Occurrence of *Salmonella* spp. and *Cryptosporidium* spp. in a French coastal watershed: relationship with fecal indicators

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**Abstract**

*Cryptosporidium* and *Salmonella* are pathogenic microorganisms that can cause severe gastrointestinal illness in humans. Because these organisms are potentially transmitted through natural waters, this study was carried out to estimate the concentrations of both pathogens in a French coastal watershed and to determine the relationships with fecal indicators. Water samples from nine wastewater treatment plants and eight rivers were analyzed. Although both pathogens and indicators are released from sewage effluents, no clear correlation was found between the two enteric pathogens nor between a given pathogen and fecal indicators. These results suggest that fecal indicators do not adequately indicate the presence of *Cryptosporidium* and *Salmonella* in natural waters and that pathogens and indicators may have different behaviors in the aquatic environment.

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**Keywords:** Cryptosporidium; Salmonella; Fecal indicators; Watershed; Relationships pathogens–indicators

1. Introduction

Natural waters are subject to occasional discharges of sewage or wastewater as a result of human activity or storm-water run-off. These effluents contain a wide range of microorganisms (viruses, bacteria or protozoa) that may pose a health hazard to human populations when they are discharged into recreational waters or into shellfish-farming areas [1,2].

*Cryptosporidium* spp. are monoxenous protozoan parasites that cause gastrointestinal diseases, such as diarrhea and malabsorption in humans and animals [3]. Since 1984, waterborne outbreaks of cryptosporidiosis have increasingly been reported and especially in the UK, USA, Canada and Japan [3–6]. The largest one occurred in 1993 in Milwaukee (WI, USA) and affected more than 400,000 people. This outbreak involved a surface water supply that received full treatment consisting of coagulation, filtration and disinfection [6]. The report of a UK group of experts on *Cryptosporidium* in water supplies identified both sewage effluents and sewage sludge as sources of oocysts that could contaminate watercourses [1].

Although *Cryptosporidium* concentrations in environmental samples may be low, the environmentally resistant form (i.e. oocysts) is ubiquitous in surface waters, and capable of surviving and remaining infectious for several weeks under various environmental pressures [7]. Moreover these robust oocysts are tolerant to disinfectants commonly used in water treatment [8].

In contrast to *Cryptosporidium*, *Salmonella* spp. are ubiquitous Gram-negative bacteria frequently associated with waterborne diseases. They can cause a large range of symptoms from mild gastroenteritis to death and are a prime example of water and shellfish transmitted pathogens [9,10].

As with most waterborne pathogens, *Salmonella* and *Cryptosporidium* are difficult to detect and enumerate with accuracy in surface waters due to methodological limitations. Particularly problematic is the low concentration of the organisms and the high cost and effort of the current detection technologies. This is probably why both pathogens are rarely analyzed in surface waters. Nevertheless, the presence of pathogenic enteric microorganisms in water constitutes a potential threat to human health.
Therefore, standard and recommended guidelines based upon microbial indicator concentrations (e.g. thermotolerant coliforms (FCs) and enterococci (FSs)) are used to protect the environment and to prevent exposure of the public to pathogenic microorganisms. Pathogenic microorganisms, such as *Salmonella*, are only searched for when an inspection in the bathing area denotes fecal pollution. However, the use of indicator microorganisms to detect all pathogens in water is still a matter of debate.

We are not aware of any study comparing at the same time the concentrations of *Cryptosporidium*, *Salmonella* and indicators in surface waters. The aims of this study were (i) to evaluate the impact of sewage treatment plant effluents on the dissemination of *Salmonella* spp. and *Cryptosporidium* oocysts in a French coastal river (Le Tech, Pyrénées-Orientales, France), (ii) to determine the relationship between these two enteric pathogens in wastewater influents, treated wastewater effluents and river waters, and (iii) to evaluate the accuracy of two fecal indicators (i.e. FCs and FSs) to assess water safety.

