future analysis (10).

Membranes have traditionally been used to separate substances based on size (13). Filtration is a physical concentration method that consists of removing organisms from a sample, passing the sample through filters with different sizes pores. Despite its easy realization, filtration is very sensitive to the matrix, where large particles tend to clog the filter, inhibitory compounds of PCR could be concentrated with the microorganisms and some filters could inhibit the nucleic acids amplification, which limits the use of this technique (10). However, tangential flow filtration is a technique that reduces filter clogging through parallel flows of tangent fluids to the filter surface, which allows the microorganisms to be concentrated in an efficient, aseptic and rapid manner using a recirculating filtration closed system that allows to handle high volumes of sample that in the case of the water is between 1-1000 L (14,15).

The particles electrokinetic movement refers to the migration of these charged or non-electrically charged particles into a medium in the presence

of an electric field (16). Dielectrophoresis uses the electrical properties of bacteria to separate them from other components under a non-uniform electric field (10). Cells with different physical and chemical characteristics act differently in an electric field, which allows the separation of different bacterial species (11,12). Dielectrophoresis uses a high frequency electric field (0.1-10 MHz) that attracts towards the electrode the bacteria which have a negative electric charge and thus are separated from the matrix (11). Bacteria or other cells with similar charge that are attracted to the electrode are then eluted using a conductivity gradient (10).

Many microorganisms have a negative net charge at pH ≥ 5 due to the ionization of the carboxyl and amino groups of the cell wall (11). The ion exchange resins are composed of small porous polymer spheres that are bonded with positively or negatively charged groups (10). Positive charge electrical exchange resins attract negatively charged bacteria that are adsorbed and can subsequently be eluted by altering pH (10).

Immunomagnetic separation is a specific concentration technique consisting of beads with a core of magnetic material and a polymer group conjugated to an antibody on the surface, known as immunomagnetic beads (17). The immunomagnetic beads can bind to the bacterium and form a bead-bacteria complex which can subsequently be easily separated from the matrix where it is located and concentrated to a smaller volume by applying a magnetic field (18). The immunomagnetic separation specificity is based on the fact that the monoclonal antibodies bound to the immunomagnetic beads are directed against a specific antigen of the bacterium of interest (11).

Although flow cytometry is not a concentration method per se, it is a technique that provides information about a single-cell culture by analyzing intrinsic and extrinsic parameters based on the scattering of

light or fluorescent signals (19). The cells are suspended in a liquid and produce a signal when they pass individually through a beam of light; some cytometers are able to physically separate cell subsets based on their cytometric characteristics, so for specific cell detection these must be labeled with fluorescent molecules (20,21).

BACTERIAL DNA EXTRACTION

In microbiological diagnosis by PCR, DNA extraction is the first step once the bacteria are concentrated, isolated or grown from a sample. Currently, there are several extraction methodologies and it is necessary for molecular biologists to select the technique that best suits their needs (22).

In the case of the PCR technique, the DNA corresponds to be the analyte; therefore, the starting point in the diagnostic methods is the realization of a correct obtaining process of this molecule from the biological material (23), so that a high good quality DNA amount is reached; which means, not being degraded and is free of compounds that may be inhibitors of the technique (24).

The general procedure for extracting DNA from the bacterial concentrate consists of three consecutive steps: the first one is cell lysis, the second corresponds to the degradation of the DNA-associated protein fraction and the last to the DNA purification (23).

In cell lysis the degradation of the bacterial wall and the cell membrane is carried out and in this way the nucleic acid is released from the cells; for this purpose, detergents such as Triton and sodium docedil sulfate (SDS) (22), chaotropic salts that help to denature membrane proteins (23) and enzymes such as lysozyme are used, which act to destabilize the cell wall by breaking the glycosidic bonds between the polysaccharides N-acetyl-glu-

cosamine (NAG) and N-acetylmuramic acid (NAM). Incubation with the enzyme is performed for 1-2 hours at 37 °C (25). The lysis stage is critical and must be aggressive enough to achieve rupture of the cell membrane without damaging the DNA (22).

The second extraction step corresponds to degradation of the DNA-associated protein fraction, which can be done by the addition of an enzyme such as proteinase K (23), as well as using organic solvents such as phenol and chloroform that allow proteins denaturation. Phenol must be double distilled, equilibrated and protected from oxidation by adding 8-hydroxyquinoline. On the other hand, isoamyl alcohol is added to the chloroform in a ratio of 24: 1 (v/v) in order to prevent foaming and facilitate separation of the aqueous and organic phases (26). Proteins separation is enhanced by the use of salts such as ammonium acetate, sodium acetate and sodium chloride at high concentrations (22).

Chloroform-isoamyl alcohol (24: 1) is widely used in extraction methods because of the quantity and quality of the obtained DNA, in this case the extraction is considered liquid-liquid, which is based on the separation of a mixture of molecules based on the solubility difference of each one of them in two immiscible liquids (27). With the use of chloroform-isoamyl alcohol after cell lysis and proteins denaturation, an organic phase is generated in the lower part of the tube where lipids, proteins and cellular debris are found and an aqueous phase in the upper part where nucleic acids and other small water-soluble molecules are. At the interface, most of the proteins are located, due to their content in hydrophobic and hydrophilic amino acids (22).

The final purification step in turn consists of three phases; the first is the DNA precipitation that being insoluble in alcohol, precipitated with cold

ethanol or isopropanol and then recovered by centrifugation (23). When isopropanol is employed the process can be accelerated and precipitation can be performed at room temperature (22). Some techniques improve precipitation by using monovalent cations at concentrations of 0.1 to 0.5 M (26). The second stage is the washing of DNA with 70 % ethanol, at this concentration the DNA is kept precipitated and the remaining water allows the dissolution of the salts. After washing, the obtained pellet is dried for alcohol evaporation (22). In the last phase the resuspension of the sediment obtained is carried out with solutions that ensure its preservation as sterile and DNA se-free water, or with Tris EDTA (TE) buffer. The amount of solution used should be minimal to maintain adequate but sufficient concentration to achieve complete DNA dissolution (22).

Due to the laborious processes of bacterial DNA extraction and the toxicity of organic solvents, extraction kits are now widely used that are based on the same principles, allowing the process to be simpler and faster and obtainable a higher quality DNA (28).

An example of a commercial kit is PureLink ™ Genomic DNA mini Kit, which allows rapid and efficient purification of genomic DNA from breast, tissue, mouse, blood and bacteria samples. This kit is based on the selective binding of DNA to a silica membrane in the presence of chaotropic salts. For lysis of cells and tissues proteinase K at 55 °C is used with a digestion buffer that helps protein denaturation and enzyme activity and RNase is also used to remove RNA; then this cell lysate is mixed with ethanol and a binding buffer that allows the DNA to adhere to the silica membrane located on a column, the impurities are removed with two washing buffers and the DNA is eluded in a low salinity buffer (29).

REAL-TIME PCR

For years, science has focused its research on finding tools and mechanisms directed to the study of DNA, from this have been developed numerous techniques based on molecular bases, such as the PCR technique, whereby millions of specific DNA copies sequence are obtained through the DNA polymerase enzyme for the purpose of using them for a wide variety of purposes (30).

Real-time PCR is a variant of PCR; where the term "real time" refers to the detection of amplified products in each cycle of the reaction (31). This variant is of the quantitative type, since it is possible to quantify the amount of DNA in the sample as the amount of fluorescence of the amplified product labeled (32). Thus, the real-time PCR technique combines amplification and detection in the same step, by relating the amplified product generated in each cycle with a fluorescence intensity signal (33). The emission of produced fluorescence in the reaction is proportional to the amplified DNA amount, which allows knowing and recording the kinetics of the amplification reaction (34).

This technique has the advantage of reducing the diagnostic time, since the amplification and the visualization of the products are carried out simultaneously; obtaining the results in two hours, unlike the conventional PCR that requires 2-3 hours to complete the cycles of amplification, added to the time for products visualization by the electrophoresis. Another advantage is the high specificity and the wide detection range that can be from 2.5 fg to 25 pg (35,36). However, it should be recognized that the presence of inhibitors in different environmental samples and the lack of data on the viability of microorganisms may be some of the problems that must be taken into account when implementing this technique (31). To solve the

problem of inhibitors, an internal control is used that is added at the time of DNA extraction and is amplified while the target segment is detected independently (37).

For the development of real-time PCR, in addition to the reagents used in conventional PCR, it is necessary to use a fluorophore and a device capable of detecting fluorescent signals, a fluorometer to monitor the progress of the amplification reaction, as well as hardware and software for data capture and analysis (35). Fluorescence monitoring generates a typical curve consisting of an exponential phase where the product doubles exactly in each cycle and a linear plateau phase where the reagents are consumed and the reaction is stopped. There is also a baseline that corresponds to the basal level or background of fluorescence during the first cycles of the PCR and the threshold that is the fixed fluorescence level above the baseline where the early exponential phase begins (37).

In this kinetics it is important to highlight the Ct value (Cp or Cq), which is the cycle number at which the fluorescence exceeds the threshold and is detected, this is inversely proportional to the initial concentration of target DNA or RNA present in the Sample (38). From this principle can be generated quantitative data, producing a standard curve that relates the value of Ct obtained with the initial amount of DNA or number of gene copies to be detected. In the case of the microbial load determination of a sample, the standard curve can be made from serial dilutions of established bacterial density (UFC/mL), known concentrations of synthetic oligonucleotides (copies/mL) or cloned plasmids concentrations. The curve is then generated with the amplification data obtained from these standards and the linear regression analysis of them (38).

The fluorescence detection systems used in real-time PCR can be inter-

calators and probes specific for DNA fragments (39). Intercalating agents are fluorochromes that increase the fluorescence emission when bound to double-helical DNA; one of the most used agents is SYBR Green, which provides the advantage of optimizing reaction conditions, in addition to being more economical, but has the disadvantage of being less specific (37).

The specific probes are based on the FRET (Fluorescence Resonance Energy Transfer) principle, which is based on the transfer of energy between two fluorophores: a donor (reporter) and an acceptor (quencher), which emit fluorescence at different wavelengths. When the reporter and the quencher are close, the quencher absorbs all of the reporter's fluorescence, but when separated, the reporter's fluorescence cannot be absorbed by the quencher and can therefore be detected (30).

The most commonly used probe is the taqMan probe, also known as the 5' nuclease probe, because it uses the 5' 3' exonuclease activity of the Taq polymerase to generate the fluorescence signal. The system consists of probe specific use for the gene of interest marked with two fluorophores, a reporter attached to the 5' end such as 6-carboxyfluorescein (FAM) and a 3' terminator such as 6-carboxytetramethylrodamine (TAMRA); Furthermore, it possesses a phosphate molecule which binds to the 3'-thymine terminal residue to prevent the extension of the bound probe during amplification. The 5' exonuclease activity of the DNA polymerase cuts nucleotides from the probe during amplification, whereby the fluorophores are separated and the fluorescence signal is observed (40).

IMPLEMENTATION OF PCR FOR THE DETECTION OF PATHOGENIC BACTERIA IN WATER

Waterborne diseases outbreaks are often not reported in developing countries due to the lack of systematic studies. Microorganisms presence

such as *Salmonella* spp. in water samples provide undeniable evidence of the poor microbiological quality of an urban public service, thus demonstrating the lack of a systematic and regulated mechanism for surveillance and pathogens monitoring in potable water supply, which is a necessity for minimizing waterborne diseases problems (41).

In developing countries, pathogens as *Salmonella* spp. are rarely reported in waterborne outbreaks despite being frequently detected in surface waters, including recreational waters and waters used for irrigation or as a source of drinking water. Increasingly, more and more resistant strains to multiple drugs are present, which represent a greater risk to human health and may contribute to the spread of drug resistance (42). These limitations can place public health at significant risk, leading to substantial monetary losses in health care, as well as costs associated with reduced productivity in the area affected by the outbreak, and the costs held by water quality control departments (43).

Pathogenic bacteria have been detected for a long time using methods based on classical culture, including growth in selective media followed by morphological, biochemical and immunological confirmation; however, these culture methods are time consuming and many bacteria transmitted by water can enter a viable but non-cultivable state in the environment without losing their virulence. In order to solve this problem, methods based on DNA amplification, such as the PCR, have been developed that can improve pathogens detection in the environment (44). For example, in most cases, culture is the standard method and the most specific diagnostic procedure for legionellosis; however, this technique requires a lot of time due to the slow growth rate of this bacterium, the inability to detect non-cultivable viable bacteria and the difficult isolation in contaminated samples with high levels of accompanying flora (45,46).

New technologies based on real-time PCR (RT-PCR) have emerged in recent years as a leading technology for the rapid detection and quantification of pathogenic microorganisms in different media: food products, clinical samples and water, all due to their high degree of sensitivity and specificity, introducing the possibility of a faster detection of the target microorganism in real time, without the need for additional time to detect the PCR products by electrophoresis (43,47); however, molecular identification is used under circumstances such as the species characterization with aberrant biochemical characteristics, recurrent diseases, resistance patterns, for purposes of public health surveillance and publications (48).

