Cryptosporidium incidence and surface water influence of groundwater supplying public water systems in Minnesota, USA

Supporting Information

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Supporting Information contains 10 pages, 2 tables, and 1 figure.

Sample processing and nucleic acid extraction

Filters were backflushed following Smith and Hill (*1*). Desiccated beef extract (1% m/v) was added, and the eluate was frozen until further processing. For secondary concentration, polyethylene glycol 8000 (8% m/v) and NaCl (0.2M) were added to the elute before stirring at 4° C for a minimum of two hours and incubating at 4° C overnight. Samples were centrifuged for 45 min at 4700 x g at 4° C, and the resulting pellet was resuspended in Tris-EDTA buffer and stored at -80° C until nucleic acid extraction.

A QIAcube® and QIAamp DNA blood mini kit with buffer AVL (Qiagen, Valencia, CA) were used to extract nucleic acids from 140 µL of concentrated sample from secondary concentration; 65 µL of nucleic acid template were eluted.

qPCR

The 20-µL reaction contained 6 µL of template from the extraction step and 14 µL of master mix, with primers and probe at concentrations of 300 and 50 nM, respectively; sequences are shown in Table S1. A hydrolysis probe was used for quantification, and a standard curve was created from a gBlock® oligo of the target amplicon (Integrated DNA Technologies, Coralville, IA) with the sequence altered so it could be distinguished from wildtype amplicon. Standard concentrations were regressed against cycle of quantification values using the LightCycler® 480 software, and unknowns were quantified using the second derivative maximum method provided by the LightCycler® 480 instrument. All samples were analyzed in duplicate. The average concentration of positive replicates was reported, while zero was reported if both were negative.

For qPCR inhibition, samples with a cycle of quantification (Cq) value at least 2 or 6 cycles greater than the inhibition control were diluted 1:5 or 1:10 with AE buffer, respectively.

Species and subtype identification

Nested PCR amplification was used to produce amplicons for sequencing following Xiao et al. (*2*), Glaberman et al. (*3*), and Strong et al. (*4*). For analysis of the 18S gene to determine species, both the primary and nested PCR reactions consisted of 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, with an initial hot start at 94°C for 3 min and a final extension at 72°C for 7 min. For analysis of the *GP60* gene to determine subtype, both the primary and nested subtyping PCR reactions consisted of 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min, with an initial hot start at 95°C for 3 min and a final extension at 72°C for 10 min. Nested PCR products were sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Products were submitted for sequencing to the University of Wisconsin-Madison Biotechnology Center DNA Sequencing Facility (Madison, WI). Consensus sequences, constructed and aligned with Lasergene (DNASTAR, Madison, WI), were compared with available sequences in NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for species identification.

Table S1. Primer and probe sequences and references for qPCR assay (*Cryptosporidium* spp.) and sequencing reactions for species and subtype identification.

Analysis primer or probe	**Primer/probe sequence**	**Amplicon size (bp)**	**Reference**
qPCR			*5*
Forward primer	CATGGATAACCGTGGTAAT	178	
Reverse primer	TACCCTACCGTCTAAAGCTG		
Probe	CTAGAGCTAATACATGCGAAAAAA		
18S rRNA sequencing			*2*
Primary forward primer	TTCTAGAGCTAATACATGCG	ca. 1325	
Primary reverse primer	CCCATTTCCTTCGAAACAGGA		
Nested forward primer	GGAAGGGTTGTATTTATTAGATAAAG	819-835	
Nested reverse primer	AAGGAGTAAGGAACAACCTCCA		
GP60 sequencing			*3*, *4*
Primary forward primer	ATAGTCTCCGCTGTATTC	ca. 1000	
Primary reverse primer	TCCGCTGTATTCTCAGCC		
Nested forward primer	GGAAGGAACGATGTATCT	ca. 900	
Nested reverse primer	GAGATATATCTTGGTGCG		

Direct immunofluorescent assay (IFA)

Secondary concentrate (100 µL) was vacuum filtered through a 1-µm pore size, 25-mm pre-blackened polycarbonate membrane (Maine Manufacturing, Sanford, ME). Membranes were saturated with 80 µL of a 1:2 dilution of fluorescein isothiocyanate (FITC)-labelled anti-*Cryptosporidium* monoclonal antibodies (Merifluor *Cryptosporidium/Giardia* Detection Kit, Meridian Biosciences, Inc., Cincinnati, OH). The entire filter was counted at 200x using a Nikon 50i Eclipse epifluorescent microscope. Presumed oocysts were confirmed at 400x based on external morphology.

