**Giardia Cysts in Wastewater Treatment Plants in Italy**

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Reductions in annual rainfall in some regions and increased human consumption have caused a shortage of water resources at the global level. The recycling of treated wastewaters has been suggested as a solution to this problem. In Italy, there are few published data on the prevalence of parasites in wastewaters. The objectives of this study were to evaluate the prevalence of these parasites in four wastewater treatment plants, to estimate the efficiency of treatment plants in removing these parasites, to develop a reliable method for DNA extraction from contaminated water samples, and to determine the species and genotype of these parasites by means of a molecular assay.

**MATERIALS AND METHODS**

Sample collection and processing. Samples were collected at four wastewater treatment plants. One plant was located in northern Italy (plant 1, located in the city of Bergamo, Lombardy region), and three plants were located in southern Italy (plant 2, city of Naples, Campania region; plant 3, city of Cagliari, Sardinia region; and plant 4, city of Palermo, Sicily region). Samples (15 to 20 liters) of untreated wastewater (influent) and of primary, secondary, and final effluent were collected during the spring, summer, autumn, and winter of the year 2000.

To be able to examine the same wastewater at various points in the treatment...
process, when collecting the samples, the holding times of each step in the process were respected.

The specific steps in the process to remove Giardia cysts were described in Table 1. The treatment carried out at plant 1 differed from that at the other three plants. Specifically, primary treatment did not include sedimentation, and secondary treatment consisted of oxidation with O₂ and sedimentation, whereas in the other three plants it consisted of activated sludge and sedimentation. Furthermore, no disinfection was used in plant 1. In plant 1, since it was not physically possible to collect samples at the end of the primary treatment, the first sample of treated wastewater was collected shortly after the oxidation process had begun. In plant 4, samples were not collected after the primary treatment.

The water samples were filtered through a 50-mesh sieve (300 μm) to remove large particles and then concentrated by filtration on cellulose-acetate filters (0.8-μm pore size, 142-mm diameter; Nuclepore-Whatman, Clifton, N.J.) (2). The filter was placed in a 50-ml conical polypropylene centrifuge tube and dissolved with acetone. After centrifugation at 4,620 × g for 10 min at 4°C, the supernatant was discarded, and about 5 ml of pellet was left at the bottom of the tube. The pellet was resuspended in 50 ml of 95% alcohol, centrifuged, resuspended in 50 ml of 70% alcohol, centrifuged, and resuspended in 50 ml of phosphate-buffered saline containing 0.1% Tween 80, 0.1% sodium dodecyl sulfate, and 0.001% antifoam agent B (Sigma, St. Louis, Mo.), and centrifuged, leaving a 5-ml pellet. An aliquot of 50 μl of the pellet was serially diluted (1:10, 1:50, and 1:100) and examined by immunofluorescence with anti-Giardia and anti-Cryptosporidium monoclonal antibodies conjugated to fluorescein isothiocyanate according to the manufacturer’s protocol (Meridian Diagnostics, Inc., Cincinnati, Ohio).

Giardia cysts were identified based on their size, shape, and the pattern and intensity of immunofluorescent assay staining (i.e., bright green fluorescence of the cyst wall). Cryptosporidium oocysts were identified based on their size, shape, and the presence of a suture on the oocyst wall at a magnification of 1,000×. The number of oocysts was counted for each sample in triplicate.

**Results**

**Prevalence of protozoa in wastewater samples.** Giardia cysts were found in the influents of all plants throughout the year. The estimated mean number of cysts per liter ranged from 2.1 × 10^3 to 4.2 × 10^4. In all plants, the highest number of cysts was detected in autumn and winter (Fig. 1). Cryptosporidium oocysts were only detected in two plants: twice in the influent of plant 1 (40 and 2.5 oocysts/liter) and once in the influent of plant 4 (277 oocysts/liter), always during the spring and only before primary treatment.

The removal efficiency of Giardia cysts after primary treatment, which was evaluated at plants 2 and 3, was 50.2 and 65.2%, respectively (geometric means for the four seasons). At plant 1, where the first treated sample was collected shortly after the oxidation process had begun, the number of cysts was observed to have increased by 17.5% in the spring and by 132.9% in the summer.

