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Research on the methodology of spatial-temporal distribution of fecal coliform and human adenoviruses in bathing beaches

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Abstract: [Objective] Human adenoviruses (40/41) have been related to acute gastroenteritis, and used as an index of human viral pollution in bathing waters. Traditionally, fecal coliform (FC) was used as an bacterial indicator by using cultivation techniques to estimate risks posed by pathogen in environmental samples. The spatial-temporal detection of waterborne pathogens is of great importance to public health and the prevention of illness. **[Methods]** In this study, a total of 30 bathing water samples were collected from ten representative bathing beaches of China from May to October, 2008. The quantification of human adenoviruses and FC were analyzed. **[Results]** The concentrations of adenoviruses ranged from 1.7×10^6 to 1.1×10^8 genomic copies/L detected by real-time PCR assay. Occurrence of adenoviruses was determined by real-time PCR and compared to that of common PCR, the positive rate was 30% and 26.7%, respectively. The FC values in seven sampling beaches were higher than 2 000 CFU/L. The temporal distribution trend of adenoviruses presented from August to October were much more than that of other months (P<0.05). Under this experimental conditions, when the sample areas changed in spatial scale, including not only among these ten bathing beaches, but also among different sites of each beach, adenovirus distribution had no obvious difference (P>0.05). Results also showed that spatial and temporal variation of FC were not significant (P>0.05). While there was a correlation between the concentration of FC and the distance from the seashore (P<0.05). The result further confirmed that bacterial and viral indicators were not correlated with each other in the chosen beaches. **[Conclusion]** In order to prevent a major outbreak of gastroenteritis disease in the swimming season, the monitoring of viral and bacterial indicators as well as sanitation management must be strengthened.

Keywords: Bathing beaches, Indicator, Adenovirus, Fecal coliforms, Real-time PCR

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时空监测海水浴场粪大肠菌群和人类腺病毒方法的探讨

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摘　要: 【目的】人类腺病毒(40/41)与人类急性胃肠炎显著相关, 被用作娱乐水体中人类病毒污染的指示生物。粪大肠菌群(FC)作为传统的细菌指示生物, 用来估计水环境中病原微生物的潜在风险。了解水传播的病原微生物的时空分布对公众健康和疾病的预防具有十分重要的意义。【方法】于 2008 年 5 月到 10 月, 在中国 10 个典型海水浴场共采集 30 个表层海水样品, 分别用定量 PCR 和细胞培养的方法分析人类腺病毒和 FC。【结果】腺病毒的含量为 1.7×10^{6}–1.1×10^{8} 基因拷贝/L, 其阳性检出率为 30%, 而普通 PCR 的阳性检出率为 26.7%。其中 7 个海水浴场的 FC 超出了景观娱乐水质标准(2 000 CFU/L)。时间分布趋势表明, 人类腺病毒从 8 月份到 10 月份的污染较其他月份严重(P<0.05)。在该实验条件下, 不论是在同一浴场的不同站位还是在不同浴场, 腺病毒的空间分布差异都不明显(P>0.05)。同样, FC 在不同浴场的时空分布也无明显差异(P>0.05), 但是其分布与离岸距离的远近显著相关(P<0.05)。此外, 在我们所研究的浴场, 细菌和病毒这两种指示生物之间并没有相关性。【结论】为避免在游泳季节胃肠道疾病的大规模爆发, 必须加强卫生设施建设和肠道细菌、病毒两种指示生物的监测。

关键词: 海水浴场, 指示生物, 腺病毒, 粪大肠菌群, 实时定量 PCR

1 Introduction

It has been generally perceived that adenoviruses (AdV) serotype 40 and 41 (species F) were second only to rotaviruses as a leading cause of acute gastroenteritis, especially among children[1]. Adenoviruses have broadly roused public concern because of its public health implication and the year-round occurrence in many aquatic environments[2−3]. In addition, adenoviruses and polyomaviruses are stable in environmental waters as the only two types of DNA viruses in the enteric virus family. It is reported that adenoviruses are 60 times more resistant to UV irradiation than RNA viruses, such as enteroviruses and hepatitis A virus[4]. Studies also have shown that adenovirus can be used as an index for human viral pollution because of its concomitancy with other human enteric viruses[5−6].

