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OCCURRENCE OF VIRUSES AND PROTOZOAN PARASITES IN SURFACE, GROUND AND TREATED WATER IN THE CITY OF MASHHAD, IRAN

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ABSTRACT

A research plan was developed to evaluate the occurrence of enteroviruses, giardia cysts and cryptosporidium oocysts in thirty-five different sites in the city of Mashhad, Iran. The occurrence of these pathogenic microorganisms was evaluated to investigate a possible health risk in the communities.

For the detection of enteroviruses in water samples, the conventional method of cell culture, using Buffalo Green Monkey (BGM) cell line, was used. The minimum sample volume for viruses was 500 liters, collected using a positively charged MK cartridge filter, eluted with beef extract and concentrated by organic flocculation. The method of detection for cysts and oocysts relied on microscopic observation of water samples by the immunofluorescence assay (IFA). For this study, water samples were collected using a cartridge filter, eluted by washing the filter using a detergent-based medium, concentrated by centrifugation, clarified by a percoll-sucrose density gradient, stained by an indirect fluorescent antibody, and examined by epifluorescence microscopy.

A total of seventy surface, ground or treated water samples were collected from 35 different sites in the metropolitan city of Mashhad. Thirty-five samples were assayed for the presence of enteroviruses by cell culture and 35 samples were assayed by IFA technique for the detection of cysts and oocysts. Two surface water samples tested positive for virus presence and three surface water samples tested positive for giardia cysts. Based on the results of this research project, no microbial contamination of finished water was documented, suggesting proper treatment of surface water at the time of sampling.

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INTRODUCTION

The enteroviruses (poliovirus, coxsackie A and B viruses, echovirus) can cause a variety of illnesses ranging from gastroenteritis to myocarditis and aseptic meningitis.[1] Numerous studies have documented the presence of enteroviruses in raw and treated drinking water,[2,3] wastewater[4] and sludge.[5] Enteroviruses in the environment pose a public health risk because these viruses can be transmitted via the fecal-oral route through contaminated water[6] and low numbers are able to initiate an infection in humans.[7]

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Giardia cysts and cryptosporidium oocysts are environmentally resistant forms of intestinal parasites that can cause gastroenteritis in humans if ingested. These organisms can be transported by water and numerous waterborne outbreaks of giardiasis through contaminated public water supply have been documented.[8] Several outbreaks of waterborne cryptosporidiosis have been reported[9] with possibility of producing illnesses in thousands of individuals.[10] The method of detection for giardia cysts and cryptosporidium oocysts in water samples relies primarily on microscopic observation of water samples by an immunofluorescent technique.[11] For this study, water samples were collected using a 1 micron porosity cartridge filter. The procedure was then continued by eluting the microorganism from the filter, concentrating the elution media by centrifugations, clarifying the concentrate by a density gradient, labeling the cysts and oocysts with specific antibodies and microscopic examination of the sample.

The purpose of this study was to evaluate the microbial quality of water used as a source of drinking water for the city of metropolitan Mashhad, and to evaluate the process involved in one of the drinking water treatment plants serving a large portion of the city. The water is treated in a treatment plant involving chlorination followed by sedimentation and slow sand filtration. Mashhad is a city of 1.2 million people with most of its drinking water at the present time being provided from surface water. Results from this field survey could be applied to the development of a database of virus and parasite occurrence in drinking water of the metropolitan area. This type of database can be used to provide useful guidelines for water treatment processes.

MATERIALS AND METHODS

Protozoan stock

Giardia lamblia cysts, generated by passage through Mongolian gerbils, were obtained from Swabby Gerbco, Inc. (Diane Swabby, Phoenix, AZ). *Cryptosporidium* oocyts were obtained from Dr. Bruce Anderson, University of Idaho. The cysts and oocysts were received in a fecal suspension and partially purfied on a 1 M sucrose (Sigma, St. Louis, MO) density gradient followed by a Percoll (Pharmacia, Upsala, Sweden) step gradient. Recovered cysts and oocysts were suspended in distilled water and identified and counted by using an indirect immunofluorescence procedure on membrane filters with antibodies (Meridian Diagnostic Inc., Cincinnati, OH) and fluorescein isothiocyanate-labeled, affinity purified goat anti-mouse immunoglobulins A, G, and M (heavy and light chains) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). Epifluorescence was observed with a BHTU epifluorescence microscope (Olympus, New Hyde Park, NY).

A-Immunofluorescence Assay (IFA) for Detection of Giardia Cysts and Cryptosporidium Oocysts

Sample collection

Water samples were collected using a portable gasoline-driven water pump or pressurized tap and filtered through ten-inch, yarn-wound polypropylene cartridge filters (Micro Wynd II; AMF/CUNO Division, Meriden, CT) with a nominal porosity of 1 μm.[12] Flow rates were adjusted to one to two gallons per minute and 100 to 400 gallons of water were filtered.

