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.

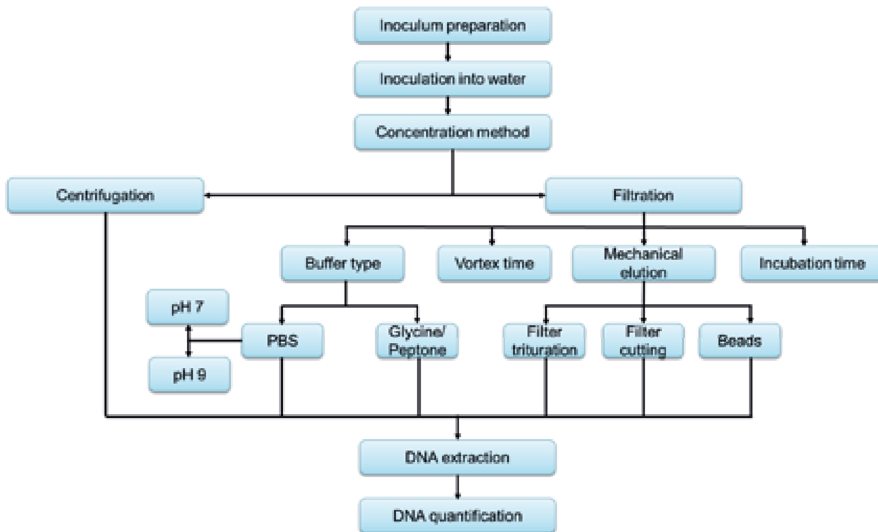

Figure 3.1.
Flowchart of *E. coli* concentration and elution methods standardization for DNA extraction

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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