Bathing beaches are attracting more and more visitors to the shoreline and providing recreational activities, such as swimming, surfing, snorkeling and etc. Water quality is a key issue for the sustainable and healthy development of bathing beaches. Traditionally, fecal coliform (FC) was used as indicators of fecal pollution to protect human health during water recreation[7]. However, it is becoming clear that the common bacterial indicators used to evaluate health risks frequently fail to alert for the

presence of enteric viruses. The contamination of water with human enteric viruses is gradually gaining more interests[8−10]. Provided it is impossible to detect all kinds of human enteric viruses in environmental waters, taking adenoviruses as an index to indicate the human viral contamination is more feasible and worthwhile. However, so far only very little work has been done on the detection of fecal contamination in bathing beach waters by combing these two kinds of indexes, namely fecal coliforms and adenoviruses, especially in China.

Therefore, the overall goals of this study were to 1) use bacterial and viral indicators simultaneously to evaluate the coastal fecal pollution, 2) establish a method to find out the spatial-temporal distribution of adenovirus and fecal coliforms, 3) gain insight into the occurrence of human adenoviruses during peak usage of the beaches, and 4) compare the sensitivity of real-time PCR with common PCR. The AdV quantitation data provided in this study could be used as basic data in the future for assessing risks associated with recreational activities in Chinese bathing beaches, as well as preventing the transmission of AdV-related diseases.

2 Materials and methods

2.1 Sample sources

Many researchers have observed that the outbreak of human enteric viruses was in summer and early fall seasons, which coincides with increased water recreational activities and human-water contact[11]. Therefore, we selected August 2008 as the time to collect water samples to study the spatial distribution of adenovirus and FC. Ten representative bathing beaches were selected, from north to south of China, including Beidaihe (BDH), Dalian (DL), Yantai (YT), Qingdao (QD), Lianyungang (LYG), Ningbo (NB), Xiamen (XM), Shenzhen (SZ), Beihain (BH), and Sanya (SY) (Fig. 1). By using FC index to judge, DL is the cleanest beach in these ten beaches, but adenoviral pollution still exists there. Therefore, between May and October in 2008, DL was chosen as a typical bathing beach to investigate the spatial distribution of human adenovirus and FC during recreational season monthly. At each beach, samples were collected at two points, 100 m and 1 000 m beyond the shore, where the water is easily and rarely affected by human activities, and marked as site 1 and 2 respectively. At each site, ten liters of seawater were collected for adenovirus analysis, while 100 mL was assayed for FC, which means totally 30 samples were got.

Water temperature and dissolved oxygen (DO) were measured by a portable calibrated DO meter, and the pH value was measured using pH meter, the results were shown in Table 1.

2.2 Adenovirus concentration

Viruses were concentrated using ultra-filtration method as described previously[12]. In brief, ten liters of water was sequentially filtered through a 0.8-μm diameter fiber filter and a 0.22-μm-pore-size filter to remove particulates, in case of clogging of the spiral filter cartridge. Then, the filtrates were concentrated to 100 mL using the Millipore virus concentration device (Centricon Plus-70). Finally, a volume of 350 μL sample was enriched by using a Centriprep-30 ultra-concentration unit.

