Detection of human fecal pollution in environmental waters using human mitochondrial DNA and correlation with general and human-associated fecal genetic markers

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ABSTRACT

Human mitochondrial DNA (mtDNA) genetic markers are abundant in sewage and highly human-specific, suggesting a great potential for the environmental application as human fecal pollution indicators. Limited data are available on the occurrence and co-occurrence of human mtDNA with fecal bacterial markers in surface waters, and how the abundance of these markers is influenced by rain events. A 1-year sampling study was conducted in a suburban watershed impacted by human sewage contamination to evaluate the performance of a human mtDNA-based marker along with the bacterial genetic markers for human-associated Bacteroidales (BacHum and HF183) and Escherichia coli. Additionally, the human mtDNA-based assay was correlated with rain events and other markers. The mtDNA marker was detected in 92% of samples (n = 140) with a mean concentration of 2.96 log_{10} copies/100 ml throughout the study period. Human mtDNA was detected with greater abundance than human-associated Bacteroidales that could be attributed to differences in the decay of these markers in the environment. The abundance of all markers was positively correlated with rain events, and human mtDNA abundance was significantly correlated with various bacterial markers. In general, these results should support future risk assessment for impacted watersheds, particularly those affected by human fecal pollution, by evaluating the performance of these markers during rain events.

Key words | fecal source tracking, human mitochondrial DNA, human-associated Bacteroidales, quantitative PCR, rainfall, water quality

INTRODUCTION

Identifying the sources of fecal pollution in environmental surface waters used for human recreation and/or fish breeding is the first step for reducing the potential for human contact with enteric pathogens (Simpson et al. 2002; Jeong et al. 2019). Human waste can be introduced into environmental waters in a variety of ways, including leaking septic tanks, untreated sewage, sewer malfunctions, contaminated storm drains, urban runoff, and other sources (Marsalek & Rochfort 2004; Bakir et al. 2006; Badgley et al. 2010). Hence, the accurate detection of human fecal pollution is imperative for mitigating human and environmental health risks, reducing economic losses, and maintaining water quality. For over a century, microbiological water quality has been assessed by measuring the densities of two fecal indicator bacteria (FIB), Escherichia coli and Enterococcus spp. As monitoring tools, these fecal bacterial indicators often provide a useful baseline for establishing public health risks. However, many studies have recognized the limitations of conventional bacteria-based fecal source tracking, including the facts that most of these microorganisms are able to

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inhabit the intestines of several hosts, including humans, livestock, and wildlife, and are able to grow and survive naturally in the environment (Gordon 2007; Anderson et al. 2005; Ishii et al. 2006). Recently, new microbial source tracking assays, such as the human-associated Bacteroidales genetic markers, have been developed using the quantitative polymerase chain reaction (qPCR) and aim to detect bacteria derived specifically from human feces (Seurinck et al. 2005; Kildare et al. 2007; Sauer et al. 2011; Layton et al. 2013; Kapoor et al. 2015). Nonetheless, an alternative to these human fecal microbial targets is the use of approaches that detect the presence of human DNA itself, derived from human fecal waste, specifically in the form of mitochondrial DNA (mtDNA) (Caldwell et al. 2007; Vuong et al. 2015). In geographic regions where Bacteroidales markers are not abundant in human hosts due to differences in diet, culture, or other environmental influences, mtDNA-based markers may be particularly useful in determining human fecal pollution (Yatsunenko et al. 2012; Nshimyimana et al. 2017). In addition, few studies have demonstrated the cross-reactivity of human-associated Bacteroidales assays with samples from other host feces (Johnston et al. 2013; Layton et al. 2013). A direct marker in the form of human mtDNA could add a layer of confirmation to current source tracking studies guiding mitigation efforts, which require strong stakeholder support and are often very expensive to implement.

