

# DETECTING SALMONELLA IN HYDRIC ENVIRONMENTS ADVANTAGES OF REAL-TIME PCR

Detection of Salmonella in hydric environments is usually performed using traditional microbiological methods that are excessively long and complex. To optimize response times and limit operational steps, the Biology Department of Hera Laboratories has introduced use of the Polymerase Chain Reaction (PCR) technique. A study of this method has demonstrated the high degree of sensitivity and specificity of this technique.

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The Biology Department of Hera (Holding Energia Risorse Ambiente) Laboratories performs microbiological testing of water quality during the various phases of the Integrated Water Service [service formed pursuant to the Italian «Galli Law» 36/1994 to implement an integrated reform of water services in Italy]. Of these various tests, testing for Salmonella is particularly important, as the presence of this bacteria in hydric environments indicates fecal contamination. Salmonella (Enterobacteria family) is a genus that includes aerobic and facultative anaerobic, gram-negative, C8-estrase positive, non-lactose fermenting microorganisms. This genus includes species that are pathogenic to humans, which may cause infections with serious symptoms, such as typhoid fever. These pathogens are spread to humans in water and contaminated food products. Quality characteristics for surface water used to produce drinking water are defined in table 1/A of Annex 2 of Legislative Decree 258/2000: «Supplementary and corrective provisions of Legislative Decree 11 May 1999, no. 152, regarding water safeguard from pollution, according to article 1, paragraph 4 of Law 24 April 1998, no. 128». The guidelines require the absence of Salmonella spp. in 5,000 ml for category A1 water, which has been physically treated and disinfected, as well as the absence of this bacteria in 1,000 ml for category A2 water, which has been both physically and chemically treated and disinfected.

The absence of Salmonella is also the limit value set for waste water by Ministerial Decree no. 185/2003, which sets out requirements for the reuse of waste water: «Regulation containing technical standards for the reuse of waste water to implement article 26, paragraph 2, of Legislative Decree 11 May 1999, no. 152». With regard to drinking water, detection of disease-causing enterobacteria, which includes Salmonella, is considered as supplementary in Legislative Decree no. 31/2001 Implementation of EC Directive 98/83/EC relative to the quality of water destined for human consumption». Salmonella must be consistently absent in 1,000 ml. For many years, the Biology Department of Hera Laboratories used traditional microbiological methods to detect Salmonella, including the ISS A 011A method - Istisan reports 07/5 for drinking water [1] and the Apat Irsa Cnr 7080 method for waste and surface water [2]. Both methods are extremely long and complex: they require a pre-enrichment step, followed by a selective enrichment step, extraction and biochemical and/or serologic identification of suspect colonies. A positive Salmonella detection requires approximately 6 -7 days. The number of «man-hours» in the lab is significantly high, and because said analysis involves continuous hands-on activity, technicians are required to work on weekends and holidays. These constraints gave rise to the need to assess an alternative reliable, fast, sensitive and easy-to-use method that would limit involvement of human resources. Of the various alternate biomolecular methods based on the study of specific genetic sequences of the target pathogen, we selected the Polymerase Chain Reaction

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(PCR) technique, currently used in the food industry [3], for its high sensitivity and specificity, speed and potentially automated analysis. The enrichment, DNA extraction and amplification phases that compose this method require a total of two days to obtain a final result, with less technician involvement. PCR is an enzymatic reaction to amplify, *in vitro*, a segment of DNA obtained using a Taq polymerase and two specific primers, which are single-strand DNA sequences required to initiate DNA synthesis. This technique enables amplification of a DNA fragment of the target microorganism. Amplification signifies creating an exponential number of copies (up to several millions) *in vitro*, thereby allowing the pathogen to be absolutely and specifically detected. The real-time PCR technique used by Hera Laboratories allows DNA sequences to be amplified and the product of this amplification to be followed in real time. In the fully automated system, the amplified DNA fragments are identified by recording the fluorescence generated during the amplification reaction, as a result of the annealing of specific molecular beacons marked with fluorescent reporters (attached to fluorescent molecules) (Figure 1 [4]). Molecular beacons feature a hairpin structure, composed of a loop and a stem; when linked to the complementary sequence, they open and generate a fluorescence signal,

as a result of the fluorophore and its quencher moving away from each other. Consequently, each moment in the detection of the pathogen can be followed in real time. This system therefore uses two levels of specificity, classic primers and beacons, to provide a unique and highly sensitive detection system.