Salmonella spp.

The genus *Salmonella*, is a member of the family Enterobacteriaceae (49). Are Gram-negative bacilli, facultative anaerobes, with a size from 2 to 3 by 0.4 to 0.6 microns, non-spore forming, mobile, oxidase negative and catalase positive, producing gas from D-glucose and can be used citrate as the sole source of carbon (50,51). The genus is composed of two species that are *S. bongori* and *S. enterica*, and the latter is divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae,* and *indica*. S. enterica subsp. *enterica* includes over 1,500 serotypes, which despite their high genetic similarity vary greatly in their wide range of hosts and diseases, ranging from enteritis to typhoid fever. The degree of adaptation to the hosts varies between the serotypes of *Salmonella* spp and determines the pathogenicity. Serotypes adapted to humans, such as S. Typhi and S. Paratyphi A, B, C, cause systemic typhoid fever (52). The genome of Salmonella spp. contains about 4.8 to 4.9 million base pairs, with approximately 4400-5600 coding sequences (50).

One of the main pathways for *Salmonella* spp. transmission is contaminated water (53); therefore, the increased risks of salmonellosis are associated

with the ingestion of water with human or animal feces (51). Likewise, contamination with sewage, freshwater and coastal marine waters is also a source of fecal microorganisms, including *Salmonella* spp. (51,54). In the productive field, water with presence of *Salmonella* spp. has the potential to contaminate fresh products such as fruits and vegetables at various points in the production cycle (55). In view of the above, it is necessary to guarantee the microbiological quality of the water from all areas in order to avoid salmonellosis outbreaks (55). The best strategy for prevention is to sample and detect this microorganism with a rapid, sensitive, specific and simple method. Several rapid detection methods have been developed to detect *Salmonella* spp. in water and food, including PCR. This method has significantly reduced the test time required for confirmatory results from 4-6 days to less than one day (56).

Several papers have been developed that seek the standardization and optimization of PCR for *Salmonella* spp. detection in water, one of them optimized the detection of *invA* in drinking and surface water, this gene is indispensable for the entry of this pathogen to the epithelial cells and is used as a reference marker for *Salmonella* spp. by PCR. In this study it was shown that the sensitivity of PCR can be influenced by the physical dilution of the bacteria in the aquatic environments, so a culture stage must be developed before applying the technique, thus avoiding the problem of DNA amplification from dead cells, in addition to preventing the effect of PCR inhibitory substances present in water. The incorporation of a non-selective pre-enrichment step of 6 h incubation increased the detection limit to 26 CFU/mL in comparison to methods that not applied this step which was 2.6 x 10^4 CFU/mL. Likewise, it was demonstrated that the development of the PCR directly on environmental waters samples did not detect Salmonella spp. But, when the PCR is performed after an enrichment step; the analyzed samples containing *Salmonella* spp. were positive by PCR (57).

The PCR technique has been implemented in several studies related to water quality control. Thus, in the urban water systems of Nepal, the occurrence and diversity of *Salmonella* spp. serotypes was determined. The isolated Salmonelas were analyzed by PCR by the detection of the *invA* gene and the *spvC* gene, the latter encoded a virulence plasmid, important for this pathogen survival in host cells. The results of the study revealed that *Salm.* Paratyphi A and *Salm.* Typhimurium isolates were positive for the *invA* gene and negative for the *spvC* gene; however, one isolate of *Salm.* Typhimurium and two of *Salm.* Entiritidis were positive for both the *invA* gene and the *spvC* gene, the presence of the latter probably due to horizontal gene transfer (41).

As well, in Iran, the microbiological tap water and bottled drinking water quality was evaluated in 448 samples, making the comparison between the traditional culture method and the PCR. The culture method showed that 4 (2.77 %) of 144 tap water samples and no sample of 304 bottled drinking water samples were positive for *Salmonella* spp; While 5 (3.47 %) of 144 analyzed samples of tap water were detected through PCR. Therefore, with these results it can be considered that the PCR technique has a greater precision for the detection of this bacterium in water samples (1).

Other variants of PCR have also been applied in the diagnosis of *Salmonella* spp. in water samples; such as multiplex PCR (m-PCR) for the detection and quantification of this bacterium simultaneously with other pathogens such as *Campylobacter* spp. and *Escherichia coli* O157:H7 in the same reaction. Specifically, for the detection of *Salmonella enterica* Typhimurium a pair of primers were used for the detection of the *invA* gene at a concentration of 960 nM. Three types of uniplex, duplex and triplex PCR were performed in the study. The quantitative modality was developed through real-time PCR using SYBR Green and determining for

each target a different melting temperature, which allowed distinguishing and quantifying the three pathogens at the same time in the same reaction. In uniplex PCR the detection limit for S. *enterica* Typhimurium was 7.33 x 10^2 copies, while m-PCR showed a detection limit of 7.33 x 10^3 copies in mixed culture samples, this could be due to the competition of primers, as well as the dimers formation (58).

The application of real-time PCR variant been carried out in home water distribution systems, where a qPCR evaluated the microbiological quality of tap water from 24 households fed from rainwater tanks in Queensland, Australia. Among the 24 households, 4 % of samples from the rainwater tank contained *Salmonella* spp., being attributed as a possible source of contamination to bird feces (59).

Although qPCR is considered as a rapid and specific method to detect and quantify pathogens, it is unable to distinguish DNA signals that originate from living or dead cells; thus, dead cells can produce false positive results (26). To overcome these limitations, mono-acid propidium (PMA) combined with qPCR (PMA-qPCR) has been used as a strategy to achieve the most efficient exclusion of DNA amplification from dead cells. PMA is a DNA/RNA intercalation dye that can selectively penetrate through the damaged cell membrane of dead cells by covalently binding to DNA. PMA treatment is applied prior to DNA extraction, it is a simple method for the quantification of viable cells since after photoactivation it avoids the PCR amplification of genetic targets from non-viable cells (59).

The table 1 summarizes some of the PCR based techniques reported in the literature as methods for evaluating the microbiological quiality of water; where indicated the technology applied, the type of sample, the target gene, the prime or primers used and/or sequence, amplicon size and detection limit.

Table 2.1.
***Salmonella* spp. PCR-based techniques reported for monitoring the microbiological quality of the water**

Method	Type of sample	Target gen	Primers and/or sequence (5'-3')	Amplicon size(bp)	Detection limit	Reference
qPCR	Rain water	Inv A	F: ACAGTGCTCGTTTACGACCTGAAT R: AGACGACTGGTACTGATCGATAAT	244	7.3 X 10^3 cells/L	(59)
PCR	Tap water and bottled water	IpaB	F: GGACTTTTTAAAAGCGGCGG R: GCCTCTCCCAGAGCCGTCTGG	NR	NR	(1)
PMA-PCR	Drinking and environmental water	ttr	F: CTCACCAGGAGATTACAACATGG R: AGCTCAGACCAAAAGTGACCATC	95	1 CFU / PCR ó 10 cells/ mL	(5)
Multiplex-PCR	River water	16S rDNA	Sal-F: CGGGCCTCTTGCCATCAGGTG Sal-R: CACATCCGACTTGACAGACCG	396	NR	(61)
qPCR using SYBR Green	Lake water	invA	139: GTGAAATTATCGCCACGTTCGGGCAA 141: TCATCGCACCGTCAAAGGAACC	284	10^3 cells mL^{-1}	(62)
qPCR	Drinking, Surface, rain water and treated waste water	invA	F: CACCAAGAAGGTGACTTTATTGTG R: GAACTTATAACCACCCGCG	284	1 CFU/ mL	(6)
PCR	Raw water from treatment plant	invA	invA1: ACAGTGCTCGTTTACGACCTGAAT invA2 AGACGACTGGTACTGATCGATAAT	244	200 cells/PCR	(63)

NR: not reported. F: forward. R: reverse

Escherichia coli

The enterobacteria *Escherichia coli* represents one of the best studied microbial organisms in the world (64,65). In the year of 1885 the pediatrician German Theodor Escherich described in detail Bacterium coli commune now known as *Escherichia coli* in honor to his name (66). With regard to the taxonomy of this microorganism are details in table 2.2.

Table 2.2.
Taxonomy of *Escherichia coli*

Taxonomia *E. Coli*	
Domain:	Bacteria
Kingdom:	Eubacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Escherichia*
Specie:	*E. coli*

Source: Second edition of the handbook of bergey's (67).

E. coli is a gram-negative bacterium in the form of straight cylindrical rods of 1.1 -1.5 μm in diameter and 2.0 - 6.0 μm in length can be alone or in pairs and have mobility by perimeter flagella, although some strains are not motile. *E. coli* strains are catalase positive and oxidase-negative fermenters of glucose with gas production and most also use lactose, except for some serotypes such as *E. coli* O157:H7. It is able to grow both aerobically and anaerobically, with a wide temperature range between 15-45 °C and an optimum temperature of 37 °C. This bacterium have a pH between 5-9, which confers resistance to adverse environmental conditions (68,69).

According to Van Elsas and collaborators, data suggest that *E. coli* may persist for varying periods of time in terrestrial and aquatic habitats, which is why an important aspect of some strains of *E. coli* is their ability to acquire nutrients producing structures filaments that extend from the cell surface and allow the cell to adhere to plant surfaces, it is thanks to this peculiarity that *E. coli* from contaminated soils, manure, irrigation water or seeds can colonize some plants such as radish or Lettuce. Pathogenic strains of *E. coli*, such as *E. coli* O157:H7, represent a threat to the food chain and an environmental risk still underestimated (70).

The size of the *E. coli* genome varies between 4.6-5.5 Mb (millions of base pairs) depending on the strain and the serotype. For example: for strain K12 is 4,639,221 base pairs (pb) and the chromosome of strain O157:H7 has 5,528,445 bp being the largest size (71).

E. coli is present in the normal microbiota of the intestine of humans and animals, but of the strains of *E. coli* are not pathogenic and live harmLessly in the colon; Although in some cases *E. coli* may become an opportunistic pathogen in immunocompromised individuals; however, it should be noted that some serotypes or clones can cause intestinal and extraintestinal diseases in healthy people due to a series of virulence factors (69).

These virulence attributes are frequently coded in mobile genetic elements such as transposons, plasmids, pathogenicity islands or phages that can be mobilized between the different strains by horizontal gene transfer to create new variants of virulence factors or in some cases these genetic elements, that in some time they were mobile have, evolved to become part of their genome. These factors of virulence have persisted in some strains and have become 'Pathotypes' of *E. coli*, as is the case of the famous serotype O157:H7 or others less common as the variant of *E. coli* O104: H4 capable of causing diseases in healthy individuals (64,72,73); but without a doubt as to public health the most important serotype is *E. coli* O157:H7 which was first isolated in 1975 (74).

A large part of these pathotypes cause public health problems and high morbidity and mortality throughout the world. This is because they have low infectious doses and are transmitted through ubiquitous media, including food and water. Outbreaks are common in both developed and underdeveloped countries, sometimes the final resolution of the disease has fatal outcomes; hence the importance of international and national surveillance programs for *E. coli* infections in humans, animals, food and the environment (68,75).

There are six main diarrheogenic *E. coli* pathophyses: enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC) and Diffuse adhesion *E. coli* (DAEC) (76).

Recently, new strains have been described as the case of the EPEC/ETEC hybrid strain in a child with acute diarrea (77) and in patients with hemolytic uremic syndrome and diarrhea the STEC/ETEC hybrid (70) and the particular case of a shigatoxin-producing E. coli identified in Germany that

caused one of the largest outbreaks of uremic-hemolytic syndrome in the world, with the suggestion of the name of *E. coli* entero-aggregative-hemorrhagic (EAHEC), thus demonstrating the plasticity of the *E. coli* genome (67).

There is enormous diversity within the species, which differs in subgroups based on various physiological, morphological and antigenic characteristics (65). *E. coli* has four surface antigens, although it is true that polysaccharide O and flagellin are the two major antigens of gram-negative bacteria, also known respectively as antigens O and H (79), the capsular antigen (K) and Fimbrial antigen (F) have also been used to serotype strains (80).

Classical serotyping is based on the Kauffman classification scheme, where polysaccharides (O) (somatic), surface antigens (H) (flagellar) and capsular antigens (K) are determined (81). Molecular methods such as PCR, where the identification of genes involved in the biogenesis of antigen (O) (eg, wzx and wzy genes) and fliC for antigen (H), can also be used to identify the serotype (65,68).

