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# High prevalence of *Salmonella* spp. in wastewater reused for irrigation assessed by molecular methods

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ABSTRACT

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

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

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

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

## 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 (Sanchez-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., 2017).

Although the treatment processes of wastewater are developed to

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

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

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

At present, public concern about the risks of using reclaimed water for agriculture irrigation is arising, due to the risk of re-entrance of pathogens in the food chain. Irrigation represents up to 33% of the total water use in EU. In Spain, near 80% of reused wastewater is intended for irrigation (European Environmental Agency, 2012). Consistent contamination with irrigation waters is a common route of crop contamination in produces related to *Salmonella* outbreaks (Levantesi et al., 2012).

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

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

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

Ribosomal rRNA probe hybridization without culturing (Fluorescent *in situ* Hybridization, FISH) has become widely adopted for detection of specific bacterial groups in mixed populations (Garcia-Hernandez et al., 2012; Moreno et al., 2011). The FISH assay is less sensitive to inhibitory substances than PCR and has shown to be a very useful tool for 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, 2010; Girones 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.

**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<br>BTC1, 2, 3, 4 | + | + |
| S. Virchow | 2 | CECT 64<br>BTC 5 | + | + |
| S. Derby | 3 | ATCC 6960<br>BTC 6, 7 | + | + |
| S. Bredeney | 6 | CECT 99<br>BTC 8, 9, 10, 11, 12 | + | + |
| S. Enteritidis | 6 | CECT 50, CECT 4300<br>BTC 13, 14, 15, 16 | + | + |
| S. Goldcoast | 1 | CECT 56 | + | + |
| S. Branderburg | 3 | CECT 207<br>BTC 17, 18 | + | + |
| S. Muenchen | 3 | CECT 16<br>BTC 19, 20 | + | + |
| S. Newport | 3 | CECT 116<br>BTC 21, 22 | + | + |
| S. Paratyphi | 1 | CECT 554 | + | + |
| S. Cholerasuis | 1 | CECT 915 | + | + |
| S. Anatum | 6 | CECT 176<br>BTC 23, 24, 25, 26, 27 | + | + |
| S. Seftenberg | 4 | CECT 37<br>BTC 28, 29, 30 | + | + |
| S. Indiana | 4 | CECT 92<br>BTC 31, 32, 33 | + | + |
| S. Agona | 2 | ATCC 5 51957<br>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 | – | – |

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