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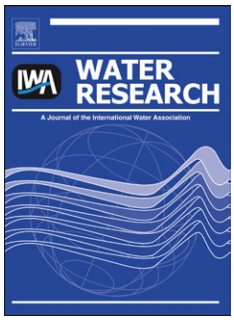

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Specific Detection of viable *Listeria monocytogenes* in Spanish Wastewater treatment plants by Fluorescent *In Situ* Hybridization and PCR.

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Running title: Detection of viable *L. monocytogenes* in wastewater by DVC-FISH and PCR

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Abstract

Listeria monocytogenes detection in wastewater can be difficult because of the large amount of background microbiota and the presence of viable but nonculturable forms in this environment. The aim of this study was to evaluate a Fluorescent *In Situ* Hybridization (FISH) assay combined with Direct Viable Count (DVC) method for detecting viable *Listeria monocytogenes* in wastewater samples, as an alternative to conventional culture methods. 16S rRNA sequence data were used to design a specific oligonucleotide probe. In order to assess the suitability of the method, the assays were performed on naturally (n= 87) and artificially (n=14) contaminated samples and results were compared to those obtained with the isolation of cells on selective media and with a PCR method. The detection limit of FISH and PCR assays was 10^4 cells/mL without enrichment and 10 cells/mL after enrichment. A total of 47 samples, including 3 samples from effluent sites, yielded FISH positive results for *L. monocytogenes*. Using DVC-FISH technique, the presence of viable *L. monocytogenes* cells was detected in 23 out of these 47 FISH positive wastewater samples. PCR and culture methods yielded 27 and 23 positive results, respectively. According to these results, FISH technique has the potential to be used as a sensitive method for the detection and enumeration of *L. monocytogenes* in environmental wastewater samples.

L. monocytogenes / FISH / PCR / Wastewater / Detection

1. Introduction

Listeria monocytogenes is the causal agent of one of the most important foodborne diseases worldwide, with a case-fatally rate of about 30% (Newell et al., 2010). Infection has been associated with the ingestion of a great variety of food products (Lunden et al., 2004). *Listeria monocytogenes* is widely distributed in the natural environment (Fenlon, 1999) and can be found in wastewater at high levels (Paillard et al., 2005; Odjadjare and Okoh, 2010). Some authors have observed that *L. monocytogenes* is able to survive wastewater physical secondary treatment (Paillard et al., 2005). Therefore, the organism could re-enter the human food chain via sludge applications to land as fertilizer or by irrigating crops with treated water. Although when present in low concentrations it should not pose a risk to human health, several cases of *Listeria* outbreaks associated with raw and treated wastewater have been reported (Paillard et al., 2005) and there is a need for more extensive studies to assess the real risk for public health (Odjadjare et al., 2010).

Isolation of *L. monocytogenes* may require about 4-5 days. Besides, under unfavourable environmental conditions *Listeria* cells can enter into a Viable But Nonculturable (VBNC) state, in which they cannot be detected by traditional culture methods (Bersnard et al., 2000). Molecular techniques such as PCR have been applied as a rapid alternative to conventional detection methods (Liu, 2008; Shanon et al., 2007). However, when applying PCR-based methods to environmental samples a complication arises: inhibitory substances, such as humic acids, can have a significant effect on the activity of the *Taq* polymerase enzyme (Lemarchand et al., 2005). Ribosomal RNA probe hybridization without culturing (Fluorescent *in situ* Hybridization, FISH) is less sensitive to inhibitory substances than PCR and has shown to be a very useful tool for phylogenetic, ecological, diagnostic and environmental studies in

microbiology (Bottari et al., 2006). It has been successfully used for the detection and identification of different pathogens in foods, surface water, drinking water and wastewater (Moreno et al., 2007; Piqueres et al, 2006; Schmid et al*.,* 2003).

FISH and PCR techniques do not allow discrimination between dead and viable cells (Okoh et al., 2007), which is a limitation that can lead to false positive results. The Direct Viable Count (DVC) procedure (Kogure et al., 1979) involves exposing bacterial cells to a revival medium which contains antibiotics that prevent cellular division; elongated cells are then enumerated as viable cells (Servais et al., 2009). The combination of DVC, which rises intracellular rRNA levels and increases cell length (Doudu and Colquhoun, 2010), with FISH has been proposed for monitoring viable cells in different environments (Baudart et al., 2002; Piqueres et al., 2005).

