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Santiago Cuellar, P.; Jiménez Belenguer, AI.; García Hernández, J.; Montes Estellés, RM.; Hernández Pérez, M.; Castillo López, MÁ.; Ferrús Pérez, MA.... (2018). High prevalence of Salmonella spp. in wastewater reused for irrigation assessed by molecular methods. International Journal of Hygiene and Environmental Health. 221(1):95-101. https://doi.org/10.1016/j.ijheh.2017.10.007

The final publication is available at

http://doi.org/10.1016/j.ijheh.2017.10.007

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Additional Information

1 **High prevalence of *Salmonella* spp. in wastewater reused for irrigation assessed by**

2 **molecular methods.**

3

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40

41 **Running head**

42 *Salmonella* detection in reused wastewater

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52

53 **Abstract**

54

55 *Salmonella* spp. is one of the most important causal agents of food-borne illness in developed
56 countries and its presence in irrigation water poses a risk to public health. Its detection in
57 environmental samples is not easy when culture methods are used, and molecular techniques
58 such as PCR or ribosomal rRNA probe hybridization (Fluorescent *in situ* Hybridization,
59 FISH) are outstanding alternatives.

60 The aim of this work was to determine the environmental risk due to the presence of
61 *Salmonella* spp. in wastewater by culture, PCR and FISH. A new specific rDNA probe for
62 *Salmonella* was designed and its efficiency was compared with the rest of methods. Serotype
63 and antibiotic resistance of isolated strains were determined.

64 Forty-five wastewater samples (collected from two secondary wastewater treatment plants)
65 were analysed. *Salmonella* strains were isolated in 24 wastewater samples (53%), two of them
66 after disinfection treatment. Twenty-three *Salmonella* strains exhibited resistance to one or
67 more antimicrobial agent. Analysis of wastewater samples yielded PCR positive results for
68 *Salmonella* in 28 out of the 45 wastewater samples (62%). FISH analysis allowed for the
69 detection of *Salmonella* in 27 (60%) samples. By using molecular methods, *Salmonella* was
70 detected in four samples after disinfection treatment.

71 These results show the prevalence of *Salmonella* in reclaimed wastewater even after U.V.
72 disinfection, what is a matter of public health concern, the high rates of resistance to
73 antibiotics and the adequacy of molecular methods for its rapid detection. FISH method, with
74 SA23 probe developed and assayed in this work provides a tool for detecting *Salmonella* in
75 water within few hours, with a high rate of effectiveness.

76

77

Keywords

Salmonella; FISH; wastewater; molecular detection; reclaimed water

Significance and Impact of the Study

In this study, a new specific nucleotide probe for *Salmonella* has been developed. *In situ* hybridization, more rapid and sensitive than culture, is proposed for the detection of *Salmonella* in environment, as an alternative or in combination with PCR.

Public health risk is demonstrated, as antibiotic resistant *Salmonella* strains are present in wastewater reclaimed for irrigation use.

1 Introduction

Salmonella spp. is one of the most important causal agents of food-borne illness in developed countries. The presence of *Salmonella* in water poses a risk to public health, since it is one of the most frequently encountered pathogenic microorganisms in surface waters. Even if disease is not directly caused by its consumption, contaminated water can be considered an important source of transmission on food (Sánchez-Vargas *et al*., 2011).

One of the problems of most concern from standpoint of environment and health is bacterial resistance to antibiotics, and the possible spread of antibiotic resistance among microorganisms in environment. Antimicrobial drug resistance in *Salmonella* is an almost inevitably effect of the use of antimicrobial drugs in food producing animals and human medicine. Resistant strains can enter various stages of the urban water cycle (Pruden, 2014) and, at present, the presence of multidrug-resistant *Salmonella* in the environment is considered a public health hazard (Ferri *et al*., 2015).

104 Although the treatment processes of wastewater are developed to remove successfully
105 pathogens from the influent, some bacterial pathogens are able to resist the disinfection
106 process and can be present in the irrigation water. Several factors as concentration of
107 disinfectant, exposition time to disinfection, influence of suspended particles on the action of
108 disinfectants as UV, or chlorine and pathogen ability to resist the treatment, are involved in
109 the success of the tertiary treatment (Hijnen *et al*., 2006; Moreno *et al*., 2003; Ndiaye *et al*.,
110 2011). Physical (heat, radiation, freezing) and chemical agents (chlorine) are the main
111 disinfection methods for tertiary treatment of sewage. Chlorine has shown its efficiency for
112 eliminating a wide variety of pathogens, including *Salmonella* Typhimurium, *Yersinia*
113 *enterocolitica,* and *Listeria monocytogenes*. UV disinfection, investigated in a full-scale plant
114 in Ontario has shown to be as efficient as chlorination with respect to the inactivation of total
115 coliforms, faecal coliforms and faecal streptococci (Zhou and Smith, 2002).

