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▣ ORIGINAL ARTICLE

Occurrence of *Cryptosporidium* and *Giardia* and the Relationship between Protozoa and Water Quality Indicators in Swimming Pools

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Abstract: A total of 60 samples were collected from 35 swimming pools in Beijing, China, and the presence of *Cryptosporidium* and *Giardia* were investigated. The results showed that 16.7% and 15.0% of samples were positive for *Cryptosporidium* oocyst and *Giardia* cysts, respectively, with a mean concentration of 0.30 oocysts/10 L and 0.27 cysts/10 L. The oocysts and cysts were found to have higher rates of occurrence in August than in May. Genotyping confirmed the presence of *Cryptosporidium hominis*, *C. parvum*, and *Giardia* assemblages A and B, all of which were associated with human infections. The predominant species/assemblages were *C. hominis* and *Giardia* assemblage A. Analyses of the relationships between parasite oocysts/cysts, indicator bacteria, and physical-chemical parameters revealed that there was no correlation between 2 parasites and fecal bacterial indicators, whilst there was a significant correlation between protozoa and urea concentration, which indicates that urea concentration rather than fecal bacterial indicators might be an appropriate index for chlorine-resistant protozoa in swimming pools. This study provides useful information to improve the safety of swimming pool water and deduce the risk of protozoan infections.

Key words: *Cryptosporidium*, *Giardia*, bacterial indicator, swimming pool, water quality

INTRODUCTION

Swimming is one of the popular recreational activities worldwide. It offers health and social benefits, and it is suitable for a wide age range of people from children to aged persons. However, health risks for swimmers may arise from exposure to pool water of poor quality [1]. They may suffer from various diseases, such as gastroenteritis caused by bacteria, viruses, or parasites of fecal origin, which may be released by the bathers or, in the case of outdoor pools, by animals, such as birds and rodents [2].

Many studies were carried out to investigate the presence of *Cryptosporidium* and *Giardia* in swimming pool water. It was found that 8.1% of swimming pools in Georgia, USA and 11.8% in the Netherlands were contaminated with *Cryptosporidium* and/or *Giardia* [3,4]. In China, *Cryptosporidium* and *Giardia* were detected in surface water which was used for drinking water production [5-7], but there was no data available for recreational water.

Several indicators were used to assess the microbiological quality of swimming pool water. Some consider that bacteria from fecal contamination affect the microbial quality of pool waters [8], while others emphasize that microorganisms derived from vomit, mucus, saliva, and skin of bathers rather than fecal contamination contributed to the risk of infection [9,10]. Nevertheless, heterotrophic plate count bacteria (HPC), total coliforms (TC), and fecal coliforms were still regarded as the best microorganisms to indicate hygienic conditions [1,11,12]. In China, HPC and TC are applied as the microbial indicators in the Hygienic Standard for Swimming Place [13] and physical-chemical properties, such as urea, free available chlorine, pH, and turbidity are also used in this standard as indicators to predict the quality of the pool water.

Previous studies found that fecal bacterial indicators could well indicate contamination of *Cryptosporidium* and *Giardia* in surface water [14-16]. However, there was no data available that bacterial and physical-chemical indicators can be used to indicate contamination of the protozoa in artificially controlled

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water, such as swimming pool waters.

In the present study, the presence and genotype of *Cryptosporidium* and *Giardia* in public swimming pools was investigated. The correlations between parasitic pathogens and basic quality of the water such as bacterial indicators, physical-chemical properties were also assessed.

MATERIALS AND METHODS

Water sampling

A total of 60 water samples from 35 swimming pools in Beijing, China were collected. The swimming pools were selected at random, and the type of pool was classified based on its location. In May 2015, 27 samples were collected from 27 pools. In August, 25 pools were re-sampled and another 8 pools were sampled, amounting to 33 samples. All of the sampling was conducted in the evening when the swimmer numbers usually reached the highest point of the day. The sampling point was at the central area of each pool, in order to avoid the hydraulically stagnant zone.