In this work, the development of a rapid DVC- *in situ* hybridization combined protocol using a 16S rRNA probe to detect and identify viable *L. monocytogenes* in raw and treated wastewater samples for investigating the occurrence and the survival to wastewater treatments of this pathogen has been reported. Results were compared with those obtained by a culture on selective media and by a PCR method.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Seven reference *Listeria* strains, i.e. 5 *L. monocytogenes* (CECT 911, CECT 4031, CECT 4032, CECT 933 and CECT 936), 1 *L. ivanovii* (CECT 913), 1 *L. innocua* (CECT 910) were used in experiments. Fourteen non-*Listeria* strains (*Vibrio vulnificus* CECT 529, *Vibrio parahaemolyticus* CECT 511, *Staphylococcus aureus* CECT 240, *Micrococcus luteus* CECT 245, *Citrobacter freundii* CECT 401, *Escherichia coli* CECT

349, *Salmonella enterica* CECT 915, *Enterobacter cloacae* CECT 194, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter faecalis* DSMZ 20478, *Campylobacter jejuni* NCTC 11828, *C. coli* NCTC 11366, *Helicobacter pylori* NCTC 11637 and *Arcobacter butzleri* NCTC 12481) were also used to evaluate the specificity of the assays. All of them were rehydrated and cultured according to their Culture Collections instructions (CECT; Spanish Type Culture Collection, Valencia, Spain. ATCC; American Type Culture Collection, Rockville, Md. USA. DSMZ; German Type Culture Collection, Braunschweig, Germany. NCTC; Health Protection Agency Culture Collections**,** Salisbury, UK). *Listeria* strains were grown on TSA (Casein – peptone soy meal - peptone agar for microbiology, Merck, Darmstadt, Germany) for 24 h at 37 ºC and subcultured overnight in Brain-Heart-Infusion (BHI) broth (Merck, Darmstadt, Germany) for specificity and sensitivity assays.

2.2. Fluorescent *in situ* hybridization assays

An oligonucleotide probe complementary to a 16S rRNA region of *Listeria monocytogenes* was designed (*Lmon* probe: 5´-CTATCCATTGTAGCACGTG-3´). The probe targeted position 1242 to 1260 in *L. monocytogenes* 16S rRNA. The specificity of the probe was assessed by a gapped BLAST search (Altschul et al., 1997). Specificity of *L. monocytogenes* probe was also evaluated by *in situ* hybridization with the *Listeria* and non-*Listeria* reference strains included in this study, by whole-cell hybridization according to Wagner et al. (1998). The probe was synthesized and labelled by MGW Biotech (Mannheim, Germany) with 5 (6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) and CY3.

For FISH analysis, a volume of 1 mL of each sample was centrifuged (8000 rpm, at 4 ℃ for 10 min). Resulting pellet was resuspend ed in PBS buffer (130 mM sodium

chloride, 10 mM sodium phosphate, [pH 7.2]), and then fixed with ethanol/PBS (50:50) mixture (Amann et al., 1995). Fixed samples were stored at -20 ºC until their hybridization.

Ten μL of each fixed sample were spotted on a gelatin-coated slide, allowed to air dry, treated with lysozyme and dehydrated (50, 80, 100% ethanol) as previously described (Wagner et al., 1998). A concentration of 20% formamide in the hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl, pH 7.6) was enough to reach the specificity of the whole cell hybridization. In the case of wastewater FISH analysis, the percentage of formamide was increased to 30% to avoid cross-hybridization with other non-culturable genus of bacteria present in the samples. The EUB338 universal probes mixture, complementary to a region of 16S rRNA of the domain *Bacteria* (Amann et al., 1995) was used as a positive control. Slides were mounted in FluoroGuard Antifade Reagent (Bio-Rad, Madrid, Spain) and visualized by epifluorescence Olympus microscopy BX50 with 460-490 nm (U-MWIB) and 520-550 nm (U-MWIG) exciter filters.

2.3. DVC-FISH assay optimization

An overnight culture of *L. monocytogenes* was serially diluted from 10^1 to 10^8 CFU/mL. An aliquot of 1 mL of each dilution was added to 9 μL of BHI broth supplemented with yeast extract (2.5 mg/mL) and different concentrations (0.8, 1, 1.5 μg/mL) of the antimicrobial agent Ciprofloxacin (SIGMA Chemical Co., St. Louis, Mo.), as described by Besnard et al. (2000). DVC broths were incubated at 37 ºC in aerobic conditions and aliquots from each dilution were immediately taken after inoculation and after 7, 16 and 24h and fixed for FISH as described above.