116 Advanced treatment technologies and disinfection process are regarded as a major tool to
117 control the spread of antibiotic resistant strains into the environment. However, in spite of all
118 the efforts made over the last years to provide solutions to antibiotic resistance spread in the
119 environment, the question is far to be solved (Rizzo *et al*., 2013).

120 Usually, indicator bacteria such as faecal coliforms are used to assess the efficiency of
121 pathogen removal in water purification processes. However, some pathogens are more
122 resistant to conventional wastewater treatment, including chlorination (Salgot *et al*., 2006;
123 Wéry *et al.*, 2008; Fernandez-Cassi *et al.*, 2016). In this sense, the suitability of these bacteria
124 as indicators of the occurrence and concentration of *Salmonella* in wastewater has been
125 questioned (Ashbolt, 2015).

126 At present, public concern about the risks of using reclaimed water for agriculture irrigation is
127 arising, due to the risk of re-entrance of pathogens in the food chain. Irrigation represents up
128 to 33% of the total water use in EU. In Spain, near 80% of reused wastewater is intended for
129 irrigation (European Environmental Agency, 2012). Consistent contamination with irrigation

130 waters is a common route of crop contamination in produces related to *Salmonella* outbreaks
131 (Levantesi *et al.*, 2012).

132 Investigation of *Salmonella* in reclaimed water is not required by either WHO (Blumenthal *et*
133 *al.*, 2000), U. S. Environmental Protection Agency (Bastian and Murray, 2012) or European
134 Directives (Council Directive 91/271/EC). However, many studies demonstrate its presence in
135 reused water (Li *et al.*, 2014; Lopez-Galvez *et al.*, 2014; Levantesi *et al.*, 2010). Detailed
136 scientific studies on the quality of re-used effluents are needed to aid in making informed
137 decisions concerning future uses of recycled water to ensure the health safety.

138 On the other hand, important problems concerning the detection of *Salmonella* in
139 environmental samples arise when culture methods are used. These processes are time-
140 consuming and laborious, requiring at least 5 days for obtaining a positive confirmation
141 (Waage *et al*., 1999). Moreover, as other waterborne pathogens, *Salmonella* can survive
142 disinfection treatments by several strategies as integrating into biofilms (Solano *et al*., 2002),
143 as a host of a protozoa (Wildschutte *et al.,* 2007) or adopting the viable but non-cultivable
144 (VBNC) state (Zeng *et al*., 2013). Thus, the actual prevalence of *Salmonella* in reused water
145 may be underestimated.

146 An alternative to conventional detection methods is PCR. However, when environmental
147 samples are analyzed difficulties arise, since inhibitory substances, such as humic acids can
148 have significant effect on the activity of the Taq polymerase enzyme (Lemarchand *et al*.,
149 2005; Shanon *et al*., 2007).

150 Ribosomal rRNA probe hybridization without culturing (Fluorescent *in situ* Hybridization,
151 FISH) has become widely adopted for detection of specific bacterial groups in mixed
152 populations (Garcia-Hernandez *et al.*, 2012, Moreno *et al.*, 2011). The FISH assay is less
153 sensitive to inhibitory substances than PCR and has shown to be a very useful tool for
154 phylogenetic, ecological, diagnostic and environmental microbiology studies (Bottari *et al.*,

2006). It has been successfully used for detection and identification of different pathogens, including *Salmonella*, in foods, surface water, drinking water and wastewater (Zadernowska *et al*., 2014; Sha *et al.*, 2013; Almeida *et al*., 2011; Almeida *et al*., 2010; Gironés *et al*., 2010).

The aim of this study was to determine the suitability of a new FISH method for rapid and accurately detecting *Salmonella* in wastewater samples, in order to determine the environmental risk due to the presence of the pathogen. The presence of antibiotic-resistant strains or main pathogenic serotypes was determined. Especial attention was paid to the presence of *Salmonella* in treated water intended for irrigation, due to the risk of its re-entrance in the food chain.

2 Materials and Methods

2.1 Bacterial strains and culture conditions

A total of seventy-six *Salmonella* strains (16 reference strains and 50 strains from our collection, isolated from river and wastewater), representing 25different serotypes, and nine additional strains belonging to other bacterial genera were used to examine primers and probe specificity (Table 1). *Salmonella enteritidis* CECT 50 (Colección Española de Cultivos Tipo, Spain) was used for inoculating samples and for sensitivity tests. All strains were cultured under CECT recommended conditions.

2.2 Probe

A 23S rRNA oligonucleotide probe complementary to *Salmonella* spp. was designed (SA23 probe: 5´-CACTTCACCTACGTGTCA-3´). The probe targeted position 1725 to 1742 in *Salmonella* spp. 23S rRNA. The specificity of SA23 probe for *Salmonella* spp. detection was

181 confirmed by a gapped BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi
182 http://www.genome.jp/tools/blast/). SA23 probe specificity was also evaluated by *in situ*
183 hybridization with different *Salmonella* and non-*Salmonella* species prior to its use. The probe
184 was synthesized and labelled by MGW Biotech (Mannheim, Germany) with CY3.