### Materials and methods

The principles behind the validation protocol for selecting a new method are based (UNI IEC EN ISO/IEC 17025:2005 [5]) on a comparative study of the alternate method and the reference method, conducted both at the laboratory and/or in ring studies. At Hera Laboratories, the traditional cultural method of detection for Salmonella was compared to the real-time PCR technique, which uses the Stratagene Mx3005P thermocycler and the Adiafood Rapid-Pathogen Detection system for Salmonella Kit from AES CHEMUNEX (Figure 2).

### Traditional reference method

Pre-enrichment: consists in enriching 100 or 1,000 ml of sample, according to standard procedure, in buffered peptone water (Bpw) for 18 - 24 hours at 36±1°C, after filtration through a sterile membrane filter. Selective enrichment: an aliquot of pre-enrichment broth is transferred into Rappaport-Vassiliadis (RV) selective broth and incubated for 24+24 hours at 42±1°C. Extraction and identification: using the RV broth, two successive multiple-swipe subcultures are made on an Hektoen Enteric Agar (HEA) isola-

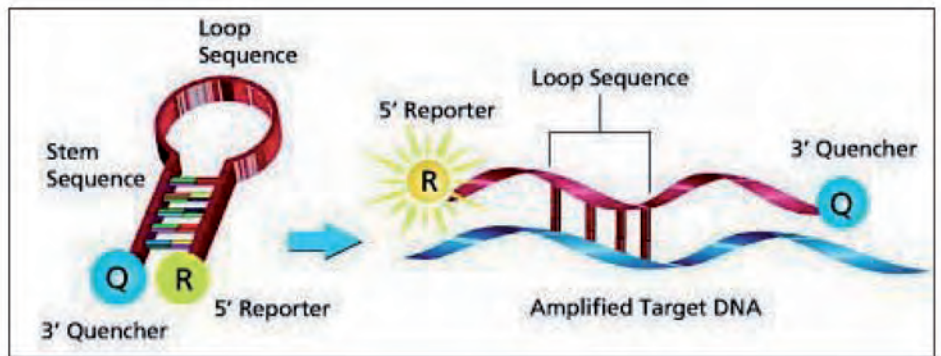


Figura 2 - Termociclature e PC con software integrato.

tion medium; the first after 24±2 hours of incubation, and the second after 48±2 hours of incubation; the isolation microplates are then incubated for 24 hours at 36±1°C. For presumed colonies of Salmonella, green colonies with or without black nuclei, a cytochrome oxidase test is performed, as is C8-esterase testing and biochemical identification using miniaturized systems.

### Alternate method using real-time PCR

Pre-enrichment: identical to the traditional method. DNA Extraction: for each sample, 10 microlitres BPW are aliquoted in the 96-well microplate in 90 microlitres of the «extraction mix» included in the kit. The extraction reaction takes place over 20 minutes in the Stratagene Mx3005P thermocycler, and involves a first phase of 10 minutes at 40°C and a second phase of 5 minutes at 95°C. The extraction microplate is then centrifuged for 5 minutes at 1,800 rpm. Amplification and Detection: 10 microlitres of each aliquot of the extracted DNA are transferred to the Flexiwell strips provided in the kit, after reconstitution with 15 microlitres of detection reagent, containing Taq polymerase, nucleotides and the detection buffer. Each sample is processed in duplicate in both the extraction microplate, as well as the detection strip: the first well contains the primers and the specific molecular beacon for the pathogen, while the second well is used for reaction inhibition control (both the primers and

