Detection of *Cryptosporidium* species and sources of contamination with *Cryptosporidium hominis* during a waterborne outbreak in north west Wales

Rachel M. Chalmers, Guy Robinson, Kristin Elwin, Stephen J. Hadfield, Euron Thomas, John Watkins, David Casemore and David Kay

ABSTRACT

As part of investigations into the cause of a waterborne outbreak of *Cryptosporidium hominis* infection linked to a mains water supply, surface waters and wastewater treatment plants were tested for *Cryptosporidium* spp. Oocyst counts in base flow surface water samples ranged from nil to 29 per 10 l. Oocyst counts in effluent from a community wastewater treatment plant were up to 63 fold higher and breakout from one septic tank five logs higher. There were no peak (storm) flow events during the investigation. *C. hominis,* four named genotypes (cervine, muskrat II, rat, W19) and six new small subunit ribosomal RNA gene sequences were identified. Four of the new sequences were closely related to *Cryptosporidium* muskrat genotype I, one was closely related to the fox genotype and one to *Cryptosporidium canis*. *C. hominis* was found extensively in the catchment, but only at sites contaminated by wastewater, and in the treated water supply to the affected area. All were gp60 subtype IbA10G2, the outbreak subtype. Multiple routes of contamination of the reservoir were identified, resulting in persistent detection of low numbers of oocysts in the final water. This work demonstrates the utility of genotyping *Cryptosporidium* isolates in environmental samples during outbreak investigations.

Key words | *Cryptosporidium hominis*, genotypes, source, species, wastewater

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INTRODUCTION

Waterborne outbreaks of the diarrhoeal disease cryptosporidiosis caused by the protozoan parasite *Cryptosporidium* have been linked to both human (wastewater) and animal (agricultural and wildlife) sources of contamination (Meindhart *et al.* 1996; Glaberman *et al.* 2002; Smith *et al.* 2006; Karanis *et al.* 2007; Chalmers *et al.* 2009*a*). The taxonomy of *Cryptosporidium* is under continual review but of the numerous species and genotypes (isolates of uncertain taxonomic status) identified, *Cryptosporidium parvum* and *Cryptosporidium hominis* predominate in human cases of cryptosporidiosis, and their epidemiology is monitored in an on-going typing scheme in the UK (Chalmers & Pollock 2008; Chalmers *et al.* 2009*b*). The natural host range of *C. hominis* is mainly limited to humans with only rare, mainly single, reports of infection in animals (Xiao *et al.* 1999*a*; Morgan *et al.* 2000; Zhou *et al.* 2004; Ryan *et al.* 2005; Smith *et al.* 2005; Giles *et al.* 2009). Analytical epidemiologic studies confirm a human transmission cycle (Hunter *et al.* 2004). In contrast, *C. parvum* has a broad mammalian host range, particularly ruminants and humans, and there appear to be independent human and animal as well as zoonotic transmission cycles (Mallon *et al.* 2003; Hunter *et al.* 2007; Morrison *et al.* 2007). Most other *Cryptosporidium* species and genotypes cause human disease

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more rarely (Chalmers *et al.* 2009*b*). Many exhibit some degree of host specificity, providing some indication of potential source when detected in water (Feng *et al.* 2007). Notable exceptions appear to include the cervine genotype which has been found in a variety of hosts (Santin & Fayer 2007).

Application of PCR-based methods for the characterisation of oocysts in water samples has been undertaken either from microscope slides as part of routine raw water testing (Ruecker *et al.* 2005; Ruecker *et al.* 2007) and treated water monitoring (Nichols *et al.* 2006) or from raw water concentrates (Xiao *et al.* 2000; Jellison *et al.* 2002; Jiang *et al.* 2005*a*). This has enabled the identification of the species and genotypes present and provides additional information about possible sources of contamination and the risk to public health. However, when source and storm waters were sampled, only some of the isolates detected matched named *Cryptosporidium* species or genotypes, while others were also found in animals in the catchment thus generating a common name for the genotype (Feng *et al.* 2007). Isolates found only in water by Feng and colleagues in watersheds in New York, USA, have been given temporary sequential genotype numbers prefixed by the letter W (water) until a host is identified (Feng *et al.* 2007).

Typing of clinical isolates during waterborne outbreak investigations has provided valuable information about potential sources of contamination (Glaberman *et al.* 2002; Smith *et al.* 2006) and confirmation of the presence of the infecting isolate in drinking water strengthens the evidence for linkage to water (Tillett *et al.* 1998; Glaberman *et al.* 2002). However, investigation of waterborne outbreaks in the past has not routinely involved timely characterisation of *Cryptosporidium* oocysts detected in water samples. Here, we describe extensive hydrological catchment investigations undertaken during the investigation of an outbreak of *C. hominis* (Mason *et al.* 2010) to establish whether the same isolate was present in the treated water, identify possible sources of contamination, whether this was ongoing, and indicate possible interventions.

STUDY CATCHMENT AND MAINS WATER SUPPLY

Initial descriptive epidemiology of the outbreak indicated that the mains drinking water supply was the possible cause of the outbreak. The source water is from a reservoir (Llyn Cwellyn) in the north west of Wales, UK, within the Snowdonia National Park. Llyn Cwellyn (grid reference of centre OS 256000 355000) is approximately 2 km long, 0.75 km wide and 37 m at its deepest point, and orientated north west–south east at approximately 142 metres above sea level. The lake is in an area of high annual rainfall (> 1,500 mm) with various inputs to the lake (streams and ditches). It is fed at the south eastern end by the River Gwyrfai, and the river flows out of the lake over a dam at the north western end. The regional south west to north east wind direction is funnelled by the mountainous topography along the lake's main axis from inlet to outlet, creating wind driven surface streaming. The raw water abstraction point for the water treatment works is at the outflow dam. Although no data have been presented, lakes of similar characteristics in the UK have been shown to be stratified with a distinct thermocline developing during the summer period and a likely turnover in the autumn (Kay & McDonald 1980; De Cesare *et al.* 2006; George *et al.* 2007; Baldwin *et al.* 2008; Elci 2008; Wilhelm & Adrian 2008; Caliskan & Elci 2009).

The hydrological catchment covers 20.1 km^2 of rural upland, and is characterised by steeply sloping topography with a maximum altitude of 1,085 m (Mount Snowdon summit), with extensive landforms characteristic of glaciation and subsequent periglacial activity. The characteristically glacial 'U' shaped valleys are bounded by steep valley sides, mainly covered by scree deposits that encourage rapid hillslope throughflow of rain to the valley floor. The catchment is sparsely populated with mainly single dwellings, a small village (Rhyd Ddu, permanent population ~ 200) and attracts year-round tourism for outdoor activities. Tourist accommodation is provided in the village public house, a Youth Hostel and camp sites. In agricultural terms, the holdings in the catchment are upland/hill, predominantly sheep farms. Cattle farming of small suckler herds on the hills and fattening on improved land is practised adjacent to the lake side.

The village is served by mains drinking water and a wastewater treatment plant, comprising primary settlement, percolating filter and final settlement. There is no storm separation at the plant. Due to its small size, flows are not continually measured. Secondary treated effluent is

discharged into the R. Gwyrfai ~ 1,100 m upstream of the lake. Premises out-with the village have either a pre-fabricated package plant for the biological decomposition of wastewater and discharging treated effluent or a septic tank facilitating settlement and anaerobic digestion to break down the organic component of wastewater, with effluent soakage into the ground by sub-surface irrigation pipes.

