Effect of Sample Holding Time on Recovery of Cryptosporidium Oocysts and Giardia Cysts from Water Samples

L. J. ROBERTSON* AND B. GJERDE

Seksjon for Parasitologi, Institutt for Farmakologi, Mikrobiologi og Næringsmiddelygiene, Norges Veterinærhøgskole, 0033 Oslo, Norway

Received 30 November 1999/Accepted 20 January 2000

U.S. Environmental Protection Agency methods for analysis of water for Cryptosporidium and Giardia stipulate maximum sample holding times which are not always practical to comply with. A spiking experiment indicated that holding times of up to 2 weeks had no significant effect on recovery of these parasites from 10-liter samples of raw water in plastic carboys.

In 1996 the U.S. Environmental Protection Agency initiated an effort to identify technologies for protozoan monitoring and analysis. Method 1622 (2) and Method 1623 (3) were developed as a response to that initiative. These methods, which include validated techniques for analyzing 10-liter samples of raw water for Cryptosporidium (2) and Cryptosporidium and Giardia (3), are described in detail.

In 1998 a survey was commenced in Norway for analyzing background levels of Cryptosporidium and Giardia in raw water supplies across the country. When this survey commenced, only a draft format of Method 1622 was available (1). This draft format was used as a basis for deciding the technique that would be used for the Norwegian survey. For this survey, 10-liter grab samples of raw water were collected and analyzed by membrane filtration, immunomagnetic separation (IMS) (Dynal GC combo, Dynal AS, Oslo, Norway), and immunofluorescence assay (IFA) (AquaGlo G/C direct, Waterborne Inc., New Orleans, La.). Method 1622 and Method 1623 both give precise details about sample collection, storage, and holding times. In particular, they stipulate that samples must be shipped on the day of collection, must arrive at the laboratory within 24 h of collection, and must be stored between collection and shipment and upon arrival at the laboratory at between 0 and 8°C. Filtration, elution, and concentration of the sample must be completed within 72 h of sample collection, and, if not continuing directly to IMS, the concentrate must be stored at between 0 and 8°C. IMS and sample staining must be completed within 24 h of sample concentration, and stained slides should be stored at between 0 and 8°C in the dark. Up to 72 h is allowed for screening of the stained sample. While it is obviously preferable to complete analysis as soon as possible after sample collection, we found that in many instances it was practically impossible to comply with the sample holding times specified in the Environmental Protection Agency methods, particularly those specified in the description of sample collection and storage; samples frequently failed to reach the laboratory within 24 h of collection, and samples often could not be shipped on the day of collection. In many of these instances, the temperature at which the sample was held could not be regulated. In order to ascertain whether increased holding times in the 10-liter collection carboy had any effect upon parasite recovery, a simple seeding experiment was conducted.

Three hundred liters of water was collected from a raw water source in 30 10-liter plastic (high-density polyethylene) carboys (Dynoplast AS, Oslo, Norway). The raw water source had been monitored over the previous 15 months and found to have very low levels of both Cryptosporidium and Giardia (39 10-liter samples had been analyzed; 37 were negative, a single Giardia cyst was detected in 1 sample, and a single Cryptosporidium oocyst was detected in 1 sample). The turbidity of the water was 0.75 nephelometric turbidity unit (Hach 2100A turbidimeter). At the laboratory, 2 of the water samples were analyzed immediately. The remaining 28 water samples were spiked with a 50-μl spike containing Cryptosporidium parvum oocysts and Giardia intestinalis cysts. The Cryptosporidium oocysts were a bovine isolate, approximately 6 months old, sucrose purified and stored at 4°C in distilled water without fixatives or preservatives. The Giardia cysts had been obtained from a commercial supplier (Waterborne Inc.), were approximately 6 months old, and had been stored at 4°C in distilled water without fixatives or preservatives. Spike size had been estimated by six replicate hemocytometer counts of a stock suspension followed by serial dilutions. Microscopic analysis of the spike suspensions revealed that, on the basis of standard morphological criteria, approximately 60% of the Cryptosporidium oocysts were intact at spiking and approximately 30% of the Giardia cysts were intact at spiking. Four test spikes were placed on slides and enumerated directly by IFA.