molecular beacons specific to the pathogen are absent). Each analysis cycle includes 6 controls provided in the kit, consisting of 3 negative controls (to exclude contamination), 2 positive controls (to verify DNA amplification) and 1 inhibition control (to verify inhibitions as a result of the matrix effect). After being sealed with the optical-grade caps, the strips are shaken manually, vortexed for 1 minute, then centrifuged for 1 minute at 1,800 rpm and secured in the thermocycler for 1 hour and 30 minutes. Reaction conditions are: 1 activation cycle of 15 minutes at 95°C and 40 denaturation cycles (15 seconds at 94°C), linking (30 seconds at 55°C) and extension (15 seconds at 72°C). Upon completion, the microplate and results are displayed on the screen. If the reaction has been completed correctly, controls will be visible in green. Negative samples will be indicated in green, while positive samples will be red and non-amplified or invalid samples will be yellow, and must be subjected to additional analysis (repeated analysis, etc.) According to specifications, the kit enables the amplification, and consequently, the detection of 166 strains of Salmonella. It is interesting to note that portions of enriched broth may be refrigerated for one week if the DNA cannot be immediately extracted from the samples, for whatever reason. The extracted DNA may also be kept in the refrigerator for one week if they will not be immediately amplified.

## Results

The study conducted by the Biology Department of Hera Laboratories in order to validate the alternate method that uses the real-time PCR technique was designed to assess the following parameters: sensitivity, specificity, minimum detection limit, correspondence of results with the reference method, participation in ring studies.

## Sensitivity and specificity

Assessment of the PCR technique began by verifying correct assignment of positive and negative samples to their corresponding groups, as set forth in ISO/TR 13843 [6]. Sensitivity (ratio of the total number of positive samples correctly assigned to this group) was determined by analyzing artificial samples prepared in the laboratory using standard strains of Salmonella, obtained from ring studies or the American Type Culture Collection (Atcc). These strains are listed in Table 1A. Specificity (ratio of the total number of negative samples assigned correctly) was evaluated by amplifying DNA extracted from standard, non-target strains (Table 1B). The trials conducted drew attention to the extreme sensitivity and specificity of both the technique and the corresponding kit: no false positive or false negative results were obtained, even when microorganisms similar to Salmonella were present.

## Recovery tests and detection limit

**Trials using pure cultures** - The Atcc 14028 strain of Salmonella typhimurium was progressively diluted in Brain Heart Infusion (Bhi) broth, in order to obtain solutions with theoretical values of 100 to 0.01 Units Forming Colony (UFC)/10 µl: a positive signal was obtained using PCR up to a concentration of 0.1 UFC. Another

A		
Bacterial strain	Origin	PCR result
Salmonella typhimurium Atcc 14028	Hera collection	Present
Salmonella typhimurium Atcc 14028 a titolo noto		
Salmonella oranienburg	Ring study trial Lgc	
Salmonella infantis	Afnor	
Salmonella abony	Ring study trial Senate	
Salmonella nottingham	Ring study trial Unichim	
B		
Bacterial strain	Origin	PCR result
Enterococcus faecalis Atcc 29212	Hera collection	Absent
Escherichia coli Atcc 25922		
Stafilococcus aureus Atcc 25923		
Escherichia coli	Ring study trial Unichim 4	
Klebsiella pneumoniae		
Escherichia coli Atcc 13706	Hera collection	
Pseudomonas aeruginosa Atcc 27853		
Escherichia coli	Senate	
Escherichia coli	Ring study trial Senate 04	
Escherichia coli Nctc 9001	Ring study trial Unichim 5	

Table 1 - Quality Control using certified strains

Recovery trials				
	Nominal value (UFC)	Traditional YEA (UFC)	Traditional HEA (UFC)	PCR
Trials with pure culture	1	1	0	Present
	1	1	0	
	1	1	0	
	1	1	1	Present
	0,5	0	0	
	0,5	0	0	
	0,5	0	0	
	0,5	0	0	
	0,5	0	0	
	0,5	0	0	
	0,1	0	0	Present
	0,1	1	0	
	0,1	0	0	Absent
	0,1	0	0	
Trials with artificially contaminated samples	0,5 UFC/100 ml	0	0	Present
	0,1 UFC/100 ml	0	0	Absent
	0,05 UFC/100 ml	0	0	Absent
	0,01 UFC/100 ml	0	0	Absent