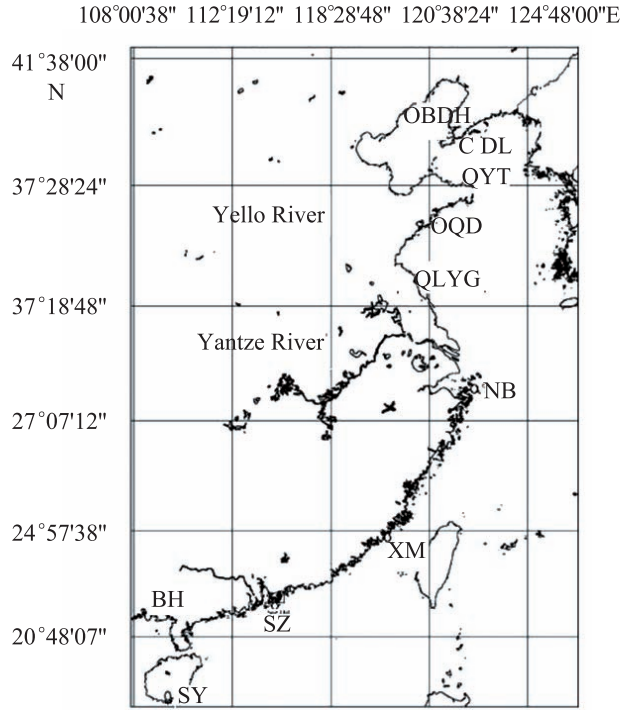

Fig. 1 The site locations of the study
图 1 本研究的站位布设图

Table 1 The parameters of water sampling
表 1 取样站位水质参数

Time 时间	Location 地点	pH	DO 溶解氧(mg/L)	*S* 盐度(‰)
2008.8	BDH	8.02	7.82	30
2008.8	YT	7.96	7.61	29
2008.8	QD	8.04	8.16	31
2008.8	LYG	7.95	8.22	28
2008.8	NB	8.08	6.98	28
2008.8	XM	8.26	6.48	26
2008.8	SZ	8.46	6.57	26
2008.8	BH	8.24	6.43	25
2008.8	SY	8.11	6.52	26
2008.5	DL	8.15	7.49	30
2008.6	DL		7.48	30
2008.7	DL		7.28	29
2008.8	DL		6.57	27
2008.9	DL		6.69	28
2008.10	DL		7.02	31

2.3 Detection of human adenovirus by TaqMan real-time PCR

Standard curve was created prior to real-time PCR detecting human adenovirus in water samples. PCR was preformed for the AdV-40 hexon gene using a pair of degenerate primers Ad1 and Ad2 (Table 2)[13] according to previously published methods[14]. The target 482 bp amplicon was cloned into the PMD18-T (TaKaRa) and confirmed by sequencing. The purified recombinant plasmid DNA was quantified by UV spectrophotometer. Then, the plasmid was serially diluted with EASY Dilution kit (TaKaRa).

The real-time PCR was performed and optimized according to He and Jiang[13]. Primers and probes were located in hexon and yield a 163-bp sequence. Samples were detected using real-time PCR with 20 μL mixture, containing dilution 2 μL, Premix *Ex Taq*™ (2×) 10.0 μL, PCR forward primer AD3 (10 μmol/L) 0.4 μL, PCR reverse primer

Table 2 Summary of primers and probes for real-time PCR detection of adenovirus
表 2 腺病毒实时定量 PCR 的引物和探针信息

Primer or probe 引物和探针	Sequence 序列(5′→3′)	Assay targets 方法靶定类型
Ad1	TTCCCCATGGCIAYAACAC	Serotypes 40, 41
Ad2	CCCTGGTAKCRATRTTGTA	
AD3	CCCTGGTAKCCRATRTTGTA	Serotypes 40
AD4	GACTCYTCWGTSAGYGGCC	
ADP	FAM-AACCAGTCYTTGGTCATGTTRCATTG-TAMRA[a]	Serotypes 1–5, 9, 16, 17, 19, 21, 28, 37, 40, 40, 41, and simian adenovirus 25

Note: FAM: 6-Carboxyfluorescein, fluorescence reporter dye; TAMRA: 6-Carboxy-tetramethylrhodamine, fluorescence quencher dye.
注: FAM: 6-羧基荧光素, 荧光报告基团; TAMRA: 6-羧基二乙酸荧光素, 荧光淬灭基团.

AD4 (10 μmol/L) 0.4 μL, ADP (3 μmol/L) 1.0 μL, ROX Reference Dye Ⅱ (50×) 0.4 μL, template DNA 2 μL, and DEPC 5.8 μL. The final thermocycling profile was 95 °C for 10 s, 58 °C for 15 s, and 62 °C for 1 min for 40 cycles. Thirty samples were run in triplicate by using ABI 7500 sequence detection system. Each reaction was run in triplicate.

2.4 AdV detection by PCR assay

Common PCR was arranged to detect the adenoviral genes in the water samples, in order to compare the results with that of the real-time PCR. The primer Ad1 and Ad2 was assigned. The PCR protocol followed the method mentioned above[14].

2.5 Samples for fecal coliforms (FC) analysis

FC analysis was performed in the unconcentrated 100 mL water samples, using the most probable number (MPN) approach and fermentation method in microplates. Thirty water samples were enumerated according to the standard procedures[15]. FC plates were incubated for 24±2 hours at the temperature of 44 °C.

2.6 Statistical analysis

In order to analyze the relationship between the distance off the seashore and the occurrence of pathogens (adenovirus and FC), as well as spatial and temporal distributions of these pathogens, SPSS 16.0 and Fisher exact test were used.