The mains drinking water supply was treated by pressure filtration (aimed primarily at reduction of naturally occurring manganese) and disinfection by chlorination, and is more fully described in the accompanying paper by Mason *et al.* (2010).

METHODS

Cryptosporidium sampling, detection and enumeration

Preliminary descriptive epidemiological findings presented at an incident management team (IMT) meeting on 7th November 2005 suggested a possible link with the drinking water supply. At the request of the IMT, an environmental investigation was initiated to identify possible sources of *Cryptosporidium* contamination. A detailed visual survey of the catchment was undertaken on 22nd and 23rd November 2005 to locate obvious inputs and potential sampling points. At least 13 septic tanks were identified at properties within the catchment. Base-flow sampling of the wastewater treatment plant (influent and effluent) and four surface waters (one upstream of human inputs, and three downstream of the wastewater treatment plant discharge) was established at twice weekly intervals with provision for high flow condition sampling. Six other sites were sampled on single occasions. The sampling covered a 39 day period from 19th November to 28th December 2005, during which time there were no rainfall events leading to high flow conditions.

Surface water samples were taken where possible by bankside filtration using FiltaMax™ (IDEXX) or Envirocheck™ HV filters (Pall Corporation). If filtration was not possible, 10 l grab samples were taken. In addition to regularly sampled sites, *ad hoc* samples of water and/or effluent were taken from the package plants, septic tanks and a defective sewer, sample sizes ranging from 1 to 10 l.

All samples were sent by courier to accredited *Cryptosporidium* testing laboratories (Severn Trent Laboratories, Coventry or CREH Analytical Limited in Leeds). Filter samples were eluted and concentrated by immunomagnetic separation (Isolate™, TCS Biosciences or Dynabeads® anti-*Cryptosporidium*, Invitrogen) according to the Drinking Water Inspectorate approved protocol (Anon 2005). Grab samples and treated wastewater samples were processed by membrane filtration using a 142 mm cellulose acetate membrane. Membranes were eluted in 0.1% Tween 80 in distilled water, concentrated by centrifugation and subjected to IMS (Anon 1999; Anon 2005). Sewage samples which could not be filtered were centrifuged at 1,100 × *g* for 15 minutes in 50 ml conical centrifuge tubes, pellets combined to a single tube and the particulate material concentrated by further centrifugation. Pellets were re-suspended and subjected to immunomagnetic separation (Anon 2005). Solid material was processed by immunomagnetic separation as described for animal faecal material by Smith *et al.* (2009). For all samples, *Cryptosporidium* oocysts were detected by epifluorescent microscopy in accordance with the approved protocol (Anon 2005) using an immunofluorescent test (TCS Water Sciences) and 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma) staining.

Cryptosporidium genotyping

Cryptosporidium-positive microscopy slides generated as described above, and from routine monitoring and tap samples as described by Mason *et al.* (2010), were sent overnight to the UK *Cryptosporidium* Reference Unit in Swansea for typing. Coverslips were removed from slides, and DNA extracted from the recovered material as described by Chalmers *et al.* (2009*a*) and validated by Robinson (2005). Briefly, acetone was used to loosen the coverslip which was carefully removed from the slide using a scalpel blade. 50 μl Buffer AL (Qiagen Ltd) was applied, the material scraped from the slide using a plastic pipette tip, and transferred to a 1.5 ml sample tube. The slide was rotated through 90° and the scraping process repeated three more times. Oocysts were disrupted by three freeze thaw cycles (cardice – methanol/100°C) and DNA extracted and purified using the QIAamp® DNA Mini Kit (Qiagen Ltd) according to the manufacturer's instructions. The final

elution volume was 50 μl, and extracted DNA was stored at − 20°C prior to use.

Cryptosporidium spp. and genotypes were identified by analysing DNA extracted from all water and environmental samples and from 6 human *C. hominis* case samples (Mason *et al.* 2010) by repetitive nested PCR-RFLP and bi-directional sequencing of small subunit ribosomal (SSU) rRNA gene. Briefly, five replicate PCRs (Xiao *et al.* 2006*a*) of a ~ 840 bp product of the SSU rRNA gene were performed using internal and external primers and PCR conditions and subjected to restriction endonuclease digestion by *Ssp* I and *Vsp* I as described previously (Xiao *et al.* 2000). The digested products were separated by agarose (3% w/v) gel electrophoresis, visualised using SYBR Green I (1 × solution) and recorded using a digital imaging system (AlphaImager, Alpha Innotech). Product sizes were confirmed by comparison with a DNA molecular weight standard marker (Invitrogen).

Following the original work during the outbreak investigation, the SSU rRNA gene PCR assay was re-optimised, incorporating the use of a modified secondary PCR reverse primer and the anti-inhibitory properties of 500 ng/μl non-acetylated bovine serum albumin (Jiang *et al.* 2005*b*). Previously negative samples for which sufficient DNA remained were retested using the new assay and results incorporated into the study dataset shown here.

Identification was confirmed by bi-directional sequencing of purified PCR products (Qiaquick, Qiagen, Crawley, UK) using SSU rRNA gene forward and reverse secondary PCR primers (Xiao *et al.* 2000; Jiang *et al.* 2005*a*) and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, UK) on an ABI3730 automated sequencer (Applied Biosystems, UK) by GeneService, Cambridge, UK. Nucleotide sequences were edited and assembled using ChromasPro 1.4a (Technelysium Ltd, Australia), aligned using ClustalX 2.0 (http://www.clustal.org/download/current/) and compared using the Basic Local Alignment Sequence (BLAST) tool (US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) against all sequences on GenBank. Sequences were verified, and those showing > 99.5% similarity in the region targeted by the PCR to a published sequence were assigned to that species or genotype (Xiao *et al.* 2006*b*). To determine the relationship between the SSU rRNA gene sequences found in this study with reference sequences from GenBank across the genus, distances were calculated using the Kimura two-parameter model and a phylogenetic tree was produced using neighbour-joining analyses (Jiang *et al.* 2005*a*). Branch bootstrap values were determined from 1000 re-samples. A SSU rRNA gene sequence from *Eimeria tenella* was used to root the tree.

Exceptionally (see Table 1), PCR-RFLP and bi-directional sequencing the *Cryptosporidium* oocyst wall protein (COWP) gene provided identification in the absence of SSU rRNA gene PCR products. Briefly, a 550 bp region of the COWP gene was amplified using primers cry-15 and cry-9 according to Spano *et al.* (1997). Due to the limited volume of DNA available, a single aliquot was amplified and digested with *Rsa* I. Gel electrophoresis and sequencing using cry-15 and cry-9 was undertaken as described above.

To identify subtype families and their subtypes, *C. hominis* isolates were further investigated by DNA sequence analysis of the gp60 gene using internal and external primers as described by Alves *et al.* (2003) producing final fragments of 800 to 850 bp. Bi-directional sequencing of PCR products using forward and reverse primers AL3532 and AL3534 was undertaken as described above. After identifying subtype families, subtypes were identified by counting the number of trinucleotide repeats (TCA, TCG or TCT) coding for the amino acid serine in the microsatellite region (Cama *et al.* 2007).