Table 2 – Recovery trials using pure cultures and artificially contaminated samples

series of trials was then conducted to verify recovery in the interval between 1 and 0.1 UFC (theoretical), comparing the traditional method to the real-time PCR technique. The following were analyzed using the PCR technique: 4 replicas of the solution containing 1 UFC, 7 replicas of the solution containing 0.5 UFC and 4 replicas of the solution containing 0.1 UFC. Multiple-swipe cultures of 3 solutions were simultaneously conducted in Yeast Extract Agar (YEA) non-selective growth medium and in Hektoen Enteric Agar (HEA) selective growth medium. As shown in Table 2, using PCR at the theoretical concentration of 0.1 UFC, a positive response was obtained in 75% of cases, while in YEA, only 25% of responses were positive and in HEA, which is more selective, 0% of cases were positive. At concentrations of 1 and 0.5 UFC/10 µl, real-time PCR provided 100% positive results, unlike other methods. The trials conduc-

Detection limits: sterile water contaminated with certified strain of <i>Salmonella typhimurium</i>				
Nominal value	No. Replicates	No. positive results PCR	No. positive results PCR	Note
0,5	10	10	0	positive samples = 100%
0,1	10	7	3	positive samples = 70%
Detection limits: Surface matrix contaminated with certified of <i>Salmonella typhimurium</i>				
Valore nominale	N° Repliche	N° Risultati positivi PCR	N° Risultati negativi PCR	Note
0,5	20	20	0	Campioni positivi = 100%
0,1	20	11	9	Campioni positivi = 55%

**Table 3 - Limits of detection**

**Tabella 3 - Limiti di rilevazione.**

ted therefore underscore the high degree of sensitivity offered by the alternate technique: the amplification signal nearly attains the theoretical limit of 0.1 UFC. An additional trial using ten replicas of a sample of sterile water contaminated with 0.5 and 0.1 UFC of certified bacterial strain, confirmed that the detection level (95% positive replicas) falls between these two concentrations (Table 3). Trials using artificial samples - In order to also assess the matrix effect, trials were conducted using artificial samples composed of 100 ml of surface water, which had produced a negative result using the traditional technique, and this same sample, contaminated with the Atcc 14028 strain of *Salmonella typhimurium* and using the PCR technique. Levels of contamination were

Comparison: traditional method of detection HEA (APAT IRSA CNR 7080) vs. real-time PCR			
Sample-#	Sample matrix	HEA traditional method	Rt - PCR
1	Drinking water	Absent	Absent
2		Absent	Absent
3		Absent	Absent
4		Absent	Absent
5		Absent	Absent
6	Natural underground water	Absent	Absent
7		Absent	Absent
8		Assente	Assente
9	Natural surface water	Present	Present
10		Present	Present
11		Present	Present
12		Present	Present
13		Absent	Absent
14		Present	Present
15		Absent	Present
16		Absent	Absent
17		Absent	Absent
18		Absent	Absent
19		Absent	Absent
20		Absent	Absent
21		Absent	Present
22	Waste water	Present	Present
23		Present	Present
24		Absent	Absent
25		Absent	Absent
26		Absent	Absent
27		Absent	Absent
28		Absent	Absent
29		Absent	Absent
30		Absent	Absent
31		Absent	Absent
32		Absent	Present
33		Absent	Absent
34		Absent	Absent
35		Absent	Present
36		Absent	Absent
37		Present	Present

**Table 4 - Comparison between traditional method using HEA isolation and real-time PCR**

intentionally kept low (theoretical number between 0.01 and 0.5 UFC/100 ml). Following enrichment, a portion of broth was collected for analysis with the real-time PCR technique, while another portion was analyzed using the traditional method (isolation in both a YEA non-selective growth medium, and an HEA selective growth medium.) Results indicate that only PCR detects the presence of *Salmonella* at the theoretical concentration of 0.5 UFC (Table 2). Using artificial samples, the PCR technique was used to analyze twenty replicas of varying concentrations (0.5 and 0.1 UFC), and confirmed that the detection limit (95% positive replicas) is situated between these two concentrations (Table 3).