The detection of fecal bacterial indicators may be influenced by rainfall events (Santiago-Rodriguez et al. 2012; Stachler et al. 2018). It has been previously reported that rainfall may lead to an increase of fecal indicators and pathogens in environmental surface waters and, thus, may represent a heightened risk to human health (Shehane et al. 2005; Santiago-Rodriguez et al. 2012). Specifically, rainfall events may result in stormwater runoff, resuspension of sediments, and sewage overflows that can contribute to an increase of indicators in surface waters (Parker et al. 2010; Sidhu et al. 2015). Nevertheless, the contribution of fecal pollution due to rainfall is often not considered when assessing water quality, and limited information is available on the correlation between rainfall and source tracking markers, including human mtDNA. Studies have examined the occurrence of human mtDNA in environmental waters; however, there is still little observational data on the occurrence and co-occurrence of human mtDNA with human-associated Bacteroidales and general E. coli markers in surface waters over extended temporal and spatial scales (Martellini et al. 2005; Kapoor et al. 2013, 2014). In this study, qPCR assays targeting human mtDNA, human-associated Bacteroidales markers (HF183 and BacHum), and E. coli were applied to identify and quantify human fecal contamination in environmental surface waters. The Cibolo Creek in the Upper Cibolo Creek (UCC) watershed (Texas, USA) was used for sampling due to a history of significant human fecal contamination (Bass & Burger 2013). Surface waters of the Cibolo Creek receive discharge from two wastewater treatment systems and are subject to occasional stormwater runoff from nearby agricultural fields during rainfall events.

The objective of this study was to identify the sources of human fecal pollution in a suburban watershed by using general and human-associated fecal genetic markers, as well as human mtDNA as a direct and robust human fecal marker. The study aimed to understand indicator prevalence in environmental surface waters and what that might suggest about the sources of these indicators. The influence of rainfall events on the abundance of these indicators was also evaluated. In addition, the correlation between human mtDNA, human-associated Bacteroidales markers, and E. coli was studied to better understand the efficacy of each marker for the detection of human fecal pollution. The results of this study provide valuable information on the sources of fecal pollution in the UCC watershed and important insights on the distribution of traditional (E. coli) and more novel indicators (human mtDNA and human-associated Bacteroidales).

MATERIALS AND METHODS

Study area and sampling sites

The Cibolo Preserve in the UCC watershed covers approximately 40 hectares of land in the southern part of Kendall County, Texas, USA, and was chosen for sampling due to a history of significant human fecal contamination (Bass & Burger 2013). The primary surface water body of the UCC is the Cibolo Creek, which is formed from the contributions of Brown and Champee springs in southern Kendall County and flows in a northwest to southeast direction for
161 km through five other counties before its confluence with the San Antonio River. The creek drainage area covers 200 km² and is characterized as rural/suburban with light ranch areas. Although it has a history of bacterial contamination often exceeding the Texas water quality standards for safe contact recreation, the creek is commonly used for recreational activities (Bass & Burger 2013). A previous study reported bacterial impairments in the Cibolo Creek as well as a high concentration of orthophosphorus and other nutrients; however, since 2013, no studies have been conducted to examine fecal contamination in the Cibolo Creek (Bass & Burger 2013). The geology of the area is fractured karst limestone, resulting in significant groundwater recharge through the streambed, and decreases in the measured flow of the lower 30 km of the creek during the dry season. As an effort to maintain the flow, the South Texas Wastewater Treatment Plant (STWT) and Wastewater Treatment and Recycling Center (WWTRC) in Boerne, TX, began discharging their treated effluent into the creek. Wastewater effluent has been suggested as a potential source of fecal pollution of surface waters (Servais et al. 2007; Naidoo & Olaniran 2014). Thus, the occurrence of human fecal contamination in the Cibolo Preserve may likely be linked to the effluent discharges from the two wastewater treatment plants (WWTPs).

A total of nine sampling sites were selected within the study area based on proximity to the WWTPs, discharge to the Cibolo Creek and in an attempt to cover the extent of the preserve area (Figure 1). Site 1 is located on the Currey Creek downstream of the STWT; since no flow upstream of the STWT was observed over the study period, no sampling site was selected in the upstream region of the Currey Creek. Site 2 is located approximately 1 km downstream of site 1 and is the only recreational site in the study area. Sites 7 and 8 are located before and after the contribution from the WWTRC, respectively, and site 3 is located just after the confluence of the Cibolo Creek and the Menger Creek. The WWTRC discharges effluent water into the Menger Creek, which converges with the Cibolo Creek at the preserve boundary. Sites 4 and 5 are located before and after the converging point of the Browns Creek with the Cibolo Creek. Site 6 is located at the eastern edge of the preserve area and was selected to observe the quality of the water before leaving the preserve area. Site 9 is located on the Browns Creek, which converges with the Cibolo Creek inside of the preserve area.