Detection of parasitic pathogens

Cryptosporidium oocyst and *Giardia* cyst concentrations were measured following the previously described method, including filtration, flotation, labeling with monoclonal antibody, and microscopic analysis [17]. Briefly, 10-liter water samples were filtered through membrane filters of 142 mm diameter with a 1.0 µm pore size. After filtration, the membrane filter was dissolved in acetone solutions to recover oocysts/cysts. Then, the recovered oocysts/cysts were separated from other particulate materials by flotation on Percoll-sucrose gradients. Finally, the pellets from the purification were stained with a combined fluorescein isothiocyanate (FITC) conjugated anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies (Waterborne Inc., New Orleans, Louisiana, USA) and examined microscopically for the detection of *Cryptosporidium* oocysts and *Giardia* cysts. This method permitted a mean recovery of 41.3% for initial recovery tests, which meets the acceptance criteria of the US Environmental Protection Agency (USEPA) Method 1623 (24-100% recovery) [17].

To determine the species/genotypes of the protozoa, another 10-liter samples from each pool, in the second campaign, were collected and concentrated. Genomic DNA was extracted from each Percoll-sucrose flotation-purified pellet by using a FastDNA SPIN kit for soil (MP Biomedicals, Illkirch, France), according to the manufacturer's instructions, and eluted in 50 µl of reagent-grade water as described previously [17]. *Cryptosporidium* was genotyped by a nested PCR amplification of a 435-bp fragment of the small subunit (SSU) rRNA locus, and *Giardia* assemblages were identified by using another nested PCR to amplify of a 292-bp fragment of *Giardia* SSU rRNA gene. Primers and amplification conditions were employed as described by Plutzer et al. [18]. All positive secondary PCR products were purified and cloned. Clones were sent to Beijing Augct Co., Ltd. for sequencing using ABI 3730 automated DNA sequencer (BigDye Terminator Chemistry, Applied Biosystems, Foster City, California, USA). Nucleotide sequences obtained in the study, with reference sequences downloaded from the GenBank database, were aligned using the Clustal W programs and analyzed to determine *Cryptosporidium* species and *Giardia* assemblages using phylogenetic trees.

Analyses of bacterial indicator and physical-chemical quality

Colony counts and 5-tube most probable numbers (MPN) procedure was used, respectively, to enumerate HPC and TC according to Chinese standard examination method for drinking water-microbiological parameters [19]. Briefly, a 10-fold serial dilution of each sample was carried out. For HPC, 1.0 ml each of serial dilutions were inoculated in sterile nutrient agar plates and incubated at 36°C for 48 hr, counting colonies as they developed. While for TC, another 1.0 ml each of serial dilutions were transferred to 5 tubes of lactose peptone broth (10.0 ml) with inverted Durham tubes, which were then incubated at 37°C for 24±2 hr. All positive presumptive tubes that demonstrated an acidic reaction or gas production were submitted to the confirmed phase with total coliform test by using eosin methylene blue agar medium according to the above standard [19]. The physical-chemical quality of the water in terms of turbidity, pH, urea, and free residual chlorine was on site measured for each sample with portable photometer.

Statistical analysis

Data were tabulated and compared with local guidelines [13]. The chi-square test was used to evaluate possible significant differences in the seasonal pattern of the prevalence of *Cryptosporidium* and *Giardia*. Whereas the concentrations of the parasites in different time points were compared using paired-samples t-test, the association between parasite concentrations, the concentration of microbiological indicators, and physical-

chemical properties was correlated using the nonparametric Spearman's correlation 2-tailed test. Differences with *P*-values of <0.05 were defined as being statistically significant. All statistical tests were performed using PASW Statistics 18 computer software package.

RESULTS

Occurrence and genotyping of *Cryptosporidium* oocyst and *Giardia* cyst in water samples

Of the 60 swimming pool water samples collected, 10 (16.7%) were positive for *Cryptosporidium* and 9 (15.0%) were positive for *Giardia*. The mean concentration of *Cryptosporidium* and *Giardia* were 0.30 oocysts/10 L and 0.27 cysts/10 L, respectively (Table 1). Although the detection percentages of the cysts of *Giardia* changed little (14.8-15.2%) for the 2 sampling campaigns (chi-square, $P>0.05$), the positive rate of *Cryptosporidium* oocyst was higher in August (24.2%) than in May (7.4%).