2.4. PCR analysis

Cells were harvested by centrifugation at 14000 x g for 30 s, washed twice with sterile phosphate buffer and suspended in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). DNA isolation was performed using Realpure genomic DNA isolation kit (Durviz, Paterna, Spain) according to the manufacturer´s instructions. Concentrated DNA was stored at -20ºC. *L. monocytogenes* detection was performed by multiplex PCR using the primers described by Bansal *et al*. (1996), which amplify a genus specific 938 bp fragment of 16S rDNA of *Listeria* sp. and a species specific 750 bp fragment of *hly*A gene of *L. monocytogenes*, according to Zamora *et al*. (2000). A final reaction volume of 30 µl was obtained by addition of 3 µl of sample, 20 pmol of each primer, 0.2 mM of each deoxynucleotide, 2.5 mM $MgCl_2$ and 2 U of *Taq* polymerase (New England Biolabs, U.K.). The amplification consisted of an initial DNA denaturing step at 95 ºC for 1 min, followed by a 40-cycle reaction (94 ºC for 30 s, 51ºC for 20 s and 74ºC for 20 s) and a final extension step at 72 ºC for 2 min. PCR reactions were performed with an automatic thermal cycler (PHC-3 Thermal Cycler, Techne Corporation, Cambridge, UK). PCR products were analyzed by electrophoresis at 100 V for 1 h through 1% (wt/vol) SeaKem LE agarose (FMC Bioproducts, Denmark) gels. Amplimers were visualized by ethidium bromide staining under UV light. A 100 bp DNA ladder was used as a molecular weight marker.

2.5. Detection of *L. monocytogenes* in inoculated samples

An overnight culture of *L. monocytogenes* CECT 911 was serially diluted from 100 to 10^8 CFU/mL, and used to inoculate 300 mL of PBS buffer and 300 mL of two *Listeria*-free (*Listeria*-negative by PCR and by culture) wastewater samples with different origins (one from each plant, A and B). The number of cells in each dilution was

calculated from the colony count on Tryptone Soy Agar plates (Merck, Darmstadt, Germany). For direct detection, cells from 100 mL of each inoculated water sample were harvested by centrifugation (1000 xg) and the pellet was resuspended in 3 mL of PBS buffer. For detection after enrichment, one hundred mL of each inoculated water sample were filtered through 0.45 μm cellulose nitrate membrane filters (Whatman, Maidstone, England), the membranes were aseptically rolled, transferred to 100 mL of Modified Fraser broth (Garrec *et al*., 2003) and incubated in aerobic conditions at 37 ºC for 24 h. One mL aliquots of each PBS suspension and each enrichment broth were fixed, inoculated for DVC incubation and processed for PCR analysis respectively.

2.6. Detection of *L. monocytogenes* in natural wastewater samples

A total of 261 samples were collected in 87 sampling campaigns from two secondary wastewater treatment plants during a two-year period: 45 sample collections (M1 to M45) were taken from plant A (899,000 population equivalents), and 42 (M46 to M87) from plant B (262,000 population equivalents). Both plants, located in Valencia, Spain, collect urban and industrial wastewater and apply biological secondary treatment (activated sludge tank), and tertiary UV disinfection treatment. Final effluent is discharged into the sea or used for irrigation purposes. On each occasion, samples were taken from three sites: the influent, the secondary treatment effluent and after the tertiary treatment. All samples were placed into sterile glass bottles, refrigerated and processed for culture, FISH, DVC-FISH and PCR analysis within 6 h of collection. All the assays, except the DVC-FISH one, were performed with and without enrichment, as described for inoculated samples. To confirm the results, each sample was tested twice in different experiments.

Aliquots of 100 μL of each sample, before and after enrichment, were plated onto selective Palcam media (OXOID SA, Madrid, Spain) and Chromogenic Listeria Agar (OCLA, OXOID) and incubated at 37 ºC for 24 h. Presumptive colonies were purified and identified by API-Lis biochemical system (Biomerieux, Mercy L'Etoile, France) and PCR, as described above.