Filter elution

Filters were back–washed with 2,700 mL of eluent (distilled water containing 0.1% Tween 80 to facilitate desorption). The filter was cut longitudinally, separated from the core, teased apart, and washed three times; each time in one-third of the eluent. The washing was done on a shaker for 10 minutes in a one-gallon container. The sample was concentrated and combined into a single pellet by centrifugation (1,200 g for 10 minutes). The final pellet was divided in half and resuspended in 10% formalin.

Pellet processing

Pellets suspended in formalin were washed and resuspended in a solution containing 1% Tween 80 and 1% sodium dodecyl sulfate (SDS) solution and then homogenized (setting, 30) in a VirTis homogenizer (The

Table I. Total microbial samples collected.

	Virus		Parasite	
Total Samples 70	**Surface Water** 28	**Ground Water** 7	**Surface Water** 28	**Ground Water** 7
Raw Water	17	7[1]	17	7
Treated Water[2]	11	0	11	0

[1]Samples collected prior to any disinfection.
[2]Treated through the water treatment plant.

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Table II. Microbial analysis results.

	Surface Water	Ground Water
Enterovirus	2/28 (7.1%)	0/7 (0.0%)
Giardia Cyst	3/28 (10.7%)	0/7 (0.0%)
Cryptosporidium Oocyst[1]	0/28 (0.0%)	0/7 (0.0%)

[1]Two raw water samples contained oocyst looking objects (1 oocyst/sample) with characteristic fluorescence and size, although no suture line was observed.

Table III. Microbial analysis results of raw and treated water.

	Raw	Treated
Enterovirus	2/17 (11.8%)	0.0%
Giardia Cyst	3/17 (17.6%)	0.0%
Cryptosporidium Oocyst	0/17 (0.0%)	0.0%

VirTis Co., Inc., Gardiner, NY) for 3 minutes. One drop of antifoam (Sigma) was added to facilitate total sample recovery. Next, the sample was washed and resuspended in distilled water for clarification with potassium citrate (1.24 g/mL). After sonication (25 kHz, Branson Ultrasonic Cleaner; Branson Sonic Co., Shelton, CT), 10 mL of each sample was layered onto 30 mL of the density gradient solution. The gradient was centrifuged at 800 g for 10 minutes. The entire supernatant from each sample was recovered, diluted 1:3 with 1% Tween 80, and centrifuged (1,200 g for 10 min). The pellets were suspended in 1 to 5 mL of 1% Tween 80.

Cyst and oocyst detection

Samples (0.1 to 1 mL) were filtered, either directly or after 1:10 dilutions, through 13 mm diameter cellulose nitrate membrane filters with a pore size of 5.0 µm. Cellulose nitrate membrane filters were housed in stainless steel filter holders (Millipore Corp., New Bedford, MA). Filters were rinsed twice with 5.0 mL of 1 X PBS, using a 5-mL syringe attached to the filter holder. The bottoms of the housings were plugged with Parafilm (American National Can, Greenwich, CT), and the appropriate antibodies were applied directly onto the filters (approximately 0.1 to 0.2 mL). After 30 minutes of incubation at room temperature, the filters were rinsed as described above. The secondary antibody was applied and incubated in the same manner as described above and was then rinsed a second time. Filters were then removed from the housings and placed on glass slides in 50% glycerol-phosphate-buffered saline solution (pH 8.0), and cover slips were applied. The entire filter was examined at a magnification of 200 × by using a BHTU epifluorescence microscope equipped with a 100-W high-pressure mercury lamp (Olympus, New Hyde Park, NY) for enumeration of the microorganisms. Cysts and oocysts were identified by their shape, size (8-15 microns for cyst and 4-7 microns for oocyst) and green-apple color under the microscope.

B-Cell Culture Assay for Detection of Enteroviruses

Virus and cell culture

Poliovirus type 1 (LSc strain) for controls was obtained from Dr. Charles P. Gerba at the University of Arizona, Tucson. It was propagated in Buffalo Green Monkey kidney (BGM) cells and virus infectivity was determined by a plaque assay.

Water sample collection for virus

Water samples were collected using an electropositive MK cartridge filter (AMF CUNO, Meriden, CT) placed within a plastic filter housing and connected to a flow meter. Following sampling, the filters were placed in ziploc bags and shipped on ice to the laboratory for processing. Separate filters, filter housing, and tubing were used for each sample.