It was revealed that 2/5 of outdoor pools, 3/6 of school pools, 3/5 of community pools, 2/7 of hotel pools, and 3/6 of commercial pools were positive for *Cryptosporidium*, *Giardia*, or both (data not shown). However, no oocysts or cysts were detected in any of the sampled waters from 6 bath pools. The counts of parasites ranged from 0 to 4 oocysts and 0 to 3 cysts per 10 L (Table 1). A higher detection percentage of both parasites were found in samples from outdoor swimming pools than that from indoor pools. Nevertheless, the concentration of oocysts or cysts in the samples was at the same level.

DNA sequencing of PCR products revealed the presence of the following 2 species of *Cryptosporidium* and 2 *Giardia* assemblages; *C. hominis*, *C. parvum*, and *Giardia* assemblage A and B. The most common *Cryptosporidium* species and *Giardia* assemblage were *C. hominis* and *Giardia* assemblage A, which were found in 5 and 3 positive samples, respectively (Table 2).

Fecal bacterial indicator and physical-chemical analyses

The results in Table 3 show the average values, median values, and ranges of the fecal bacterial indicator and physical-chemical parameters of swimming pool water. HPC and TC were found positive in 30 and 22 out of 60 samples, respectively. However, only in 1 commercial pool and 1 hotel pool, the values of bacterial indicator violated the guideline limits (HPC, >1,000 CFU/ml or TC, >18 MPN/L) [13].

As for urea, 22/60 of samples have a value over 3.5 mg/L. It was obvious that all the surveyed turbidity values (0.1-0.8 NTU) in swimming pools were consistent with the standard (not more than 5 NTU) [13]. Similarly, no pH value was out of the

Table 2. Distribution of *Cryptosporidium* species and *Giardia* assemblage in water samples collected from swimming pools in August 2015

Sample location	Detection rate	Type of parasites (no. of samples)
Outdoor pool	2/5	*C. parvum* (1); *Giardia* assemblage A (1)
School pool	3/6	*C. hominis* and *Giardia* assemblage A (1); *C. hominis* (2)
Community pool	2/4	*C. hominis* (1); *Giardia* assemblage B (1)
Hotel pool	1/6	*C. hominis* (1)
Bath pool	0/6	
Commercial pool	2/6	*C. hominis* (1); *Giardia* assemblage A (1)
Total	10/33	*C. parvum* (1); *C. hominis* (6); *Giardia* assemblage A (3) and B (1)

Table 1. Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in water samples collected from swimming pools in Beijing, China

	No. of sample	*Cryptosporidium* (no. of oocysts/10 L)				*Giardia* (no. of cysts/10 L)			
		No. of positive (%)	Mean±SD	Min.-Max.	95% UCL[a]	No. of positive (%)	Mean±SD	Min.-Max.	95% UCL[a]
Time of sampling									
May 2015	27	2 (7.4)	0.07±0.27	0-1	0.20	4 (14.8)	0.22±0.64	0-3	0.50
August 2015	33	8 (24.2)	0.48±1.00	0-4	0.85	5 (15.2)	0.30±0.77	0-3	0.58
Pool type									
Outdoor pool	5	2 (40.0)	0.60±0.89	0-2	1.50	2 (40.0)	0.80±1.30	0-3	2.00
School pool	12	4 (33.3)	0.58±1.00	0-3	1.17	2 (16.7)	0.25±0.62	0-2	0.67
Community pool	9	1 (11.1)	0.22±0.67	0-2	0.80	2 (22.2)	0.33±0.71	0-2	0.88
Hotel pool	13	1 (7.7)	0.15±0.38	0-1	0.38	1 (7.7)	0.23±0.83	0-3	0.75
Bath pool	12	0 (0.0)	0.00±0.00	0-0	0.00	0 (0.0)	0.00±0.00	0-0	0.00
Commercial pool	9	2 (22.2)	0.44±1.33	0-4	1.45	2 (22.2)	0.33±0.71	0-2	0.80
Total	60	10 (16.7)	0.30±0.79	0-4	0.50	9 (15.0)	0.27±0.71	0-3	0.45

[a]UCL, upper confidence limit which was calculated based on 1,000 bootstrap samples using PASW statistics 18 software.