3. Results

3.1. FISH control assays

Alignment of the *Lmon* designed probe with the sequences deposited in GenBank for *Listeria* and other related organisms showed that the studied probe completely and exclusively matched the target region of *L. monocytogenes* 16S rRNA. The *Lmon* probe hybridized with all the *L. monocytogenes* strains, whereas none of the other tested species showed any fluorescent signal (Fig. 1).

The detection limit of the FISH assays for *L. monocytogenes* in inoculated PBS buffer was 10^4 CFU/mL. In inoculated wastewaters, it was ten-fold higher than in PBS (10^5 CFU/mL). However, the 24-hour enrichment step enabled a detection limit of 10 CFU/mL in both PBS and wastewaters (Fig. 2).

The optimal concentration of Ciprofloxacin for DVC analysis was 2 mg/L in both pure bacterial cultures and spiked samples. A seven hour-incubation generated a significant elongation of viable cells. DVC-FISH specific analysis showed elongated and not elongated *L. monocytogenes* cells in the inoculated wastewater samples but, in accordance with Besnard et al. (2000), only those elongated at least twice their original size were estimated as viable.

3.2. PCR control assay

Alignment of the sequences of *L. monocytogenes* deposited in GenBank with those of other related organisms showed that the two sets of primers used in this study were suitable for multiplex PCR detection of *L. monocytogenes* species. All the reference *Listeria* strains tested were positive by multiplex PCR (mPCR) reaction. No amplification was obtained for the remaining bacterial genera included in this work.

The detection limit of mPCR assay for *L. monocytogenes* in both inoculated wastewater samples and PBS buffer without enrichment was 10^4 CFU/mL and after 24 h of incubation it decreased to 10 CFU/mL. Incubation periods longer than 24 h did not improve the detection limit in any type of sample.

3.3. *L. monocytogenes* detection in wastewater samples

Forty-eight out of the 261 samples tested were found to be positive for the presence of *L. monocytogenes* using any of the assays, including one PCR positive sample, which was *Listeria* negative by FISH and culture detection (M44). By FISH analysis, 47 samples yielded positive results for *Lmon* probe hybridization (Fig. 3), 37 of them allowing *L. monocytogenes* detection prior to enrichment (Table 1). Twenty-three out of these 37 FISH positive samples were also positive by DVC-FISH (presence of elongated viable *L. monocytogenes* cells).

Positive PCR results for *L. monocytogenes* were obtained in 27 samples, although in 25 only after the enrichment step. By culture, 23 samples were positive, although in 25 cases, only after enrichment. Biochemical tests and multiplex PCR identified all the isolates as *L. monocytogenes.*

When compared, 15 samples yielded concordant results for all three methods. FISH and culture results were concordant for 23 samples and discordant for 24 samples

(table 2). FISH and PCR yielded concordant results for 26 samples, while discordances were observed in 22 cases. Finally, culture and PCR results were concordant for 31 samples and discordant for 17. Thirteen FISH positive samples yielded negative results by both, PCR and culture techniques. In eleven cases, samples were positive by FISH and PCR, but not by culture. PCR was negative for eight FISH and culture positive samples.

When comparing results from the two wastewater treatment plants, percentage of positive samples by any technique was 49% (22/45) in plant A and 62% (26/42) in B. Although there were two positive samples after secondary treatment in plant A and 6 in B, only three samples (2 in plant A and one in plant B) were *Listeria*-positive after tertiary treatment.

4. Discussion

In this study, a specific probe for *L. monocytogenes* FISH detection has been developed. The FISH method has the advantage of not being inactivated by inhibitors, even when a large amount of sample is processed (Moreno et al., 2001). Besides, a protocol to obtain the DNA from bacteria is not necessary, and positive results may be directly observed in the sample without culture. A combination of FISH with a DVC step allowed the direct *in situ* identification and visualization of viable cells of *L. monocytogenes* within the sample. A positive response to the DVC procedure indicates that a cell is substrate-responsive in the incubation conditions used for DVC. This does not mean that a DVC-positive cell is active in the natural environment, but it demonstrates that its cellular machinery is intact and that it can be considered viable and, therefore, potentially infective (Servais et al., 2009).