Sample collection and DNA extraction

Surface water samples \((n = 140)\) were collected over a period of 1 year (March 2017 to March 2018) from nine different sites within the study area. All samples were collected using sterilized 1-L bottles (Nalgene, Rochester, NY) and transported on ice to the laboratory at the University of Texas at San Antonio (San Antonio, TX) within 2–4 h of collection. The water samples were then filtered (300–500 ml) onto 0.45-μm pore size, 47-mm diameter mixed cellulose ester membranes (Millipore, Billerica, MA), after which the membranes were immediately frozen at \(-80^\circ\text{C}\). Extraction controls with autoclaved distilled water were used during filtration to monitor for potential extraneous DNA contamination. The manufacturer’s protocol was followed to extract DNA from the sample membranes using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germantown, MD). The purity and concentration of DNA were determined using the NanoDrop OneC Spectrophotometer (Thermo Scientific, Wilmington, DE). DNA extracts were stored at \(-20^\circ\text{C}\) for subsequent qPCR analyses.

qPCR analyses

TaqMan qPCR assays were used to measure both the presence and relative abundance of the four genetic markers in environmental water samples (Table 1). The human mtDNA genetic marker using primer sets targeting the human mitochondrial gene NADH dehydrogenase subunit 5 (ND5) was used to detect human mtDNA (Caldwell et al. 2007). The remaining three markers used qPCR assays targeting the fecal bacterial groups: \(E.\ coli\) (EC235857 assay) (Chern et al. 2011) and the two human-associated Bacteroidales (HF185 and BacHum assays) (Kildare et al. 2007; Haugland et al. 2010).

The methodology for the qPCR assays performed is described in detail in a previous study (Kapoor et al. 2018). Briefly, reaction mixtures (25 μL contained 1× SsoAdvanced Universal Probes Supermix (Bio-Rad, Hercules, CA), 0.2 μM final concentration of forward and reverse primers, a 6-FAM (6-carboxyfluorescein)-labeled hydrolysis probe, and 2 μL of the extracted DNA template from the water samples. All reaction mixtures were prepared in duplicate, and qPCR assays were performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The qPCR
data were analyzed using the Bio-Rad's CFX Manager Software (version 3.1). Standard curves were generated by using plasmids containing the sequences for each of the targeted genes. No template controls were used to check for
cross-contamination for each qPCR run. In addition, undiluted and 10-fold dilutions for each DNA extract were used as the DNA template in qPCRs to test for PCR inhibition.

**Data analyses**

All data for each genetic marker were calculated as the copy number per 100 ml of water for every sample analyzed using qPCR with a cycle threshold ($C_T$) value above background. Before performing statistical analyses, all data were transformed to $\log_{10}$ copies per 100 ml of the water sample. The difference in the detection frequency for human mtDNA, *E. coli*, and human-associated Bacteroidales markers was determined using cross-tabulation by means of Venn diagrams. Differences in marker concentrations among the study sites were examined using a nonparametric Kruskal–Wallis one-way analysis of variance. Spearman’s rank correlation coefficients ($r$) between qPCR indicators were calculated using the means of $\log_{10}$ transformed marker concentrations in GraphPad Prism 8 (La Jolla, CA) using two-tailed 95% confidence intervals. For comparison purposes, Spearman’s coefficients are ranked according to the following scale: 0.1–0.39 (weak correlation), 0.4–0.59 (moderate correlation), 0.6–0.79 (strong correlation), and 0.8–1 (very strong correlation). All statistical analysis results were regarded as significant at $p < 0.05$. Precipitation data reported 24 h before sample collection was obtained from the US Geological Survey (USGS) station number 08183890, located on the Cibolo Creek within the Cibolo Preserve.