3 Results

3.1 Adenovirus occurrence in the recreational beaches

Ten of 30 (33%) water specimens were adenovirus positive detected by real-time PCR. The average value of adenoviral genomes of all bathing beach water samples was 1.7×10^6 to 1.1×10^8 genomic copies/L. Using agarose gel electrophoresis, amplicon from positive tests were proved to be the expected amplicon size of 163 bp. 30 samples also were examined for adenovirus genomes by the common PCR system; the positive rate was 26.7% (8/30), which was similar to the previous report[16]. The comparison of detected results between TaqMan real-time PCR and common PCR is summarized in Table 3.

3.2 Standard curve of TaqMan real-time PCR

AdV40 was used as the model adenovirus for establishing the standard curve. The results showed a positive log linear correlation between hexon gene copy number and PCR threshold cycle number with the detected value of 10^2–10^6 genome equivalent copies/L. The correlation coefficient of the standard curve was 0.996 3, while the efficiency of amplication was 99.6%, and the slope was −3.0 (Fig. 2). These profiles collectively indicated an ideal condition for real-time PCR.

3.3 Indicator bacteria

3.3.1 August 2008, fecal coliforms counts in ten representative bathing beaches: In August 2008, twenty water samples in ten representative bathing beaches were examined for FC analysis by the MPN approach. The Landscape Recreational Water quality standard for fecal coliforms is the geometric mean of 2 000 CFU/L with upper limit equal to or greater than 24 000 CFU/L[17]. If the value is less than 20 CFU/L, it was defined as not detected. The numbers of fecal coliforms in our study ranged from less than 20 to more than 24 000 CFU/L.

Table 3 The comparison of detective results between TaqMan real-time PCR and common PCR in 30 shares of seawater samples in China (copies/L)
表 3 中国近岸 30 份海水样品的 TaqMan 实时定量 PCR 和普通 PCR 检测结果对比(拷贝/L)

DL	DL	DL	DL	QD	LYG	LYG	YT	NB	NB
July, site 1	August, site 1	September, site 2	October, site 2	August, site 1	August, site 1	August, site 2	August, site 1	August, site 1	August, site 2
−	+	+	+	+	+	−	+	+	+
1.7×10^6	1.9×10^7	1.1×10^8	2.0×10^7	2.2×10^7	2.6×10^7	2.8×10^7	1.1×10^7	2.3×10^7	1.6×10^7

Note: +: Positive for AdV; −: Negative for AdV.
注: +: 腺病毒阳性; −: 腺病毒阴性.

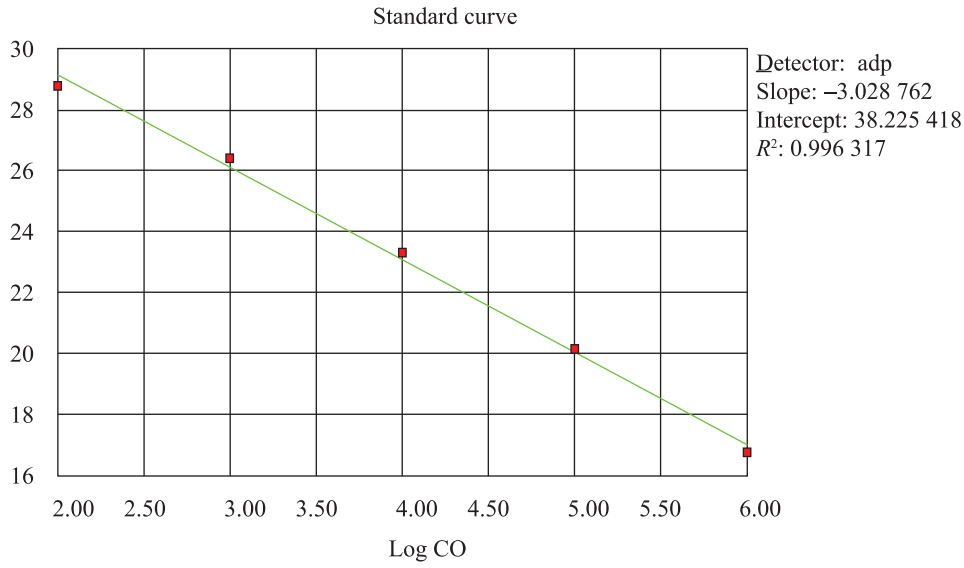

Fig. 2 Standard curve of TaqMan real-time PCR detection of adenoviruses
图 2 腺病毒 TaqMan 实时定量 PCR 标准曲线

Seven samples were detected out of 2 000 CFU/L of fecal coliforms, which were found outnumbering the established thresholds. In ten representative bathing beaches in our study, DL, YT, XM, SZ bathing beaches have water qualities passed according the criterion of recreational water quality, except QD bathing beach was seriously polluted by feces (including control site 2).