185

186 **2.3 FISH preliminary assays**

187

188 Overnight cultures of *Salmonella enteritidis* CECT 50 were serially diluted in water to obtain
189 1 to 10^8 CFU/ml. Dilutions were inoculated into 200 ml of sterile water and 200 ml of
190 *Salmonella*-free influent wastewater samples (negative detection by PCR and by culture). To
191 determine enrichment step effect, 100 ml of the inoculated samples were filtered through 0.45
192 µm membrane filters (Whatman, Maidstone, England). The membranes were aseptically
193 removed from the filtration unit, rolled, and transferred to 100 ml of Rappaport-Vassiliadis
194 Broth (Oxoid) and incubated at 42ºC. Aliquots were taken after 6 and 24h.

195 FISH analysis was performed according to Moreno *et al*. (2003). Briefly, a volume of 1 ml of
196 each sample was centrifuged (8000 rpm, at 4°C for 8 min). Resulting pellet was resuspended
197 in PBS buffer (130 mM sodium chloride, 10 mM sodium phosphate, [pH 7.2]), and fixed with
198 three volumes of 4% paraformaldehyde for 2 h at 4ºC. Fixed samples were centrifuged,
199 washed with PBS buffer and finally resuspended in 1:1 PBS/ethanol (v/v). An aliquot of 20 µl
200 fixed sample was placed on a gelatine-coated slide, air-dried and dehydrated (50, 80, 100%
201 ethanol). To provide a specific hybridization to the target organisms, 50 ng of probe and
202 different concentrations of formamide (0% to 35%) were tested in the hybridization buffer
203 (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl, pH 7.6). Unbinding probe was removed by
204 washing with prewarmed washing buffer (20 mM Tris-HCl, 0.01% SDS, 5 mM EDTA, 80
205 mM NaCl) for 20 min. Slides were mounted with FluoroGuard Antifade Reagent (Bio-Rad,
206 Spain) and visualized by Olimpus BX50 microscopy system with filters U-MWIB and U-

207 MWIG. Digital colour micrographs were taken by DP10 camera (Olympus Optical CO.,
208 Germany).

209

210 **2.4 PCR preliminary assays**

211

212 For PCR preliminary assays, sterile and wastewater samples were inoculated and processed as
213 described above for FISH assays. Aliquots of 1 ml of each centrifugated sample and
214 enrichment broths were obtained and DNA was extracted following the CTAB method
215 (Wilson, 2001). PCR was performed by using an amount of 2 μl from each DNA extract and
216 primers ST1-1 and ST1-5 (ST1-1: 5´-GCCAACCATTGCTAAATTGGCGCA-3´; ST1-5: 5´-
217 GGTAGAAATTCCCAGCGGGTACTGG-3´), which amplify a *Salmonella* sp. specific
218 chromosomal fragment of 429 bp. (Aabo *et al*., 1993).

219 PCR was performed according to Soumet *et al*. (1999), with a slight modification in annealing
220 temperature for increasing specificity. Briefly, samples were amplified in a reaction volume
221 of 50 μL containing 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM MgCl2 1% glicerol
222 (v/v), 2 μl from each primer, 100 μM from each dNTP, 0.2 μL of DNA polymerase
223 (BIOTAQTM Bioline M9581B) and 2 μL of DNA sample. An initial denaturation at 95ºC
224 form 10 min was followed by 35 cycles of amplification at 95ºC for 30 sec, 1 min. at 60 ºC
225 and 72ºC for 30 sec., with a final extension cycle of 72ºC for 10 min. PCR products were
226 analysed by electrophoresis at 100 V for 1 h through 1% (w/v) agarose gels in TBE buffer pH
227 8.3 and visualized by staining with ethidium bromide under U.V. light. A 100 bp DNA ladder
228 was used as a molecular weight marker.

229

230 **2.5 Wastewater samples**

231

232 Forty-five wastewater samples were collected from two secondary wastewater treatment
233 plants located in Valencia, Spain. Both plants collect urban wastewater and apply biological
234 secondary treatment (activated sludge tank) and tertiary chlorination (Plant A) or UV
235 disinfection treatment (Plant B). Final effluent is mainly reused after disinfection step for
236 irrigation purposes. Sampling was performed between October 2015 to February 2016,
237 alternating collections from the two plants over the sampling period. Twenty-one samples,
238 from 7 different collections (A1 to A7) were taken from plant A (351.198 population
239 equivalents), and 24 (B1 to B6) from plant B (166.942 population equivalents). Samples were
240 obtained at the entry (raw sewage, R), at secondary treatment effluent (S) and at tertiary
241 treatment effluent (T). For samples taken at plant B, another sampling point, consisting in
242 water submitted to sand treatment filtration process (F) just after secondary treatment, was
243 included (Table 2).