Sequence data for representative isolates from this study were deposited in GenBank, accession numbers GQ183505 to GQ183528.

RESULTS

Cryptosporidium oocyst detection

Cryptosporidium oocysts were present at multiple sites with hydrological links to the reservoir within the catchment. Oocyst numbers are shown in Table 1. Base-flow sampling of the wastewater treatment plant and four surface waters at twice weekly intervals demonstrated that counts were highest in wastewater treatment plant effluent (range 30 to 1824 oocysts per 10 l), and lowest in the reservoir outlet at

Table 1 | *Cryptosporidium* oocyst counts and identity in surface and wastewaters in the Llyn Cwellyn catchment

Location	Sample date	Sample type	Sample volume	Number of oocysts seen	Oocysts per 10 l	Isolate -replicate number(s)	*Cryptosporidium* species/genotype* (named differences are in brackets where identified; see Tables 2 and 3)	*C. hominis* gp60 subtype
Llanwnda distribution	07/11/2005	Filter	1,800 l	6	0.03	#11765-1, -3, -4	*C. hominis* (A)	NA
Reservoir outlet at dam	02/12/2005	Filter	103 l	3	0.3	#12212-2, -4, -5	*C. hominis*	IbA10G2
	14/12/2005	Filter	104 l	4	0.4	#12323	NA	
	16/12/2005	Filter	95 l	4	0.4	#12327	NA	
	19/12/2005	Filter	102 l	0	0			
	21/12/2005	Filter	66 l	19	2.9	#12368	NA	
	28/12/2005	Filter	97 l	0	0			
					Mean = 0.7			
River at inflow to reservoir	25/11/2005	Filter	38 l	30	7.9	#12129-3, -4, -5	*C. hominis*	IbA10G2
	02/12/2005	Filter	93 l	42	5.3	#12213-1, -2, -4, -5	*C. hominis*	NA
	14/12/2005	Filter	78 l	10	1.3	#12324-4, -5	*C. hominis* (C, A)	NT
	16/12/2005	Filter	75 l	12	1.6	#12328-2, -4	*C. hominis* (A)	NA
	19/12/2005	Filter	63 l	3	0.5	#12365-2	Cervine genotype (A)	
	21/12/2005	Filter	63 l	1	0.2	#12369	NA	
	28/12/2005	Filter	98 l	6	0.6	#12394	*C. hominis*†	NA
					Mean = 2.5			
River 250 m down stream of wastewater treatment plant outfall	19/11/2005	Grab	10 l	29	29	#12040	*C. hominis*†	NA
	23/11/2005	Grab	10 l	14	14	#12043	NA	
	14/12/2005	Filter	42 l	12	2.9	#12325-4	*C. hominis* (D)	NT
	16/12/2005	Filter	53 l	12	2.3	#12329	NA	
	19/12/2005	Filter	47 l	18	3.8	#12366-2, -5	*C. hominis* (A)	NA
						#12366-4	UK E1	
	21/12/2005	Filter	49 l	17	3.5	#12370-1, -4, -5	*C. hominis* (A)	NA
						#12370-2	UK E2	
	28/12/2005	Filter	64 l	5	0.8	#12395-4	*C. hominis* (B)	NA
						#12395-2	Cervine genotype (A)	
					Mean = 8.0			

Table 1 | (*continued*)

Location	Sample date	Sample type	Sample volume	Number of oocysts seen	Oocysts per 10 l	Isolate -replicate number(s)	*Cryptosporidium* species/genotype* (named differences are in brackets where identified; see Tables 2 and 3)	*C. hominis* gp60 subtype
River upstream of wastewater treatment plant outfall and stream near public toilets	23/11/2005	Grab	10 l	1	1	#12044	NA	
	28/11/2005	Filter	29 l	2	0.7	#12128-4	UK E3	
	14/12/2005	Filter	36 l	2	0.6	#12326	NA	
	16/12/2005	Filter	44 l	0	0			
	19/12/2005	Filter	46 l	5	1.1	#12367-1	Cervine genotype (B)	
	21/12/2005	Filter	38 l	19	5	#12371-5	Cervine genotype (A)	
	28/12/2005	Filter	49 l	2	0.4	#12396-1, -2, -3, -4	Cervine genotype (C, A, B, A)	
					Mean = 1.3			
Wastewater treatment plant effluent	19/11/2005	Grab	10 l	100′s	>100	#12041-1, -2, -4, -5	*C. hominis* (A)	IbA10G2
	23/11/2005	Grab	10 l	100′s	>100	#12042-1, -2, -4, -5	*C. hominis* (A)	NA
	25/11/2005	Grab	10 l	100′s	>100	#12125-1, -2, -3, -4, -5	*C. hominis* (A)	IbA10G2
	14/12/2005	Grab	10 l	1,824	1,824	#12494-1, -2, -3, -4, -5	*C. hominis* (A)	IbA10G2
	16/12/2005	Grab	10 l	1,542	1,542	#12495-1, -2, -3, -4, -5	*C. hominis* (A)	IbA10G2
	19/12/2005	Grab	10 l	786	786	#12374-1, -2, -3, -5	*C. hominis* (A)	NA
	21/12/2005	Grab	10 l	48	48	#12376-1, -3, -4, -5	*C. hominis* (A)	NA
	28/12/2005	Grab	5 l	15	30	#12397-1, -2, -3, -4, -5	*C. hominis* (A)	NA
Wastewater treatment plant influent	14/12/2005	Grab	2 l	3	15	#12372-1, -2, -3, -4, -5	*C. hominis* (A)	NA
	16/12/2005	Grab	1 l	13	130	#12330-1, -2, -3, -4, -5	*C. hominis* (A)	NA
	19/12/2005	Grab	2 l	90	450	#12373-1, -2, -3, -4, -5	*C. hominis* (A)	NA
	21/12/2005	Grab	2 l	22	110	#12375-1, -2, -3, -4, -5	*C. hominis* (A)	NT
	28/12/2005	Grab	1 l	48	480	#12398-1, -2, -3, -4, -5	*C. hominis* (A)	IbA10G2
Property 1								
Septic tank	21/12/2005	Grab	250 ml	2	400	#12496	Cervine genotype†	
Property 5								
Package plant sludge	28/11/2005	Grab	10 ml	3	3,000	#12124	NA	
Package plant effluent	28/11/2005	Grab	5 l	0				
Stream below property	28/11/2005	Filter	44 l	1	0.2	#12127-5	Muskrat genotype II	
Property 7								
Stream beside property	28/11/2005	Filter	32 l	1	0.3	#12132-2	UK E4	
Property 11								

Table 1 | (*continued*)

Location	Sample date	Sample type	Sample volume	Number of oocysts seen	Oocysts per 10 l	Isolate -replicate number(s)	*Cryptosporidium* species/genotype* (named differences are in brackets where identified; see Tables 2 and 3)	*C. hominis* gp60 subtype
Ditch water	25/11/2005	Filter	26 l	1	0.4	#12131	NA	
Property 12								
Package plant sludge	14/12/2005	Grab	1 l	0	0			
Property 13								
Septic tank	25/11/2005	Grab	5 ml	38	7.6×10^4	#12122-1, -2, -3, -4, -5	*C. hominis*	IbA10G2
Soakaway breakout	25/11/2005	Grab	10 ml	5,630	5.6×10^6	#12123-1, -2, -3, -4, -5	*C. hominis*	IbA10G2
Culvert to stream	25/11/2005	Filter	130 l	28	2.2	#12214-2, -5	Cervine genotype (B, A)	
						#12214-1	W19 genotype	
						#12214-4	UK E5	
Stream below property	25/11/2005	Filter	26 l	36	13.8	#12130-2	Cervine genotype (D)	
						#12130-3	Rat genotype	
Blocked sewer at public toilets in Rhyd Ddu	08/12/2005	Grab	2 g	6	3×10^4	#12493-5	UK E6	