**RESULTS AND DISCUSSION**

**Performance of qPCR assays**

The linear range and amplification efficiencies of the qPCR assays were determined by plotting the standard curves generated using serial dilutions of known copy numbers of each marker. The linear range of quantification for all the qPCR assays was between 10 and $10^6$ copies per reaction. The qPCR amplification efficiencies for all the assays ranged from 86 to 105%, with $r^2$ values above 0.9. To determine PCR inhibition, reactions were performed with 10-fold dilutions of each DNA extract as described in a previous study (Kapoor et al. 2015). In these tests, PCR inhibition did not interfere with the amplification efficiency, since a $C_T$ value proportional to a 10-fold dilution relative to the undiluted DNA templates was observed for the reactions. DNA extraction controls and no template controls indicated the absence of contamination in the qPCR assays.

**Detection of human mtDNA and fecal bacterial markers**

The concentrations of human mtDNA, human-associated Bacteroidales, and *E. coli* were measured for the water samples using TaqMan qPCR assays. The human mtDNA marker was detected in 92% of water samples (Table 2), which indicates that human fecal pollution was predominant in the study area. Both human-associated Bacteroidales (HF183 and BacHum) markers were present in the majority of the water samples, while *E. coli* showed the maximum detection frequency (99%) for samples in this study. The mean marker abundance for the human mtDNA marker was measured the highest among all the markers, followed by *E. coli*.

The detection of human mtDNA was compared with the bacterial markers (Table 3). Specifically, 92% (129 out of 140) of the samples detected both *E. coli* and human mtDNA markers. For human-associated Bacteroidales, 50% (70 out of 140) of the samples were positive for both HF183 and BacHum markers, while 7 and 18% were positive for only HF183 and BacHum, respectively. In comparison, human mtDNA was detected in 96% (91 out of 95) of water samples positive for BacHum, and 95% (76 out of 80) of water samples positive for HF183. However, when comparing total samples, both human mtDNA and BacHum were detected concurrently in 65% (91 out of 140) of water samples.

| Table 2 | Distribution of molecular markers ($\log_{10}$ copies per 100 ml) used in this study detected via qPCR assays (n = 140) |
|---------|-------------------------------------------------------------------------------------------------------------|
| **Assay** | **Mean** | **Range** | ** +% (%)** |
| Human mtDNA | 2.96 | 0.00–6.69 | 92 |
| Human-associated Bacteroidales (HF183) | 1.57 | 0.00–4.89 | 57 |
| Human-associated Bacteroidales (BacHum) | 1.80 | 0.00–5.17 | 68 |
| *E. coli* (EC23S857) | 2.90 | 0.00–4.96 | 99 |

+% (%) – percentage of samples detected positive for the marker.
samples, and both human mtDNA and HF183 were detected concurrently in 54% (76 out of 140) of water samples.

**Spatial distribution of markers**

The spatial distribution of the levels of markers across the study sites is represented in Figure 2. The two human-associated Bacteroidales markers, BacHum and HF183, displayed a similar spatial distribution pattern across the sampling sites, although the mean marker level of the BacHum marker was higher than HF183. The levels of the human-associated Bacteroidales markers were statistically different ($p < 0.05$, Kruskal–Wallis one-way analysis of variance) from each other among the study sites using qPCR assays. Site 1, located just downstream of the STWT, had the highest mean copy number for the human-associated Bacteroidales markers, while site 9 had the lowest copy numbers. Site 2, located downstream of site 1, after the convergence of the Currey Creek with the Cibolo Creek, showed a decrease in copy numbers for both of the human-associated Bacteroidales markers. A gradually decreasing trend in the concentration was observed for both BacHum and HF183 in a downstream direction, from site 2 to site 6 on the Cibolo Creek, suggesting that the human fecal contamination is coming from upstream sources, including the outfall of WWTPs. Previous studies have reported a high abundance of fecal indicators in treated waters at the outlet of WWTPs (Servais et al. 2013). Moreover, it has been reported that the persistence of human-associated Bacteroidales in the environment begins to decrease after 4 days of separation from its host and is completely decayed after 7 days, although the decay rate depends on multiple variables such as temperature, sunlight, and nutrients (Walters & Field 2009). Surprisingly, there were no notable differences in human mtDNA marker levels among the study sites, except for site

| Detection                  | Human mtDNA vs. BacHum | Human mtDNA vs. HF183 | Human mtDNA vs. E. coli |
|----------------------------|------------------------|-----------------------|-------------------------|
| Only marker 1              | 38                     | 53                    | 0                       |
| Only marker 2              | 4                      | 4                     | 10                      |
| Both 1 and 2               | 91                     | 76                    | 129                     |
| None                      | 7                      | 7                     | 1                       |
| Total                     | 140                    | 140                   | 140                     |

