Supporting Information

Table S1 Characteristics and physicochemical parameters of four WWTPs

Plant	Capacity (m^3/d)	HRT (h)	Effluent water quality characteristics (mg/L)				
			BOD	COD	Total N	Ammonia	TSS
A	2×10^4	12	9	35	15.7	1.1	25
B	2×10^4	16	7	24	3.39	1.32	10
C	15×10^4	16	10	42	10.2	1.28	10
D	39×10^4	15	4	24	4.39	0.69	10

Notes: HRT denotes hydraulic retention time. TSS denotes total suspended solid. The four WWTPs collected sewage from municipal pipe network and served approximately 3.60 million inhabitants. All plants mainly discharged the final effluents into the Yitong River through the city from south to north, sometimes reclaimed water from these plants was also used to irrigate public parks, for street sweeping, or agricultural irrigation

Table S2 Number of water samples where *Cryptosporidium* were detected (total number of samples was 70). Major and minor known hosts for species and genotypes of *Cryptosporidium* detected and potential human health concern

Species	Detections number	Major known hosts	Minor known hosts	Potential human health concern*
C. andersoni	59	Cattle	Humans, Bactrian camels, sleep	Nil
C. bovis	45	Cattle	Sleep, humans	Low
C. ryanae	41	Cattle		Nil
C. meleagridis	14	Avians	Cattle	Nil
C. balleyi	14	Chickens	Turkey, cockatiels, quails, ducks	Nil
C. cuniculus	9	Rabbits	Humans	Medium
C. felis	9	Cats	Humans, cattle	Low
C. canis	9	Dogs	Humans	Medium
C. muris	9	Rodents	Humans, bactrian, camels	Medium
C. parvum	5	Humans,cattle	Pigs, goats	High

Notes: * High rating-common association; medium rating-sporadic association; low rating-individual reports; nil rating-no association reported, summarized from Ryan and Power (2012), Swaffer et al. (2014)

Table S3 Zeta potential of *Cryptosporidium* oocysts under different hydro-chemical conditions

Species	pH	IS (mM)	Solution	Zeta potential (mV)	References
C. parvum	5.7	0.1	NaCl	–13.27	(Abudalo et al., 2010)
C. parvum	6.5	3.0	NaCl	–20.64	(Searcy et al., 2005)
	7.0	3.0	NaCl	–19.13	
	8.0	3.0	NaCl	–30.70	
	7.5	3.0	$CaCl_2$	–7.50	
	8.5	3.0	$CaCl_2$	–11.00	
C. parvum	4.0	1.0	NaCl	–25.03	(Bustamante et al., 2001)
	5.7	1.0	NaCl	–27.01	

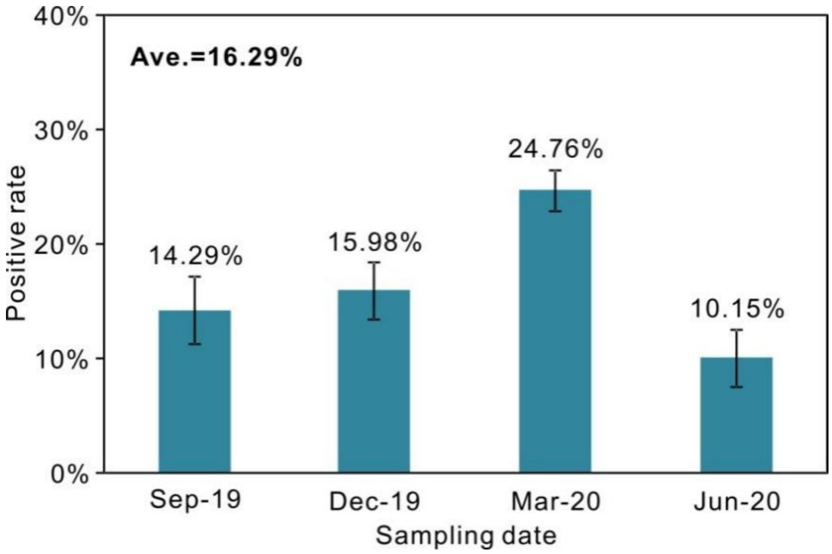

Fig. S1 Positive rates of *Cryptosporidium* infection in calves from seven livestock farms in Changchun city (China) during 2019–2020. The seven livestock farms are Yangjia livestock farm, Guangze livestock farm, Weilong livestock farm, Jiyuan livestock farm, Haotian livestock farm, Haoyue livestock farm, and Luxi livestock farm. The total test number of calves was n=230 at Sep-19, n=220 at Dec-19, n=210 at Mar-20, n=220 at Jun-20. The positive rates were determined by analyzing the faeces of tested calves using the standard method put forward in “Nested PCR method for detection of *Cryptosporidium* spp.” (GB/T 35942-2018, China). These data were collected from these seven livestock farms. Herein, the average positive rate (16.29%) was closed to the levels in previous investigation in China (Gong et al., 2017; Cai et al., 2019)