244 All samples were placed into sterile glass bottles, refrigerated and processed for culture,
245 FISH, and PCR analysis within 6 h of collection. All the assays were performed before and
246 after 24 h enrichment, as described for inoculated samples. To confirm the results, each
247 sample was tested twice in different experiments.

248

249 **2.6 Faecal coliforms enumeration**

250

251 Presumptive faecal coliforms concentrations were measured after tertiary treatment (T
252 samples), by using standard methods based on membrane filtration (UNE-EN ISO 9308-1:
253 2014): a total of 100 ml of each water sample obtained was filtered; each membrane was
254 deposited in plates containing mFC agar (Merck) and incubated at 44 ºC. Enumerations were
255 expressed as colony-forming units (CFU) per 100 ml.

256

257 **2.7 Detection of *Salmonella* in wastewater samples**

258

259 For direct detection, 200 mL of each sample were centrifuged (8000 rpm), and resuspended in
260 2 mL of PBS buffer. For detection after enrichment, 100 mL of sample were filtered through
261 0.45 µm-pore-size membrane filters (Whatman, Maidstone, England) and processed as
262 described for inoculated samples.

263 For cultural detection, 0.1 ml aliquots of Rappaport-Vassiliadis enrichment broth were plated
264 onto both, XLD and Hektoen Agar (OXOID), incubated at 37ºC and examined for the
265 presence of characteristic colonies after 24 and 48 h of incubation.

266 Two to four *Salmonella* typical colonies were randomly picked from each of the respective
267 isolation media and were biochemically confirmed by Biochemical tests using API20E system
268 (Biomérieux, France), transferred to agar slants and stored for further analyses. Serotypes
269 were identified by standard agglutination in Centro de Calidad Avícola y Alimentación
270 Animal de la Comunidad Valenciana (CECAV).

271 For PCR and FISH detection, aliquots of 1 mL were taken from PBS containing centrifuged
272 sample and after the enrichment step, and processed as described for inoculated samples.

273

274 **2.8 Antibiotic resistance test**

275

276 Antibiotic resistance tests were performed by disk diffusion tests (Antimicrobial
277 Susceptibility Test Disc, OXOID Ltd., England, UK), according to the Clinical Laboratory
278 Standards Institute guidelines (CLSI, 2014). Susceptibility to twelve commercially available
279 antibiotics was determined: amikacin (AK: 30µg), ampicillin (AMP: 10µg),
280 amoxicillin/clavulanic acid (AMC: 20/10µg), sulfamethoxazole-trimethoprim (STX:
281 1.25/23.75µg), ceftriaxone (CRO: 30µg), ciprofloxacin (CIP: 5µg), chloramphenicol (C:
282 30µg), carbenicillin (CAR: 100µg), gentamicin (CN:10µg), nalidixic acid (NAL: 30µg),

283 tetracycline (TE: 30µg) and cephalothin (KF: 30µg). *E. coli* ATCC 25922 was used as a
284 control strain. The levels of resistance were determined according to the recommendations of
285 the Clinical Laboratory Standards Institute (CLSI, 2014).

286

287 **3. Results and discussion**

288 **3.1 Molecular preliminary assays**

289 Optimized final formamide concentration was stablished at 20 %. Under these stringent
290 conditions, the designed SA23 probe was able to detect all *Salmonella* strains tested, while
291 other bacteria yielded negative results (Table 1). Despite the fact that wastewater samples
292 showed a moderate non-specific fluorescence background, cells of *Salmonella* could be easily
293 detected, even when they were included into bacterial flocks.

294 Alignment of GeneBank published sequences of *Salmonella* with other related organisms
295 showed that the pair of primers used in this study was suitable for PCR detection of
296 *Salmonella* species. PCR reactions using primers ST11 and ST15 were positive for the 76
297 *Salmonella* strains and negative for the remaining bacterial genera tested.

298 Detection levels of FISH method in inoculated wastewater samples yielded 10^2 CFU/ml in
299 sterile tap water and 10^3 CFU/ ml in wastewater, decreasing in both type of samples until 100
300 CFU/ml after 6h enrichment and 1 CFU/ml after 24 h of enrichment.

301 Otherwise, detection limits of PCR assays in both, inoculated sterile water and wastewater
302 samples, were 10^2 CFU/ml without enrichment, 10 CFU/ml after 6h enrichment and 1
303 CFU/ml after 24 h of enrichment in Rappaport-Vassiliadis broth. Based on these results, a 24
304 h enrichment step was always performed when environmental samples were analyzed.