Surface water influence

The USEPA's definition of GWUDI (40 CFR part 141.2) directs States to establish criteria to determine each system's status. The National Primary Drinking Water Regulation (*6*) states: "Ground water under the direct influence of surface water (GWUDI) means any water beneath the surface of the

ground with significant occurrence of insects or other macroorganisms, algae, or large-diameter pathogens such as *Giardia lamblia* or *Cryptosporidium*, or significant and relatively rapid shifts in water characteristics such as turbidity, temperature, conductivity, or pH which closely correlate to climatological or surface water conditions. Direct influence must be determined for individual sources in accordance with criteria established by the State. The State determination of direct influence may be based on site-specific measurements of water quality and/or documentation of well construction characteristics and geology with field evaluation."

Determination methods for GWUDI status therefore vary by state. Consistent with the USEPA's directive, the State of Minnesota continues to develop assessments of potential surface water influence based on many factors, including those used for this study. This determination methodology does not necessarily include all possible factors that will be used for regulatory assessments of GWUDI in Minnesota.

For the purposes of assessing the relationship between surface water influence and *Cryptosporidium* incidence in this study, we focused on two aspects of surface water influence, evidence of an evaporative surface water signature and rapid infiltration, as both are associated with GWUDI status. Wells were first classified as having evidence for an evaporative surface water signature or not, and those wells without such evidence were evaluated for the potential to be subject to rapid infiltration, as shown in Figure S1. These two classifications are not mutually exclusive as wells with evidence of evaporative water may also have evidence of rapid infiltration; however, such wells are not counted in the latter category given that the two-step classification process started with the former. This is consistent with the primary goal of this process, which was to identify the presence of surface water as indicated by evaporative water or rapid infiltration. The individual criteria used for the classification of evaporative water and rapid infiltration are described below. Sample collection and

analysis for the parameters used to determine surface water influence are described in the manuscript Methods and Table S2.