2. Materials and methods

2.1. Sampling strategy

The Tech is a typical coastal river on the western Mediterranean coast with a drainage area of 780 km². The upstream area of the watershed is characterized by agriculture and forests with extensive rearing activities (cattle and boar). Some small poultry farms are also located in this area [12]. The Tech river has several tributaries in the western area. Twenty-one wastewater treatment stations are located within the watershed with their outfalls located in the river or in its tributaries (Fig. 1).

For microbial analyses, samples were collected in sterile flasks and analyzed within 4 h after sampling. The samples analyzed include nine wastewater treatment plants (seven located inside the Tech river watershed and two outside) and eight river water samples located along the Tech river (Fig. 1). All the treatment plants analyzed run the same process (activated sludge treatment) and no malfunctions were reported during the sampling period.

2.2. Cryptosporidium spp. analysis

The Envirocheck capsule filter (Pall Gelman Laboratory, MI, USA), a 1-µm nominal pore-sized pleated polyethersulfone filter in a polycarbonate housing, was used to concentrate *Cryptosporidium* oocysts from water samples. The volumes analyzed were dependent on the nature of the sample, 1 l was used for untreated wastewater analyses, 10–20 l for activated sludge treated wastewater analyses and 50–100 l for river water analyses.

The samples were filtered at a flow rate of 2 l min⁻¹ through the capsule filter. Then, the filter was rinsed with an elution buffer (1.5% Tween 80, 1.5% antifoams B in PBS, pH 7.4) and treated according to the manufacturer’s instructions.

After this first concentration step, an immunomagnetic separation (IMS) was carried out on water concentrates (500-µl-packed pellet volume) according to the manufacturer’s instructions (Chemunex, France) to remove debris prior to oocysts capture through a 2.0-µm filter membrane (CB 2.0, Chemunex). Then, each membrane was incubated with a labeling solution that contained a fluorescent monoclonal antibody specific for *Cryptosporidium* (Chem-Id 1, Chemunex) and 6-diamino-2-phenylindole (DAPI). Oocysts labeled with the monoclonal antibody were detected by solid-phase cytometry and subsequently confirmed by a microscopic examination of the membrane. Due to the high cost of the IMS procedure, only two IMSs were carried out for each concentrated water sample. When the pellet volume was greater than 1 ml, no significant difference was found between the results obtained from these two IMS (i.e. 1 ml analyzed), and those obtained from the analysis of the total pellet volume (data not shown).

2.3. Bacterial analyses

The most probable number (MPN) procedure was carried out to enumerate *Salmonella* spp. Three replicates of three different volumes (1 l, 100 ml and 10 ml) were used to determine the MPN. When the presence of *Salmonella* spp. was confirmed, the MPN value and confidence interval of 95% were determined using the De Man table [13].

Each water sample was filtered through one or more 47-mm-diameter, 0.45-µm-pore-sized nitrocellulose membrane filters (Millipore, Corp., Bedford, MA, USA). Then, each set of filters was incubated in one enrichment tube and all the tubes (three per volume) were used to determine the MPN. For river samples, *Salmonella* spp. counts were obtained after a pre-enrichment step in peptone-water broth without indole (Bio-Rad, Marnes-la-Coquette, France) at 37°C for 24 h. Then, a selective enrichment step was realized in Rappaport-Vassiliadis broth (RV10 broth, Dcho Laboratories) at 43°C for 24 h. For wastewater samples, the filters were immediately incubated in RV10 broth at 43°C for 24 h. After this selective enrichment step, colonies were isolated by spread plating aliquots onto *Salmonella-Shigella* agar (Bio-Rad) and incubating at 37°C for 24–48 h. Biochemical tests were used to confirm the identity of suspected *Salmonella* spp. colonies (i.e. clear colonies with black centers). Isolates were tested for their inability to produce oxidase (DrySlide oxidase, Becton Dickinson), their ability to produce H₂S and to degrade 4-methylumbelliferyl caprilate (Research organics, Inc., OH, USA).
Fig. 1. Map of the Tech river watershed and localization of sampling area. (Circles) River water, (circles with diamonds inside) wastewater treatment plants.
The enumeration of FCs and FSs was carried out following both spread plate (for wastewater analyses) and membrane filtration (47-mm-diameter, 0.45-μm pore-sized nitrocellulose membrane filters [Millipore Corp., Bedford, MA, USA]) (for river water analyses) procedures. In each case appropriate dilutions (or concentrations) were analyzed in triplicate. FCs were enumerated on TTC agar (Bio-Rad) supplemented with Tergitol after 24 h of incubation at 44.5°C. Enterococci were enumerated on d-Cosseol agar (BioMérieux, France) after 48 h of incubation at 37°C. For the spread plate procedure, serial dilutions were realized in sterile physiological water (9% NaCl, pH 7.5) and 100 μl of each were spread on appropriate agar. The incubation conditions were the same as previously described.