Filter elution and reconcentration

Viruses adsorbed to the filter were eluted by passing one liter of 1.5% beef extract V (Becton Dickinson, Cockeysville, MD) with 0.05 M glycine (pH 9.5), under pressure, through each filter. The eluates were immediately adjusted to neutral pH with 1 N HCl. The pH of the eluate must be adjusted immediately after elution to prevent virus inactivation due to the high pH of the eluent. The one liter volumes of eluate were reconcentrated by organic flocculation and resuspended in 20-30 mL of buffer.[13] The pH of the final sample was adjusted to 7.2. Bacteria were removed from the final samples through centrifugation at 15,000 g and treated with kanamycin, gentamicin sulfate, penicillin G sodium (United States Biochemical Co., Cleveland, OH) and nystatin (Sigma) antibiotics each at a final concentration of 100 units/mL.

Cell culture assay

The standard cell line used to assay environmental samples for enteroviruses is the BGM cell line. BGM cells were grown to confluent monolayers in 75 cm^2 plastic flasks. Before exposure to the sample, the growth medium was poured off and the cell monolayer was wahsed twice with Tris (Sigma Chemical Co., St. Louis, MO) buffered saline solution.[2] To prevent toxicity, growth medium with 8% fetal bovine serum was added to the flasks prior to inoculation. For each sample, a 3 mL volume of the final

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concentrate was inoculated into each of three flasks. The flasks were incubated at 37°C for 60 min and rotated every 15 min to allow virus adsorption to the cells. Twenty mL of maintenance medium with 2% fetal bovine serum and one mL of gentamicin (50 mcg/mL) was added to each flask. The flasks were incubated at 37°C and examined daily for 14 days for viral cytopathic effect (CPE). Any flask with suspected viral CPE was confirmed by passage into a fresh monolayer of BGM and observed for CPE. At least half of the reconcentrated sample was assayed.

Sample shipment

After the samples were collected, the filters were processed at the University of Mashhad. The concentrates for the cell culture and IFA analyses were shipped to the University of Arizona in Tucson. The sample concentrates were kept cold during the 48 hours in transit from the University of Mashhad to the University of Arizona, Tucson.

RESULTS

The possibility of presence of enteroviruses, giardia cysts, and cryptosporidium oocyst in surface, ground, and treated water was evaluated for the city of Mashhad. A total of seventy surface, ground or treated water samples were collected from 35 different sites in the metropolitan area (Table I).

Thirty-five water samples were assayed for the presence of enteroviruses by cell culture and 35 samples were assayed by IFA technique for the detection of cysts and oocysts. Two surface water samples tested positive for virus presence and three surface water samples tested positive for giardia cysts (Table II). Based on the results of this research project, no microbial contamination of finished water was documented, suggesting proper treatment of surface water at the time of sampling (Table III).

DISCUSSION

The water treatment plant serves drinking water to a large portion of the city with a production of 96,000 m^3/day. The treatment process involves prechlorination, coagulation-flocculation, settling, sand filtration, and disinfection with chlorine. The plant is using chlorine gas for the prechlorination process and ferric chloride is used as the coagulant. Before coagulation-flocculation processes, neutralization is applied if needed to adjust the flocculation pH to its optimum value and to obtain the equilibrium pH in the treated water. Flocculation is further enhanced by adding flocculant aid, such as synthetic flocculants or polyelectrolytes. A pulsator is used for the flocculation-settling process which is a sludge blanket clarifier made up of a flat bottomed tank equipped with a system of perforated laterals and baffles. The treated water at this stage is recovered at the top in clarifier water collection channels. Sand filtration is used to remove particles suspended in water from the coagulation proces. The AQUAZUR "V" filters are used for this purpose with the following characteristics:

-A filter bed of uniform grain size, which stays homogeneous after washing,
- Backwashing is done simultaneously by air and water accompanied with surface sweep followed by a water rinse with the same flow rate, and
-The depth of water above the filter is 1.2 meters.

The treatment processes are completed by final disinfection using chlorine before it is pumped into the distribution system.

In addition to surface water sites, seven ground water sources were sampled for the pathogens. The depth of the seven wells ranged from 110-200 meters with an average of 122.4 meters. Microbial analysis for these samples tested negative for cysts, oocyts, and enteroviruses. However, the sources are disinfected prior to distribution.

This work can determine the occurrence of virus and parasite contamination in water used as a source of drinking water. In addition, it evaluates the processes involved in a drinking water treatment plant. Based on the results of this research project, no microbial contamination of finished water was documented, suggesting proper treatment of water at the time of sampling. However, results from this field survey could be applied to the development of a database of virus and parasite occurrence in drinking water of the city of Mashhad. This type of database should be expanded for drinking water source characterization and to provide useful guidelines for water treatment processes. The main objective of the work was to develop such a database and analyze the results in a manner that will permit an assessment of the applicability of the water quality parameters such as microbial or physical/chemical indicators for providing safe drinking water. We would like to point out the importance of a pathogen monitoring program for the source characterization and for the evaluation of the quality of treated drinking water.

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