According to other authors (Moreno et al., 2001; Liu, 2008), we included an enrichment step in order to reduce the use of large volume samples to reach the detection limit of pathogens in low contaminated samples. In contrast with other works (Piqueres et al., 2005) in which enrichment diminished the effectiveness of detection, enrichment increased the level of *L. monocytogenes* cells present in the samples, thus avoiding interference of competitive microbiota. The increase of positive samples after an enrichment step shows the possible underestimation of this pathogen when samples are directly analyzed by FISH.

The FISH assay was shown to be more sensitive than culture: of the 48 samples positive for the presence of *L. monocytogenes* detected by any technique, half were positive by the FISH test and negative by culture. Thus, as expected, DVC-FISH yielded more positive results than culture before enrichment. There were four DVC-negative samples in which culture was positive, but only after enrichment, indicating that the number of cells in the sample was too low to be directly detected by this technique. However, in two samples (M53 and M61) culture was positive without enrichment, while DVC-FISH yielded false-negative results. It must be considered that differentiation between live and dead *Listeria* based on the elongation is a subjective test, which contributes to increase the subjectivity of any fluorescent assay (Coallier et al., 1994). Furthermore, fluoroquinolone MIC can vary according to the strain and subMIC concentrations for one isolate can inhibit and even kill other ones.

The comparison of results obtained using the molecular methods described in this study shows that FISH allowed direct detection of this pathogen in a high percentage of naturally contaminated wastewater samples, although in inoculated samples it showed the same detection limit as the PCR technique. It has been reported that FISH sensibility can be affected by some environmental factors, such as contamination (Bouvier and del

Giorgio, 2003). However, in our work, background fluorescent signals due to non-specific probe attachment to flocks microbiota did not interfere with the FISH specific signal. In contrast, microbial background represented a problem for *L. monocytogenes* isolation from this complex bacterial community due to overgrowth in selective media.

In six samples, viable *Listeria* cells were directly detected from the sample, while culture was negative, even after enrichment. This could indicate the presence of viable but nonculturable (VBNC) cells. This fact may be important from a sanitary point of view; some authors (Pommepuy et al. 1996; Asakura et al. 2002; Jolivet-Gougeon et al. 2006) have suggested that pathogenic VBNC bacteria can maintain their virulence, becoming a potential reservoir of disease.

Although PCR method is considered to be more sensitive than FISH or culture methods, more *L. monocytogenes* positive samples were obtained by FISH technique than by culture or PCR analysis, even when an enrichment step was performed. Similar results have been previously found for the detection of other pathogens such as *Helicobacter pylori* in wastewater (Piqueres et al., 2005). Lepueuple et al. (2003) also reported the problem of cultural techniques and the higher number of positive samples by FISH techniques to enumerate *E. coli* in water.

In our work, an enrichment step was necessary for obtaining multiplex PCR positive results in most samples. To confirm this, each sample was tested twice and, for all samples, repeated PCR analysis yielded consistent results. Some authors have suggested that this could be due to inhibitory substances present in wastewater as humic acids that can have a significant effect on the activity of the *Taq* polymerase enzyme, yielding false negative results (Lemarchand et al., 2005). An enrichment step dilutes inhibitors of the sample, thus improving detection rates (Liu, 1998).

Although FISH seems to be more effective than PCR for *L. monocytogenes* detection in wastewater samples, and including a DVC step has allowed us to distinguish between live and dead cells, its combination with PCR could be an excellent tool to avoid false negative results. However, for detection purposes in environmental samples, the cost-benefit ratio must be taken into account.

Thirty out of 87 samplings of wastewater showed the presence of *L. monocytogenes* when directly examined by FISH, what means that concentrations were at least 10^4 ufc/mL. Percentage of positive samples by any technique was similar for both wastewater treatment plants (49% in A and 62% in B). Although there were more positive samples after secondary treatment in plant B, differences were not significant. These results are in agreement with those reported by other authors, which have previously shown a great prevalence of *L. monocytogenes* in wastewater (Paillard et al., 2005).

Despite an official analysis to detect the presence of *L. monocytogenes* on final effluents is not usually required, the spread of this pathogen in the environment should be controlled since these treated waters could be used in agriculture, reaching humans or animals.

Conclusions

Viable *L. monocytogenes* cells are present in wastewater samples, including final treated water. These findings indicate that *L. monocytogenes* is able to survive tertiary wastewater treatment. The DVC-FISH combined method developed in this work is a quick and specific tool for the detection, identification and direct visualization of viable *L. monocytogenes* in complex mixed communities such as wastewater samples.