There is an extensive genetic substructure within *Escherichia coli* species. Clermont and collaborators described a triplex PCR where *E. coli* isolates fit into one of four known A, B1, B2 or D phylogroups (82) but in 2012 Clermont developed a new method based on a quadruplex PCR that allows to assign an isolation of *E. coli* to one of the eight phylogroups that are currently recognized: seven (A, B1, B2, C, D, E, F) belong to *E. coli* Sensu stricto while the eighth is the Escherichia cryptic clade I. This new differentiation between strains occurred due to increasing body data from multiple locus sequences and genome data for *E. coli*. This new quadruplicate PCR allows correctly assigning more than 95 % of the isolates of *E. coli* to a phylogroup (83).

Traditional culture methods for pathogenic *E. coli* serotypes are time consuming and laborious. For this reason, molecular techniques that allow a faster identification of the different pathotypes (68) are commonly used today; thus the available evidence suggests that the chromogenic means for the detection of strains of the Shigatoxigenic Escherichia coli (STEC) group do not have sufficient sensitivity or specificity to replace methods that directly detect toxin or toxin genes in samples. Accordingly, most Perry (2017) studies have concluded that the optimal use of such media is for the isolation of STEC from samples that are determined to be positive using more sensitive methods of detection, eg PCR (84).

The table 2.3 summarizes some of the PCR-based techniques reported in the literature as methods for evaluating the microbiological quality of water, selected because of the interesting approaches used in the development of a new protocol or the promising results obtained. The technology applied, the type of sample, the target gene, the primer or primers used and/or the sequence, amplicon size and detection limit are indicated.

Tabla 2.3.
***E. coli* PCR-based methods for monitoring the microbiological quality of the water**

Type of PCR	Type of sample	Target gene	Primer sequence (5'→ 3')	Amplicon size (bp)	Detection limit	Reference
Multiplex PCR	Water samples	aaiC	F: AGAGCGTCCACTGTCAGAGCGT R: GCGACCTGCTCTGGCGTGAAAT	183	5.2 ng/µL	(85)
		escV	F: TAACGCCTGCGCGCATATCACC R: GTTGATGCGCCTGTCGCTAGT	266		
		bfpA	F: TCTGCAATGGTGCTTGCGCTTG R: CAGTTGCCGCTTCAGCAGGAGT	478		
		stx1	F: AGCGATGCAGCTATTAATAA R: GAAGAGTCCGTGGGATTACG	130		
		ipaH	F: CAGGTCGCTGCATGGCTGGAAA R: GGCAGTGCGGAGGTCATTTGCT	383		
		stx2	F: TTAACCACACCCCACCGGGCAGT R: GCTCGGATGCATCTCTGGT	346		
		Elt	F: AGGCGTATACAGCCCTCACCCA R: ACCTGAAATGTTGCGCCGCTCT	550		
Multiplex PCR	Artificial water samples and Sample city water distribution network	uidA	UAL: TGGTAATTACCGACGAAAACGG UAR: ACGCGTGGTTACAGTCTTGCG	147	NR	(86)
		lacZ	LZL-F: ATGAAAGCTGGCTACAGGAAGGCC LZR-R: CACCATGCCGTGGGTTTCAATATT	876		
qPCR and PMA-qPCR	Drinking and process water samples	uidA	ECN1254-F: GCAAGGTGCACGGGAATATT ECN1328-R: CAGGTGATCGGACGCGT	75	12-120 bacterial cells	(87)
Multiplex PMA-qPCR	Irrigation water	stx1	stx1-F GACTTCTCGACTGCAAAGAC stx1-R TGTAACCGCTGTTGTACCTG	306	1 CFU / 100 mL	(88)
		stx2	stx2-F CCCGGGAGTTTACGATAGAC stx2-R ACGCAGAACTGCTCTGGATG	482		
		eae	eae-F GCGCGTTACATTGACTCCCG eae-R CCATTTGCTGGGCGCTCATC	245		
		ehxA	ehxA-F TCTGTATCTGCGGGAGTTAG ehxA-R CAACGTGCTCAAACATAGCC	136		
		uidA	PT-2 GCGAAAACTGTGGAATTGGG uidA-R TCGTCGGTAATCACCATTCC	382		

NR: *Not Reported.* ***F:*** *Forward.* ***R:*** *Reverse*

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Chapter 3

Development of a methodology for the standardization of a real time PCR for the *Escherichia coli* quantification and *Salmonella* spp detection

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The methodology will be presented in two sections, the first one will describe the standardization process of *Escherichia coli* quantification method and the second one section will refer to the process to standardize the method for *Salmonella* spp. detection using real-time PCR. As well, the first section will cover two numerals; the first one corresponds to the standardization of the concentration and bacterial elution process for DNA extraction and the second to the standard curve elaboration for *Escherichia coli* quantification. The second section presents two numerals, the first comprises the DNA extraction method evaluation from pre-enrichment cultures for *Salmonella* spp. detection and the second to the Ct value definition to establish when a sample is positive.

CONCENTRATION AND BACTERIAL ELUTION TECHNIQUES STANDARDIZATION FOR DIRECT DNA EXTRACTION FROM HUMAN DRINKING WATER AND SEA WATER

A series of tests were carried out in which concentration and bacterial elution methods were tested, each of which consisted primarily in the preparation of an *E. coli* inoculum, followed by its addition into the water matrix, then the concentration and the elution method continued and finally the DNA extraction by the organic methodology with chloroform: isoamyl alcohol (24: 1).

The proposed methodologies evaluation was performed by microbial counts on Tryptic Soy Agar-TSA agar and the DNA quality and concentration measured by spectrophotometry. The selected strategies were evaluated verifying the assay reproducibility using the real-time PCR technique. The general scheme of work is presented in figure 3.1.

Inoculum preparation

For each assay the inoculum was prepared by culturing a single *Escherichia coli* ATCC 25922 colony in Brain Heart Infusion-BHI broth and incubated at 37 °C for 24 hours. After incubation, the inoculum optical density was adjusted by spectrophotometry and tenfold dilutions were performed in peptone water (0.1 %).

Inoculation

The inoculation in each assay was performed by taking different volumes of 10^{-6}, 10^{-7} and 10^{-8} dilutions, according to the amount of cells required in each assay; these were added to 100 mL volumes. The number of inoculated cells was corroborated by plate counts on TSA agar.

Bacterial concentration methods

Centrifugation

Inoculated samples were added to 50 mL Falcon type tubes and centrifuged at 5,800 rpm for 20 minutes, in a later step the supernatant was discarded and the rest of the sample was centrifuged under the same conditions. The supernatant was again discarded and the pellet obtained was resuspended in 3 mL of buffer Phosphate-Buffered Saline-PBS pH 7, vortexed for 3 minutes and cultured in TSA agar. The petri dishes were incubated at 37 °C for 24 hours, and culture plate counts were performed.

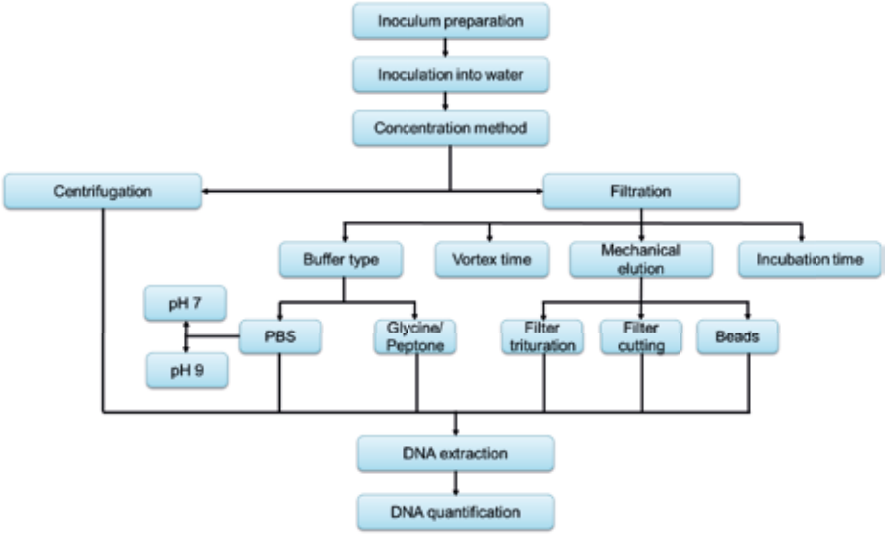

Figure 3.1.
Flowchart of *E. coli* concentration and elution methods standardization for DNA extraction

Filtration

The inoculated water samples were filtered through® Millipore stainless steel filtration equipment and 250 mL capacity polysulfone vessels using 0.45 µm pore size cellulose acetate filters. Initially a flushing with 100 mL

of sterile water was performed to purge the system, then each membrane filter was placed and 100 mL of the inoculated water was filtered for each test. After each filtration two washes were performed with sterile water in each replicate with 200 mL of sterile distilled water.

Methods of elution

Different strategies were tested to perform the cells elution from the filter, evaluating various variables such as buffer type, vortexing time, incubation time and mechanical elution strategies such as filter cutting, filter trituration and the use of zirconia beads.

Buffer type and vortex time

Three buffer were tested: glycine/peptone buffer (0.05 M glycine and 3 % peptone water), PBS buffer pH 7 and PBS buffer pH 9, for each buffer 3 replicates were made. The process consisted that after filtration the filter was placed in 50 mL Falcon type tubes with 5 mL of the buffer to be tested and then vortexed for 3 and 5 minutes, 1 mL of each tube was taken and cultured in TSA agar; as well, after vortex time the filter was taken and placed in a Petri dish with TSA agar. Petri dishes were incubated at 37 °C for 24 hours.

Incubation time

This test consisted of evaluating two different filter immersion times (10 and 20 minutes) into two different PBS buffer (pH 7 and pH 9). To perform this, after filtration, filters were placed in a 50 mL Falcon type tubes with 5 mL of PBS buffer pH 7 or pH 9, leaving them immersed according to the two evaluated times. Three replicates were performed for each time and evaluated buffer. After incubation time, each tube was vortexed for 3 minutes and 1 mL of each tube was taken and cultured in TSA, at the same time the filter was taken and placed in a Petri dish with TSA.

Mechanical elution

Three strategies were evaluated to perform a mechanical elution, among them are: filter cutting, filter trituration and zirconia beads. For each of them three replicates were made.

- **Filter cutting:** After filtrations, each filter was taken and cut into pieces with sterile scissors and dropped into 50 mL Falcon type tubes with 5 mL of each buffer (PBS pH 7 and glycine/PBS buffer), then vortexed for 10 minutes, then the entire supernatant was centrifuged at 12000 rpm for 10 minutes (3 centrifugations in the same tube), the supernatant was discarded, from the pellet obtained the extraction of DNA was carried out by the organic method.

- **Filter trituration:** After filtration, each filter was taken and putted into an Eppendorf® tube with 600 µL of glycine/peptone buffer, each filter was triturated with a sterile, alcohol-flamed forceps and vortexed for 10 minutes; the supernatant was then taken and added to a new Eppendorf® tube and this was subjected to centrifugation at 12,000 rpm for 5-10 min, then the supernatant was discarded and followed the organic DNA extraction protocol with the obtained pellet.

- **Zirconia Beads:** After filtration, each filter was placed in an Eppendorf tube with 600 µL of Buffer TE (1 X), 30 µL SDS (10 %) and Zirconia beads (approximate amount measured with 0.2 mL Eppendorf® tube). The tubes were vortexed for 10 minutes for mechanical lysis and then DNA was extracted to the obtained lysate by the organic method. A control was performed with no addition of zirconia beads.

DNA extraction

600 µL of 1X TE Buffer (10mM Tris-HCl, 1mM EDTA pH 8.0), 30 µL of

SDS (10 %) and 12 µL of lysozyme (10 mg / mL) were added to each pellet in a reaction tube and vortexed for 1 minute. The tubes were incubated for an hour and a half at 37 °C. After the incubation time, 80 µL of sodium acetate (3M) and 100 µL of sodium chloride (5M) were added to each tube and shaken vigorously for 30 seconds. Afterwards, the tubes were heated to 65 °C in a water bath for 10 minutes. Then, the volume of each tube was divided into equal parts and double volume of chloroform: isoamylic alcohol (24: 1) was added. They were centrifuged for 5 minutes at 12.000 rpm. The two supernatants from each tube were added into a new reaction tube, and twice the volume of cold isopropanol were added and mixed by inversion. Subsequently, they were centrifuged for 5 minutes at 12.000 rpm and the supernatant was discarded. 500 µL of 70 % cold ethanol was added to each tube and centrifuged for 5 minutes at 12.000 rpm and the supernatant discarded. The tubes were left open for overnight ethanol residues evaporation. The DNA was hydrated with 15 µL of MilliQ water and heated in a bath at 55 °C for 20 minutes to resuspend it and proceed to quantify it.