*Identified by SSU DNA sequence analysis unless otherwise indicated.
†Identified by COWP sequence analysis.
NA, Not Amplified; NT, Not Tested (insufficient DNA).

the dam (range 0 to 2.9 oocysts per 10 l). The highest oocysts counts in surface water were from the river down stream of the wastewater treatment plant outfall (range 0.8 to 29 oocysts per 10 l). Counts from river water upstream were lower (range 0 to 5 oocysts per 10 l) but more consistent, compared to downstream where they declined over time. Counts also declined over time in the effluent and further downstream where the river flows into the reservoir, probably because the humus tanks had been emptied by Dwr Cymru/Welsh Water (DCWW) and the contents removed from the site by tanker as a measure to control this source of contamination. Oocyst detections at the dam, which is near the off-take for the water treatment works, were less frequent.

Oocyst counts in effluent from a community wastewater treatment plant ranged from 30 to 1824 per 10 l and were up to 63 fold higher than those in base flow surface water samples, indicating a major source of contamination. Although three other septic tanks, package plants or sewers contained oocysts (400, 3000 and 3×10^4 oocysts per 10 l respectively) the other significant source of environmental contamination with a hydrological link to the reservoir was breakout from one septic tank where oocyst counts of up to 5.6×10^6 oocysts per 10 l, five logs higher than those in base flow surface water samples, were detected.

Cryptosporidium species

The phylogenetic and genetic relationships at the partial SSU rRNA gene of *Cryptosporidium* species and genotypes found in this study compared with reference isolates are shown in Figure 1, and nucleotide differences in Tables 2 and 3.

C. hominis was confirmed only in tap water (Llanwnda distribution sample) and at sites under the influence of human wastewater but these were multiple (Table 1): the reservoir outlet at the dam, the river inflow to the reservoir, downstream of the wastewater outlet into the river, in the wastewater treatment plant effluent and influent and in the septic tank and effluent breakout at property 13. *C. hominis* was confirmed upstream of the reservoir throughout the study period, but only once at the dam.

More than one species/genotype was detected in 5 samples, at 3 sampling points, and sometimes included *C. hominis*. Other named *Cryptosporidium* species/genotypes identified in the catchment were the *Cryptosporidium* cervine genotype (W4), muskrat genotype II (W16), the rat genotype and W19. Of these, only the cervine genotype was detected in the regularly sampled sites: the river flowing into the reservoir, downstream of the effluent outfall and, most frequently, upstream of the outfall. The cervine genotype was also detected in a septic tank sample from property 1. Sequence heterogeneity was observed in the cervine genotype at the partial SSU rRNA gene (Table 2). Of the 11 cervine isolates, two groups of 6 and 3 isolates each were homologous within each group but differed by one nucleotide transition and two deletions. These are denoted cervine genotype (A) and (B) respectively. The other two isolates each had unique transitions, called cervine genotypes (C) and (D).

Muskrat genotype II was detected in a stream below property 5, and differed from the reference isolate (GenBank accession number AY545548) by 3 nucleotide (nt) substitutions (Table 2). The cervine genotype and many new sequences (see below) were detected in a culvert to a stream below property 13. The cervine genotype and the rat genotype were detected in that stream. Also detected in the culvert was W19 which differed from the reference isolate (AY737585) at 3 nt positions (2 substitutions and a deletion) (Table 2).

Six different SSU rRNA gene sequences, present in 6 samples from 5 sites, had only 97% to 98% sequence similarity to any previously published species or genotype. We regard these sequences to be new, and designate UK environmental (UK E) sequences 1 to 6. UK E1, UK E2, UK E3 and UK E5 all clustered in the phylogenetic tree (Figure 1) close to muskrat genotype I (W7) but with reasonable bootstrap support for them to form a separate clade. There is strong support for branching within the clade, although the branches are small since the sequences were 99.54% to 99.85% similar to each other. UK E1, UK E2 and UK E3 were found in the river water whereas UK E5 was found in the culvert to the stream below property 13. UK E4 was most similar to but distinct from the fox genotype, and was found in a stream beside property 7; UK E6 was most similar to but distinct from *Cryptosporidium canis* and was