Text S1 Method for detecting *Cryptosporidium* oocysts in water samples and the recovery assays

The method coupling membrane filtration, sucrose gradient purification, and immunofluorescence assay was used in this study to determine the prevalence and concentration of *Cryptosporidium* oocysts in target water samples.

The membrane filtration method using mixed cellulose est*ers with p*orosity of 0.45 μm (42 mm diameter, Millipore, USA) was used for concentrating as described in previous studied (Haramoto et al., 2012). The matter on membrane was dissolved in 40 mL of acetone and the solution and centrifuged for 10 min at 1000×g. Then the centrifuged precipitate was dissolved in 40 mL of PBS (pH 7.0) and centrifuged once again. The pellet was resuspended in 5 mL distilled water and washed three times and layered on specific sucrose gradient (density ρ=1.110 g/cm^3) for purification and concentration (Arrowood and Sterling, 1987; Falohun et al., 2021). The oocyst-containing fraction was diluted to 50 mL of PBS (pH 7.0) and centrifuged at 1500 g for 10 min. The centrifuged precipitate was resuspended in 5 mL PBS and stored at −20℃ for further use. Each time 1 mL aliquots were centrifuged again (1500 g, 10 min) and resuspended in 100 μL of PBS, then 100 μL of targeted suspension were placed on a glass slide for microscopy. Primary identification of the (oo)cysts in targeted samples was achieved by modified acid-fast staining (NY/T 1949-2010, China), then further confirmed by immunofluorescence assay to method 1623 (USEPA, 2005) using a commercially-available fluorescein isothiocyanate (FITC)-conjugated antibody kit (A100FLK, Waterborne, USA) (Jenkins et al., 2010). The positive samples confirmed with FITC were stained by 4’, 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) according to a method previously described (Sammarro Silva and Sabogal-Paz, 2021), and then the number of live *Cryptosporidium* oocysts (DAPI+/PI- and DAPI-/PI-) counted by fluorescence microscopy (BX63, Olympus, Japan). The concentration of (oo)cysts per litre in target sample was calculated according to the numbers of the live oocysts.

To examine the reliability and feasibility of this method, the recovery efficiency of the method was determined by seeding 10 L background solution with different turbidity values (0 and 10 NTU) with a known number of oocysts. A total of 200 L of the background solution was obtained by filtering the effluent from WWTP A by 0.45 μm membrane, and this helped to approach the hydro-chemical condition of real water samples. The desired final concentrations of test samples were 0 (blank group, only pure water), 1×10^1, 2.5×10^1, 5.0×10^1, 1.0×10^2, 2.5×10^2, 5.0×10^2, 1.0×10^3 oocysts/L. The concentration of each sample was determined using the method described aforementioned. This procedure was repeated three times for each sample. The mean percentage recovery efficiencies were 26.67%–38.67% at 0 NTU and 20.00%–32.73% at 10 NTU, which meets the acceptance criteria described by method 1623 (USEPA, 2005).

Text S2 Nested-PCR approaches for detecting *Cryptosporidium* in water treatment plants

To evaluate whether nested-PCR could detect *Cryptosporidium* in water, target water samples (1 L) were spiked with final concentration of 0.1, 1, 10 or 100 oocysts/mL in triplicate. A negative control (without spiked oocysts) was included in the assays. These samples were centrifuged at 1500 g for 10 min. The pellets were accumulated in a beaker (1.5 mL) and resuspended in 50 µL distilled water. The samples were subjected to 5 freeze/thaw cycles (6 min in dry ice and 3 min at 55℃) for extracting DNA according to the kit instructions (Fast DNA® SPIN for soil Kit, Thermo, USA). Two PCR reactions were tested using two sets of primers (Table S4). These primers were selected herein based upon Xiao et al.'s works (Xiao et al., 2000).