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Legend to Figures

Fig. 1- FISH micrographs showing hybridization of a mixed *L. monocytogenes* CECT 911 and *L. innocua* CECT 910 culture by simultaneous application of probes *Lmon*-CY3 and EUB338mix-FLUOS. Green fluorescence signifies hybridization with EUBmix-FLUOS and orange signifies hybridization with both *Lmon*-CY3 and EUB338mix-FLUOS.

Fig. 2- FISH-DVC specific detection of *L. monocytogenes* CECT 911 in inoculated wastewater samples.

Fig. 3- FISH-DVC detection of *L. monocytogenes* by simultaneous application of probes *Lmon*-CY3 and EUB338mix-FLUOS in wastewater samples.

Research Highlights

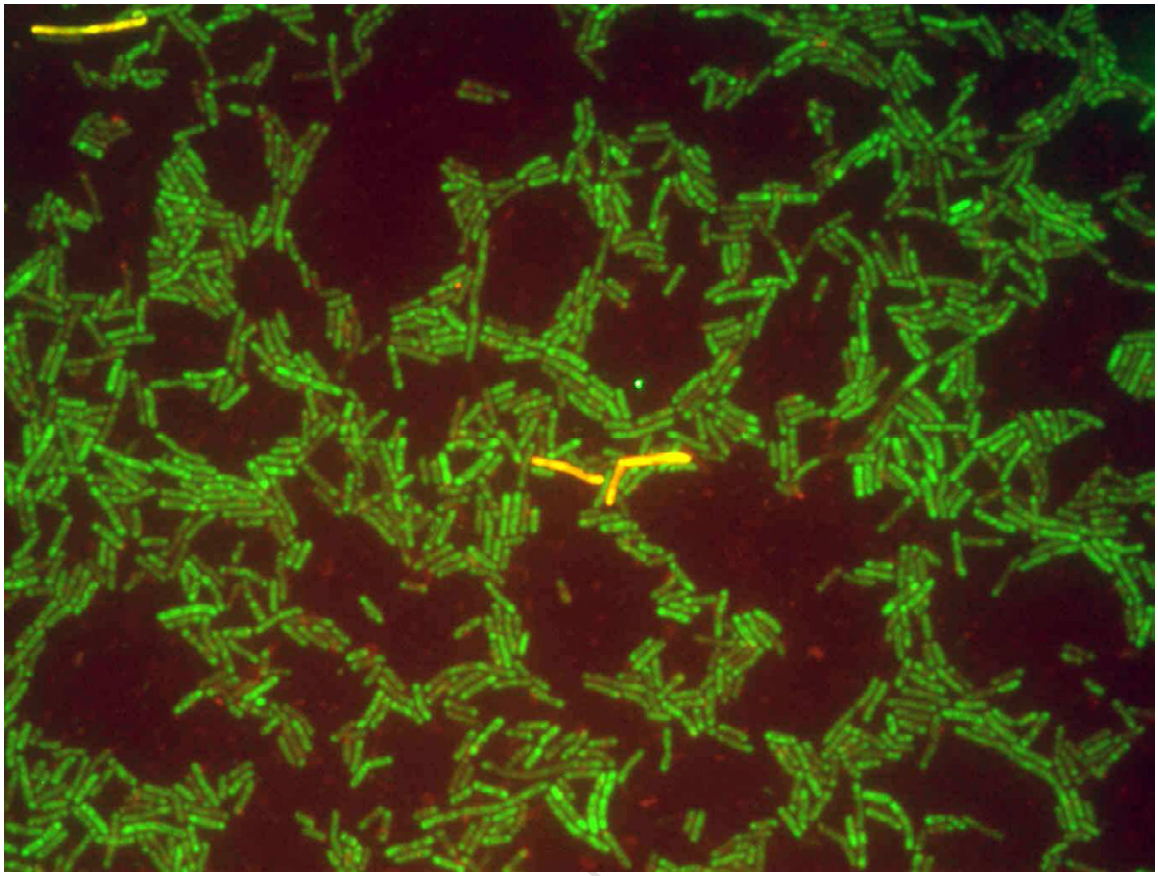
1. A high percentage of wastewater samples are positive for *L. monocytogenes*
2. FISH technique is a quick and sensitive method to detect *L. monocytogenes*.
3. Treated wastewater for agricultural use should be analyzed to detect the presence of *L. monocytogenes* .