2.4. Solid-phase cytometry

The ChemScan is a solid-phase cytometer that allows the detection of rare events in waters [14]. A rapid automated analysis of an entire 25-mm-diameter membrane is performed within 3 min. A special application was developed by the manufacturer for the detection of fluorescently labeled Cryptosporidium oocysts from surface water concentrates. At the end of the scan the results are displayed as a scan map. As the monoclonal antibody can bind non-specifically to other particles and mimic oocysts [15], a microscopic confirmation stage is necessary. Therefore, after being scanned by the instrument, the membrane is placed onto an epifluorescent microscope with a motorized stage and the stage is directed to move to the exact co-ordinate that the solid-phase cytometer detected as a potential oocyst [16]. The microscopic observation was performed to validate the detected events and to check for the presence of nuclei within the DAPI-stained oocysts.

2.5. Statistical analysis

The Spearman correlation coefficient was applied to establish the relationship between Salmonella spp., Cryptosporidium spp. and indicator microorganism concentrations in wastewater and in river water. Statistical analyses were performed using the PRISM 1.2 software. Statistical significance was assessed at P < 0.05.

<table>
<thead>
<tr>
<th>Wastewater treatment plant</th>
<th>FC (day⁻¹)</th>
<th>FS (day⁻¹)</th>
<th>Salmonella (day⁻¹)</th>
<th>Cryptosporidium oocysts (day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amélie les Bains</td>
<td>2.5 × 10¹⁵</td>
<td>8.6 × 10¹²</td>
<td>4 × 10⁹</td>
<td>&lt; 1.68 × 10⁷</td>
</tr>
<tr>
<td>Reynès</td>
<td>3.5 × 10¹²</td>
<td>4.0 × 10¹¹</td>
<td>&lt; 6.0 × 10⁹</td>
<td>4.5 × 10⁹</td>
</tr>
<tr>
<td>Céret</td>
<td>3.7 × 10¹³</td>
<td>5.9 × 10¹²</td>
<td>6.9 × 10⁸</td>
<td>2.0 × 10⁹</td>
</tr>
<tr>
<td>Prats de Mollo</td>
<td>2.6 × 10¹¹</td>
<td>1.4 × 10¹¹</td>
<td>&lt; 2.9 × 10⁸</td>
<td>&lt; 9.8 × 10⁴</td>
</tr>
<tr>
<td>Le Boulou</td>
<td>3.6 × 10¹¹</td>
<td>9.2 × 10¹⁰</td>
<td>&lt; 2.2 × 10⁸</td>
<td>3.0 × 10⁸</td>
</tr>
<tr>
<td>Maureillas</td>
<td>2.2 × 10¹²</td>
<td>1.6 × 10¹¹</td>
<td>1.4 × 10⁸</td>
<td>9.5 × 10⁷</td>
</tr>
</tbody>
</table>