Table S4 Primers used in Nested-PCR analysis

Step	Primer name	Sequence (5′-3′)
First	forward	TAG AGA TTG GGG TTG TTC CT
	reverse	CTC CAC CAA CTA AGA ACG GCC
Second	forward	AAG GAG TAA GGA ACA ACC TCC A
	reverse	GGA AGG TTG TAT TTA TTA GAT AAA G

Both primers amplify the hyper-variable region of gene encoding the 18 SSU rDNA of the genus *Cryptosporidium*. All reactions were conducted in a final volume of 25 µL, containing 200 µM of each dNTP, 1 µM of each primer and 5 µL of DNA template. The other compounds and conditions of the reaction were described in the Table S5. The products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. A remarkable sensitivity was obtained as the *Cryptosporidium* oocysts in samples spiked with 0.1 to 100 oocysts/mL could be detected with the nested-PCR (Fig. S2).

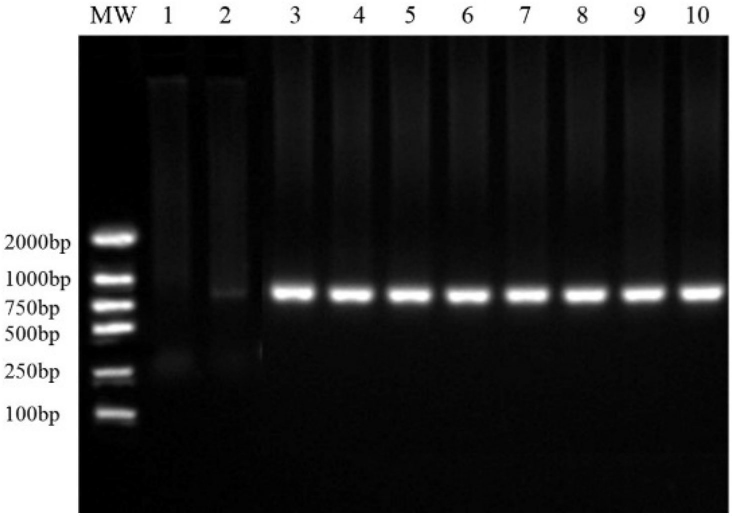

Fig. S2 Agarose gel electrophoresis (2%) showing genetic fragment amplification products from Nested-PCR with different concentrations of Cryptosporidium parvum oocysts. MW: standard molecular weight; 1. negative control; 2. PBS; 3. 1 oocyst; 4. 2 oocysts; 5. 5 oocysts; 6. 10 oocysts; 7. 50 oocysts; 8. 100 oocysts; 9. 10^3 oocysts; 10. 10^4 oocysts.

Table S5 Nested-PCR cycling conditions for each gene

Set	Cycling step	Temperature (℃)	Time	Ramp rate	Number of cycles
First	Enzyme activation	94	3 min	2°C/s	1
	Denaturation	94	40 s		35
	Annealing	55	1 min		35
	Extension	72	1 min		35
	Signal stabilization	72	6 min		1
	Hold (optional)	4	Infinite		1
Second	Enzyme activation	94	3 min	2°C/s	1
	Denaturation	94	45 s		35
	Annealing	45	50 s		35
	Extension	72	1 min		35
	Signal stabilization	72	7 min		1
	Hold (optional)	4	Infinite		1

Text S3 Detection gp60, COWP2, and HSP70 gene of *Cryptosporidium parvum* by a sensitive droplet digital PCR (ddPCR) method

In all PCR methods, droplet digital polymerase chain reaction (ddPCR) has been shown to generate accurate results in low copy number quantification (Tang et al., 2016) and is less susceptible to PCR inhibitors (Zhang et al., 2019). In this study, in order to detect target gene (gp60, HSP70 and COWP2) in different samples with a high level of sensitivity, we used a ddPCR method for the detection and absolute quantification of target gene.