Table 3. Fecal indicator bacteria and physical-chemical parameters of water samples collected from swimming pools in Beijing, China

Pool type	Statistics	HPC[a] (CFU/ml)	TC[b] (MPN/L)	Urea (mg/L)	Turbidity (NTU)	pH	Free chlorine (mg/L)
Outdoor pool	Mean±SD	232.0±228.0	3.0±4.1	3.7±3.3	0.3±0.1	7.3±0.2	0.4±0.1
	Median	140.0	3.5	3.7	0.3	7.4	0.4
	Range	50-600	<3-8	0.3-7.5	0.2-0.4	7.1-7.5	0.3-0.5
School pool	Mean±SD	90.8±199.7	2.5±3.8	2.0±1.6	0.3±0.1	7.2±0.2	0.5±0.1
	Median	8.6	1.9	1.1	0.3	7.2	0.5
	Range	<1-700	<3-12	0.4-4.3	0.2-0.6	7.0-7.4	0.3-0.8
Community pool	Mean±SD	13.3±40.0	0.3±1.0	2.6±2.4	0.4±0.2	7.2±0.1	0.8±0.3
	Median	13.3	0.3	1.6	0.5	7.1	0.6
	Range	<1-120	<3-3	0.2-6.5	0.1-0.8	7.1-7.4	0.5-1.5
Hotel pool	Mean±SD	197.7±356.1	4.6±7.5	1.7±1.6	0.3±0.2	7.2±0.2	0.4±0.2
	Median	30.0	3.0	1.4	0.3	7.2	0.4
	Range	<1-1,100	<3-27	0.1-4.3	0.1-0.6	7.0-7.6	<0.02-0.6
Bath pool	Mean±SD	63.3±119.9	0.9±1.7	2.3±1.8	0.3±0.2	7.2±0.1	0.7±0.4
	Median	35.6	0.9	2.8	0.3	7.1	0.6
	Range	<1-400	<3-4	0.3-5.7	0.1-0.7	7.0-7.4	0.3-1.3
Commercial pool	Mean±SD	297.8±517.3	5.0±9.8	2.4±2.1	0.4±0.2	7.2±0.2	0.4±0.2
	Median	80.0	2.0	0.9	0.4	7.2	0.5
	Range	<1-1500	<3-30	0.4-5.8	0.1-0.7	7.0-7.6	<0.02-0.7
All types	Mean±SD	139.7±292.1	2.7±5.7	2.3±2.0	0.3±0.2	7.2±0.2	0.6±0.3
	Median	9.4	1.5	1.5	0.3	7.2	0.5
	Range	<1-1,500	<3-30	0.1-7.5	0.1-0.8	7.0-7.6	<0.02-1.5

[a]HPC, heterotrophic plate count.
[b]TC, total coliform.