3.3.2 May to October 2008, fecal coliforms (FC) detection in DL bathing beach: Between May and October, 2008, 12 samples were tested for fecal coliforms (CFU/L). According to the Landscape Recreational Water quality standards[17], none of these samples were above the stipulated bacterial standard (data not shown).

3.4 The spatial distribution of waterborne adenovirus and fecal coliforms

The spatial distribution of AdV and FC, from northern bathing beaches to that of south of China, was compared in Fig. 3. In these ten different beaches, the statistical analysis of correlationship concerning the prevalence of adenovirus between north and south bathing beaches were analyzed by Fisher exact test. The amount of FC exceeding standard in the north bathing beaches was also compared with that of south by this method. Results were shown in Table 4, which showed there was no significant spatial difference.

In each bathing beach, the relationship between the distance of sampling from the seashore and the pathogens (adenovirus and FC) was analyzed by Fisher exact test and SPSS 16.0. Results were presented in Table 5, which showed that the incidence of adenovirus in the water samples collected from 100 m off the seashore was found not to be correlated with those from 1 000 m (P>0.05). While there was a correlation between the number of FC and the distance off the seashore (P<0.05).

3.5 The temporal distribution of waterborne adenovirus and fecal coliforms

The results of temporal distribution trend about adenovirus and FC from May to October 2008, were shown in Fig. 4. The relevance of adenovirus in different months was compared by SPSS 16.0 (t test). The results were presented in Table 6, which indicated that the value of adenoviruses in August, September and October were higher than other three months (P<0.05). Because FC in these six months all complied with the bacterial standards of the Chinese recreational water quality, there was no need to discuss the temporal distribution of this index.

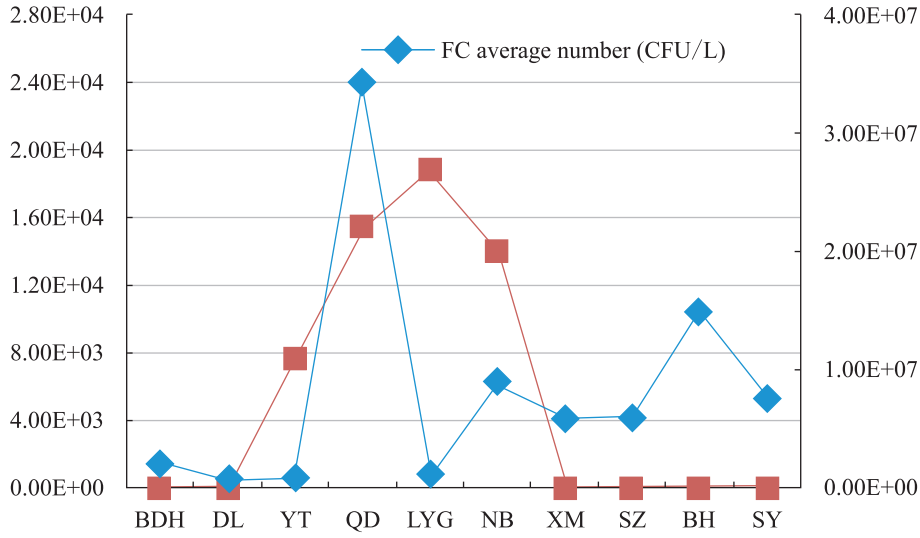

Fig. 3 The spatial distribution of adenovirus and fecal coliforms in ten representative bathing beaches from north to the south of China in August, 2008

图 3 2008 年 8 月中国十大典型海水浴场腺病毒和粪肠球菌从北到南的空间分布

Table 4 Prevalence of adenovirus and FC in the north and south bathing beaches of China
表 4 中国海水浴场腺病毒和粪大肠菌群从北到南的流行情况

	Adenovirus[a] 腺病毒 [a]		FC[b] 粪大肠菌群 [b]	
	Positive	Negative	Positive	Negative
North	5	25	4	26
South	2	28	3	27
P value	0.211 9		0.5	

Note: [a]: No. of samples positive/No. tested; [b]: Positive, out of limits (2 000 CFU/L). Negative, up to standard.
注: [a]: 样品阳性或未检出的数量; [b]: 高出水质标准(2 000 CFU/L)的为阳性; 符合标准的为阴性.