Figure 2 | Spatial variation in levels of markers across study sites as determined using qPCR assays. Bars represent averages, and error bars represent standard deviations ($n = 15$).
Correlation of human mtDNA and fecal bacterial markers

Spearman’s rank correlation coefficients were calculated between each pair of human mtDNA and fecal bacterial markers as measured by the qPCR (Table 4). All Spearman’s correlation coefficients were positive and showed statistical significance based on the p-value of 0.05. Human mtDNA exhibited a moderate correlation with fecal bacterial markers (E. coli, HF183, and BacHum). When compared with human-associated Bacteroidales, human mtDNA was detected with higher abundance (approximately 1–2 orders of magnitude) than HF183 and BacHum. This could be attributed to differences in the decay of human-associated Bacteroidales and human mtDNA markers in the environment. As discussed above, human-associated Bacteroidales can be completely decayed in 7 days after release from a host, whereas human mtDNA can be detected for up to 15 days in the environment (Martellini et al. 2005). There was a strong correlation between both the human-associated Bacteroidales markers, HF183 and BacHum. The human-associated Bacteroidales and E. coli marker showed the moderate-to-strong
correlation, indicating that human fecal pollution was predominant in the study area. A similar correlation has been previously reported between *E. coli* concentration and the concentrations of the human-associated Bacteroidales markers in environmental water samples (Kapoor et al. 2013).

The mitochondrial marker and the fecal bacterial markers targeting human were tested on more than 100 water samples. These samples were collected over a period of 1 year from nine different sites in a fast-growing suburban watershed, representing natural areas with minimal human impact (Cibolo Preserve) and small urban areas close to the natural environment or surrounded by agricultural activities. The preserve area was overrepresented to observe the potential impact of discharging treated wastewater effluent into the creeks in the preserve. High incidences of the human mtDNA and human-associated Bacteroidales markers were observed, especially for human mtDNA with a 92% occurrence in the water samples. Among the 140 samples tested with the human markers, human mtDNA marker and BacHum were concurrently positive in 91 samples and negative in seven samples, representing a 70% convergence for these markers. The remaining samples were detected by either human mtDNA (27.1%) or BacHum samples. These samples were collected over a period of 1 year from nine different sites in a fast-growing suburban watershed, representing natural areas with minimal human impact (Cibolo Preserve) and small urban areas close to the natural environment or surrounded by agricultural activities. The preserve area was overrepresented to observe the potential impact of discharging treated wastewater effluent into the creeks in the preserve. High incidences of the human mtDNA and human-associated Bacteroidales markers were observed, especially for human mtDNA with a 92% occurrence in the water samples. Among the 140 samples tested with the human markers, human mtDNA marker and BacHum were concurrently positive in 91 samples and negative in seven samples, representing a 70% convergence for these markers. The remaining samples were detected by either human mtDNA (27.1%) or BacHum samples.

Table 4 | Spearman’s rank correlation coefficient matrix for human mtDNA, HF183, BacHum, and *E. coli* markers as measured by qPCR

|                  | Human mtDNA | HF183 | BacHum | *E. coli* |
|------------------|-------------|-------|--------|-----------|
| Human mtDNA      | 1           |       |        |           |
| HF183            | 0.571       | 1     |        |           |
| BacHum           | 0.468       | 0.664 | 1      |           |
| *E. coli*        | 0.561       | 0.610 | 0.548  | 1         |
(2.8%). Given that the BacHum marker has a bacterial origin and the human mtDNA marker is derived from human cells themselves, we expected this variability due to the differential persistence of mitochondria and/or mtDNA vs. Bacteroidales in surface water.