The total DNA was extracted from the same number of oocysts (1×10^3) according to consistent procedures and performed 2 μL in a 20 μL reaction volume accordance with the manufacturer’s instructions (Bio-Rad, USA). The primers and probe were synthesized according to previous publications (Alves et al., 2003; Lee et al., 2008, Liang and Keeley, 2011) by Kumei Biological Technology (Changchun, China). The probe was labeled with Fluorescein (FAM) as the fluorescent reporter and Eclipse (a minor groove binder-MGB) as the fluorescence quencher (Table S6). And the concentration of probe was selected the optimal concentration of qPCR (500 nmol) and the reactions included 10 μL of 2×ddPCR™ Supermix for Probes (Bio-Rad, USA), 1 μL of probe, 7 μL of DNase/RNase-free H_2O and 1 μL of DNA template. Each sample was loaded into a well of a disposable DG8™ cartridge (Bio-Rad, USA) and 70 μL of Droplet Generation Oil (Bio-Rad, USA) were placed into each of the adjacent oil wells in the cartridge (Bio-Rad, USA). Droplets were produced in each well using a QX200™ droplet generator (Bio-Rad, USA). The droplets were then transferred to a 96-well PCR plate (MLL9601, Bio-Rad, USA). To differentiate the amplitude between the negative and positive droplets and to reduce the background of the negative droplets, we performed a temperature gradient in the annealing step. The PCR amplifications were performed with an initial step of 95℃ for 10 min, followed by 40 cycles of 94℃ for 30 s, 55℃–60℃ for 60 s and 1 cycle of 98℃ for 5 min, with a final hold at 4℃. To achieve the best results for the method, a range of annealing temperatures (55℃ to 66℃) was tested. After PCR amplification of the DNA target in the droplets, the microdroplets from each well were read individually using a QX200 Droplet Reader (Bio-Rad, CA, USA). A threshold was set between the positive and negative microdroplet clusters and the copy number of each well was evaluated automatically by QuantaSoft™ version 1.7 (Bio-Rad, CA, USA). Next, we identified the optimal annealing temperature for the ddPCR method by testing temperatures of 65℃, 64℃, 63℃, 62℃, 61℃, 60℃, 59℃, 58℃, 57℃, 56℃ and 55℃. As shown in Table S7, as the annealing temperature rose, the distinction between the intensity signals of the positive (blue) and negative (gray) droplets became smaller. An annealing temperature of 60℃ for gp60, 58℃ for COWP2, 56℃ for Hsp70 was chosen as the optimal annealing temperature for the ddPCR method. All statistical analyses and data plotting were performed using GraphPad Prism software (Version 5.0, La Jolla, USA). Kappa statistics were used to compare the detection results from the ddPCR.

In this study, we used aforementioned ddPCR method for quantitative and automatic analysis of the HSP70, GP60 and COWP2 in *C. parvum* oocysts incubated at days 0, 1, 3, 5, and 7 at different environmental temperature (0℃, 5℃, 10℃, 15℃, 20℃ and 25℃). We have described these scenarios in section 2.5 in the main text.

ddPCR reaction for each sample was run in triplicate for each gene, HSP70, GP60 and COWP2, and each run was repeatedly tested three times to get the average. For each gene, we finally obtained five groups of data (five sampling time), and each group contained six data (at six temperature). The relative standard deviations of the mean gene copies for all groups were in the range of 0.45%–3.44%. This level of uncertainty is much lower than values typically observed for quantification of specific DNA target sequences using currently commercially available real-time PCR technology (Pinheiro et al., 2012). Thus the ddPCR in this study allowed very precise copy number test and the data we obtained were valid.

Moreover, in the one dimensional plots of ddPCR assay (Figs. 8(a), 8(c), and 8(e)), more than 10000 effective droplets were generated in each ddPCR reaction to ensure accurate quantification, and showed clear separation with an approximately 12–20 times increase in fluorescence of positive compared to negative drops. These also indicated the reliability of ddPCR method in this study (Nshimyimana et al., 2019).

Table S6 Primers used in ddPCR analysis

Gene	Primer name	Sequence (5′-3′)
gp60	forward	GATGTTCCTGTTGAGGGCTC
	reverse	TTACAACACGAATAAGGCTGC
COWP2	forward	TTCAATTTGTCAGGACAACA
	reverse	GGTTGAGTTGGAGCAGAAC
HSP70	forward	CGTAATACAACTATCCCAGCAA
	reverse	CAATATCAAAGGTGACTTCAATTT

Table S7 ddPCR cycling conditions for each gene

Gene	Cycling step	Temperature (℃)	Time	Ramp rate	Number of cycles
gp60	Enzyme activation	95	5 min	2°C/s	1
	Denaturation	95	30 s		35
	Annealing/extension	60	2 min		35
	Signal stablization	98	5 min		1
	Hold (optional)	4	Infinite		1
COWP2	Enzyme activation	95	5 min	2°C/s	1
	Denaturation	95	30 s		35
	Annealing/extension	58	1 min		35
	Signal stablization	90	5 min		1
	Hold (optional)	4	Infinite		1
HSP70	Enzyme activation	95	5 min	2°C/s	1
	Denaturation	95	30 s		35
	Annealing/extension	56	1 min		35
	Signal stablization	95	5 min		1
	Hold (optional)	4	Infinite		1

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