3. Results and discussion

3.1. Daily flows of the different microorganisms released from wastewater plant systems

The discharge of pathogens in the environment should be analyzed not only from the concentration of pathogens in a given source of pollution but also in terms of daily flow since the impact on natural ecosystems is directly related to the flows. The daily flows of FC and FS were only determined in six of the nine wastewater treatment plants analyzed. The daily flows of FC and FS ranged from 2.6 × 10¹¹ to 3.7 × 10¹³ FC day⁻¹ and from 9.2 × 10¹⁰ to 8.6 × 10¹² FS day⁻¹ (Table 1). In contrast, great variations were reported for pathogen flows. Salmonella flows showed 3 log unit variations between the different wastewater treatment plants and ranged from less than 6 × 10⁵ to 6.9 × 10⁸ Salmonella day⁻¹ (Table 1). Cryptosporidium flows ranged from less than 9.8 × 10⁶ to 9.5 × 10⁷ Cryptosporidium day⁻¹ (Table 1). Data on Cryptosporidium flows are very scarce [1], but those obtained for Salmonella are in the range of values reported by Baudart et al. [12].

3.2. Relationships between pathogens and indicators in wastewater treatment plants

Despite the absence of serious outbreaks of cryptosporidiosis in France, our study demonstrated that Cryptosporidium oocysts occurred in 78% of untreated wastewaters and in 52% of activated sludge effluents. Cryptosporidium oocysts concentrations ranged from 1 to 87.13 oocysts l⁻¹ and from 0.4 to 209.2 oocysts l⁻¹ in untreated wastewaters and activated sludge effluents, respectively (Table 2). These data are congruent with those reported in England by Bukhari and coworkers in seven wastewater treatment plants [1]. Nevertheless, these authors observed 63.3% positive samples in untreated wastewaters and 46.6% of positive samples in activated sludge effluents. These results were lower than those from our investigations. The difference is probably due to the detection limit of the protocol used for the numeration of Cryptosporidium oocysts in water. In our study, the automatic numeration system combined with an immunomagnetic purification of water concentrates allowed a lower detec-
tion limit than concentration by centrifugation and a direct microscopic examination [16].

Surprisingly, in two treatment plant effluents (i.e. Reynes and Maureillas) the oocyst concentrations were higher than in their respective in£uents. A similar trend was found in Ceret for Salmonella concentrations and in Amélie-les-Bains for FC concentrations. This phenomenon has previously been observed by Bukhari et al. [1] and could have occurred because sample collection was not temporally related to take account of the holding time of treat-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Wastewater treatment plant</th>
<th>River water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FC (l⁻¹) (×10⁶)</td>
<td>FS (l⁻¹) (×10⁸)</td>
</tr>
<tr>
<td>Canet influent</td>
<td>2800 (4800)</td>
<td>440 (85)</td>
</tr>
<tr>
<td>treated effluent</td>
<td>37 (32)</td>
<td>20 (18)</td>
</tr>
<tr>
<td>Sainte-Marie influent</td>
<td>1100 (790)</td>
<td>560 (500)</td>
</tr>
<tr>
<td>treated effluent</td>
<td>2.8 (4.5)</td>
<td>1.9 (3.6)</td>
</tr>
<tr>
<td>Amélie les Bains influent</td>
<td>100 (5.8)</td>
<td>1200 (300)</td>
</tr>
<tr>
<td>treated effluent</td>
<td>150 (70)</td>
<td>51 (2.1)</td>
</tr>
<tr>
<td>Arles sur Tech influent</td>
<td>350 (170)</td>
<td>180 (15)</td>
</tr>
<tr>
<td>treated effluent</td>
<td>450 (40)</td>
<td>160 (15)</td>
</tr>
<tr>
<td>Reynes influent</td>
<td>1200 (570)</td>
<td>1500 (1400)</td>
</tr>
<tr>
<td>treated effluent</td>
<td>140 (110)</td>
<td>57 (15)</td>
</tr>
<tr>
<td>Ceret influent</td>
<td>1900 (520)</td>
<td>840 (570)</td>
</tr>
<tr>
<td>treated effluent</td>
<td>110 (55)</td>
<td>68 (50)</td>
</tr>
<tr>
<td>Prats de Mollo influent</td>
<td>2400 (620)</td>
<td>720 (66)</td>
</tr>
<tr>
<td>treated effluent</td>
<td>2.7 (0.47)</td>
<td>1.4 (0.61)</td>
</tr>
<tr>
<td>Le Boulou influent</td>
<td>2200 (300)</td>
<td>530 (72)</td>
</tr>
<tr>
<td>treated effluent</td>
<td>4.8 (1.9)</td>
<td>1.2 (0.38)</td>
</tr>
<tr>
<td>Maureillas influent</td>
<td>2800 (24)</td>
<td>800 (430)</td>
</tr>
<tr>
<td>treated effluent</td>
<td>49 (41)</td>
<td>17 (18)</td>
</tr>
<tr>
<td>River water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>La Preste influent</td>
<td>0.001 (0.0004)</td>
<td>0.003 (0.0004)</td>
</tr>
<tr>
<td>Le Graffoul</td>
<td>0.65 (0.33)</td>
<td>0.29 (0.07)</td>
</tr>
<tr>
<td>Le Riuferrer</td>
<td>0.014 (0.007)</td>
<td>0.064 (0.003)</td>
</tr>
<tr>
<td>Le Mondony</td>
<td>1.5 (0.3)</td>
<td>0.59 (0.03)</td>
</tr>
<tr>
<td>Le Tech (downstream Ceret)</td>
<td>2.5 (0.5)</td>
<td>0.71 (0.41)</td>
</tr>
<tr>
<td>Maureillas river</td>
<td>6.2 (0.4)</td>
<td>2.7 (0.62)</td>
</tr>
<tr>
<td>Le Tech (Le Boulou)</td>
<td>0.15 (0.03)</td>
<td>0.024 (0.001)</td>
</tr>
<tr>
<td>Le Tech (Ortaffa)</td>
<td>0.03 (0.004)</td>
<td>0.011 (0.008)</td>
</tr>
<tr>
<td>Le Tech (Ortaffa+rainfall event)</td>
<td>0.4 (0.04)</td>
<td>0.36 (0.14)</td>
</tr>
</tbody>
</table>