The spiked water samples were mixed thoroughly. Ten of the spiked carboys were then transferred to a cold room and held at between 0 and 4°C in the dark. The remaining 18 spiked carboys remained at room temperature (between 18 and 22°C) in the light. Analysis of 2 of the spiked carboys at room temperature was commenced within 3 h of spiking (after 2 and 2.5 h). The water in the carboys was mixed immediately before analysis. Processing was done by membrane filtration, IMS, and IFA. The membrane filtration was performed as described elsewhere (1), using 142-mm-diameter Isopore membrane filters with a 2.0-μm pore size (Millipore, Bedford, Mass.) in a standard 142-mm membrane filter housing (Millipore). IMS and IFA were conducted as described elsewhere (3).

The remaining carboys were processed at defined intervals as follows: (i) approximately 24 h after spiking (three held at room temperature were processed after 21 to 22 h and three held in the cold were processed after 22.5 to 23.5 h), (ii) approximately 48 h after spiking (three held at room temper-
ature were processed after 45 to 46 h and three held in the cold were processed after 46.5 to 47.5 h), (iii) approximately 72 h after spiking (three held at room temperature were processed after 69 to 70 h and three held in the cold were processed after 70.5 to 71.5 h), (iv) approximately 1 week (168 h) after spiking (three held at room temperature were processed after 165 to 166 h and three held in the cold were processed after 166.5 to 167.5 h), and (v) approximately 2 weeks (336 h) after spiking (two held at room temperature were processed after 333 to 333.5 h).

Processing, from commencement of filtration to drying of the final concentrate to microscope slides, was completed within 5 h for each sample. Slides were screened within 72 h of staining.

Neither Cryptosporidium oocysts nor Giardia cysts were detected in the two unspiked samples. The results for the spiked samples are given in Table 1. The four test spikes enumerated directly by IFA showed spike sizes of approximately 166 Cryptosporidium oocysts (range, 159 to 172) and approximately 231 Giardia cysts (range, 216 to 258). Recovery efficiencies for the parasites, as estimated from the results for the spiked samples processed within 3 h of spiking, were 55% for Cryptosporidium and 57% for Giardia. No significant difference in numbers recovered could be detected between the samples held for differing periods of time and at different temperatures (Table 1).

These results indicate that the holding times described in Method 1622 (1, 2) and Method 1623 (3) for the initial sample may not be as critical as suggested and may be extended, if necessary, for at least up to 2 weeks. Furthermore, keeping the sample cool may not be as critical as suggested.

In general, when the sample has been received at the laboratory it should be feasible to process it within the time limits stipulated in Method 1622 and Method 1623. If extension of these times is required, then spiking experiments appropriate for the particular stage of the procedure should be conducted to ascertain any impact on recovery efficiency. While the holding time for the initial sample was demonstrated here to be a possible candidate for extension, other stages may also be appropriate candidates which could be tested. One point which we believe might prove worthy of testing is the interval between methanol fixation of the final sample concentrate to the welled slide and staining with monoclonal antibody.

This work was supported by a grant from the Norwegian Food Control Authority.

REFERENCES


<table>
<thead>
<tr>
<th>Approx period between seeding and analysis</th>
<th>2 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>1 wk</th>
<th>2 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holding temp</td>
<td>RT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>RT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cold&lt;sup&gt;b&lt;/sup&gt;</td>
<td>RT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cold&lt;sup&gt;b&lt;/sup&gt;</td>
<td>RT&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>n</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>No. of Cryptosporidium oocysts recovered&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Mean</td>
<td>92</td>
<td>84</td>
<td>99</td>
<td>97</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>86–97</td>
<td>66–96</td>
<td>85–125</td>
<td>96–98</td>
<td>76–93</td>
</tr>
<tr>
<td>No. of Giardia cysts recovered&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Mean</td>
<td>132</td>
<td>122</td>
<td>132</td>
<td>133</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>130–133</td>
<td>120–126</td>
<td>115–145</td>
<td>128–141</td>
<td>119–140</td>
</tr>
</tbody>
</table>

<sup>a</sup> RT, room temperature (between 18 and 22°C).
<sup>b</sup> Cold, 0 to 4°C.
<sup>c</sup> Cryptosporidium oocyst spike size was 166 ± 6 (mean ± standard deviation).
<sup>d</sup> Giardia cyst spike size was 231 ± 19 (mean ± standard deviation).