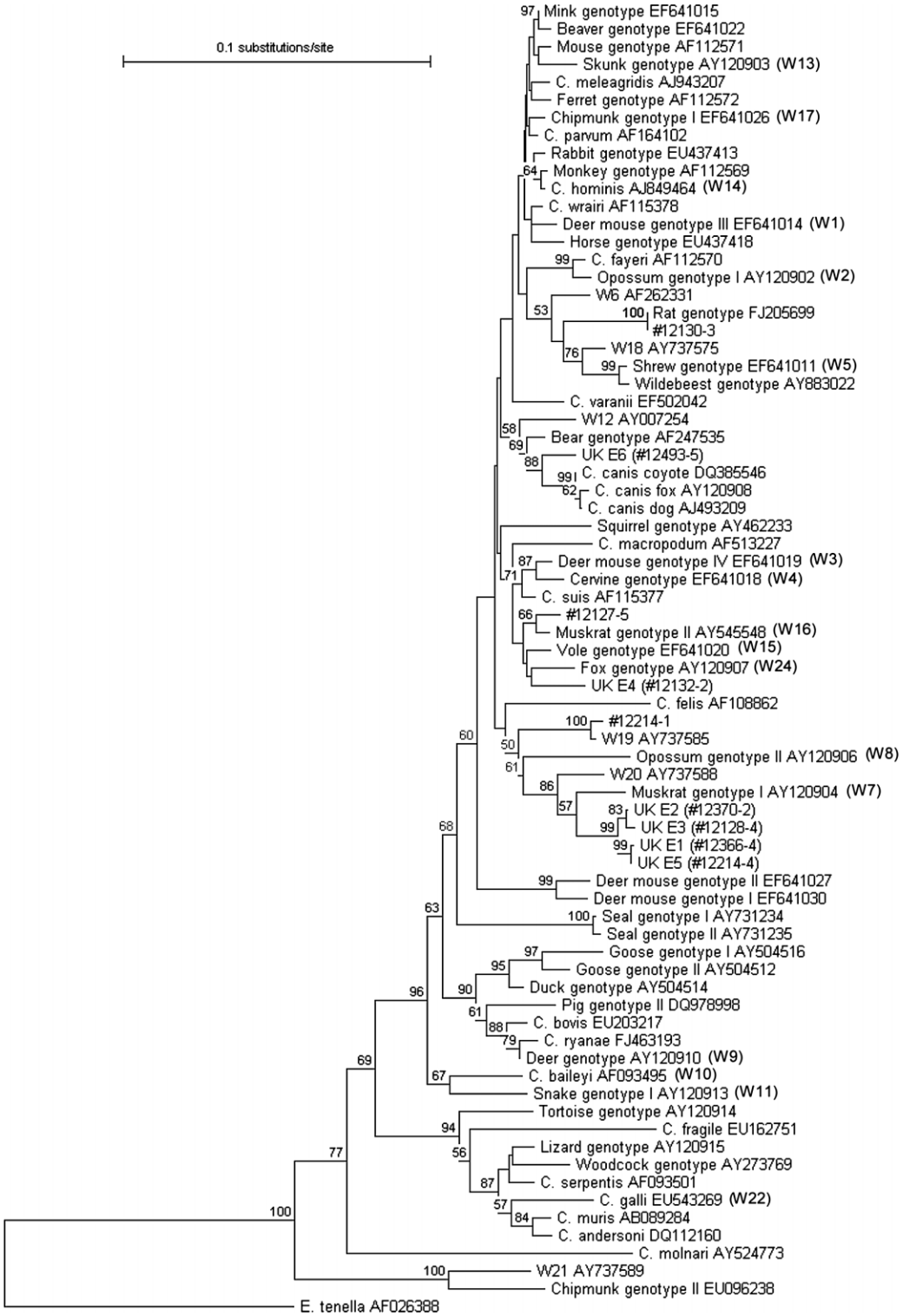

Figure 1 | Phylogenetic relationships among *Cryptosporidium* isolates from this study and known species and genotypes reported on GenBank, as inferred by neighbour-joining analysis. Water genotype names are given in brackets after their animal hosts, according to Feng *et al.* 2007. Numbers on branch nodes are bootstrap values (>50%) obtained from 1000 re-samples. *C. hominis* and cervine genotype sequences from this study are omitted but partial alignments are presented in Tables 2 and 3.

Table 2 | Nucleotide differences in the *Cryptosporidium* partial SSU rRNA genes seen in isolates identified as named genotypes from environmental samples in this study. Dots denote nucleotide identity to the reference sequence from GenBank. Dashes denote sequence deletions

Reference isolate (GenBank accession number)	Location of nucleotide differences	
Replicate number(s), this study		
	W19 genotype	
	NT 407 to 479	
W19 (AY737585)	TTTTATATTATTAATTTATTATTATTAATATAATATTAACATAATTCATATTACTTTTTTTGAGTATATGAAA	
#12214-1	.....-.....G.....A.....	
	Muskrat genotype II	
	NT 453 to 525	
Muskrat genotype II (AY545548)	ATTACTATTATTATTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATTTGCCTTGA	
#12127-5	.....AT.....G.....	
	Cervine genotype	
	NT 457 to 529	
Cervine genotype (AF442484)	AAATAACAATACAGGACTTTAAATAGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAA	
(A) #12365-2, #12395-2, #12371-5, #12396-2, #12396-4, #12214-5	.....	
(B) #12367-1, #12396-3, #12214-2	.....G.....	
(C) #12396-1	....G.....	
(D) #12130-2	.....G.....	
	NT 661 to 696	NT 718 to 752
Cervine genotype (AF442484)	ATATAGTATTAACATAATTCATATTACTATATTTTA	GAGAAAATTAGAGTGCTTAAAGCAGGCATTAGCCT
(A) #12365-2, #12395-2, #12371-5, #12396-2, #12396-4, #12214-5	.....	.....
(B) #12367-1, #12396-3, #12214-2	.....-.....	.....
(C) #12396-1	.....	.....A...
(D) #12130-2	..G.....	.....

Table 3 | Nucleotide differences in the partial SSU rRNA genes of *Cryptosporidium hominis*, comparing human outbreak and environmental isolates. Dots denote nucleotide identity to the reference sequence from GenBank

Reference Isolate (GenBank accession number)	Location of nucleotide differences in the partial *C. hominis* SSU rRNA gene			
Isolate-DNA replicate number(s), this study				
	NT 135-152	NT 379-390	NT 496-509	NT 669-681
C. hominis (AJ849464)	GGGTTCGATTCCGGAGAG	TAAAAAGCTCGT	CTTTGAGAAAATTA	GACAAACTAATGC
(A) 6 outbreak case samples, 3 final water and 62 environmental isolate DNA replicates	. . . . .	. . . . .	. . . . .	. . . . .
(B) #12395-4	. . . . . T	. . . . . T	. . . . .	. . . . .
(C) #12324-4	. . . . .	. . . . .	. . . . . A	. . . . .
(D) #12325-4	. . . . .	. . . . .	. . . . .	. . . . . G . . . . .

found in the blocked sewer at the public toilets in Rhyd Ddu, although this site may have also been subject to environmental or wildlife contamination.

Variation within *C. hominis*

Of the 95 replicates from the 27 environmental samples in which *C. hominis* was found in this study (Table 1), full-length consensus SSU rRNA gene sequences were constructed for 65. Variable numbers of thymine (T) bases were observed in the hypervariable polyT region, ranging from 8T to 13T (except 9T). This variation is mainly attributable to the presence of heterogeneous copies of the SSU rRNA gene (Xiao *et al.* 1999*b*). Otherwise, *C. hominis* sequences from the human outbreak cases, the final water and 62/65 (95%) of the environmental sample replicates were homologous, and are here referred to as *C. hominis* (A) (Tables 1 and 3). However, three replicates from two isolates at two different sample sites had sequences which contained unique single nucleotide polymorphisms (SNPs) and are referred to as *C. hominis* (B), (C) and (D) (Tables 1 and 3). Although these variants were found at sites downstream of the wastewater treatment plant, only *C. hominis* (A) was detected in the influent and effluent. The source of the other isolates is not known.

Of the 27 environmental samples containing *C. hominis*, 9 amplified with the gp60 primers, 15 failed to amplify and 3 were insufficient to test. All amplified samples were IbA10G2, which is the same subtype as the outbreak case isolates (Mason *et al.* 2010). Those that did not amplify were the *C. hominis* (B), (C) and (D) and some of the (A) isolates. The significance of this is not known, as the numbers of (B), (C) and (D) isolates were small and the oocyst numbers low.

DISCUSSION

By undertaking a catchment-based study during the outbreak investigation, information confirming the presence of *C. hominis* in treated water, its continuing presence in the source water and at multiple points in the catchment was provided to the outbreak control team. Deterioration in raw water quality from the original classification of Llyn Cwellyn as A1 (Council Directive 1975) (Mason *et al.* 2010) was also confirmed by the detection of *Cryptosporidium* oocysts in the source water, originating from natural (wildlife) contamination, septic tanks and the wastewater treatment plant. This is not surprising given the basic nature of the wastewater treatment, number and location of septic tanks, wildlife, agricultural and tourist activities around the lake. Critically for the outbreak, *C. hominis* entered Llyn Cwellyn through one or more of the routes identified from wastewater treatment systems in the catchment area. Effluent, even after standard treatment, frequently contains *Cryptosporidium* oocysts (Smith *et al.* 1991; Bukhari *et al.* 1997), and here there were no effective barriers to prevent oocysts reaching the mains water supply. Few studies of comparable wastewater treatment systems have been undertaken in rural areas, and it is difficult to compare

oocyst counts between studies since the sample size, matrix, preparation and detection methods differ. However, the finding of *Cryptosporidium* oocysts is not unexpected since systems are generally not designed for their removal, particularly where only primary and secondary treatment is present (Bowman 2007). Despite being in a rural area, only *C. hominis* was detected in both influent and effluent from the wastewater treatment plant, and in a failing septic tank, suggesting the entry of little animal derived material and that there were people shedding *C. hominis* in the locality. Again, few previous studies have genotyped isolates from effluent but animal-derived isolates appear to have been detected more commonly than human, although this will depend on the inputs into the plant (Bowman 2007). One septic tank in this study was found to contain the cervine genotype, which has been found in numerous animal hosts and very rarely in humans.