In the case of DNA extraction with zirconia beads, the process started after cell lysis; from the obtained lysate the filter was removed and the salt precipitation step was carried out with sodium acetate and sodium chloride and the DNA extraction process was continued. In the case of controls, lysis was performed directly with the filter in the Eppendorf® tube without the beads, which contained 600 µl of 1X TE Buffer and 30 µl of SDS (10 %), incubated for one hour and half at 37 °C, the filter was then removed and continued with the DNA extraction process.

DNA quantification

The DNA was quantified by spectrophotometry at λ260 nm and λ280 nm on Eppendorf® Bio-spectrometer.

Real-time PCR amplification

A real-time PCR was performed with diluted DNA at 10 ng/µL which was extracted in the mechanical elution assays. The amplification was performed on the Biorad® CFX 96 equipment following the indications of the advanced kit for *E. coli* of Primer Desing® according to the following protocol and using the amounts described in table 3.1:

I. 95 °C for 2:00 min.
II. 95 °C for 10 sec.
III. 60 °C for 1:00 min (reading).
IV. From the third step, it goes to the second step 50 times.

Tabla 3.1.
Primer Design® advance kit master mix for *E. coli*

Reagent	Quantity
Master Mix OasigTM or Precision PLUSTM 2x qPCR	10 µL
Primer/Probe for E. coli	1 µL
Primer/Probe internal extraction control	1 µL
RNAsa/DNAsa free water	3 µL
Final volume	15 µL

Protocol verification in drinking water and sea water

A verification and optimization test of the direct method for *E. coli* quantification in drinking water was carried out, using the filter trituration protocol with glycine/peptone buffer in a volume of 600 µL. Centrifugation time was increased from 5 to 10 minutes after bacterial cells elution from the filter. The number of inoculated cells was 56 CFU, which were added directly in the 100 mL of water into the filtration vessels, seven replicates of this procedure were performed. In addition, a test with different CFU concentrations was carried out, performing theoretical calculations to obtain the quantities of 50, 100, 500 and 1000 CFU. It should be clarified that according to the microbial count, where 50 CFUs were expected, a count of 13 CFUs was obtained and where 100 CFUs were expected, a count of 56 CFUs was obtained.

Also, the standardized direct protocol was verified in sea water samples, 100 mL samples were inoculated directly into the filtration vessel to have an approximate amount of 500 CFU in the total volume to be filtered. Seven replicates were made. The extracted DNA was quantified and then diluted to give a final concentration of 10 ng/µL. Replicates R2 and R4 were also diluted to a final concentration of 20 ng/µL and 50 ng/µL.

DEVELOPMENT OF A STANDARD CURVE FOR *E. coli* QUANTIFICATION IN WATER SAMPLES

Initially two preliminary tests were developed for calibration curves elaboration using *Salmonella* spp and *E. coli*, in order to adjust the working methodology. In the first test, the results were obtained until DNA quantification step and in the second real time PCR amplifications are performed but only for the case of *E. coli*. Once preliminary results were analyzed and confirmed, seven replicates were carried out, which enabled the standard curve for *E. coli* to be elaborated from obtained data analysis. The activities carried out for this purpose were the following:

Inoculum preparation

Two test tubes with BHI broth were inoculated with a colony of *Salmonella enteritidis* ATCC 13076 and *Escherichia coli* ATCC 25922 respectively and incubated at 37 °C for 24 hours. Afterwards tenfold dilutions were made in 9 mL of 0.1 % peptone water.

In the first preliminary test, it was worked with both bacteria by taking 100 µL and 50 µL from dilutions 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} and added to a reaction tube by triplicate, and the same inoculum volume was plated in TSA agar to establish the count of them.

In the second preliminary test, 1000 µL were taken from dilutions 10^{-0} to 10^{-8}

in the case of *Salmonella* spp. and added to 1.5 mL reaction tubes and for *E. coli* 100 µL was added from dilutions 10^{-2} to 10^{-8}. All assays were performed by triplicate and the same amount was also cultured on TSA agar. The final assays for the *E. coli* standard curve development were carried out according to the second preliminary test with 100 µL from dilutions 10^{-0} to 10^{-8}.

DNA extraction

In the first preliminary test each reaction tube with inoculum was added with buffer TE (1X): the tubes with 50 µL were added with 550 µL and tubes with 100 µL were added with 500 µL. In the case of second preliminary test 1 mL of *Salmonella* spp. culture was centrifuged at 12000 rpm for 5 minutes and 600 µL of TE buffer was added to the obtained pellet.

Subsequently, 30 µL of SDS (10 %) and 12 µL of lysozyme (10 mg/mL) were added to each tube and the extraction protocol was followed as described in the page 44.

The DNA was hydrated with 20 µL of Milli Q water and heated in a water bath at 55 °C for 20 minutes to resuspend it and proceed to quantify it by spectrophotometry at λ260 nm and λ280 nm in an Eppendorf® Bio-spectrometer.

Real-time PCR

E. coli DNA from the second preliminary assay and the replicates made to elaborate the standard curve was used for real-time PCR amplification following the instructions from the Primer Design® advance kit for E. coli following the protocol described in the page 47.

METHOD DEVELOPMENT FOR *Salmonella* spp. DETECTION BY REAL TIME PCR IN DRINKING WATER AND SEA WATER

The developed methodology for *Salmonella* spp. qualitative detection method standardization encompasses first the realization of three preliminary tests to evaluate the pre-enrichment process of the filter using conventional PCR; which differed in the matrix and in the inoculum amount used. After this, the protocol was verified using real-time PCR in the drinking water and sea water matrices and through these tests the DNA extraction method was evaluated and finally an assay was performed for the determination of the Ct value to define the whether a sample is positive for *Salmonella* spp.

Preliminary tests

- The first one was developed with two 500 mL samples of distilled water inoculated with 95 CFU/100 mL and 10 CFU/100 mL.
- The second assay was developed with two 1L samples of drinking water taken directly from the tap and inoculated with 327 CFU and 9.5 CFU. In the case of the 9.5 CFU sample, DNA extraction was done by both the organic method with chloroform: isoamyl alcohol (24:1) and using the Invitrogen brand Pure Link® Genomic DNA mini kit.
- The third test was performed with two seawater samples, one of 100 mL inoculated with 485 CFU and the second of 1000 mL inoculated with 156 CFU.

In the three assays the common stages of the process were:

Inoculum preparation

The inoculum was prepared by taking a *Salmonella entirica* serovar Enteritidis ATCC 13076 colony, cultured in BHI broth and incubated at

37 °C for 24 hours. After incubation time, the inoculum optical density was adjusted to 1.9 by spectrophotometry with sterile BHI broth, afterwards 10 folds dilutions were made in 9 mL of 0.1 % peptone water.

Inoculation

The inoculation was performed by taking different volumes of the 10^{-6}, 10^{-7} and 10^{-8} dilutions according to the amount of cells required, which were added to the water volumes indicated for each assay. The inoculated cells amount was corroborated by plaque count on TSA agar.

Filtration

The water samples were filtered through Millipore® stainless steel filtration equipment and 250 mL capacity polysulfone vessels using 0.45 µm pore size cellulose acetate filters. Initially a wash with 150 mL of sterile water was performed before the filtration process started; each filter was placed and the filtration of 100 mL or 1 L of the inoculated water was performed according to each test. After each filtration, two washes were performed with 250 mL of sterile distilled water.

Pre-enrichment

After the filtration process, each filter was introduced into 250 mL of buffered peptone water and allowed to incubate at 37 °C for 18-24 hours.

DNA extraction

For DNA extraction, 1.5 mL of each pre-enrichment culture was taken, placed in a reaction tube and centrifuged for 5 minutes at 12000 rpm to obtain the cellular biomass, subsequently the supernatant was discarded from the tube. To each pellet, 600 µL of buffer TE (1X), 30 µL of SDS (10 %) and 12 µL of lysozyme (10 mg/mL) were added and vortexed for 1 minute. Later, the process was carried out as described in the page 46.

The DNA was hydrated with 20 µl of Milli Q water and heated in a bath at 55 °C for 20 minutes to resuspend it and proceed to quantify it by Spectrophotometry λ260 nm and λ280 nm in a Eppendorf® Bio-spectrometer.

Polymerase chain reaction

Obtained DNAs were amplified by conventional PCR for *InvA* gene detection, according to the protocol described by Villarreal and collaborators in 2008, which is shown in Table 3.2.

Table 3.2.
Master mix for *InvA* gen amplification

Reagent	Final concentration
Magnesium chloride	2mM
Buffer	1X
dNTPs	0.2 mM
Primers	0.5 µM
Taq polimerase	0.75 U

It was completed for a final volume of 20 µL and 5 µL of each DNA sample. Amplification conditions were initial denaturation for 2 minutes at 95 °C, followed by 30 cycles comprised of one minute at 95 °C, one minute at 59.9 °C, one minute at 72 °C and one final step of extension of 5 minutes at 72 °C.

Electrophoresis

The amplified DNA was run on a 2 % agarose gel at 50 V for an hour and a half.

Evaluation of the Real Time PCR Method for the detection of *Salmonella* spp. in water

Once the three preliminary assays with different water matrices and amounts of inoculated bacteria were developed, a complete test was carried out for the evaluation of the real-time PCR technique with seven replicates for drinking water and sea water.

Water sampling and sample preparation

For these tests, in the case of drinking water samples were taken directly from tap water, for that purpose the tap was disinfected with alcohol at 70 % and water was allowed to run for 3-5 minutes. Sterile 1 L flasks were added with 2mL thiosulphate at a concentration of 20 mg/L and filled with 1 L of tap water. In relation to sea water, the samples were taken at three different beaches along the Colombian Caribbean region: Arrecife beach (Tayrona National Park), Salgar and Puerto Colombia. Samples were taken to the research laboratory and added into 1 liter sterile flasks.

Inoculation

Drinking water samples were inoculated with 4 CFU and sea water samples with 10 CFU. A control without inoculation was performed to each sea sample and the presence of *Salmonella* spp. in these samples was verified by ISO method for *Salmonella* spp. detection in water, which yielded a negative result for each one of the samples.

Filtration

In the sea water filtration process, for each sample, the total volume of 1 L was divided in four filtrations of 250 mL, because the filter was clogged, changed at each filtration and added to the pre-enrichment culture medium.

DNA extraction

DNA extraction was performed from the pellet obtained from the pre-enrichment culture as indicated in the preliminary tests by both the organic method with chloroform: isoamyl alcohol (24:1) and the commercial Invitrogen® Pure Link Genomic DNA mini kit, following the manufacturer's instructions.

The extracted DNA quality was measured using the Eppendorf® Biospectrophotometer and before performing the real time PCR, in each replicate the DNA was diluted to a concentration around 10 ng/µL.

Real-time PCR

Real-time PCR was developed following the manufacturer instructions of Primer Design® All Pathogenic *Salmonella* species Kit, which is provided with a TaqMan® probe that is read by the FAM channel and also has an internal extraction control, which was added at the moment of cell lysis and amplified at the same time as the target sequence using another TaqMan® probe detected on the VIC channel. Amplification was performed on Bio-Rad Laboratories® CFX96 CFX96™ equipment following the protocol: 95 °C for 2:00 min, followed by 50 cycles of 95 °C for 10 sec and 60 °C for 1:00 min. The Master mix was prepared according to table 3.3.

Tabla 3.3.
Master Mix. Kit All pathogenic *Salmonella* species from Primer Design in a CFX96 Biorad equipment

Reagent	Quantity
Master Mix OasigTM or Precision PLUSTM 2x qPCR	10 µL
Primer/Probe for Salmonella-sp	1 µL
Primer/Probe internal extraction control	1 µL
RNAsa/DNAsa free water	3 µL
Final volume	15 µL

Definition of the value of Ct to determine the positivity of water samples for *Salmonella* spp.

The obtained data from seven replicates of an one assay were statistically analyzed, inoculating 1 CFU of *Salmonella* spp. in 1 L of drinking water and sea water, from which the obtained Ct values were analyzed statistically according to the standardized *Salmonella* spp. protocol.

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Chapter 4

Results obtained in the process of standardization of a real time PCR for the *Escherichia coli* quantification and *Salmonella* spp detection

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The results will be presented according to the methodology developed; therefore, first will be described the results of concentration and bacterial elution process standardization for *Escherichia coli* DNA extraction from potable water and seawater samples. The second section will aboard the results of the standard curve development process for *Escherichia coli* quantification by real-time PCR and finally the standardization results of the *Salmonella* spp. detection method by real-time PCR in drinking water and sea water.

CONCENTRATION AND BACTERIAL ELUTION TECHNIQUES STANDARDIZATION FOR DIRECT DNA EXTRACTION FROM HUMAN DRINKING WATER AND SEA WATER

Concentration methods

About the concentration method, according to the obtained results, it was evidenced that the filtration method is the most adequate for bacterial concentration, since as shown in table 4.1, 82 % of the inoculated bacterial load was recovered, unlike of the centrifugation that under the conditions evaluated only recovered 1 % of the inoculated bacterial load.