Standard error in parentheses. < DL: Less than the detection limit of the method. ND: Not determined.

Table 3
Correlations between Cryptosporidium, Salmonella and indicator microorganisms in wastewater influents, treated wastewater effluents and river waters

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean concentration (l⁻¹)</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cryptosporidium</td>
<td>Salmonella</td>
</tr>
<tr>
<td>Wastewater influent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>23.4</td>
<td>0.56*</td>
</tr>
<tr>
<td>Salmonella</td>
<td>328.04</td>
<td>−0.56*</td>
</tr>
<tr>
<td>FC</td>
<td>1.65E+08</td>
<td>−0.27</td>
</tr>
<tr>
<td>FS</td>
<td>7.5E+07</td>
<td>−0.33</td>
</tr>
<tr>
<td>Treated wastewater effluent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>26.05</td>
<td>0.23</td>
</tr>
<tr>
<td>Salmonella</td>
<td>110.04</td>
<td>0.23</td>
</tr>
<tr>
<td>FC</td>
<td>1.05E+07</td>
<td>0.44*</td>
</tr>
<tr>
<td>FS</td>
<td>4.16E+06</td>
<td>0.39</td>
</tr>
<tr>
<td>River water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>0.91</td>
<td>0.37</td>
</tr>
<tr>
<td>Salmonella</td>
<td>79.59</td>
<td>0.37</td>
</tr>
<tr>
<td>FC</td>
<td>1.27E+05</td>
<td>0.83*</td>
</tr>
<tr>
<td>FS</td>
<td>5.28E+04</td>
<td>0.71*</td>
</tr>
</tbody>
</table>

Spearman correlation. *P > 0.05.
ment works. Furthermore, sewage influents contain higher concentrations of contaminating particulates than sewage effluents and these are more likely to interfere with oocysts or specific bacteria detection.