In plants 2 and 3, the removal efficiency of Cryptosporidium oocysts after the winter period was 43.9 and 61.0%, respectively. In a single sample collected during the winter at plant 2, an increase of 4% in the number of cysts was observed (Fig. 1). The removal efficiency of the disinfection process, which was carried out at plants 2, 3, and 4, was 53.2, 70.8, and 87.0%, respectively (Fig. 1).

The overall removal efficiency of Giardia cysts was 94.5, 87.0,
96.0, and 98.4% in plants 1, 2, 3, and 4, respectively (Fig. 1). The removal efficiency when comparing untreated wastewater samples to those after secondary treatment was 94.5, 72.1, 86.4, and 88.0% for plants 1, 2, 3, and 4, respectively. At plant 1, the secondary treatment consisted of active oxidation with O₂ and sedimentation (i.e., the final treatment), because a disinfection process was not applied at this plant. This treatment resulted in a higher removal efficiency in comparison to that observed in the other three plants, and the difference was significant (P = 0.05, analysis of variance).

**Molecular identification of parasites.** PCR amplification of the 753-bp fragment of the β-giardin gene was performed on these templates, and products of the expected size were obtained with 1 to 2 μl of template, which corresponds to 10 to 50 cysts (Fig. 2). At least one PCR product from each plant was sequenced (Table 2), whereas all 16 influent samples were analyzed with a PCR-restriction fragment length polymorphism assay (Fig. 3).

As shown in Table 2, cysts of assemblage A were detected in eight samples, whereas in the other eight samples cysts of both assemblages A and B were detected by sequencing and/or by PCR-restriction fragment length polymorphism (Fig. 3). Amplification of *Cryptosporidium* DNA from the three positive samples was not obtained.

**DISCUSSION**

*Giardia* and *Cryptosporidium* spp. can be transmitted to humans through contaminated water and food, in addition to the classical oral-fecal route. Transmission is sustained by both a zoonotic and an anthropotonic cycle (14, 38). The infected hosts, whether animals or humans, shed very large numbers of oocysts with their feces, thereby increasing the environmental contamination. Moreover, oocysts can withstand normal water disinfection processes, and they have been found in significant quantities in the final effluents of sewage treatment works (e.g., see reference 31).

Our investigation of the four plants revealed that *Giardia* cysts were ubiquitous, whereas *Cryptosporidium* oocysts were quite rare. Similar prevalence rates were reported in wastewater collected at a treatment plant in Bari, a city in southern Italy, where the number of *Giardia* cysts was 100-fold than the number of *Cryptosporidium* oocysts (5). Since the parasites detected in our study were probably of human origin, given that the wastewater was from cities and not from agricultural areas, these results suggest that the prevalence of cryptosporidiosis is lower than that of giardiasis; this is also supported by the results of surveys of intestinal parasites in Italy’s general population (3, 10, 23). That the prevalence of cryptosporidiosis is relatively low in Italy compared to other countries is supported by the results of previous studies, in which the prevalence among persons with AIDS before the introduction of highly active antiretroviral therapy, which is considered to reflect the prevalence among the general population, was 1.9% (27), compared to 5 to 6% in the United States (9). A prevalence of 1.9% was also reported among immunocompetent children in Italy (4).

Although *Giardia* cysts were found in all of the wastewater samples in the four treatment plants throughout the year, the greatest number of cysts was found in the autumn and winter.
Although a similar seasonal pattern has been reported by some authors (16, 41), it has not been confirmed by others (15, 30); thus, it is not clear whether or not seasonality is a general feature of *Giardia* contamination.

As shown in Fig. 1, an increase in *Giardia* cysts was observed three times during the purification process in the plants. As *Giardia* does not reproduce outside the host, this was probably due to the fact that the aggregated protozoa desegregated before sedimentation, thus increasing the concentration of free parasites in the sample, as also observed by other authors (6).