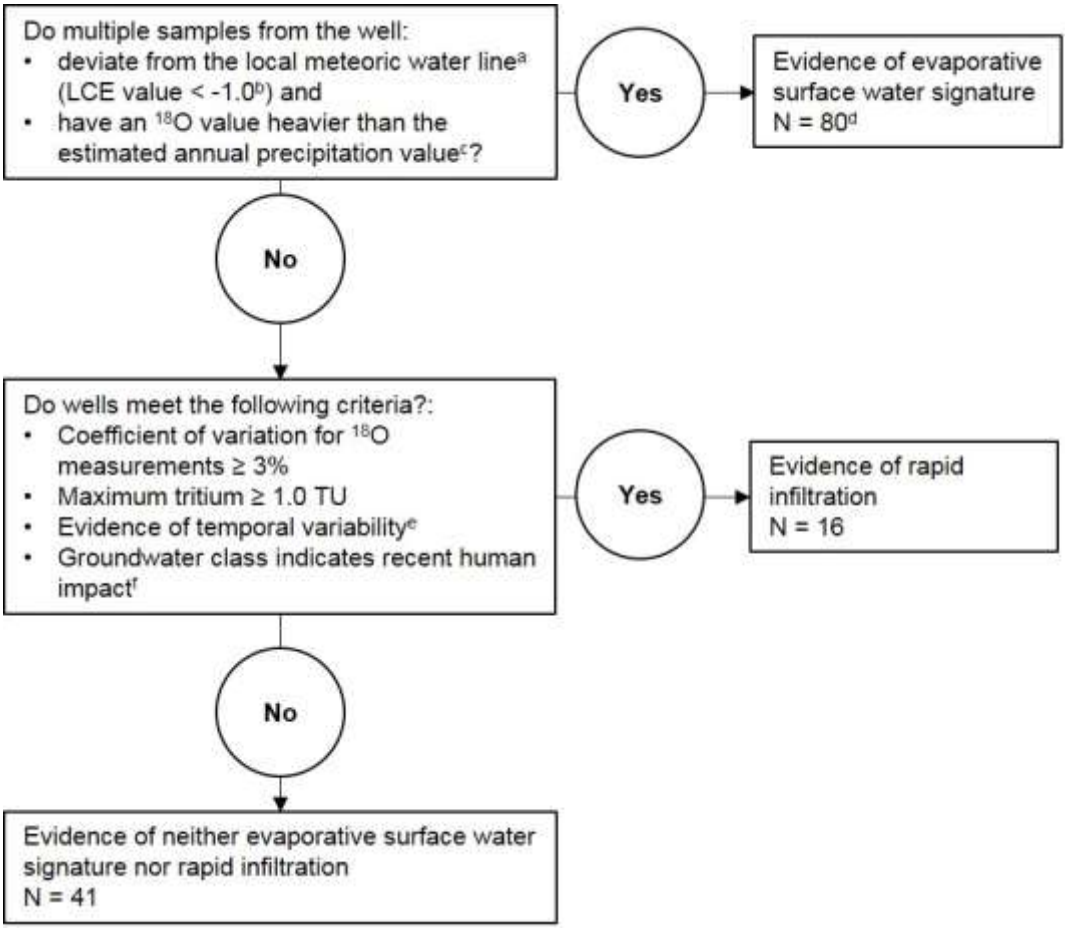

Figure S1. Flowchart for the classification of whether wells showed evidence for surface water influence based on an evaporative water signature or rapid infiltration. LCE, line-conditioned excess; TU, tritium units

[a]Reference 7

[b]Reference 8

[c]Reference 9

[d]8 wells had too few samples to make the determination

[e]Assessment of whether variation in chemical, isotopic, and/or physical parameters indicates water of variable composition

[f]Groundwater class is based on the presence or concentration of chemical, biological, and isotopic parameters

Table S2. Standard methods used for laboratory analyses.

Parameter	Method	Reference
Nitrate + nitrite nitrogen	SM4500-NO_3F	*10*
Total coliform/*E. coli*	SM 9223	*10*
Total organic carbon	SM 5301C	*10*
Bromide	EPA 300.1	*11*
Chloride	EPA 200.7	*12*
Total ammonia	EPA 350.1	*13*

The initial classification step included two criteria (Figure S1). First, isotope pairs (oxygen-18 and deuterium) that deviated from the meteoric water line (line-conditioned excess values < -1.0) indicated evaporative surface water. The analytical uncertainty associated with the isotope measurements was included, and only wells with two or more samples meeting the criterion were classified as having evidence of evaporative surface water. Second, the oxygen-18 values for a well's samples were compared to the estimated annual precipitation values for its geographic location. Only values isotopically heavier than the estimated value were considered supportive of the presence of evaporated surface water. Applying two criteria and requiring multiple samples from the well to meet them minimized the risk of spurious designation of an evaporative surface water signature.

Evidence of rapid infiltration was evaluated in the second classification step and was based on groundwater age and chemical characteristics. Temporal variability is an assessment of whether the chemical, isotopic, and/or physical data for the well suggests that it has captured water of variable composition based on the coefficient of variation (CV) of measurements. Specifically, temporal variability was determined by one of the following: 1) two or more chemical parameters exceeding a CV of 10%, 2) at least one chemical parameter exceeding a CV of 10% and at least one of the stable isotopes exceeding a CV of 3%, or 3) one of the chemical or isotopic parameters exceeding the CV stated above and either the CV for specific conductance exceeding 5% or that of water temperature exceeding 10%.

Lower CV threshold values were used for specific conductance and oxygen-18 due to the sensitivity of the latter and the commonly recognized well stabilization value of the former (*14*).

Groundwater class reflects the recharge characteristics and relative levels of human impact to the groundwater and are arrived at by assessing the chemical and isotopic data for each well. Wells must have some indication of rapid recharge (chemically dilute and temporally variable signature) and/or recent human impact to be considered subject to rapid infiltration. Recent impact was based on the presence and/or concentration (including some concentration ratios) of bromide, chloride, nitrate, ammonia, boron, total organic carbon, indicator bacteria, dissolved oxygen, and tritium, and the assessment was supported by ^{2}H and ^{18}O.

Vertical hydraulic gradient was used as an additional characteristic, with a negative value supporting a determination of rapid recharge. Vertical hydraulic gradient was determined by comparing each study well's static water level (depth to water from ground surface) and top of well screen elevation to 5 – 10 nearby wells (within one mile). The elevation of each nearby well (top of screen) was categorized as above, below, or within the study well's screen interval, and the vertical gradient was determined based on the static water level measurements. Negative values represent the potential for downward flow relative to the elevation of the study well. In addition to nearby wells, surface water features (rivers and lakes) were included in the comparison for wells that showed an evaporative isotopic signature or that had fewer than 5 wells within one mile. The evaluation was completed using an ArcMap gradient analysis tool developed by the Minnesota Department of Health.

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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