### Correspondence between the traditional method and PCR

Thirty-seven samples from various matrices (surface, underground, drinking and waste water) were analyzed in parallel to compare the PCR method to the traditional method used to detect *Salmonella* in water. As shown in Table 4, the traditional technique and PCR correspond in 89% of samples; in the remaining 11% (4 samples) positive results were obtained using PCR and negative results were obtained using the traditional method. This confirms the superior recovery of PCR, which was previously pointed out in the trials described earlier.

### Ring study trials

UNI IEC EN ISO/IEC 17025:2005 [5], p 5.4.5, includes ring trials conducted to compare the performance of techniques used in order to validate a detection method. Participation in the ring study trials «Microbiological agents in waste water», managed by Unichim, «Quality in Water Analysis scheme», managed by Lgc, and «Senate Water Microbiology scheme», managed by Did, has provided fully satisfactory results (Table 5).

Cultural confirmation of PCR positive samples All pre-enrichment broths from real samples that were positive using the Real-time PCR technique (38 samples to date) were isolated to be confirmed culturally using selective growth media (HEA), and suspect colonies were confirmed by the presence of C8 esterase using the 4-methylumbelliferyl caprylate reagent (MUCAP) test. These confirmation trials involving 6 samples of natural, underground water, 23 samples of surface water and 9 samples of waste water produced corresponding results in 100% of cases.

### Conclusions

In addition to producing optimal results, the real-time PCR technique, using the «Adiafood Rapid-Pathogen Detection system for *Salmonella*» from Aes Chemunex and the Stratagene Mx3005P thermocycler, allowed analysis results for samples of water of varying origin (surface, drinking, underground, waste water) to be obtained in significantly less time than with the traditional method. The latter requires a response time of 4 days in the absence of *Salmonella* and 6 - 7 working days for samples with positive results; in these cases, confirmation trials involving the use of selective growth media and/or serologic tests performed on a daily basis are also required.

## ANALYTICAL

Ring study trials			
Sample	Date	PCR	Expected result
Unichim waste water	mar-10	Absent	Absent
Unichim waste water A6	mar-10	Absent	Absent
Lgc waste water surface	mar-10	Present	Present
Senate drinking W1-04-6	ott-10	Present	Present
Senate drinking1-04-65	ott-10	Presente	Present
Senate drinking W1-04-66	ott-10	Absente	Absent
Unichim waste Mias 5A	ott-10	Absent	Absent
Unichim waste Mias 5B	ott-10	Present	Present

Table 5 - Ring study results

method is used, due to the specificity of the primers and molecular beacons. The kit was easy to use, owing, in part, to the use of practical strips, and is therefore adapted to routine laboratory use. Notwithstanding, technicians must be qualified and appropriately trained in using the technique. To conclude, the real-time PCR method for detecting *Salmonella* spp. in water samples has shown to be a valid alternative to the traditional method, in its strictly technical aspects, as well as in a variety of practical assessments designed to determine the applicability of a laboratory method. The benefits of using this analytical technique include the following:

- Accuracy: high specificity and sensitivity, with detection levels superior to those of the reference method.
- Timely results (results obtained in fewer than three hours following pre-enrichment): with near elimination of growth media preparation and confirmation cultures.
- Ease of use: complete analysis kits, simple software, extraction

phases and amplification cycles on video display, results without subjective interpretation.

- Improved sample management, resulting from the possibility of storing broths and extracted DNA for one week under refrigeration. This feature allows various samples to be grouped together in a single analysis microplate, thereby limiting subsequent technician «man-hours».
- Superior data traceability as a result of computer archival of all phases of the analysis.
- Per-sample cost equivalent to that of traditional analysis methods.

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