Despite the common origin of the two pathogens, Salmonella concentrations were generally higher than Cryptosporidium oocyst concentrations in treated and untreated wastewater (Table 2). In addition, no correlation was observed between pathogens and indicator microorganisms in wastewater influents (Table 3). Nevertheless, in these samples a significant correlation was found between Salmonella spp. and Cryptosporidium oocysts. In treated wastewater effluents, no correlation could be observed between the two pathogens, but a significant correlation appeared between Cryptosporidium oocysts and FCs (Table 3). To conclude, no systematic correlation was found in contaminated waters between the two pathogens as well as between a given pathogen and indicator microorganisms. This lack of correlation with indicators in sewage influents may be partly explained by the fact that not only are the concentrations of pathogens dependent, as are the indicators, upon the size of the contributing community but also, unlike the indicators, upon the levels of infection within that community.

3.3. Occurrence of pathogens in river water and relationship with indicators

The presence of Cryptosporidium and Salmonella in sources of drinking water and/or recreational waters can be a serious public health issue. In 1996, Ong and coworkers reported that a river flowing through an area with cattle pastures had higher levels of enteric microorganisms than water sampled from a river in a protected watershed [17]. Enteric microorganisms can be shed into the water environment by infected livestock or other mammals in run-off, pet waste in storm-water run-off, treated wastewater effluent and storm water/wastewater connections. In our study, eight river waters located along the Tech watershed (Fig. 1) were investigated for the presence of FC, FS, Salmonella and Cryptosporidium. The mean concentrations of Cryptosporidium, Salmonella, FC and FS in river water are reported in Table 2. Salmonella cells were detected in all samples whereas Cryptosporidium oocysts occurred in only 66.7% of them (data not shown) and mainly in waters containing more than 2000 FC l$^{-1}$. These results are in the range of values reported by LeChevallier and coworkers (i.e. 87%) [18] and by Roach and coworkers (i.e. 14%) [19].

The oocysts concentrations ranged from 0.04 to 7.3 oocysts l$^{-1}$ and the results obtained at the Ortafa station showed that after rainfall events, the concentration of Cryptosporidium oocysts was four-fold higher than during drought periods. Salmonella concentrations ranged from 0.6 to 424.5 CFU l$^{-1}$ and were greater in downstream sewage effluents and agricultural areas according to the results of Ong et al. [17].

Two significant correlations were found in river water samples. The first one was observed between Cryptosporidium oocysts and FCs ($r=0.83$, $P>0.05$) and the second one between Cryptosporidium oocysts and enterococci ($r=0.71$, $P>0.05$) (Table 3). Nevertheless, no significant correlation could be observed between Salmonella spp. and Cryptosporidium spp., nor between Salmonella spp. and fecal indicators (Table 3). The absence of correlation between Salmonella spp. and fecal indicators was already reported by others [9,20]. More generally, the lack of correlation between pathogens and indicators is at least partly explained by the fact that the transport and behavior of the different organisms is governed by several hydrodynamic, chemical and biological factors (i.e. water flow, attachment to particles, sedimentation and resuspension, survival, etc.). For instance, enterococci survive better than coliforms in surface waters, free-living Cryptosporidium oocysts can sediment in quiet waters whereas free-living bacteria remain planktonic and are more submitted to transportation. Consequently, these factors may contribute to different behaviors of these organisms not only in natural waters but also in wastewater treatment plant systems. Therefore and although the two pathogens have similar reservoirs within the watershed, Salmonella spp. cannot be used as an indicator of the presence of Cryptosporidium.

4. Conclusions

Current quantitative techniques to enumerate Salmonella and Cryptosporidium concentrations in environmental waters are cumbersome, expensive, time-consuming and not appropriate for routine monitoring. Consequently, the European Community Bathing Water Directive (76/160/EEC) had established standard and recommended guidelines based upon microbial indicators to protect the environment and to prevent exposure of the public to pathogenic microorganisms. FCs and FSs have been used for decades as indicators of water quality and their absence was considered evidence of safe water, free of waterborne pathogens. However our results clearly demonstrated that FC and FS do not necessarily correlate well with the presence of pathogenic microorganisms such as Salmonella and Cryptosporidium. Therefore and although guidelines actually used in Europe for recreational waters will probably change (lower values) in the coming months, the detection of major pathogens remains relevant but still depends on the development of more flexible and less expensive techniques for routine applications.

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References