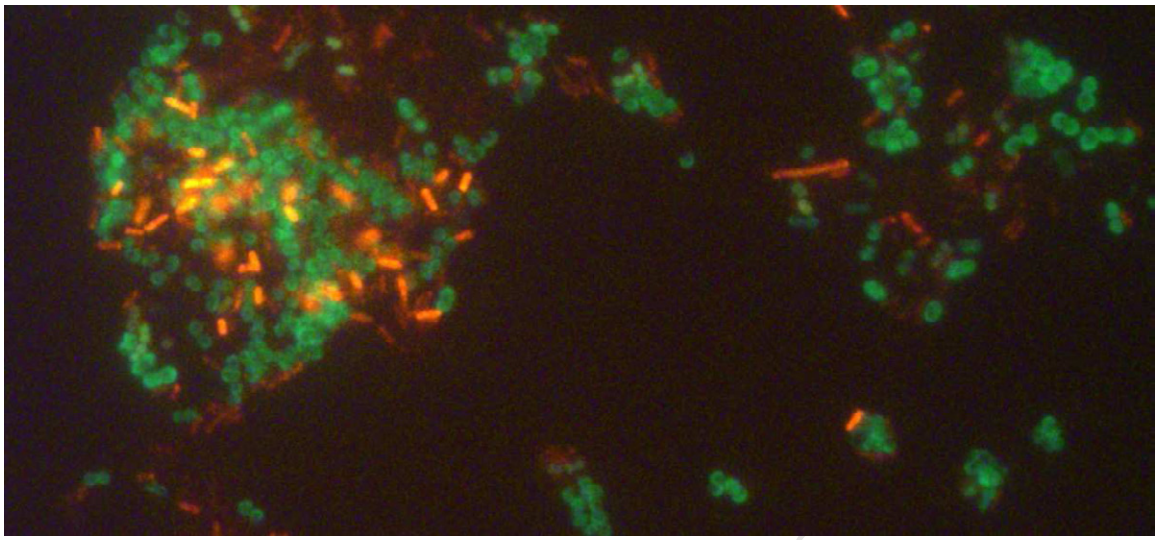
Table 1. Detection of *L. monocytogenes* in natural wastewater samples. Only positive samples for any assay are shown. Results were obtained prior to enrichment, unless indicated.

Samples	Treatment Plant	Origin	Culture[a]	FISH	DVC-FISH	mPCR
M1	A	Influent	**-**	+[b]	-	**-**
M2	A	Influent	-	+	-	+[b]
M3	A	Influent	-	+	+	+[b]
M4	A	Influent	-	+	-	+[b]
M5	A	Influent	-	+	-	+[b]
M6	A	Influent	-	+[b]	-	-
M7	A	Influent	+[b]	+	+	+[b]
M8	A	Influent	+[b]	+	+	+[b]
M9	A	Influent	+[b]	+	+	+[b]
M10	A	Influent	+[b]	+	+	+[b]
M11	A	Influent	+	+	+	+[b]
M12	A	After secondary treatment	+	+	+	+[b]
M13	A	Influent	-	+[b]	-	-
M14	A	After tertiary treatment	-	+	-	-
M15	A	Influent	-	+[b]	-	-
M16	A	After secondary treatment	-	+[b]	-	-
M17	A	Influent	+[b]	+[b]	-	-
M18	A	Influent	+	+	+	-
M19	A	After secondary treatment	-	+[b]	-	-
M20	A	Influent	-	+	-	+[b]
M21	A	After secondary treatment	-	+	-	-
M44	A	After tertiary treatment	-	-	-	+[b]
M48	B	Influent	+[b]	+	+	+[b]

M49	B	Influent	+[b]	+	+	-
M51	B	Influent	+[b]	+	+	+[b]
M52	B	Influent	-	+	-	-
M53	B	Influent	+	+	-	+[b]
M53	B	After secondary treatment	-	+	+	+[b]
M55	B	Influent	+[b]	+	+	+[b]
M56	B	Influent	+	+	+	+[b]
M56	B	After secondary treatment	-	+[b]	-	+[b]
M58	B	Influent	+[b]	+	+	+[b]
M59	B	Influent	+[b]	+[b]	-	+[b]
M59	B	After secondary treatment	+	+	+	+[b]
M61	B	Influent	+	+[b]	-	+[b]
M63	B	Influent	+[b]	+	-	-
M65	B	Influent	-	+	+	-
M66	B	Influent	+[b]	+	-	-
M67	B	Influent	-	+	-	+
M67	B	After secondary treatment	-	+	+	+
M67	B	After tertiary treatment	-	+	+	-
M70	B	Influent	+[b]	+	+	-
M70	B	After secondary treatment	+	+	+	-
M73	B	Influent	+[b]	+	+	-
M76	B	Influent	-	+	-	+[b]
M79	B	Influent	-	+	-	+
M85	B	Influent	-	+	+	-
M85	B	After secondary treatment	-	+	-	-