Oocyst counts in surface waters were up to 29 per 10 l; counts in studies using similar methods worldwide have been reported up to 176.7 oocysts per 10 l (Carmena *et al.* 2007), although counts in North American watersheds summarised by Clancy & Hargy (2007) were up to 11.7 oocysts per 10 l. Since our catchment was sampled as the suspected source in an outbreak, it is not surprising that our surface water counts were 2.5 fold higher than this. Of more importance than the absolute counts (which will be affected by factors including sampling, sample matrix and recovery rates), are the observed trends in counts. Although oocyst counts in surface water samples declined towards the dam, this was not sufficient to prevent breakthrough at the water treatment plant of *Cryptosporidium*, and *C. hominis* in particular, to the detriment of public health. The retention time and flow patterns in the lake had not been measured but a reduced mixing volume for 'dilution' and the enhanced potential for short-circuiting through surface streaming was evident as would, indeed, be expected in a stratified lake in upland Britain where thermocline depth (commonly 5–10 m) determines the mixing volume of the upper warmer water within the lake's epilimnion (Kay & McDonald 1980). Short circuiting, coupled with the buoyant density of *Cryptosporidium* oocysts which have negligible settlement in water (Badenoch Report 1990; Brookes *et al.* 2004), would effectively reduce the dilution of oocysts in the reservoir. Although oocyst viability was not measured, oocysts from catchment and treated water samples were observed during microscopical detection to be in good condition and, coupled with the low infectious dose demonstrated for *C. hominis* (Chappell *et al.* 2006) presented a significant and demonstrable public health risk.

Despite rare reports of *C. hominis* naturally occurring in animal hosts, this does not appear to be important in transmission to humans (Hunter *et al.* 2004), and in this study *C. hominis* was only found in surface waters under the direct influence of human wastewater. No other cryptosporidia were found in the influent and effluent at the wastewater treatment plant. Although the possibility of the predominance of *C. hominis* might out-compete other species in the PCR, the repetitive approach adopted during this study was designed to address this. This approach is valuable in that it reduces bias in detection and permits the identification of multiple *Cryptosporidium* species/genotypes in a single sample (Xiao *et al.* 2006*a*).

The SSU rRNA is a five copy gene in *Cryptosporidium* and sequence heterogeneity at the polyT region of *C. hominis* isolates found in this study is mainly attributable to the presence of heterogeneous copies (Xiao *et al.* 1999*b*). The significance of this heterogeneity is not yet fully understood. However, the finding of further SNPs in three replicates at two sample sites may indicate multiple sources of *C. hominis*. The gp60 nucleotide sequences showed that only subtype, IbA10G2, was detected in *C. hominis* (A) isolates, including the outbreak cases. Unfortunately, no gp60 amplicons were generated for the few *C. hominis* (B), (C) or (D) isolates. The reduced positivity rate of gp60 PCR compared with the SSU rRNA PCR may be explained by the presence of a single copy of the former gene compared with five copies of the latter.

Other cryptosporidia were detected in samples from sites likely to be influenced by diverse sources, including agricultural and wild animals. The cervine genotype has a wide host range (Feng *et al.* 2007) but the most likely origin in this catchment is sheep (*Ovis aries*), which is by far the most abundant farmed animal species, although wildlife sources cannot be excluded. The cervine genotype has been detected in sheep flocks in the UK (Elwin & Chalmers 2008; Mueller-Doblies *et al.* 2008) and is a frequent finding in surface waters in sheep farming areas in Wales (UK *Cryptosporidium* Reference Unit, unpublished data). It has

also been detected in surface or drinking waters in North America (Jiang *et al.* 2005*a*; Ruecker *et al.* 2007; Jellison *et al.* 2009) and elsewhere in the UK (Nichols *et al.* 2006). Although the cervine genotype has been found in small numbers of patients (Ong *et al.* 2002), including in the UK (Chalmers *et al.* 2009*b*), the significance for public health of its presence in source waters is not known. The sequence heterogeneity observed in the cervine genotypes in this study somewhat reflects the variation previously reported in this genotype (Santin & Fayer 2007; Elwin & Chalmers 2008). However, the biological significance of this variation is thought to be limited (Santin & Fayer 2007).

The rat genotype was found in a sample from a stream, the environs likely being infested with brown rats (*Rattus norvegicus*). This genotype has also been detected in raw wastewater in China (Feng *et al.* 2009). By contrast, the detection of muskrat genotype II is probably indicative of contamination from related wildlife members of the Family Mustelidae, such as badgers, martens and otters, which are indigenous to the British Isles whereas muskrats are not and neither have they been recorded in the wild. Muskrat genotype II has also been found in surface or drinking waters in Canada (Ruecker *et al.* 2007) and Scotland (Nichols *et al.* 2006). These results show that assumptions cannot be made about the source of named genotypes in water samples, and that host specificity or adaptation is not strictly at the host species level, but indicative of host Family or even a wildlife rather than farmed animal source. No host has yet been reported for the W19 genotype, and it is not possible to ascertain the origin of the isolate found in this study. Neither is it possible to identify the source hosts of the isolates in the phylogenetic sequence clade comprising UK E1, 2, 3 and 5. The source of UK E4, which is most closely related to the fox genotype, and UK E6 which is related to *C. canis*, may be wild foxes (*Vulpes vulpes*) or domestic dogs in the catchment. However, further work needs to be done to establish the phylogenetic relationship of these isolates and their host sources in the UK.

Action to control one source of *C. hominis* contamination was taken on 5th December when Gwynedd County Council served a statutory notice on the owner/occupier of one of the properties with a private septic tank. This required remedial works to be carried out to the defective private sewage treatment system. Actions taken by DCWW to reduce the contamination from the wastewater treatment plant included removal of sewage solids from the humus tank and, with the agreement of the OCT, the issuing of a temporary boil water notice and installation of UV treatment at the water treatment works (see Mason *et al.* 2010). However, it is harder to envisage controls over some wildlife sources of *Cryptosporidium*, although pest control and maintenance of sewerage systems are important.

CONCLUSIONS

Identification of *Cryptosporidium* species/genotype in water and environmental samples, including raw and final waters, improves information provided from oocyst numbers alone by indicating potential sources and infectivity for humans.

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REFERENCES

Alves, M., Xiao, L., Sulaiman, I., Lal, A. A., Matos, O. & Antunes, F. 2003 Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. *J. Clin. Microbiol.* **41**(6), 2744–2747.

Anon 1999 *Isolation and identification of Cryptosporidium oocysts and Giardia cysts in waters 1999. Methods for the Examination of Waters and Associated Materials*. Standing Committee of Analysts, Environment Agency.