305

306 **3.2 Faecal coliforms enumeration**

307

308 Table 3 shows the occurrence of faecal coliforms and *Salmonella* spp. in the reclaimed water
309 after disinfection for the two plants over the study period. Faecal indicators were present in
310 ten of the thirteen analyzed reclaimed water samples (100% and 50% from plant A and B,
311 respectively). Five of them yielded levels up to 100 CFU/100 ml. This is a valid level as
312 established by WHO Guidelines (Blumenthal *et al.*, 2000) when using reclaimed water for
313 unrestricted irrigation. However, it is far from limits suggested by EPA Guidelines (Bastian
314 and Murray, 2012) for food crops irrigation (no detectable faecal coliforms/100 ml). The
315 system used in plant B (sand filtration after biological treatment and UV disinfection) showed
316 a higher removal rate for indicator organisms than chlorination, used in plant A.

317 Faecal coliforms are the most commonly used indicators to evaluate the level of faecal
318 contamination and the efficiency of pathogen removal in sewage treatment processes.
319 However, several authors have questioned whether these bacteria are suitable indicators of
320 occurrence of *Salmonella* and other enteric pathogens (Harwood *et al.*, 2005; Wéry *et al.*,
321 2008). Thus, while the presence of faecal coliforms could be taken as a sign of faecal
322 contamination, their absence does not guarantee that the water was uncontaminated. In our
323 work, however, no *Salmonella* isolate was obtained from samples in which faecal coliforms
324 were absent.

325

326 **3.3 *Salmonella* isolation and antimicrobial resistance in wastewater samples**

327

328 Among the 45 water samples tested, more than 50% of samples (24 samples) yielded positive
329 results for *Salmonella* detection (Table 3). The percentage of positive samples by culture in
330 wastewater plants A and B were 61.90 (13/21 samples) and 45.8% (11/24 samples),
331 respectively.

332 Two samples from Plant A were contaminated with *Salmonella* after tertiary treatment. In
333 plant B, only one effluent sample was positive for *Salmonella*. Equally than for faecal
334 coliforms, percentage of reduction of *Salmonella*, from entry (R) (raw sewage) to after UV
335 disinfection (T), was 66.7% in A and greater in B (80.0%). Thus, our results show that
336 combination of sand filtration and UV disinfection improves the sewage depuration process.

337 Different studies have shown that UV dose for a four log reduction of *Salmonella* sp. content
338 vary from 7 mj/cm^2 in inoculated broth to 50 mj/cm^2 in a secondary treated effluent (Malayeri
339 *et al*., 2016; Brian *et al*., 2003). In wastewater treatment plants, the UV fluence received by
340 microorganisms depends on several factors as maintenance of the UV lamp, suspended
341 particles present in water and exposition time. Moreover, organisms attached to particulates in
342 wastewater may require doses of 2, 3 or more times, to achieve the same log reduction as for
343 free organisms (IWA, 2017) and several studies have reported an increased UV resistance of
344 environmental bacteria, compared to lab-grown strains (Hijnen *et al.,*2006). This means that
345 higher UV fluences are required to obtain the same level of inactivation. In this study, the
346 pathogen remained in some regenerated water samples from both plants, which indicates a
347 risk for human health and environment, which indicates a risk for human health and
348 environment (Millan-Sango *et al.,* 2017).

349 Thirty-eight *Salmonella* strains were isolated throughout the study from the two secondary
350 wastewater treatment plants: twenty from plant A and eighteen from plant B. Fourteen
351 different serotypes were identified, most of them including less than 5 % of strains. *S.*
352 *enterica* ser. Rissen was the predominant serotype (32.3 %) followed by Derby, Goldcoast,
353 Toulon and Virchow with 3.2% each. The rest of serotypes were very diverse, being unusual
354 serotypes as Bsilla. Only serotype Rissen was isolated in both plants. No serotype related to
355 enteric fever was detected.

356 Our results are in accordance with those of other authors, who have reported that the number
357 and variety of serovars found in wastewaters from different treatment plants diverge

358 considerably, and most of them are found only in one location or even in one sample from
359 the same plant (Berge *et al*., 2006; Espigares *et al*., 2006; Koivunen *et al*., 2003; Baudart *et*
360 *al*., 2000)

361 All *Salmonella* spp. isolates were tested for antibiotic resistance (Table 4). Those isolates
362 obtained from the same sample and showing the same biochemical profile, serotype and
363 antibiotic resistance pattern were considered the same strain, getting a total of 23 different
364 isolated strains. Fourteen *Salmonella* strains in plant A and 9 strains in plant B exhibited
365 resistance to one or more antimicrobial agent. Multiple resistances (≥ 3 antibiotics) were
366 observed in two isolates in plant A and in one in plant B. Although our results show a lower
367 incidence of multidrug-resistant *Salmonella* than those observed by other authors in
368 wastewater reclaimed for irrigation (Pignato *et al.,* 2009), percentage of resistant strains is
369 high and could pose a risk for public health, as sewage treatment plants are considered a main
370 point for spread of antibiotic resistances among pathogens (Sharma *et al.,* 2016).