[a] isolate identified as *L. monocytogenes*; [b]Positive results obtained only after enrichment .





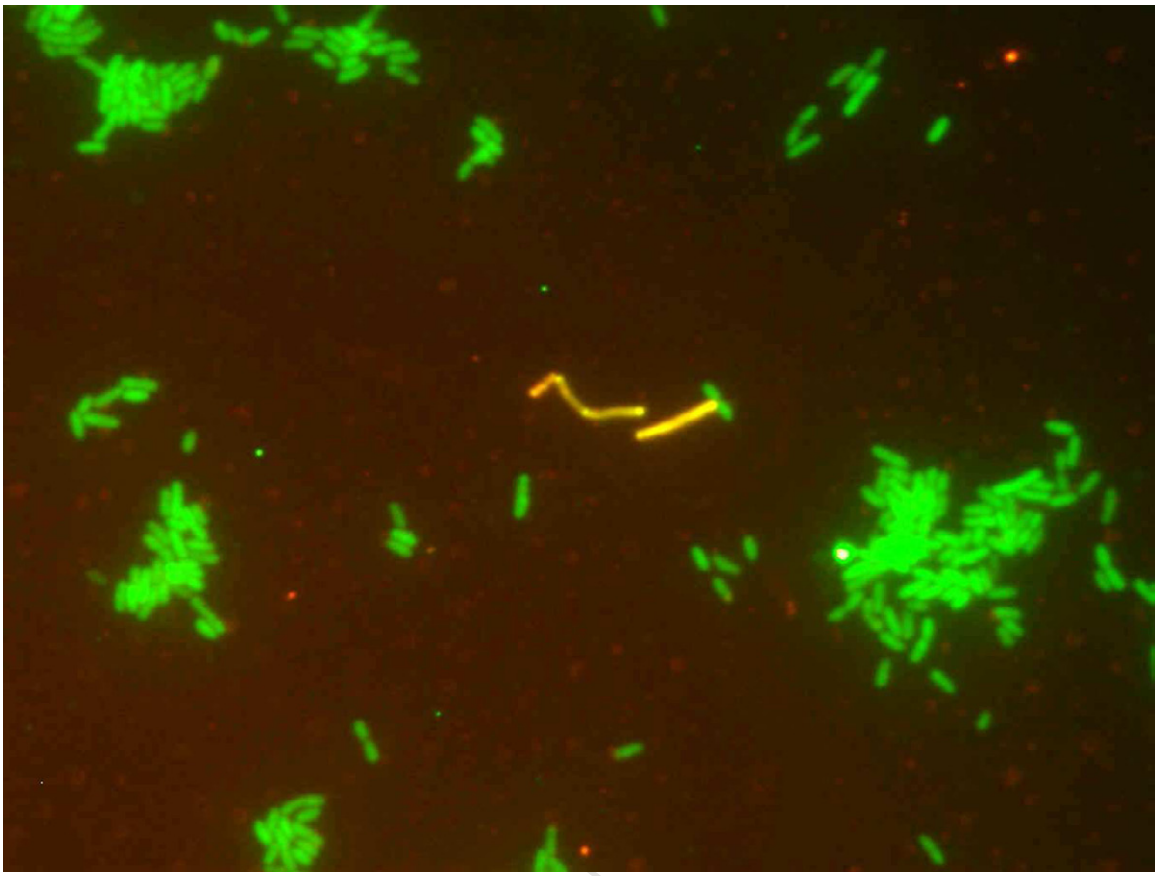


Table 2. Comparison of *L. monocytogenes* detection results obtained by the three methods used in this study

Number of samples		PCR		Culture	
		+	-	+	-
PCR	+			18	12
	-			5	13
FISH	+	26	21	23	24
	-	1	0	0	1