Table 5 Correlation between the presence of pathogens from100 m off shoreline and from1 000 m
表 5 距离岸边 100 m 和 1 000 m 与病原体流行的相关性

Presence of pathogens from 100 m off shoreline 距岸 100 m 处病原体的存在情况	From 1 000 m off shoreline 距岸 1 000 m	
	Adenovirus	FC
Positive	2/6	
Negative	22/24	
Total	24/30	
P value	0.169 1	0.028[a]

Note: The significance of the correlations was tested with the Pearson chi-square (two-sided) test.
注: Pearson 模型卡方(二元)检验显著相关性.

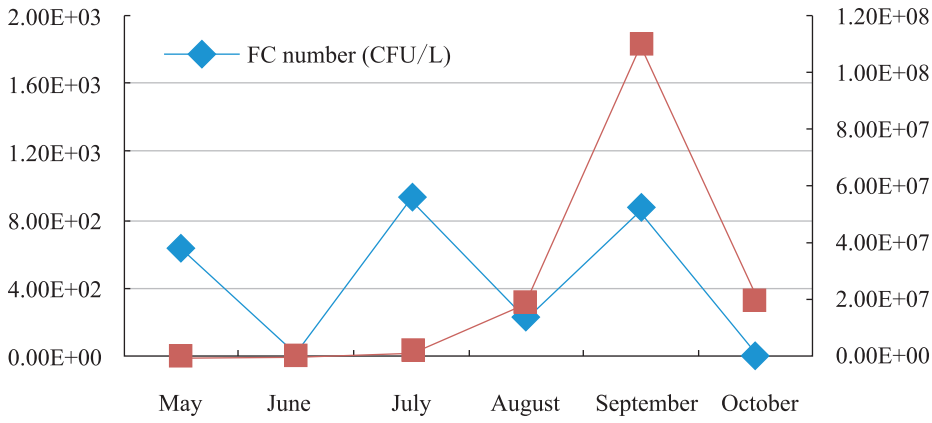

Fig. 4 The temporal distribution of adenovirus and fecal coliforms in DL bathing beaches from May to October, 2008
图 4 2008 年 5 月到 10 月期间大连海水浴场腺病毒和粪大肠菌群的时间分布

Table 6 Correlation between paired-months of adenovirus in DL bathing beach, 2008 (*P* value)
表 6 2008 年大连海水浴场腺病毒和粪大肠菌群月季分布的相关性(*P* 值)

	May	June	July	August	September	October
May		1.000	0.825	0.027[a]	0.000[a]	0.004[a]
June			0.825	0.027[a]	0.000[a]	0.004[a]
July				0.040[a]	0.000[a]	0.060
August					0.000[a]	0.309
September						0.000[a]

Note: The significance of the correlations was tested with the *t* test.
注: *t* 检验显著相关.

3.6 Correlation between bacterial indicator and human viral indicator of fecal pollution

When studied the relationship between the overproof FC and adenovirus, Fisher exact test was introduced. The result was presented in Table 7, which showed their occurrences were independent (P>0.05).

Table 7 Correlation between the presence of adenovirus and FC in all selected sites
表 7 所有站位腺病毒和粪大肠菌群之间的相关性

FC 粪大肠菌群	Adenovirus 腺病毒	
	Positive 阳性	Negative 阴性
Positive	3	6
Negative	4	17
Total	7	23
P value	0.343 1	

4 Discussions

AdV is slow-growing and often does not produce cytopathogenic effects in cells; thereby its prevalence obtained by cell culture is most likely underestimated[5]. It is reported that quantification of AdV using real-time PCR may be useful for evaluating virus occurrence in water[18−19]. In this study, 10 positive samples were detected in 30 samples by this assay, while 8 positive specimens were obtained by common PCR. The data suggest a higher sensitivity and specificity of the real-time PCR for the detection of adenoviruses than those of common PCR. In addition, the real-time PCR assay provides the advantage of speed, which effectively avoids the contamination during PCR stage.