Anon 2005 *Standard operating protocol for the monitoring of Cryptosporidium oocysts in treated water supplies to satisfy the water supply (water quality) regulations 2001, SI No. 3911 (W.323) Wales. Part 2–Laboratory and Analytical Procedures*. Drinking Water Inspectorate, Department of the Environment, Transport and the Regions, London, UK. Available online: http://www.dwi.gov.uk/regs/crypto/pdf/sop%20part%202.pdf

Badenoch, J. 1990 *Cryptosporidium in water supplies*. Report of the Group of Experts. Department of the Environment, Department of Health. London, UK, HMSO.

Baldwin, D. S., Gigney, H., Wilson, J. S., Watson, G. & Boulding, A. N. 2008 Drivers of water quality in a large water storage reservoir during a period of extreme drawdown. *Water Res.* **42**(19), 4711–4724.

Bowman, D. D. 2007 Waste management. In *Cryptosporidium and Cryptosporidiosis* (ed. R. Fayer & L. Xiao), pp. 371–385. CRC Press, Boca Raton.

Brookes, J. D., Antenucci, J., Hipsey, M., Burch, M. D., Ashbolt, N. J. & Ferguson, C. 2004 Fate and transport of pathogens in lakes and reservoirs. *Environ. Int.* **30**(5), 741–759.

Bukhari, Z., Smith, H. V., Sykes, N., Humphreys, S. W., Paton, C. A., Girdwood, R. W. A. & Fricker, C. R. 1997 Occurrence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in sewage influents and effluents from treatment plants in England. *Water Sci. Technol.* **35**(11–12), 385–390.

Caliskan, A. & Elci, S. 2009 Effects of selective withdrawal on hydrodynamics of a stratified reservoir. *Water Resour. Manage.* **23**(7), 1257–1273.

Cama, V. A., Ross, J. M., Crawford, S., Kawai, V., Chavez-Valdez, R., Vargas, D., Vivar, A., Ticona, E., Navincopa, M., Williamson, J., Ortega, Y., Gilman, R. H., Bern, C. & Xiao, L. 2007 Differences in clinical manifestations among *Cryptosporidium* species and subtypes in HIV-infected persons. *J. Infect. Dis.* **196**(5), 684–691.

Carmena, D., Aguinagalde, X., Zigorraga, C., Fernandez-Crespo, J. C. & Ocio, J. A. 2007 Presence of *Giardia* cysts and *Cryptosporidium* oocysts in drinking water supplies in northern Spain. *J. Appl. Microbiol.* **102**(3), 619–629.

Chalmers, R. M. & Pollock, K. G. J. 2008 *Cryptosporidium* in Scotland 2007: reference laboratory data. *HPS Week. Rep.* **42**(45), 385–387.

Chalmers, R. M., Robinson, G., Elwin, K., Hadfield, S. J., Xiao, L., Ryan, U., Modha, D. & Mallaghan, C. 2009*a* *Cryptosporidium* rabbit genotype, a newly identified human pathogen. *Emerg. Infect. Dis.* **15**(5), 829–830.

Chalmers, R. M., Elwin, K., Thomas, A. L., Guy, E. C. & Mason, B. 2009*b* Long-term *Cryptosporidium* typing reveals the aetiology and species-specific epidemiology of human *cryptosporidiosis* in England and Wales, 2000 to 2003. *Euro Surveill.* **14**(2), pii = 19086. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId = 19086

Chappell, C. L., Okhuysen, P. C., Langer-Curry, R., Widmer, G., Akiyoshi, D. E., Tanriverdi, S. & Tzipori, S. 2006 *Cryptosporidium hominis*: experimental challenge of healthy adults. *Am. J. Trop. Med. Hyg.* **75**(5), 851–857.

Clancy, J. L. & Hargy, T. M. 2007 Waterborne: drinking water. In *Cryptosporidium and Cryptosporidiosis* (ed. R. Fayer & L. Xiao), pp. 305–333. CRC Press, Boca Raton.

De Cesare, G., Boillat, J. L. & Schleiss, A. J. 2006 Circulation in stratified lakes due to flood-induced turbidity currents. *J. Environ. Eng.–ASCE* **132**(11), 1508–1517.

Elci, S. 2008 Effects of thermal stratification and mixing on reservoir water quality. *Limnology* **9**(2), 135–142.

Elwin, K. & Chalmers, R. M. 2008 Contemporary identification of previously reported novel *Cryptosporidium* isolates reveals *Cryptosporidium bovis* and the cervine genotype in sheep (*Ovis aries*). *Parasitol. Res.* **102**(5), 1103–1105.

Feng, Y., Alderisio, K. A., Yang, W., Blancero, L. A., Kuhne, W. G., Nadareski, C. A., Reid, M. & Xiao, L. 2007 *Cryptosporidium* Genotypes in wildlife from a New York watershed. *Appl. Environ. Microbiol.* **73**(20), 6475–6483.

Feng, Y., Li, N., Duan, L. & Xiao, L. 2009 *Cryptosporidium* genotype and subtype distribution in raw wastewater in Shanghai, China: evidence for possible unique *Cryptosporidium hominis* transmission. *J. Clin. Microbiol.* **47**(1), 153–157.

George, G., Hurley, M. & Hewitt, D. 2007 The impact of climate change on the physical characteristics of the larger lakes in the english lake district. *Freshw. Biol.* **52**(9), 1647–1666.

Giles, M., Chalmers, R., Pritchard, G., Elwin, K., Mueller-Doblies, D. & Clifton-Hadley, F. 2009 *Cryptosporidium hominis* in a goat and sheep in the UK. *Vet. Rec.* **164**(1), 24–25.

Glaberman, S., Moore, J. E., Lowery, C. J., Chalmers, R. M., Sulaiman, I., Elwin, K., Rooney, P. J., Millar, B. C., Dooley, J. S. G., Lal, A. A. & Xiao, L. 2002 Three drinking-water-associated *cryptosporidiosis* outbreaks, Northern Ireland. *Emerg. Infect. Dis.* **8**(6), 631–633.

Hunter, P. R., Hughes, L. S., Woodhouse, S., Syed, Q., Verlander, N., Chalmers, R. M., Morgan, K., Nichols, G., Beeching, N. & Osborn, K. 2004 Case-control study of sporadic *cryptosporidiosis* with genotyping. *Emerg. Infect. Dis.* **10**(7), 1241–1249.

Hunter, P. R., Hadfield, S. J., Wilkinson, D., Lake, I. R., Harrison, F. C. D. & Chalmers, R. M. 2007 Subtypes of *Cryptosporidium parvum* in humans and disease risk. *Emerg. Infect. Dis.* **13**(1), 82–88.

Jellison, K. L., Hemond, H. F. & Schauer, D. B. 2002 Sources and species of *Cryptosporidium* oocysts in the Wachusett Reservoir watershed. *Appl. Environ. Microbiol.* **68**(2), 569–575.

Jellison, K. L., Lynch, A. E. & Ziemann, J. M. 2009 Source tracking identifies deer and geese as vectors of human-infectious *Cryptosporidium* genotypes in an urban/suburban watershed. *Environ. Sci. Technol.* **43**(12), 4267–4272.

Jiang, J., Alderisio, K. A. & Xiao, L. 2005*a* Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. *Appl. Environ. Microbiol.* **71**(8), 4446–4454.

Jiang, J., Alderisio, K. A., Singh, A. & Xiao, L. 2005*b* Development of procedures for direct extraction of *Cryptosporidium* DNA from water concentrates and for relief of PCR inhibitors. *Appl. Environ. Microbiol.* **71**(3), 1135–1141.