Table 4.1.
Concentration methods evaluation

Method	Dilution	Count (cfu)	Inoculated volume (µl)	% recovery
Filtration	10-6	386	500	82 %
Centrifugation	10-6	9	900	1.0 %

Elution methods

Within evaluated elution strategies, the first one was the type of buffer with different vortexting times, which, according to the obtained results, which are shown in figure 4.1, a greater cell count was obtained with the glycine buffer when used for five minutes, because in three minutes time the number of CFUs is lower. With the PBS buffer at different pH and time there was not a very noticeable difference in cell recovery and was always much lower than the count obtained with the glycine buffer for five minutes.

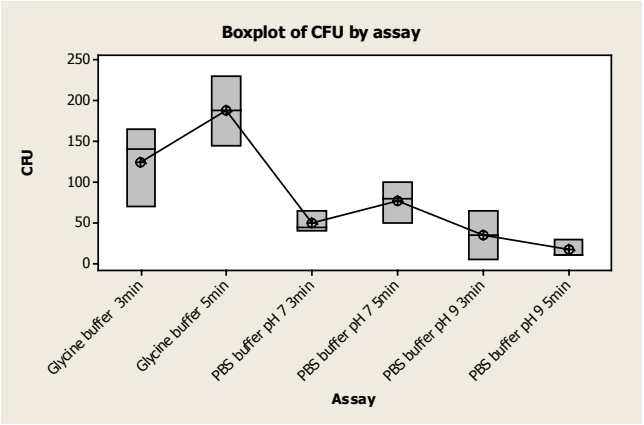

Figure 4.1.
Evaluation of different vortex times and buffers

In relation to the use of different incubation times evaluation of the filter submerged in PBS buffer at different pH results, an increase in the number of cells with respect to the inoculated was evidenced, which was more noticeable with the use of the PBS buffer at pH 7 as shown in figure 4.2.

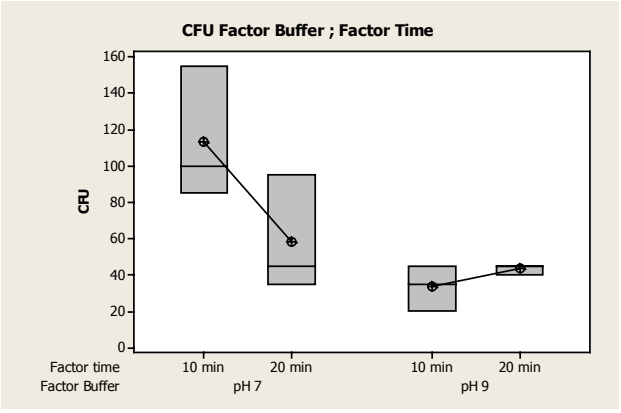

Figure 4.2.
Evaluation of different incubation and buffering times

Mechanical elution

Within the strategies of mechanical elution according to the preliminary

tests, with zirconia beads use to separate the cells of the filter was obtained good purity DNA, but in low concentration as evidenced in table 4.2.

Table 4.2.
Mechanical elution with zirconia beads

Method	Replicate	Concentration (ng/µl)	Purity (260/280)
Zirconia Beads	1	5.6	1.89
	2	5.4	2.04
Zirconia Beads Control	1	1.8	1.77
	2	2.4	1.41

On the other hand, filter trituration allowed obtaining a greater DNA amount that was able to be amplified by real time PCR, evidencing that with the use of the glycine buffer better results were obtained as presented in table 4.3. Also the filter cutting allowed obtaining greater quantity of DNA, but nevertheless the obtained Ct value in real time PCR was greater than with the filter trituration; these data are shown in table 4.3.

Table 4.3.
Mechanical elution evaluation by filter trituration and cutting

Protocol	Buffer type	Replicate	Purity (260/280)	Ct	Average
Filter trituration	Glycine	1	2.2	35.45	36.04
		2	2.19	36.91	
		3	2.2	35.78	
	PBS	1	1.45	36.5	36.63
		2	2.26	36.77	
		3	-	NO PCR	
Filter cutting	Glycine	1	2.11	36.04	36.89
		2	2.11	36.76	
		3	2.12	37.87	
	PBS	1		NO PCR	NA
		2			
		3			

Protocols testing and optimization

The assay results with the use of filtration and elution with filter trituration in glycine buffer inoculated with different CFUs showed that there is

no variation in DNA concentrations and obtained Ct values by varying the amount of inoculated CFU as is shown in table 4.4.

The results of the seven replicates inoculated with 56 CFU in drinking water are shown in table 4.5; it is shown that amplification was achieved with a low microbial load with a percentage of variance between Ct values of 12,6 %. The DNA concentrations were in the order of 10 ng/µL and the purity was maintained at a constant value between the replicates.

Table 4.4.
Protocols verification and optimization

CFU	Replicate	DNA concentration (ng/µl)	Purity(260/280)	Ct	Average
50	1	No	No	No PCR	34.03
	2	18.2	1.87	33.49	
	3	12.85	1.99	34.57	
100	1	11.2	1.91	No PCR	33.4
	2	6.15	1.64	33.92	
	3	11.45	1.92	32.88	
500	1	15.5	1.98	33.68	33.63
	2	13	1.92	33.59	
	3	19.3	1.82	No PCR	
1000	1	12.7	1.74	33.68	32.69
	2	10.75	1.64	No PCR	
	3	18.7	1.83	31.70	

Table. 4.5.
Human drinking water verification assay results

Replicate	Concentration (ng/µl)	Purity (260/280)
1	11.7	1.74
2	6.2	1.70
3	8.35	1.56
4	9	1.46
5	7.15	1.56
6	12.95	1.77
7	17.05	1.73
AVERAGE	10.34	1.64
Std. Dev.	3.802	0.11
VC %	36.8 %	7.2 %
Confidence level 95 %	3.51	0.10
Upper limit	13.85	1.75
Lower limit	6.82	1.53

On the other hand, the amplifications with sea water were achieved in the seven replicates using a DNA sample of 10 ng/mL with a percentage of variance of 2.7 %; however, when utilizing less diluted samples, in the case of the R2 and R4 replicate, the Ct value decreases without inhibition by any sea water component. The obtained DNA concentrations were higher than in drinking water and the purities above 2.0; results are shown in table 4.6.

Table. 4.6.
Sea water verification assay results

Replicate	Concentration (ng/µl)	Purity (260/280)
1	162.2	2.1
2	175.95	2.05
3	203.25	2.10
4	228	2.1
5	328.5	2.8
6	311.5	2.1
7	177.5	2.1
2 (20 ng/µL)	----	----
2 (50 ng/µL)	----	----
4 (20 ng/µL)	----	----
4/2 (50 ng/µL)	----	----
AVERAGE	226.70	2.19
Std. Dev.	67.41	0.27
VC %	0.30	0.12
Confidence level 95 %	62.34	0.25
Upper limit	289.04	2.44
Lower limit	164.36	1.94

STANDARD CURVE DEVELOPMENT FOR *E. coli* QUANTIFICATION IN WATER SAMPLES

Preliminaries *E. coli* standard curve results

The first results obtained from the calibration curve were performed using both *Salmonella* spp. and *Escherichia coli*. In the case of *Salmonella* spp. it is shown in table 4.7 that obtained DNA amount does not vary significantly when using different bacterial counts at each dilution.

Table 4.7.
First assay of preliminary *Salmonella* spp standard curve

Dilution	CFU	Average CFU	DNA conc. (ng/µl)	DNA aveg conc. (ng/µl)
-6	636	685.3	2.5	4.2
	664		5.05	
	756		5.15	
-6.5	356	374.0	1.7	2.6
	384		2.35	
	382		3.7	
-7	324	338.7	4.7	4.7
	316		4.35	
	376		4.9	
-7.5	100	127.3	2.5	7.5
	156		16.9	
	126		3.2	
-8	39	41.7	3.7	3.2
	47		1.95	
	39		4	
-8.5	18	14.3	5.85	5.1
	13		4.8	
	12		4.5	
-9	1	0.7	5.35	5.1
	0		5.35	
	1		4.65	

In the second preliminary assay with *Salmonella* spp. DNA concentration variation in the first dilutions is observed; however, from dilution 10^{-3} no difference between the obtained DNA concentrations was perceived, even in some dilutions its detection was not possible, as shown in table 4.8.

Table 4.8.
Second assay of preliminary *Salmonella* spp standard curve

Dilution	CFU	Average CFU	DNA conc. (ng/µl)	DNA aveg conc. (ng/µl)
-0	-	-	590.3	584.05
			577.8	
-1	-	-	64.2	64.55
			64.9	
-2	-	-	1.7	0.95
			0.2	
-3	-	-	2	1.65
			1.3	
-4	-	-	N	0.02
			0.02	
-5	-	-	N	N
			N	
-6	348	358	0.4	0.3
	368		0.2	
-7	31	35	2	1.85
	39		1.7	
-8	3	4	-	-
	5			

In the second preliminary assay using *E. coli*, real-time PCR amplifications were performed with the extracted DNA, linearity being observed in the first dilutions even though that DNA concentrations did not vary significantly, but from dilution 10^{-7} linearity is lost because of having lower Ct values, which is shown in table 4.9 and figure 4.3.

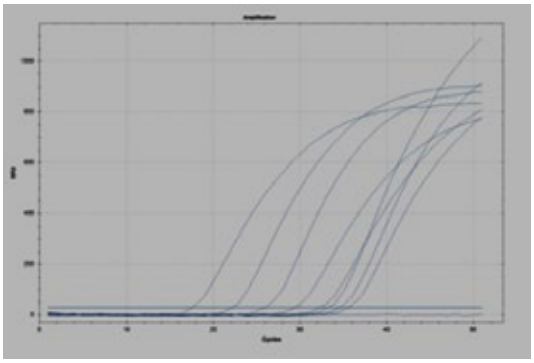

Figura. 4.3.
E. coli second preliminary assay amplification

Table 4.9.
Standard E. coli curve second assay results

Dilution	CFU	Average CFU	DNA conc. (ng/µl)		Ct*
-2	-	-	R1	5.5	22.20
			R2	5.25	
			R3	4.45	
-3	-	-	R1	1.95	26.01
			R2	5.15	
			R3	5.95	
-4	-	-	R1	4.6	29.39
			R2	2.8	
			R3	2.8	
-5	-	-	R1	9.8	32.27
			R2	7.8	
			R3	3	
-6	113	110	R1	2.9	35.49
	108		R2	2.75	
			R3	4.4	
-7	10	10	R1	3.8	34.52
	11		R2	4.1	
			R3	5.7	
-8	10	6	R1	8.65	33.23
	8		R2	5.55	
			R3	4.8	

**Correspond to replica number one*

Definitive *E. coli* standard curve results

E. coli final curve analysis was performed with the results of the seven replicates; four curves were elaborated according to the points included in each one of them with the objective of selecting the most adequate one according to R^2 value and to the efficiency of the curve. Figure 4.4 shows that the best efficiency was obtained in the six-point curve with a value of 95.71 %, and a R^2 of 0.98. It was not possible to use the nine-point curve because it has an R^2 inferior to 0.9. In all tests, linearity is lost below a bacterial density of 1000 CFU with an average Ct value of 31.11.