The number of adenoviral genomes detected in bathing beach waters was 1.7×10^6 to 1.1×10^8 ge-

nomic copies/L, which showed a much higher number of adenoviral genomes than that He and Jiang detected in Southern California sewage treatment plants (8.1×10^5 genomic copies/L). This result may contribute to the current inefficient water sterilization to virus, as well as the influence of human recreation because of large population in China. Previous work showed that the median concentration of enteric viruses in feces is 10^9/g, and the amount of feces shed in the water averages 0.14 per person[20]. It is not difficult to predict that there will be a high risk to bathing beaches if visitors are infected by adenoviruses. This result just corresponds to that in QD bathing beach, where FC is much more than 24 000 CFU/L (including control site). The beach hosts visitors most (over six million) among the 22 typical bathing beaches of China[21]. And the fecal coliforms of each infected bather may achieve 10^5 to 10^6 CFU[20]. However, we can not exclude the other possible reason, which was non-point source fecal contamination originated from the land, because there was a heavy rain before sampling time of QD bathing beach. This phenomenon is similar to previous studies conducted in the Lake Michigan and Lake Erie by Wade[22−23].

Previous report pointed out that spatial and temporal distribution of pathogens from farm-to-farm can be highly variable[24−25]. Therefore, in this work, the spatial-temporal detection of waterborne virus and bacteria were analyzed to ensure whether variation exists in bathing beaches of China. Although adenoviruses are thought to be common in the environment, insufficient data are available to evaluate their prevalence and distribution, especially in China. The gastroenteritis caused by adenoviruses happens much more during August to October than other months (Table 6). The epidemiological study showed that human adenovirus can occur in the whole year, but more common in summer and autumn[26], which was just consistent with the trend observed in this research. Besides, this result was consistent with that of the previous reports in the United States[27], Vietnam[28] and India[29]. While there were no significant report about the outbreak of diarrheal disease in the sampling local during our experiment in China.

In our study, there was no significant difference about the occurrence of adenoviruses between north and south bathing beaches. The south-north distribution trend of the FC showed no discrimination in these detected bathing beaches (Fig. 3) either. As shown in Table 3, the detection number of adenovirus in water sample collected from 100 m off shoreline were found not to be correlated with that from 1 000 meters. This conclusion reminded the conservancy of sanitation department to investigate the water quality in a large scale, from north to south bathing beaches of China and from nearshore to regions with few human activities. Because the distribution of virus could easily be influenced by environmental variables, such as nutrient concentrations, presence of predators or grazers, rainfall, stream flow, and tide[30].

Several studies have suggested that fecal coliform levels cannot be used to predict the occurrence of human enteric viruses, which is consistent with the results of our study ($P>0.05$)[5,31]. The possible reasons for this phenomenon are the conventional measure for managing bacteria indicators can not kill adenoviruses effectively[32]. In addition, the viruses' life cycles are different from that of bacteria. The survival time of viruses is longer than bacteria, because viruses can be attached to suspended solids more easily[33]. Besides, indicator bacteria maybe generated by natural sources such as birds and animal feces, even regrows in nutrient-rich soil and/or riverbed sediments[34−35]. However, enteric viruses have only human origin, which could explain why the number of FC is correlated to the distance from the seashore ($P<0.05$). In such circumstances, overreliance on fecal bacterium- based standards for water quality measurement would significantly underestimate the protection against contamination with human enteric viruses.

In conclusion, this study revealed the occurrence and quantification of human adenoviruses and the bacterial indicators in representative bathing beaches all around China. We recommend that our current recreational water quality standards be improved by adopting both bacterial, viral indicators

and the public raised safety awareness with regards to utilizing recreational beaches, especially from August to October. The aim of this study was mainly to establish a method to analyze the spatial-temporal distribution of fecal coliform and human adenoviruses in bathing beaches. Further studies and more tests are needed to confirm this trend, as well as to determine the seasonal distribution and infection rate of adenoviruses.

5 Conclusions

The data suggested a higher sensitivity and specificity of the TaqMan real-time PCR assay for the detection of adenoviruses than those of common PCR.

In this experiment, the spatial-temporal distribution of waterborne adenoviruses had no significant spatial variety ($P>0.05$). However, during six months of the sampling period, it was found more common from August to October ($P<0.05$). The spatial-temporal variation of FC was not evident ($P>0.05$), while there was a correlation between the number of FC and the distance from the seashore ($P<0.05$).

The results further confirmed that it is necessary to reevaluate both bacterial and viral indicators to monitor the fecal pollution in recreational water.

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