The overall removal efficiency ranged from 87.0 to 98.4% at the different plants, which is consistent with estimates from other treatment plants that use similar processes (8, 31). The highest removal efficiency was at plant 4 (98.4%), perhaps as a consequence of filtration, which was applied after the secondary treatment and before disinfection; although the filter had 60-μm pores and *Giardia* cysts measure 15 to 18 μm, aggregated cysts could have been trapped. However, the process of oxidation with O₂ and sedimentation used at plant 1 resulted in greater cyst reduction than that obtained by the activated sludge and sedimentation methods used at the other three plants (P = 0.05, analysis of variance). To determine whether active oxidation with O₂ is truly more effective than activated sludge in reducing the number of *Giardia* cysts, additional research will be needed. In fact, the present results may be biased by several factors, including the limited number of samples examined, the different volume of water treated in each plant (from 500 to 6,000 m³/hour), and the seasonality in the number of *Giardia* cysts.

Most studies on *Giardia* contamination of water have been limited to estimating the prevalence (15, 16, 22), and little information has been published on the specific contaminating species. However, this is of particular importance, since only *Giardia duodenalis* is associated with human infection (38), and only two of the seven *G. duodenalis* assemblages (i.e., assemblages A and B) have been found in humans (39). Therefore, the simple presence of *Giardia* cysts in the absence of data on the species or assemblage does not imply a risk of transmission to humans. Most studies have been conducted by spiking water samples with a known number of cysts, followed by evaluation of procedures for recovery and typing of the organism (32). In the few instances when nonspiked water samples were studied, the sensitivity and specificity of the PCR assays were low. In a study of drinking water samples performed after an outbreak of giardiasis in Canada, the direct typing of cysts by PCR amplification of the triose phosphate isomerase gene was unsuccessful, possibly because of the small number of cysts (25). In a study on sewage samples from Finland, nonspecific PCR amplifications were observed with primers targeting the glutamate dehydrogenase gene, and a further characterization of the *Giardia* cysts was not possible (29).

In our study, nucleic acids were efficiently extracted from concentrated wastewater samples with a method that had been developed for detecting protozoa present in fecal samples (7, 11). This method is rapid, in that it allows up to 12 samples to be simultaneously processed in about 1 h, and the DNA extracted is essentially free of inhibitors and can thus be efficiently amplified by PCR (Fig. 2). Moreover, we have shown that the β-giardin PCR assay yields robust and specific amplification products and that it allows the rapid identification of genotypes by sequence analysis or restriction analysis (Fig. 3). The better performance of this assay is probably due to the amplification target chosen, since giardin proteins are considered unique to *Giardia* (13), and primers that do not cross-react with other organisms can be designed (24).

The results indicate that water processed at the four treatment plants could be a potential source of human infection with *G. duodenalis*, although the viability of the cysts was not investigated. In Italy, about 80% of drinking water is from ground water, and only 20% originates from surface water, which is more easily contaminated with parasitic protozoa.

**TABLE 2. Genetic typing of *Giardia* cysts detected in four wastewater treatment plants in Italy**

<table>
<thead>
<tr>
<th>Treatment plant no.</th>
<th>Assemblage(s) present</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Spring</td>
</tr>
<tr>
<td>1</td>
<td>A + B</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>A + B*</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
</tr>
</tbody>
</table>

*a* Samples were sequenced.

**FIG. 2.** Electrophoretic separation of β-giardin amplification products from wastewater samples. Lanes 1 to 3, influent samples from the plant 1; lanes 4 to 6, influent samples from plant 2; lane M, 100-bp molecular ladder; lanes 7 to 9, influent samples from plant 3; lanes 10 and 11, influent samples from plant 4; lane 12, negative control; lane 13, positive control.
However, the release of contaminated effluents into the environment could increase the risk of human infection with these pathogens through the consumption of vegetables. Moreover, the results stress the importance of the microbiological control of effluents from wastewater treatment plants and the need for regulations that establish the acceptable concentrations of oocysts based on the use of wastewaters, i.e., if they should be recycled in the cities for public and/or in-house dual systems, for agricultural purposes, which could be limited to certain crops only, or for industry.

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