371 Nine antibiotic resistance patterns were verified (Table 4). *Salmonella* strains isolated from
372 plant A were resistant to eight different antibiotics (KF, TE, NAL, C, STX, AMP, CAR, and
373 CN) whereas in B *Salmonella* isolates were resistant only to four (TE, STX, AMP, and CAR).
374 Resistance to tetracycline was the most common one, reaching 66.7 and 69.2% for A and B
375 plant isolates. Percentage of resistance to ampicillin showed similar values in both secondary
376 wastewater treatment plants (11.1% and 7.8% for A and B, respectively) which is in
377 accordance to other works (Pignato *et al.,* 2009). One *Salmonella* strain isolated from plant A
378 was resistant to six antibiotics (AMP, C, CAR, NAL, STX and TE), keeping the upward trend
379 of multidrug resistance observed in recent years, including ampicillin, chloramphenicol and
380 tetracycline (Doyle, 2015). No isolate was resistant to amikacin, amoxicillin/clavulanic acid,
381 ceftriaxone or ciprofloxacin. Taking into account the critical importance for human medicine
382 of these antimicrobials (WHO, 2012) the obtained results are encouraging.

383 No strain of *Salmonella* isolated from tertiary treatment presented multiple resistances. This
384 result exhibits a low public health risk, if using like recycled water (Pruden, 2014). However,
385 four *Salmonella* isolated from tertiary treatment showed resistance to some antibiotic: two to
386 TE and two to TE and STX. Furthermore, some nalidixic acid resistant strains showed a
387 ciprofloxacin reduced susceptibility, which is in accordance with reported data (Preethi *et al.*,
388 2017)

389 Again, results proved that the sanitization treatment was more effective in B, since the
390 number of antibiotic resistant *Salmonella* after tertiary treatment was lower (1) than in plant A
391 (3).

392

393 **3.3 *Salmonella* detection in wastewater samples by molecular methods**

394

395 By using molecular methods *Salmonella* was detected in this study in 29 out of 45 (64.4%)
396 wastewater samples analyzed, four of them being effluent (after disinfection treatment)
397 samples (Table 3). For both, PCR and FISH, all positive results were obtained only after an
398 enrichment step. Negative results in direct samples were probably due to the low cell level. In
399 accordance to our results, some authors have proposed that combination of PCR with a short
400 enrichment step increases the level of viable cells, while the non-culturable or dead cells and
401 PCR inhibitors are diluted (Ahmed *et al.*, 2009; Feder *et al.*, 2001).

402 FISH method has the advantage of not being inactivated by inhibitors, independently of the
403 type of sample, even when a large amount is processed (Moreno *et al.*, 2003). In this study, a
404 new specific nucleotide probe for *Salmonella* has been developed. SA23 probe resulted
405 specific to detect all *Salmonella* strains tested and allowed a rapid and specific identification
406 and visualization of *Salmonella* species directly in the sample. Similarly as for the PCR
407 analysis, an enrichment step prior FISH increased the sensitivity of the technique, allowing

408 the detection of 1 UFC/ml. Other authors (Almeida *et al*, 2010) have shown the
409 effectiveness of an enrichment step for the recovery of *Salmonella* strains from a broad
410 spectrum of samples by using FISH method.
411 Analysis of wastewater samples yielded PCR positive results for *Salmonella* in 28 out of 45
412 wastewater samples. When FISH analysis was performed, 27 samples were positive for the
413 presence of *Salmonella* (Figure 1). No negative result was obtained by molecular methods in
414 those samples in which *Salmonella* was detected by culture. In three samples in which culture
415 was negative, discordant results were obtained for the two molecular methods assayed. In
416 samples A5S and B2S, one of each plant, PCR was positive while, by FISH, the unspecific
417 fluorescent background hindered the detection of the organism. In sample A5T, PCR was
418 negative while FISH yielded positive results, due probably to the presence of PCR inhibitors.
419 The comparison of results obtained using the molecular methods used in this study show that
420 both PCR and FISH are suitable tools for the identification of Salmonella in wastewater
421 samples.
422 FISH method has the advantage of not being affected by inhibitory substances in the
423 wastewater samples. However, in our work an enrichment step was necessary to achieve the
424 optimal sensibility. In these conditions, a dilution of fulvic and humic acids could be possible
425 and the PCR protocol could be easier. Nevertheless, FISH presents some other important
426 advantages over PCR, as positive results may be directly observed in the sample and bacteria
427 can be counted (Moreno *et al*, 2003). Unspecific fluorescent background when highly
428 complex samples, such as effluent wastewater or sludge, are analyzed can be a problem. In
429 this case, probably a combination of both methods can be an excellent tool for detecting
430 *Salmonella*.
431 There are several limitations concerning the detection of *Salmonella* in environmental
432 samples by cultural methods (Waage *et al*., 1999), such as low number, viable but non-
433 culturable (VBNC) or dead cells presence (Li *et al*., 2014). Among the *Salmonella* positive