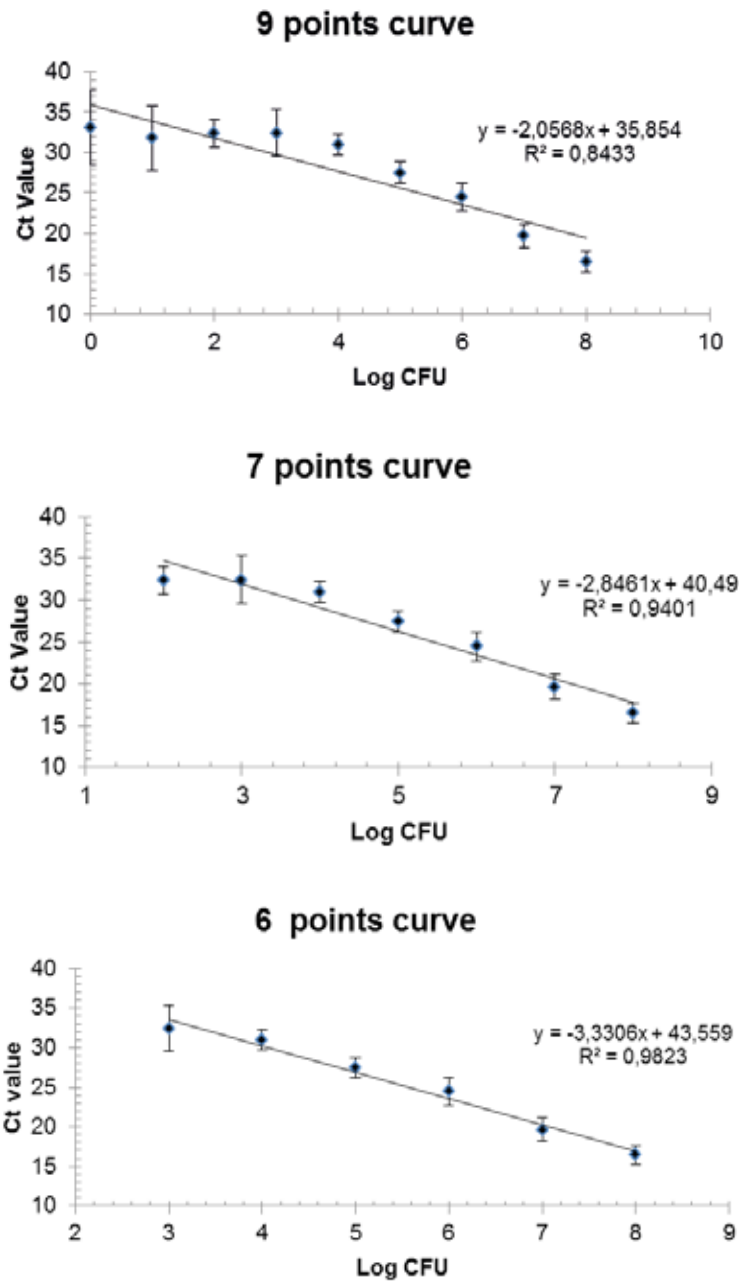

Figure 4.4 Standard curves for E.coli quantification

METHOD DEVELOPMENT FOR SALMONELLA SPP. DETECTION BY REAL TIME PCR IN WATER HUMAN DRINKING WATER AND SEA WATER

Preliminary assays

The first of the preliminary test results is shown in figure 4.5, where *invA* gene amplifications obtained by conventional PCR from a 24-hour pre-enrichment culture extracted DNA of one inoculated water sample with both 95 CFU and 10 CFU in 1L of water.

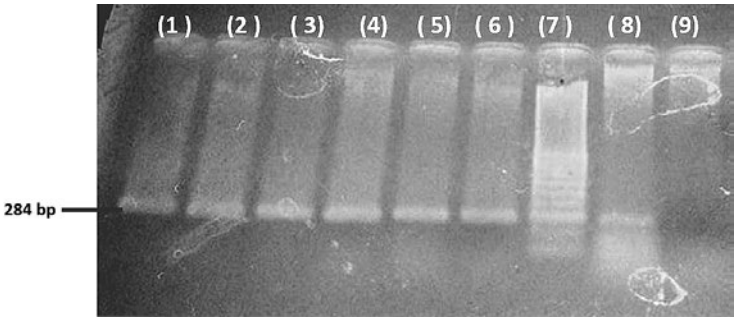

Figure 4.5.
Amplification assay results: Lane 1: Replica 1 with 95 CFU/100 mL. Lane 2. Replica 2 with 95 CFU/100 mL. Lane 3. replica 3 with 95 CFU/100 mL. Lane 4. Replica 1 with 10 CFU/100 mL. Lane 5. Replica 2 with 10 CFU/100 mL. Lane 6. replica 3 with 10 CFU/100 mL. Lane 7. Molecular weigth marker, Lane 8. Positive control. Lane 9. Negative control

As well, amplification of all tap water samples from the second test was achieved, those inoculated with 327 CFU and those inoculated with 9.5 CFU. The *invA* gene could be amplified also in the group of 9.5 CFU, including those in which DNA extraction was performed with the commercial kit, which are shown in figure 4,6 as lighter bands than those obtained with the DNA extracted by the organic method.

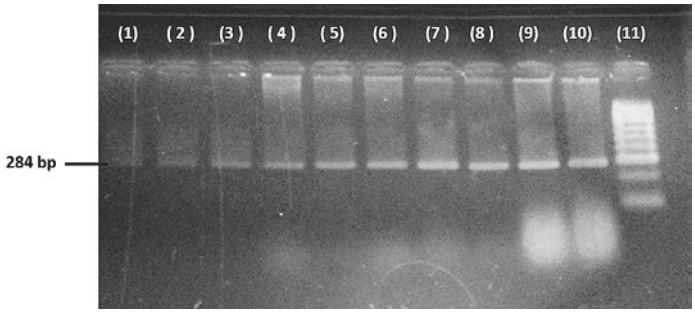

Figure 4.6.
Amplification assay 2 results. Lane 1. 9.5 CFU with kit replica 1. Lane 2. 9.5 CFU with kit replica 2. Lane 3. 9.5 CFU with kit replica 3. Lane 4. 9.5 CFU with organic extraction method replica 1. Lane 5. 9.5 CFU with organic extraction method replica 2. Lane 6. 9.5 CFU with organic extraction method replica 3. Lane 7. 327 CFU with organic extraction method replica 1. Lane 8. 327 CFU with organic extraction method replica 2. Lane 9. 327 CFU with organic extraction method replica 3. Lane 10. Control (+). Lane11. Molecular weight marker

In preliminary assay 3 was achieved the amplification in the first and third replicates of the 100 mL sea water assay and in all replicates in the 1L assay, but in both cases the bands obtained on the electrophoresis were very tenuous. The amplifications of the 100 mL assay are shown in figure 4.7.

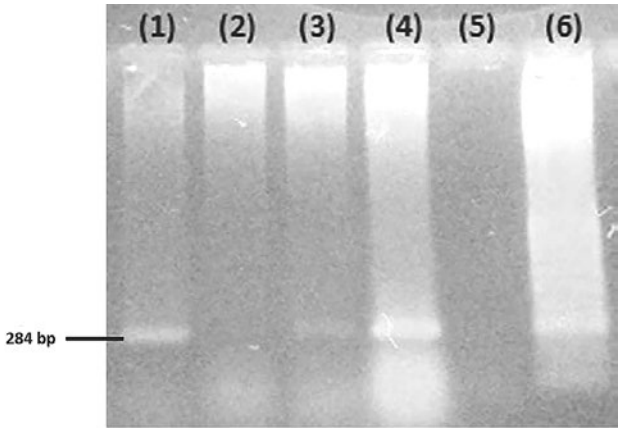

Figure 4.7.
Amplification assay results with 100 mL of sea water. Lane 1. Replica 1. Lane 2. Replica 2, Lane 3. Replica 3. Lane 4. Positive control. Lane 5.

Negative control. Lane 6. Molecular weight marker

Verification test of the *Salmonella* spp. by real-time PCR detection method

After the preliminary tests confirmed by conventional PCR, the tests were performed with real-time PCR, this way DNA extraction was verified for techniques, the commercial kit and the organic method; greater amounts of DNA were obtained with the use of the organic method; however, DNA purity was better with the commercial kit. This test showed that with this technique it is possible to detect 4 CFU of Salmonella spp. in 1L of drinking water and 10 CFU in 1L of sea water. The amplification curves obtained are shown in figures 4.8, 4.9, 4.10 by type of water and extraction method used.

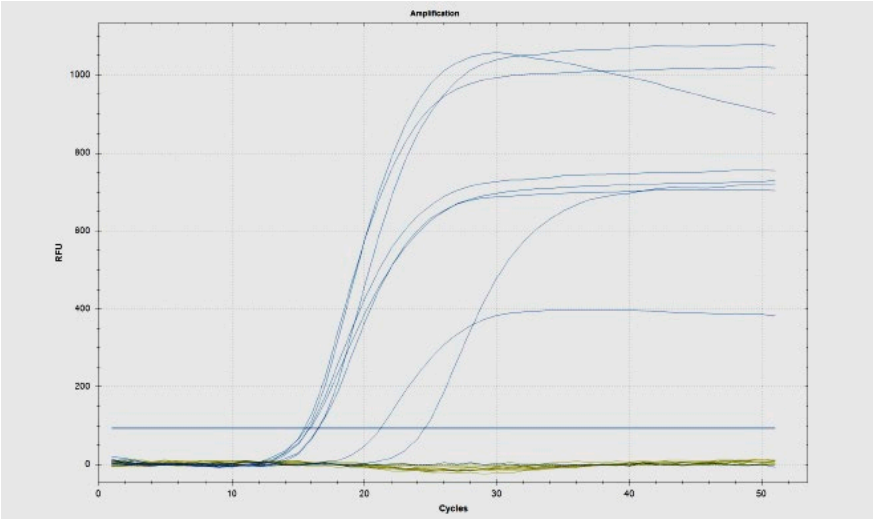

Figure 4.8.
Real time PCR amplifications with commercial kit DNA extraction method from drinking water

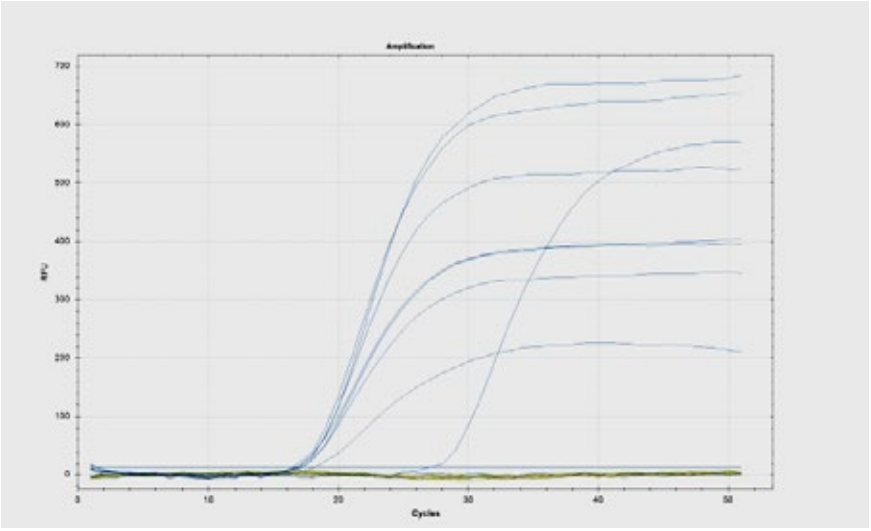

Figure 4.9.
Real time PCR amplifications with organic DNA extraction method from drinking water

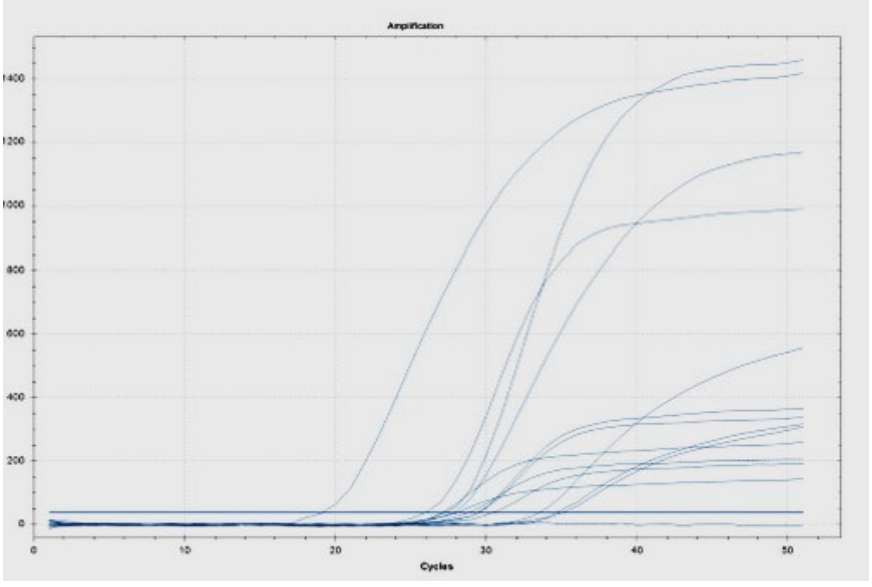

Figure 4.10.
Real time PCR amplifications with commercial kit DNA extraction method from sea water

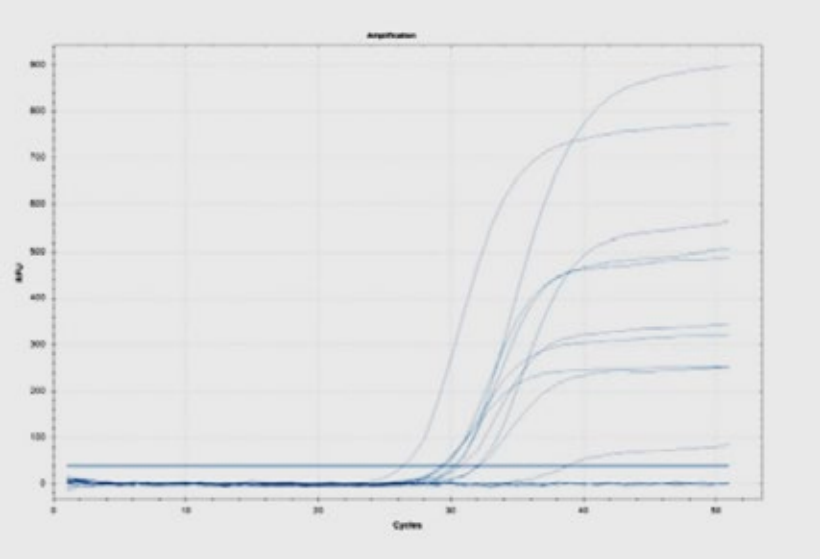

Figure 4.11.
Real time PCR amplifications with organic DNA extraction method from sea water

Definition of Ct value to determine the positivity of samples for Salmonella spp.