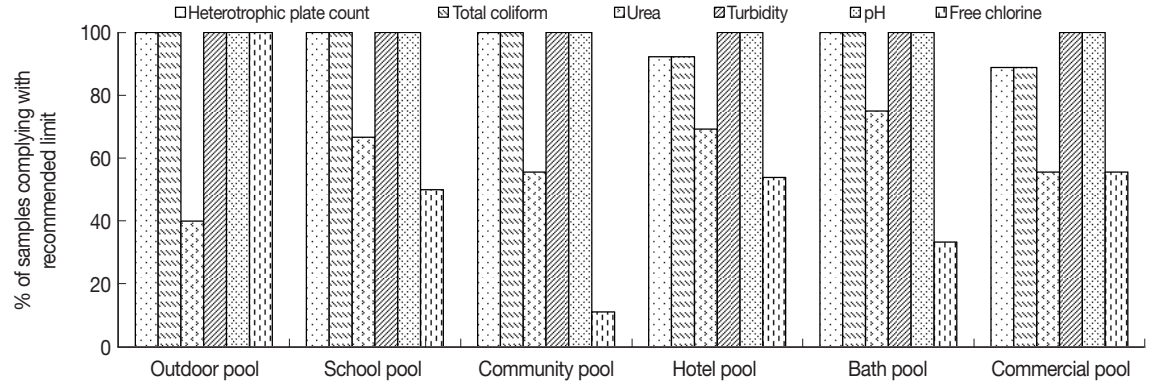

Fig. 1. Percentage of quality indicator in the swimming pool waters complying with the Chinese Hygienic Standard for Swimming Place recommended limits.

standard range (6.5-8.5) (Fig. 1). Surprisingly, nearly a half samples (29/60) contained free residual chlorine exceeding the 0.5 mg/L limit, and only 3 samples were less than 0.3 mg/L as reference to the allowable range (0.3-0.5 mg/L) (Fig. 1).

Relation between microbial quality and physical-chemical properties

Nonparametric Spearman's correlation 2-tailed test was used to assess the relation between parasitic pathogens, bacterial indicators, and physical-chemical properties. Statistical analysis showed a strong correlation between parasites and urea, but no correlation between parasites and bacterial indices (i.e., HPC and TC). Alternately, a positive correlation of bacterial indicators with pH and a negative correlation with free chlorine were found. Turbidity did not correlate to any other parameters (Table 4).

DISCUSSION

Cryptosporidium and *Giardia* were common findings in recre-

Table 4. Correlations between parasitic pathogens, bacterial indicators, and physical-chemical parameters in water from different type of swimming pools in Beijing, China

	Crypto-sporidium	*Giardia*	HPC[a]	TC[b]	Urea	Turbidity	pH	Free chlorine
Crypto-sporidium	-	0.282[c]	0.163	0.184	0.569[d]	-0.139	0.080	-0.124
Giardia	0.282[c]	-	0.012	0.009	0.343[d]	0.060	-0.072	0.029
HPC[a]	0.163	0.012	-	0.829[d]	0.251	0.039	0.642[d]	-0.859[d]
TC[b]	0.184	0.009	0.829[d]	-	0.223	0.034	0.603[d]	-0.759[d]
Urea	0.569[d]	0.343[d]	0.251	0.223	-	0.104	0.109	-0.138
Turbidity	-0.139	0.060	0.039	0.034	0.104	-	0.009	0.110
pH	0.080	-0.072	0.642[d]	0.603[d]	0.109	0.009	-	0.157
Free chlorine	-0.124	0.029	-0.859[d]	-0.759[d]	-0.138	0.110	0.157	-

[a]HPC, heterotrophic plate count.
[b]TC, total coliform.
[c]$P<0.05$ and [d]$P<0.01$ by Spearman's correlation coefficient (2-tailed test).

ation water, especially in swimming pool waters, which often caused outbreaks [3,4,20,21]. In the present study, 13/35 (37.1%) of swimming pools were tested positive for *Cryptosporidium* (14.3%), *Giardia* (14.3%) or both (8.6%). Similar contamination rates were reported from non-outbreak-related pools in other countries. Studies in Italy found that 2/7 (28.6%) of surveyed pools were positive for both *Cryptosporidium* and *Giardia* [22], and 4/10 (40%) of pools were positive for either parasite [23]. One or both parasites were found in 8.1% (13/160) of swimming pools in the United States [3] and 11.8% in the Netherlands [4], while in Greece no oocysts or cysts were found in 5 swimming pools [12]. Recently, *Cryptosporidium* oocysts and/or *Giardia* cysts were detected in 10% of samples from Egyptian swimming pools [2] and 3 out of 37 swimming pools in Belgium [24].

Relatively low *Cryptosporidium* concentrations (0.1-0.4 oocysts/L for positive sample) were found in different types of swimming pools in this study. Similar oocyst counts were obtained in swimming pools in Belgium, Egypt, and the Netherlands [2,4,24]. On the other hand, in these studies, *Giardia* cyst counts were usually higher than *Cryptosporidium* oocyst counts. However, in the present study, the counts of *Giardia* (0.1-0.3 cyst/L for positive sample) was similar to that of *Cryptosporidium*.