Karanis, P., Kourenti, C. & Smith, H. 2007 Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. *J. Water Health* **5**(1), 1–38.

Kay, D. & McDonald, A. T. 1980 Reduction of coliform bacteria in two upland reservoirs: the significance of distance decay relationships. *Water Res.* **14**, 305–318.

Mallon, M. E., MacLeod, A., Wastling, J. M., Smith, H. & Tait, A. 2003 Multilocus genotyping of *Cryptosporidium parvum* type 2: population genetics and sub-structuring. *Infect. Genet. Evol.* **3**(3), 207–218.

Mason, B. W., Chalmers, R. M., Carnicer-Pont, D. & Casemore, D. P. 2010 A *Cryptosporidium hominis* outbreak in North-West Wales, UK, associated with low oocyst counts in treated drinking water. *J. Water Health* **8**(2), 299–310.

Meinhardt, P. L., Casemore, D. P. & Miller, K. B. 1996 Epidemiologic aspects of human *cryptosporidiosis* and the role of waterborne transmission. *Epidemiol. Rev.* **18**(2), 118–136.

Morgan, U. M., Xiao, L., Hill, B. D., O'Donoghue, P., Limor, J., Lal, A. & Thompson, R. C. 2000 Detection of the *Cryptosporidium parvum* 'human' genotype in a dugong (*Dugong dugon*). *Parasitol.* **86**(6), 1352–1354.

Morrison, L. J., Mallon, M. E., Smith, H. V., Macleod, A., Xiao, L. & Tait, A. 2007 The population structure of the *Cryptosporidium parvum* population in Scotland: a complex picture. *Infect. Genet. Evol.* **8**(2), 121–129.

Mueller-Doblies, D., Giles, M., Elwin, K., Smith, R. P., Clifton-Hadley, F. A. & Chalmers, R. M. 2008 Distribution of *Cryptosporidium* species in sheep in the UK. *Vet. Parasitol.* **154**(3–4), 214–219.

Nichols, R. A., Campbell, B. M. & Smith, H. V. 2006 Molecular fingerprinting of *Cryptosporidium* oocysts isolated during water monitoring. *Appl. Environ. Microbiol.* **72**(8), 5428–5435.

Ong, C. S. L., Eisler, D. L., Alikhani, A., Fung, V. W. K., Tomblin, J., Bowie, W. R. & Issac-Renton, J. L. 2002 Novel *Cryptosporidium* genotypes in sporadic *cryptosporidiosis* cases: first report of human infections with a cervine genotype. *Emerg. Infect. Dis.* **8**(3), 263–268.

Robinson, G. 2005 *Investigating the Public Health Significance of Cryptosporidium in the Environment*. PhD Thesis, Cardiff University, UK.

Ruecker, N. J., Bounsombath, N., Wallis, P., Ong, C. S., Isaac-Renton, J. L. & Neumann, N. F. 2005 Molecular forensic profiling of *Cryptosporidium* species and genotypes in raw water. *Appl. Environ. Microbiol.* **71**(12), 8991–8994.

Ruecker, N. J., Braithwaite, S. L., Topp, E., Edge, T., Lapen, D. R., Wilkes, G., Robertson, W., Medeiros, D., Sensen, C. W. & Neumann, N. F. 2007 Tracking host sources of *Cryptosporidium* spp. in raw water for improved health risk assessment. *Appl. Environ. Microbiol.* **73**(12), 3945–3957.

Ryan, U. M., Bath, C., Robertson, I., Read, C., Elliot, A., McInnes, L., Traub, R. & Besier, B. 2005 Sheep may not be an important zoonotic reservoir for *Cryptosporidium* and *Giardia* parasites. *Appl. Environ. Microbiol.* **71**(9), 4992–4997.

Santin, M. & Fayer, R. 2007 Intragenotypic variations in the *Cryptosporidium* sp. cervine genotype from sheep with implications for public health. *J. Parasitol.* **93**(3), 668–672.

Smith, H. V., Grimason, A. M., Benton, C. & Parker, J. F. W. 1991 The occurrence of *Cryptosporidium* spp. oocysts in Scottish waters, and the development of a fluorogenic viability assay for individual *Cryptosporidium* spp. oocysts. *Water Sci. Technol.* **24**(2), 169–172.

Smith, H. V., Nichols, R. A., Mallon, M., Macleod, A., Tait, A., Reilly, W. J., Browning, L. M., Gray, D., Reid, S. W. & Wastling, J. M. 2005 Natural *Cryptosporidium hominis* infections in Scottish cattle. *Vet. Rec.* **156**(22), 710–711.

Smith, A., Reacher, M., Smerdon, W., Adak, G. K., Nichols, G. & Chalmers, R. M. 2006 Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992–2003. *Epidemiol. Infect.* **134**(6), 1141–1149.

Smith, R. P., Chalmers, R. M., Elwin, K., Clifton-Hadley, F. A., Mueller-Doblies, D., Watkins, J. & Giles, M. 2009 Investigation of the role of companion animals in the zoonotic transmission of *cryptosporidiosis*. *Zoon. Public Health* **56**(1), 24–33.

Spano, F., Putignani, L., McLauchlin, J., Casemore, D. P. & Crisanti, A. 1997 PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. *FEMS Microbiol. Lett.* **150**(2), 209–217.

Tillett, H. E., DeLouvois, J. & Wall, P. G. 1998 Surveillance of outbreaks of waterborne: categorizing levels of evidence. *Epidemiol. Infect.* **120**(1), 37–42.

Wilhelm, S. & Adrian, R. 2008 Impact of summer warming on the thermal characteristics of a polymictic lake and consequences for oxygen, nutrients and phytoplankton. *Freshw. Biol.* **53**(2), 226–237.

Xiao, L., Morgan, U. M., Limor, J., Escalante, A., Arrowood, M., Shulan, W., Thompson, R. C. A., Fayer, R. & Lal, A. A. 1999*a* Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. *Appl. Environ. Microbiol.* **65**(8), 3386–3391.

Xiao, L., Limor, J. R., Li, L. L., Morgan, U., Thompson, R. C. A. & Lal, A. A. 1999*b* Presence of heterogeneous copies of the small subunit rRNA gene in *Cryptosporidium parvum* human and marsupial genotypes and *Cryptosporidium felis*. *J. Eukaryot. Microbiol.* **46**(5), 44S–45S.

Xiao, L., Alderisio, K., Limor, J., Royer, M. & Lal, A. A. 2000 Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. *Appl. Environ. Microbiol.* **66**(12), 5492–5498.

Xiao, L., Alderisio, K. & Jiang, J. 2006 Detection of *Cryptosporidium* oocysts in water: effect of the number of samples and analytical replicates on test results. *Appl. Environ. Microbiol.* **72**(9), 5942–5947.

Xiao, L., Alderisio, K. & Singh, A. 2006 *Development and Standardisation of a Cryptosporidium Genotyping Tool for Water Samples*. American Water Works Association Research Foundation, Denver.

Zhou, L., Kassa, H., Tischler, M. L. & Xiao, L. 2004 Host-adapted *Cryptosporidium* spp. in Canada geese (*Branta canadensis*). *Appl. Environ. Microbiol.* **70**(7), 4211–4215.

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