According to the developed analysis with seven replicates inoculated with 1 CFU/1L showed in table 4.10, 33 was defined as the maximum Ct value to catalog a water sample as positive for *Salmonella* spp. This value was determined according to the mean value obtained, which was 29.23 and this was added the 95 % confidence level, which was 3.8.

Table 4.10.
Salmonella sp. data statistical summary

Cq 1 CFU	
Average	29.23142857
Typical error	1.56274092
Standard deviation	4.134623838
Sample variance	17.09511429
Minimum	20.17
Maximum	32.34
Count	7
Confidence level (95 %)	3.823889278

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Chapter 5

Discussion and conclusions of the results obtained during the standardization of a real time PCR for *Escherichia coli* quantification and *Salmonella* spp

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The development of the discussion and conclusions will be based on the present research results, seeking to contrast them with the public policies on issues related to water quality and its relation with the National Program of Science and Technology of the Sea and Hydrobiological Resources, (Resolution No. 370 of 2015) that prioritize the CTeI of water in Colombia. The findings derived from the development of the project and a review of the evolution of the National Policy on Science and Technology show a strong incidence on the main landmarks that have been associated with the integral management of water, such as diagnostic methodologies for its quality control.

The entry into force of the General System of Regalias and the respective creation of the Science, Technology and Innovation Fund has opened up an opportunity for science, technology and innovation in the country (90).

New resources will positively impact the National Program of Science, Technology and Innovation of the Sea and the Hydrobiological Resources and therefore in the application of Water National Policies and those related to its oceans and coasts (91).

At present, only projects associated with aquaculture and ports have been strengthened (92). It is necessary to present projects related to the basic sciences and their direct relation with water, e.g provide data about its quality through molecular tools such as PCR, whose results help making decisions quickly and efficiently, generating interdisciplinary knowledge that contributes to a better Colombian water resources administration.

The analysis of the microbiological indicators of water makes it evident that Colombia has an acceptable water quality, but with traditional indicators of low reliability and poor facilities in public institutions, which allow perform high quality tests and therefore international comparisons. The process of researching molecular tools in Colombia in relation to water quality has not yet been consolidated, many of the drawbacks identified in past diagnoses still persist due to the low levels of innovation of public and private institutions.

The weak institutionality of the system, the lack of focus of the policy in strategic areas, the low social appropriation of knowledge and regional disparities in scientific and technological capacities, which together generates a low capacity to generate and apply basic knowledge in most regions of the country. However, cannot ignore the progress that has been made in relation to the generation of knowledge applicable to science and its relationship with water management, reinforced on the one hand by the increasing presence of highly trained personnel level.

When implementing molecular methods to the diagnosis of water in the laboratories of microbiological can be taken into account the results

obtained in the process of standardization of a PCR technique performed in this research, from which it was evident that the filtration method is the most suitable for the bacterial concentration, because it allows to recover in a greater percentage the amount of microorganisms inoculated in a water sample, comparable with other technologies (93). With respect to the elution, it was established that a mechanical trituration of the filter submerged in Glycine/Peptone buffer obtained a better results of DNA extraction process, after a vortexing between 8 min-10 min that allows the separation and direct extraction of the DNA comparable to the results obtained with tissier and collaborators (94). The incubation time was discarded due to cell growth and therefore it is not possible to know the exact amount of bacteria present.

Based on the above, finally a method was chosen that combines concentration with filtration, elution with filter grinding with the Glycine/Peptone buffer and vortex for 10 minutes. According to this, amplification was possible in drinking water with low microbial load and in the case of sea water also the amplification was achieved but with a greater amount of CFU without being inhibited by any component of sea water. However, the quantitative results, do not allow to associate the value of Ct with the amount of inoculated bacteria, not observing a linearity when working with low number of CFU; however, other studies have shown better results in relation to the variance of real-time PCR results (59).

The need for quantitative data by microbiological methods can be solved by the real-time PCR variant, by performing a standard curve. In the preliminary tests performed with this objective, the DNA concentrations obtained do not show a trend according to the utilized dilutions of the bacterial inoculum, especially in the case of *Salmonella* spp., where some samples were negative. However, when developing the real-time PCR with *E. coli DNA*, certain linearity was observed except for the last dilutions. As found in similar studies (95), the developed curve give limited quantitative data when working with low concentrations.

In relation to preliminary results for the standardization of the qualitative type method for the detection of *Salmonella* spp. through conventional PCR amplification, they demonstrated that it was possible to detect the *Salmonella* spp. *invA* gene from DNA extracted from a 24 hours pre-enrichment culture of a sample of water with a low microbial load in both drinking water and sea water; the realization of pre-enrichment step is necessary for the detection of *Salmonella* spp. by PCR, as demonstrated by Villarreal 2008 (96). Likewise, the obtained data evidenced that greater amounts of DNA were obtained with the traditional extraction method with chloroform than with commercial extraction kit; however, the DNA obtained in the latter was of better quality, this has also been demonstrated by other studies (97).

In relation to the evaluation of the real-time PCR method for the detection of *Salmonella* spp., a good reproducibility was demonstrated with small standard deviations in both drinking water and sea water using low microbial loads. Real-time amplifications show that there were no noticeable differences between the Cqs obtained by both DNA extraction methodologies. The above demonstrates that it is possible to apply the PCR technique in real time to obtain quantitative and qualitative data; however, in the first case it is limited with low microbial loads, although it is possible to detect bacterial loads of 1 CFU as evidenced by similar studies (7,96).

From these results it is possible to infer that the molecular methods are useful for the identification of pathogenic of rapid, sensitive and specific form; the characteristics of performance of the used methods make it useful to the evaluation of the drinkable water quality and sea water in in all the regions of the country. This work can be the base so that the public health institutions are to the avant-garde in diagnosis methods, which allows improving the alertness of these pathogenic in water sources and

diminishing the risks for the human and animal health, associated with the consumption of contaminated water.

For the Colombian government, the identification of the interdisciplinary research priorities for the integral management of water in the country (98) should be a permanent task, through projects that allow the understanding and diagnosis of the complex processes of state changes, trends and impacts in spaces where the water resource is fundamental. To achieve this goal, regional governments should strengthen both the implementation of molecular tools, qualified personnel and the strengthening of their facilities within the surveillance institutions (Departmental Public Health Laboratories), which create an evident culture of science with participation of all stakeholders, without leaving aside the communities that live and depend directly on the supply of goods and services of the water resource, and achieve greater environmental awareness among citizens.

It is believed that these issues are of a structural nature to support the future of good water governance in Colombia, characterized by its social justice and environmental sustainability. This will be achieved by the design of new innovative networks in scientific research, with a greater interdisciplinary and respectful character. Such processes will require profound transformations of the educational system, whose objective is to train researchers and professionals capable of understanding, listening and dialogue with others socio-cultural groups with a vast knowledge and a diverse traditional practices to generar new strategies that improve the water quality (99).

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Chapter 6

Standardization of the PCR technique for the detection of bacterial pathogenic microorganisms inwater using as a model *Salmonella* spp. and *E. coli*.

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ABSTRACT

Traditional methods for the microorganisms identification are based on the use of strategies such culture media, isolation in selective media, biochemical identification and serological characterization. These strategies are expensive, have low specificity, low sensitivity and are time consuming. In recent years, different molecular strategies such as the Polymerase Chain Reaction (PCR) have been developed, especially quantitative real-time PCR (qPCR) for *Salmonella* spp. and *Escherichia coli* detection and quantification in different matrices. These molecular methods are simple, reproducible, flexible and allow a rapid identification. This chapter presents an analysis about methods for *Salmonella* spp. and *E. coli* detection, and make emphasis on the use of qPCR and its standardization aimed at the diagnosis of these public health importance microorganisms.

Keywords: *Salmonella* spp., *Escherichia coli*, qPCR, Foodborne diseases.

Research project which this chapter derives: Molecular detection of difficult isolation microorganisms.

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INTRODUCTION

The traditional microbiological techniques used for microorganisms detection applied in most laboratories are based on the phenotypic characteristics identification such as microscopic morphology, reaction to various types of staining, antibiotic susceptibility profile, colonies morphology and biochemical preferences. About this last case, it can be presented the fact that could exist different isotypes of the same isolated strain because the microorganisms biochemical characteristics are not totally stable or they can be influenced by genetic regulation, technical manipulation and by the loss or gain of a plasmid. However, in some cases microorganisms are not detectable in matrices that have a low number of cells (100), because they have been subjected to a high degree of injury that decreases the bacterial cells number in the applied technological processes in the food production or human drinking water processing.

In recent years, the molecular techniques application has initiated a revolution in the diagnosis and monitoring of infectious diseases. These techniques include DNA identification without prior amplification such as biochips (101), DNA probes (102), restriction enzymes and ribotyping. On the other hand, there are DNA identification techniques, which involve previous genetic material amplification such as DNA sequencing, the analysis of Restriction Fragments Length Polymorphisms (RFLP), PCR among others (103).

of all the aforementioned techniques, PCR is the most widely used in vitro amplification system, it's the main objective to obtain millions of copies from one or several target DNA fragments; is a flexible, reproducible, fast, sensitive and specific technique; which makes it the first alternative for the molecular detection of microorganisms in different types of samples without the culture media need, is also useful for the rapid detection of non-cultivable or demanding microorganisms, to detect variations at species and/or subspecies level, to determine viral load and the detection of genes or gene mutations responsible for drug resistance.

Since its discovery (104), different variations have been introduced to the technique, for example the Multiplex Polymerase Chain Reaction (MPCR), in which different types of DNA are detected in the same reaction; Coupled PCR to a previous reverse transcription reaction (RT-PCR), in which the starting nucleic acid is RNA that is retrotranscribed to DNA with the reverse transcriptase enzyme aid (105). Another widely used variant is quantitative real-time PCR (QPCR), which not only allows simultaneous and continuous detection of fluorescent signals as DNA fragments are amplified, but DNA species can be quantified in order to make accurate assessments of the microorganisms found in a given sample (106).

In a qPCR must be taken into account the factors that influence the process success. Therefore, it is necessary to find the best conditions to optimize the process (107). The factorial analysis is an excellent option to determine the optimal conditions for nucleic acids efficient amplification, since it seeks to reduce the number of tests by grouping the variables that correlate with each other, commencing from a large number of homogeneous variables.

This chapter explores the use of qPCR and its respective standardization for *Salmonella* spp. and *Escherichia coli* detection, which are important public health microorganisms.

REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

Since its creation in 1990, PCR has become the most widely used molecular technique in the world and its impact on the medical diagnostic world has been profound. Due to the high cost of the PCR system (thermocycler, electrophoresis chamber, photodocumentation system, etc.), the use of specialized locations and highly trained staff, the contamination associated with poor management and use of reagents before and after the amplification, a new PCR variant was developed that uses probes labeled with fluorochromes for amplicons detection, denominated Quantitative Real Time PCR (qPCR). This new technological development eliminated analysis contamination by developing all the reaction and quantification in the PCR tube without opening (108). In microbiology, qPCR is used for the purpose of detecting microorganisms (absence/presence), microbial quantification or microbial load, genotyping or microbial characterization and gene expression profiles.

The PCR typically employs a thermostable enzyme, a pair of oligonucleotides per fragment to be amplified which must be at a XXXpM concentration, magnesium in a range from 2.5 to 3.5 mM, triphosphate deoxynucleotides at 200nM and a buffer that favors the enzymatic action by avoiding the drastic change of pH and ionic strength. This new variant employs a non-intercalating dye that binds to double-stranded DNA and is non-specific as SYBR, which is excited at 488 nm and emits light at 522 nm (green color) when DNA molecules are amplified. Also, a probe labeled with a fluorochrome is used at one end and a quencher at the other end that absorbs the frequency that the fluorochrome emits, this format is known as Fluorescence Resonance Energy Transfer (FRET) (109). These two molecules interact with each other maintaining a low fluorescence emission.