The occurrences of *Cryptosporidium* oocysts and *Giardia* cysts were seasonal, which were more frequently detected in August than in May. This may be due to the higher density in swimmer, since August is the hottest month of the year in Beijing [25]. Similar results were found in Atlanta, Georgia, USA with higher protozoan occurrences in the high dense crowds pool [3]. Shields et al. [3] also revealed that the positive parasite prevalence was higher in pools frequented by children and adults than in pools designated for adults only. The results coincided with those from our study that a high rate of *Cryptosporidium* positive samples were found in school pools. It should be noted that no oocyst or cyst detection in any of bath pools in this study might be attributed to low dense swimmers, since few people in summer visited the bathing centre, where they incline to have bath other than swimming.

In China, animals, including pets, usually are not allowed to access to swimming pools. As expected, all the detected species of *Cryptosporidium* (i.e., *C. hominis* and *C. parvum*) and *Giardia* assemblages were associated with human infections. This indicates that anthroponotic transmission is important in cryptosporidiosis and giardiasis epidemiology in the studied area. Similar results were obtained by other authors, who reported that *C. hominis* and *Giardia* assemblage A were the predominant species/assemblages of the protozoa both in humans [26] and urban wastewaters [27] in China.

Bacterial and physical-chemical properties are conventional indicators applied to monitor water quality. Considering that both *Cryptosporidium* and *Giardia* are fecal-derived protozoa, fecal indicator bacteria could well indicate the protozoan contamination [15,16]. However, in this study, only 2 out of 35 pools did not meet the standard for bacterial indicator [13], while 13/35 of pools were contaminated with *Cryptosporidium* or *Giardia*. The low rate of violation of bacterial indicator did not indicate a low occurrence of *Cryptosporidium* or *Giardia*. It was well known that application of disinfection with chlorine has been widely used in China and many other countries to control microbial contamination in swimming pools. More than a half (32/60) of water samples were found with unqualified free chlorine, and most (29/32) of them were unqualified

because of their high chlorine concentration (Fig. 1). This finding was different from the results obtained from other countries where most of unacceptable samples were associated with lower free chlorine value [28-30]. Free chlorine at proper disinfection levels can kill most bacteria in a short time, while *Giardia* and *Cryptosporidium* are moderately even highly resistant to both environmental stress and chlorine [31]. This may be the reason why indicator bacteria (i.e., HPC or TC) were not associated with protozoa in the present study. Therefore, in the chlorine-treated water, including swimming pool waters, bacterial indicator is an inappropriate index for *Cryptosporidium* or *Giardia*.

It was interesting that *Cryptosporidium* and *Giardia* were tightly related to urea in this study. Urea in swimming pool mainly comes from urine and sweat of bathers [1]. It was estimated that 25-30 ml/bather of urine was released into swimming pools [32], and urea be released at an average of 37.1 mg/bather during 30 min of exercise [33]. During urination, feces in the anus are prone to discharged, and this would raise the contamination of *Cryptosporidium* and/or *Giardia*. This may be the reason why urea was close touch with the protozoa. The analyses of the parasite genotypes also showed that the contamination of *Cryptosporidium* and/or *Giardia* is from humans. Therefore, analysis of urea in swimming pool waters is proposed as a simple and effective method to monitor fresh man-made water pollution [34].

In conclusion, *Cryptosporidium* oocysts and/or *Giardia* cysts were present in different types of swimming pool waters in Beijing, China, with a more frequent occurrence in August than in May. Detection of species/genotype revealed that *C. hominis* and *Giardia* assemblage A were the predominant species/assemblages, and anthroponotic transmission is an important route of the protozoan diseases. Fecal bacterial indicator was not an appropriate index to monitor the contamination of *Cryptosporidium* or *Giardia* in chlorine-treated water, including swimming pool waters. The close relation between the protozoa and urea indicated that urea might be a suitable indicator for *Cryptosporidium* and *Giardia* in swimming pools.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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