Profiling Fecal Pollution in Rivers in Hanoi, Vietnam, using Host-specific Bacteroidales and crAssphage Markers

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Abstract. Microbial source tracking (MST) based on host-specific fecal microorganisms is a powerful tool for identifying sources of fecal contamination. In Vietnam, fecal contamination caused by untreated domestic wastewater as well as wastewater from the livestock sector is a major issue for the water environment. In this study, samples from different rivers in Hanoi, Vietnam, were analyzed using quantitative PCR analysis for Bacteroidales markers specific to human (HF183), pig (Pig2Bac), and ruminant (BacR) feces and a bacteriophage marker specific to human feces (crAssphage). Water samples were collected from Nhue River, To Lich River, Kim Nguu River, Day River, Duong River, and Red River during August, September, and December 2019. Groundwater samples from areas near the Nhue River and Day River were also collected. Regarding the To Lich River and Kim Nguu River, only human-specific markers (HF183 and crAssphage) were detected, indicating that the primary source of fecal contamination of these rivers was human feces. In the Nhue River, pig- and ruminant-specific markers were detected, in addition to human-specific markers. Pollution from multiple fecal sources was therefore suspected in the Nhue River. In the Day River, the concentration of pig- and ruminant-specific markers was generally higher than that of human-specific markers. Since the concentration of HF183 and crAssphage in the Day River was lower than that in the other rivers, the contribution of animal feces could be relatively higher in the Day River. The concentration of fecal markers in the Duong River and Red River was much lower than that in the other rivers. MST was successfully applied to characterize sources of fecal pollution in different rivers in Hanoi. The profiling of fecal sources is informative when considering water quality remediation in rivers.

1. Introduction

Rapid urbanization and the growth of the livestock industry caused severe pollution in the water environment. Fecal indicator bacteria (FIB), such as total coliforms and Escherichia coli, are commonly used to assess water quality. However, identifying the exact source of fecal pollution by FIB alone is difficult. Microbial source tracking (MST) is an approach that can circumvent the limitations of FIB, through the use of host-specific bacterial and viral markers [1]. Bacteroidales markers have frequently been used to identify fecal sources from humans and animals including pigs, ruminants, chickens, and ducks [2], while crAssphage is a promising bacteriophage marker of human fecal contamination [3]. In Vietnam, fecal pollution is a major problem due to the limited use of wastewater treatment. Although
many rivers suffer from fecal pollution, the sources of this pollution have not been fully evaluated. In this study, we used MST to characterize fecal pollution in different rivers in Hanoi, Vietnam.

2. Materials and methods

2.1. Sampling
Animal feces, including pig (n = 3), chicken (n = 4), duck (n = 4), and cow (n = 4) feces, were collected from livestock farms in four communes in Hanoi (My Duc, Thanh Oai, Ta Thanh Oai, and Chuong My), from April to May 2019. All samples were stored in a freezer at -20°C until DNA extraction was performed. River water samples (n = 18) were collected from the To Lich River, Kim Nguu River, Nhue River, Day River, Duong River, and Red River in Hanoi in August, September, and December 2019. The Red River is the largest river in northern Vietnam. The Duong River is one of the sources used for drinking water in Hanoi. The Day River is located in a suburban area, while the other rivers are inside the city and are severely polluted with untreated domestic wastewater. Groundwater samples (n = 6) were collected near the Day River and Nhue River. All water sampling points are shown in Figure 1.

2.2. DNA extraction
DNA extraction from fecal samples was carried out using a FastDNA Spin kit for Soil (MP Biochemicals, USA). For water samples, 25 ml of river water samples and 100 ml of groundwater samples were filtered through a 0.22-µm Isopore membrane (Merck Millipore, Germany) to harvest bacterial cells, and then DNA was extracted using a FastDNA Spin kit for Soil.

2.3. Application of MST to fecal samples from Hanoi
To check the sensitivity, specificity, and accuracy of the primers and probes shown in Table 1, the fecal samples were used as positive controls. Water samples collected from the To Lich River and Kim Nguu River were used as surrogates for human feces because these rivers receive untreated domestic wastewater from an urban district. The PCR mixture (50 µl) contained 40.6 µl nuclease-free water, 0.1
µl 100 pmol/µl primer (forward and reverse), 5.0 µl 10×PCR Buffer, 4.0 µl 10 mM dNTP Mix, 0.25 µl TaKaRa Taq EX HS (TaKaRa Bio, Japan), and template DNA. The thermal conditions consisted of 95°C for 2 min followed by 45 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s. Final elongation at 72°C for 5 min was applied. PCR products as well as negative controls were checked using agarose gel electrophoresis. To evaluate the applicability of primers, the following indices, as shown in equations (1), (2), and (3), were calculated:

\[ \text{Sensitivity} (\%) = \frac{TP}{TP+FN} \times 100 \]  
\[ \text{Specificity} (\%) = \frac{TN}{TN+FP} \times 100 \]  
\[ \text{Accuracy} (\%) = \frac{TP+TN}{TP+FP+TN+FN} \times 100 \]

where TP is the number of true positive samples, TN is the number of true negative samples, FP is the number of false positive samples, and FN is the number of false negative samples.