At the time of executing the thermal protocol, the labeled probe is located

in the region of interest by complementarity and between the pair of oligonucleotides and is displaced by the thermooresisting enzyme system, the probe deoxynucleotide which is labeled when is being displaced emits fluorescence with greater force because it is not near the quencher molecule. The fluorescence must be collected simultaneously and continuously by the optical system of a thermocycler modified for this purpose. These devices are currently coupled to a monitor that graphs the number of cycles against the Relative Fluorescence Units (RFU) or emitted fluorescence numerical values. It should be noted that many people believe that RT-PCR stands for Real Time PCR and is called real-time PCR because it can see the fluorescence as soon as it is emitted. A lie because at beginning the first computers were not coupled to a monitor.

In fact, it is called quantitative PCR because it allows us to count the generated amplicons number at the end by the use of a calibration curve.

As you can see there are many factors in a qPCR that must be taken into account and that influence its success. Therefore it is necessary to find the best conditions to optimize the process.

- Microorganisms concentration: The presence of microorganisms such as *Salmonella* spp. and *E. coli* is not accepted in drinking water, therefore the purpose of concentrating microorganisms is to increase the probability of finding them. They are in a homogeneous distribution in the matrix of interest and concentrating them in the same unit of area has as an objective to increase the detection limit and guarantees a good amount of nucleic acids. Centrifugation is the most commonly used method, especially in water. At the end of this process there is a precipitate at the bottom of the tube known as biomass to which a nucleic acid extraction is carried out.

- Another well-used method is filtration (110) with or without centrifuga-

tion and is based on the difference in microorganism's size to separate or trap them either in the membrane or filter or at the bottom of the tube. The use of a centrifuge prevents the use of a vacuum pump. In the case of solid matrices as food, non-selective enrichment culture media are employed which seek to increase the number of microorganisms by providing them with adequate growth conditions, are subsequently trapped by the aforementioned methods and subjected to nucleic acid extractions.

- DNA or RNA obtaining: Many kits for nucleic acids extraction from different types of samples are currently on the market. They are based on bacterial lysis through the use of enzymes, homogenization, sonication, or mechanical lysis with glass beads; followed by proteins selective precipitation with salts or organic solvents and finally a nucleic acids precipitation with ethanol or isopropanol. Regardless of the type of DNA or RNA extraction, quality and concentration play a key role. The kits have good results, however they are expensive and a good alternative is the use of the organic technique for DNA extraction with phenol-chloroform-isoamyl alcohol accompanied by a previous enzymatic lysis with lysozyme A. A good storage condition to avoid the fractionation, is to store the DNA or RNA at -80°C, this makes available those nucleic acids for future studies or doubtful result verification.

- Nucleic acids quantification: Although qPCR is capable of producing millions of amplicons from a low initial copy numbers, the nucleic acids concentration is particularly important. To determine the concentration and purity, spectrophotometry should be used at 260 nm for nucleic acids and at 280 nm for proteins, since after typical nucleic acids extraction DNA or RNA is usually available in sufficient quantity and with a high purity. For cases, where impurities in the DNA obtained from a PCR are suspected, fluorometry is recommended as the quan-

tification method. The dilution of the nucleic acids must be taken into account in the calculation and must be performed with the same water in which the nucleic acids have been rehydrated after the treatment to precipitate with ethanol or isopropanol. If there is little sample, it is advisable to use 1 cm cells of light path but with final volumes as small as 200 µL or to use spectrophotometers that use micro volumes of samples. Relative quantification with imaging systems may be a valid alternative as well.

- Oligonucleotides: after choosing a good pair of oligonucleotides, an important recommendation is to briefly centrifuge the tubes before opening them. They come lyophilized and the gloves electrostatic can attract them and remove them from the tube, since in the shipment they suffer from many movements. The use of talc-free gloves is also recommended. The oligonucleotides must be rehydrated in 10 mM Tris pH 8.0 and 1 mM EDTA (TE). Other users prefer DNAsas and RNAsas water free which is also a good strategy. Whether water or TE is recommended to make a stock solution at a concentration of 100µM, this is achieved by adding ten times the amount of water or TE of the oligonucleotide nanomoles. From this solution a work solution must be made, which must be at a concentration of 10 µM and its final volume will be according to the number of samples to be analyzed; this last solution will be the one used in the reactions or production of mastermix. Genes such as *hilA*, invasiveness regulatory gene (111); *ompF*, outer membrane porin f; *ttr*, tetrathionate reductase enzyme (112); *iagA*, gene activator of invasion genes expression (113); ssaN, type III secretion system of ATP synthetase (114) are frequently amplified for these pathogens detection.

- Probes: The same recommendations should be followed as with oligonucleotides but additionally because they are labeled with fluorochromes they should be worked in darkness as their activity may

be lost. The work solutions that will be subjected to several thawing must be storage in amber color tubes.

- Thermocycler: Most equipment uses plastic tubes and lids to collect fluorescence, however a few use glass tubes. An important aspect is that last generation equipment allows finding experimentally oligonucleotides best alignment temperature, by the application of thermal protocols with temperatures of gradient, which means that from a set range the equipment is able to apply different temperatures per row, making faster the standardization process. Such temperature is calculated from the information of the melting temperature or Tm which is in the information sheet of the oligonucleotides. A good recommendation is to acquire brands that can work with different types of chemistry and have the ability to turn off data collection channels so that they can be used as conventional thermocyclers to increase the life of the optical system.

- Electrophoresis: Although many do not find it convenient, electrophoresis plays a fundamental role in a standardization process. The direct verification of the expected bands, weak bands or be able to discard nonspecific bands become a means of validation in the standardization early stages.

- Positive controls: the use of a known DNA or RNA that always produces an amplification signal that validates and gives confidence about obtained results. Many people believe that an ideal positive control in the case of *Salmonella* spp. or *Escherichia coli* detection in water would be a DNA or RNA sample from feces of a sick individual. In this particular case, an ideal positive control is the DNA or RNA of a strain isolated from these microorganisms mentioned, since it will always show an amplification signal. No signal with the positive control is signal is

indicative of an error with the reagents (calculations, expiration dates) or equipment malfunction.

- Blank: Usually confused with a negative control, however in a target water is used instead of nucleic acids. Detecting a amplification signal in a target is indicative of poor technique, cross-contamination or cross-sampling. In all cases where it is used, there should be no amplification signal.

- Endogenous control: Not always used. However, in cases where the microorganism is obtained from tissue of an individual, it is known that there is also DNA or RNA of the subject, therefore a qPCR which detects a constitutive human or animal gene and that is read in another channel increases the technical rigor of a qPCR and increase confidence in the obtained results.

FACTORIAL ANALYSIS

The factorial design is an excellent option to standardize, finding the best amplification conditions in a qPCR because it allows to examine the effect of different factors or variables on one or several quality indicator responses and, in addition, it facilitates to determine the possible combinations or treatment of the different levels of these factors to finally select that combination of variables generating the desired effect, such as the most efficient amplification that allows to detect the microorganisms of interest.

The factorial analysis is performed with different statistical packages and consists, in this case, of three phases:

- The planning or a matrix design in which all variables and their different levels are combined: for example, many factors influence a qPCR success, but the hybridization temperature of the oligonucleotides

and magnesium concentration, constitute the most main points in a standardization process. Then what is wanted in this first phase is to design a matrix in which they confront the two variables of oligonucleotides hybridization temperature and magnesium concentration including their levels, that is to say, the different magnesium concentrations and the different hybridization temperatures.

- Reduction of data by default of those repeated data: when facing a factorial design with two variables and five levels for the hybridization temperature (5 °C below the lowest melting temperature of the pair of oligonucleotides to be tested, assuming a Tm of 58°C, suggested points are 56 °C, 55 °C, 54 °C, 53 °C and 52 °C) and four levels for magnesium concentration (2.5 mM, 3 mM, 3.5 mM and 4 mM), what is being proposed is a 5X4 factorial design (Table 6.1.)

Table 6.1.
Data matrix or variable combination and their respective levels

	Example of a factorial analysis matrix 5x4 – for a qPCR standardization				
Magnesium concentration	**Hybridization temperature**				
	56 °C	55 °C	54 °C	53 °C	52 °C
2.5 mM					
3 mM					
3.5 mM					
4 mM					

In the given example what is drawn or omitted are those combinations which are repeated and which will not be carried out experimentally. The obtained results are entered as numerical results. For the case of the qPCR, these values may be absolute such as the threshold point or CT or relative, for example when comparing the results with a positive control which is assumed to have no variation. A valuable alternative in the early stages of experimentation is to feed the computer program with data from an image analysis obtained with a photodocumentator. This strategy although a

little expensive will give us a record and evidence of bands presence or unwanted bands.

- Optimization of analysis: this phase directs the computer program with the different statistics that are desired to calculate and thus facilitate future results interpretation.

DISCUSSION

Many authors have devoted themselves to standardize the qPCR techniques emphasizing the concentration time or microorganisms pre-enrichment, optimizing the detection of *Salmonella* spp. in 12 hours, which includes 8 hours of pre-enrichment, followed by an genetic material automatic extraction and finally the molecular detection. Different nutrient media such as peptone water, BHI broth and soy trypticase broth were evaluated, however, no significant differences were found in any of the applied tests (115).

In another assay conducted by D'Ursoa and collaborators, who evaluated a different concentration method, which consisted of filtration for the viable bacteria recovery of *Salmonella enterica* and *Listeria monocytogenes* in 30 minutes. The filtration procedure consisted of passing the bacterial suspension on a 0.2 μm diameter filter paper for *Salmonella enterica* and 0.4 μm for *Listeria monocytogenes*. This treatment showed no effect on the viability of the cells. In the case of food the technique was able to detect 10 bacteria per 10 g of Yogurt and also able to detect viable cells in the presence of a wide range of dead cells (116).

Another way to standardize is to evaluate the selectivity that includes oligonucleotides and probes inclusivity and exclusivity in qPCR assays, since they are important elements for the design of the diagnostic methodology, such as chen and collaborators. They designed the oligonucleotides to amplify a sequence within the ATP synthetase gene of the type III secretion

system (*ssaN*), and designed an internal amplification control with their respective probe. The assay demonstrated 100 % inclusivity for the 40 Salmonella strains tested and 100 % exclusivity for the 24 unrelated strains (114).

CONCLUSION

Strict quality control of reagents, preventive maintenance of equipment and locations, technical rigor of molecular tests, biosecurity precautions and standards, good oligonucleotides and probes selection along with a statistical methodology, make the qPCR a superior tool in the pathogenic microorganisms' detection.

Costs for the microorganisms' molecular detection such as *Salmonella* spp. and *E. coli*, are high compared to traditional microbiological methods, but the benefits to the health sector by achieving a rapid and accurate diagnosis due to its high sensitivity and high specificity and the cost benefit ratio that gives to the productive sector by allowing the food products release to the market more quickly justify the implementation of this technique.

New methods and combinations of techniques make the detection process a less cumbersome, fast, sensitive and specific procedure, but the need to perform the confirmation by the traditional method is still valid and mandatory according to the legislation in some countries. These techniques have allowed discriminating between living or dead microorganisms, active or inactive molecules, allowing carrying out sanitary controls. The qPCR for *Salmonella* spp. and *E. coli* will complement but not replace traditional microbiological techniques that are necessary for epidemiological surveillance purposes.

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Simón Bolívar University, through the Microbiology program belonging to the faculty of Basic and Biomedical Sciences and its research group Bio-organizaciones, present the book entitled *Real-time PCR applied to bacterial waterborne pathogens detection and quantification*, which is a research outcome, effort of professors, researchers, students and graduated students who actively participated in the execution of the project named "*Implementación y estandarización de la técnica de qPCR en la detección de microorganismos patógenos bacterianos de importancia en salud pública como bioindicadores de la calidad del agua*", financed with resources from National Fund of Royalties of Colombia which enabled the creation of facilities for microbiology research at the Simón Bolívar University for the molecular microbiological diagnosis of water.

In this work, categorized as a research product book, the tests carried out and the results obtained in the process of implementing the real-time PCR technique to evaluate the quality of the water through the identification and quantification of Salmonella spp and Escherichia coli bacteria are detailed. The data presented will be of great contribution to the reader for the implementation of this technique in their laboratories and for students in related areas to enrich their knowledge in this subject.

This book is the product of the collaborative work of various researchers, mostly linked to the Simón Bolívar University and the Microbiology Program, such as professors Zamira Soto, David Rosado, Jairo Ceballos, Hernando Bolívar and Christian Orozco, who have a solid background in molecular and water microbiology; in addition, the professors María Badillo and Liliana Pérez supported the compilation and organization of this manuscript. Besides, two invited professors participated: José Villarreal from the Universidad Libre and Alfonso Bettin from the Universidad Metropolitana, who prepared a chapter where they express their experiences on the standardization of the PCR techniques. Also, it is worth mentioning the participation of the students Camila Pichón and Bertha Granados and the graduated student Dalidier Estrada from the Microbiology Program, who supported the execution of the project and the writing of the manuscript, being an example for those students who wish to follow the researcher career.

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