Human sewage contamination of environmental surface waters is a serious issue for populations living in fast-growing semi-urban settings and for the surrounding natural environment. Our study clearly showed a high occurrence of human markers in the water samples, even in areas with relatively low human activities (preserve area). This could be attributed to the discharge of municipal WWTP effluents containing considerable levels of fecal waste (Servais et al. 2007; Naidoo & Olaniran 2014) as well as leaking septic tanks. Other sources of fecal pollution include municipal waste from household sewage treatment systems, sanitary sewer overflows, combined sewer overflows, and stormwater and urban runoff (Bakir et al. 2006; Badgley et al. 2010). In the present study, we quantified the level of human fecal contamination in a suburban watershed by an integrated analysis of human-specific markers and FIB. The evaluation of study sites for human fecal markers demonstrates that sewage sources of fecal pollution are major contributors to water quality degradation within the Cibolo Preserve. Thus, the primary source of human mtDNA and human-associated Bacteroidales markers can be attributed to the waste influx from nearby WWTPs. Since creeks in the watershed provide critical recharge for both the Trinity and Edwards aquifers, these sources appear to represent a chronic and relatively constant source of human fecal pollution.

This study demonstrated the correlation of human mtDNA with commonly used fecal pollution indicators. The bacterial markers (E. coli, BacHum, and HF183 assays) showed a moderate correlation with the human mtDNA marker. This is expected based on the difference in survival and the persistence of human and bacterial cells in environmental waters. For instance, most Bacteroidales are obligate anaerobes and die quickly in oxygenated surface water and, therefore, are expected to survive for shorter periods in the environment after release from their hosts (Kapoor et al. 2013; Layton et al. 2013). Conversely, E. coli can grow and persist in environmental waters and sediments, can come from other nonhuman sources such as domesticated animals, and thus, are not inherently correlated with recent human fecal contamination. Based on the results of this study, human mtDNA-based markers could be an indicator of the presence of human fecal pollution in environmental waters (Yang et al. 2015; Kapoor et al. 2017). By comparing a direct marker of human feces (human mtDNA) with bacterial fecal indicators, we were able to overcome the limitations of conventional fecal indicators such as E. coli that yield false positives from bacteria already living in the environment. As our study was limited to samples collected in a localized geographic location, further research is necessary to determine if the relationships among the fecal markers noted here are significantly different when the number of geographically different samples is increased.

The detection of mitochondrial genes via PCR-based assays can be used to identify animal waste directly through its own discharged eukaryotic cells. In addition to our study, the application of mtDNA for fecal source tracking has also been demonstrated in a few recent studies. For example, qPCR assays targeting mitochondrial genes have been used to detect human-, bovine-, and swine-specific contamination (Caldwell et al. 2007; Villemur et al. 2015). A mitochondrial-based microarray (mitoArray) has been developed for rapid identification of the presence of a large number of animals potentially implicated in fecal pollution in mixed activity watersheds (Vuong et al. 2013). It should be noted that although mtDNA genes provide a reliable PCR target, it is still an indirect indicator of the presence of bacterial pathogens and the risk estimation of human health.

The 1-year-long source tracking study provided information regarding the seasonal variability of fecal indicators and their correlation with rainfall events. This study demonstrates the high abundance of the human mtDNA-based marker and their positive association with other indicators of human fecal pollution in the environment. Overall, levels of the human mtDNA marker were detected at similar concentrations to the E. coli-based assay and approximately 1–2 orders of magnitude greater than the HF183 and BacHum assays.

**CONCLUSION**

The study goal was to identify the sources of human fecal pollution in an urban watershed by using human mtDNA
as a direct marker as well as conventional fecal genetic markers. The co-occurrence of human mtDNA with the presence of bacterial fecal indicators was evaluated in sewage-impacted environmental waters. Human mtDNA abundance significantly correlated with various bacterial markers. In addition, the concentration of all the markers increased after rainfall events. By applying this human DNA as a direct indicator of contamination, we were able to overcome the limitations of using microbial markers to more adequately reflect the fecal pollution input. Further investigations are necessary to establish the association of human mtDNA with human fecal pollution in more ambient waters along with water bodies impacted by other fecal pollution sources (e.g., wildlife and agriculture). While more studies should be performed in additional water systems to further elucidate the range of correlation between human mtDNA and fecal bacterial occurrence, the correlations observed in this study broaden the range of potential applications for human mtDNA-based assays. Although limited in the surface area, these results should assist with future risk assessments for semi-urban/rural watersheds, particularly those affected by human fecal pollution, by providing the improved fecal source tracking approach.

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