434 water samples assessed by molecular methods, culture method yielded negative results in 7
435 samples. This could be due to a lack of sensitivity of cultural methods (false-negative results)
436 or to the detection of VBNC and dead cells by the molecular methods used.
437 Since crops irrigated with *Salmonella* contaminated waters have showed to be effective
438 transmission vehicles of pathogen to consumers (Ndiaye *et al*., 2011), the analyzed waters
439 represent an important human health risk. Therefore, in addition to controlling the regular
440 Microbial Indicators (FIOs), other important waterborne pathogens as *Salmonella* should be
441 monitored in treated reused waters in order to determine a correct disinfection process.
442 These results show both, the great prevalence of *Salmonella* in wastewater, even after
443 UV disinfection, and the adequacy of molecular methods for its detection instead of available
444 cultural methods. The results showed that FISH probes represents an effective tool for
445 detecting and enumerating pathogens in wastewater due to its efficiency, specifity and
446 sensivity, even when viable but non-culturable (VBNC) cells are present. FISH method has
447 been previously used with good results for identification of *Salmonella* in clinical samples
448 (Nordentoft *et al*., 1997; O'Keefe *et al*., 2001; Frickman *et al*., 2013) or in artificially
449 inoculated environmental samples (Zadernowska *et al*., 2014; Sha *et al*., 2013). Almeida *et al*.
450 (2010; 2011) reported the use of a PNA FISH probe to detect *Salmonella* in natural fountain
451 water and biofilms. However, as far as we know, this is the first time that a FISH assay
452 demonstrates its usefulness for specifically detecting the pathogen in wastewater samples.
453 New SA23 probe developed and assayed in this work provides a tool for detecting *Salmonella*
454 in environmental samples in few hours with a high rate of effectiveness.
455 The overall results obtained in this study indicate that the presence of *Salmonella* spp. in
456 reclaimed water represents a high risk for human health. These results should be considered to
457 develop improved depuration processes for the reuse of reclaimed water.
458
459 **Acknowlwdgments**

460

461 Authors gratefully acknowledge the financial support from the Ministerio de Ciencia e

462 Innovación (Project: AGL2008-05275-C03-02).

463

464 **Conflict of interest**

465

466 No conflict of interest declared.

467

468 **References**

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631

632

633 Table 1. **Strains used for primers and probe specifity tests**

Bacterium	Number of strains	Strain[a,b]	PCR[c]	FISH[d]
S. Typhimurium	5	NCTC 12117 BTC1, 2, 3, 4	+	+
S. Virchow	2	CECT 64 BTC 5	+	+
S. Derby	3	ATCC 6960 BTC 6, 7	+	+
S. Bredeney	6	CECT 99 BTC 8, 9, 10, 11, 12	+	+
S. Enteritidis	6	CECT 50, CECT 4300 BTC 13, 14, 15, 16	+	+
S. Goldcoast	1	CECT 56	+	+
S. Branderburg	3	CECT 207 BTC 17, 18	+	+
S. Muenchen	3	CECT 16 BTC 19, 20	+	+
S. Newport	3	CECT 116 BTC 21, 22	+	+
S. Paratyphi	1	CECT 554	+	+
S. Cholerasuis	1	CECT 915	+	+
S. Anatum	6	CECT 176 BTC 23, 24, 25, 26, 27	+	+
S. Seftenberg	4	CECT 37 BTC 28, 29, 30	+	+
S. Indiana	4	CECT 92 BTC 31, 32, 33	+	+
S. Agona	2	ATCC 51957 BTC 34	+	+
S. Rissen	1	BTC 35	+	+
S. Hadar	4	BTC 36, 37, 38, 39	+	+
S. Ohio	2	BTC 40, 41	+	+
S. Havana	1	BTC 42	+	+
S. Wien	1	BTC 43	+	+
S. Infantis	1	BTC 44	+	+
S. Dublin	2	BTC 46, 47	+	+
S. Thompson	1	BTC 48	+	+
S. Stanley	1	BTC 49	+	+
S. Livingstone	1	BTC 50	+	+
Campylobacter jejuni	1	NCTC 11168	-	-
Providencia stuarti	1	NCTC 10318	-	-
Proteus vulgaris	1	NCTC 4635	-	-
Citobacter freundii	1	NCTC 401	-	-
Enterobacter faecalis	1	DSM 20478	-	-
Enterobacter cloacae	1	NCTC 194	-	-
Escherichia coli	1	NCTC 12900	-	-
Klebsiella oxitoca	1	NCTC 860	-	-
Pseudomonas aeruginosa	1	ATCC 10145	-	-

634
635 [a] Abbreviations used for culture collection: ATCC, American Type Culture Collection; DSM,
636 Deutsche Sammlung Von Mikroorganismen, Germany; NCTC, National Collection of Type
637 Cultures, UK; CECT, Colección española de Cultivos Tipo, Spain.
638 [b] BTC: Strains from our collection
639 [c] With primers ST1-1 and ST1-5.
640 [d] With the probe SA23

641

642 **Table 2.** Treatment processes of secondary wastewater treatment plants and sampling sites

Sewage treatment plant	Treatment Process	Sample
A	Screening and grit removal	Raw sewage (R)
	Primary sedimentation	
	Anaerobic digestion	
	Secondary sedimentation	Secondary treatment effluent (S)
	Chlorination	Reclaimed water after disinfection (T)
B	Screening and grit removal	Raw sewage (R)
	Primary sedimentation	
	Anaerobic digestion	
	Secondary sedimentation	Secondary treatment effluent (S)
	Sand filtration	Sand filter effluent (F)
	UV disinfection	Reclaimed water after disinfection (T)

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644

645 **Table 3.** Results obtained for faecal coliforms enumeration, *Salmonella* detection by culture,

646 FISH and PCR in wastewater samples.

647

Samples	FISH	PCR	Faecal coliforms (CFU/100 mL)	Culture[a] (number of isolates)	Serotypes
A1R	+	+		+ (1)	S. Rissen
A1S	+	+		+ (1)	S. Corvallis
A1T	+	+	> 100	+ (2)	S. Rissen, S. Bsilla
A2R	+	+		+ (1)	S. Virchow
A2S	+	+		+ (1)	S. Rissen
A2T	-	-	> 100	-	
A3R	+	+		+ (2)	S. Kingston, S. Agona
A3S	+	+		+ (2)	S. Virchow, S. Virchow
A3T	-	-	> 100	-	
A4R	+	+		+ (2)	S. Rissen, S. Derby
A4S	+	+		+ (1)	S. Derby
A4T	-	-	> 100	-	
A5R	+	+		+ (2)	S. Derby, S. Derby
A5S	-	+		-	
A5T	+	-	> 100	-	
A6R	+	+		-	
A6S	-	-		-	
A6T	-	-	35	-	
A7R	+	+		+ (2)	S. Afula, S. Bredeney
A7S	+	+		+ (2)	S. Rissen, S. Rissen
A7T	+	+	25	+ (1)	S. Rissen
B1R	-	-		-	
B1S	-	-		-	
B1F	-	-		-	
B1T	-	-	> 100	-	
B2R	+	+		+ (2)	S. Rissen, S. Rissen
B2S	-	+		+ (2)	S. Enteritidis, S. Enteritidis
B2F	-	-		-	
B2T	+	+	> 100	-	
B3R	+	+		+ (2)	S. Toulon, S. Toulon
B3S	+	+		-	
B3F	+	+		+ (1)	S. Toulon
B3T	-	-	Absence	-	
B4R	+	+		+ (1)	S. Rissen
B4S	+	+		+ (2)	S. Rissen, S. Goldcoast
B4F	+	+		+ (2)	S. Goldcoast, S. Goldcoast
B4T	-	-	30	+ (1)	S. Goldcoast
B5R	+	+		+ (1)	S. Toulon
B5S	+	+		-	
B5F	+	+		+ (2)	S. Gloucester, S. Gloucester
B5T	-	-	Absence	-	
B6R	+	+		+ (2)	S. Bergen, S. Rissen
B6S	-	-		-	
B6F	-	-		-	
B6T	-	-	Absence	-	

648 A: Plant A; B: Plant B; R: Raw water; S: Secondary treatment effluent; F: Sand filter

649 effluent T: Reclaimed water after disinfection

650 [a] Isolate identified as *Salmonella* sp.

651

652 **Table 4.** *Salmonella* antibiotic resistant strains isolated and antibiotic resistant patterns depending

653 on secondary wastewater treatment plants and sewage treatment stage.

654

Sewage treatment plant	Antibiotic resistant profile**	No. Isolates (sampling point)*
A	TE	3 (R); 2 (S); 1 (T)
	TE, C	1 (R)
	TE, KF	1 (S)
	TE, STX	2 (T)
	TE, NAL, AMP, CAR,	1 (R)
	TE, NAL, AMP, CAR, C, STX	1 (R)
	NAL	1 (R)
	NAL, CN	1 (S)
B	TE	3 (R); 2 (S); 1 (F); 1 (T)
	TE, STX	1 (R)
	TE, AMP, CAR,	1 (F)

655

656 * R: Raw sewage; S: Secondary clarifier effluent; F: Sand filter effluent; T:

657 Reclaimed water after disinfection

658 ** KF: cephalothin; TE: tetracycline; C: chloramphenicol; NAL: nalidixic acid;

659 STX: sulfamethoxazole-trimethoprim; AMP: ampicillin; CAR: carbenicilline

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667 Figure caption

668

669 Figure 1. FISH showing the presence of *Salmonella* spp. cells in a wastewater sample (A4R)

670 by hybridization with probe SA23

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