2.4. Quantitative PCR
Due to the low accuracy of chicken and duck feces assays, primers and probes specific for human, pig, and ruminant feces were determined using the TaqMan probe method with a 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, USA). The PCR mixture (20 µl) contained 10 µl TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, USA), 1.0 µl of each primer (10 µmol/l), 1.0 µl of probe (5 µmol/l), 5.0 µl nuclease-free water, and 2.0 µl template DNA. The thermal cycle conditions for all assays consisted of 95°C for 20 s, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s. A serial dilution series of artificially synthesized DNA (5×10^6 to 5×10^1 copies/µl) was used as the standard. The limit of quantification (LOQ) was set at the lowest concentration of the standard, and half of the LOQ values were used for discussion.

2.5. Total coliforms and E. coli
Total coliforms and E. coli were cultivated on Chromocult® Coliform Agar (Merck Millipore, Germany) at 37°C for 24 h.

Table 1. Primers and probes used in this study.

| Target          | Primer/probe         | Sequence (5'-3')                                           | Ref.   |
|-----------------|----------------------|------------------------------------------------------------|--------|
| Human feces     | HF183F               | ATCATGAGTTCCATGTCCG-3'                                      | [4]    |
|                 | BacR287R             | CTTCTCTCTCAGAACCTATCC-3'                                    |        |
|                 | BacP234Probe         | (FAM)-CTAATGGACAGCATCCC-(NFQMGB)                            |        |
|                 | CPQ_056F1            | CAGAAGTACAAAACCTCTCTAAAAACGTAAGAG                          |        |
|                 | CPQ_056R1            | GATGACCAAAACAAAGCCATTAGC                                    | [5]    |
|                 | CPQ_056P1            | (FAM)-ATAAACGATTTACGTGATGAAAC-(MGB)                         |        |
| Pig feces       | Pig2Bac 41F          | GCATGAAATTGAGCTTGTAAATTGAT                                  | [6]    |
| Pig2Bac 163R    |                      | ACCTCATACGGTATTAATCCGC                                     |        |
| Pig2Bac 113MGB  |                      | (FAM)-TCCACGCGGATAGCC-(NFQMGB)                             |        |
| Ruminant feces  | BacR F               | GCGTATCCAACCTTCCC                                          | [7]    |
|                 | BacR R               | CATCCCCCTACGGTACC                                          |        |
|                 | BacR P               | (FAM)-CTTCCGAAAGGAGATT-(NFQMGB)                            |        |
| Chicken feces   | Chicken_qC160F-HU    | AAGGGAGATTAATCCCGGATGAG                                     | [8]    |
|                 | Chicken_qBac265R-HU  | CCGTACCCCGGCTACTAC                                         |        |
| Duck feces      | Duck_qBac336F-HU     | TTGGTCAATGGCGGCGGAAG                                       |        |
|                 | Duck_qDuck474R-HU    | GCACATCCGACACGTGAG                                        |        |

3. Results and Discussion
3.1. Sensitivity, specificity, and accuracy of each marker
The sensitivity, specificity, and accuracy of the host-specific Bacteroidales and crAssphage markers were evaluated using fecal samples collected in Hanoi. The results are summarized in Table 2. Two human-specific markers showed perfect sensitivity, specificity, and accuracy. While the pig- and ruminant-specific markers also showed perfect performance, the chicken and duck assays showed cross-reaction with other fecal samples. The use of these chicken and duck-specific primers was therefore not appropriate in this study, probably because the Bacteroidales in chicken and duck feces in Hanoi could be different from those in the original study [8].

3.2. Total coliforms and E. coli
Figure 2 shows the results of total coliforms and E. coli from the different river and groundwater samples. In the Nhue River, E. coli and total coliform levels were almost equivalent to or slightly lower than those in the To Lich River and Kim Nguu River. Although E. coli were not detected in the groundwater samples collected from near to the Nhue River, total coliforms (6 and 9 CFU/ml) were detected from some groundwater samples. The To Lich River and Kim Nguu River, which directly receive domestic wastewater, exhibited higher concentrations of total coliforms and E. coli.

Table 2. Sensitivity, specificity, and accuracy of target markers.

| Fecal source   | HF183 | crAssphage | Pig2Bac | BacR | Chicken | Duck |
|----------------|-------|------------|---------|------|---------|------|
| Human* (n = 3) | 3     | 3          | 0       | 0    | 3       | 3    |
| Pig (n = 3)    | 0     | 0          | 3       | 0    | 2       | 3    |
| Cow (n = 4)    | 0     | 0          | 0       | 4    | 4       | 3    |
| Chicken (n = 4)| 0     | 0          | 0       | 4    | 4       | 4    |
| Duck (n = 4)   | 0     | 0          | 0       | 4    | 4       | 4    |
| Sensitivity (%)| 100   | 100        | 100     | 100  | 100     | 100  |
| Specificity (%)| 100   | 100        | 100     | 100  | 7.1     | 7.1  |
| Accuracy (%)   | 100   | 100        | 100     | 100  | 27.8    | 27.8 |

*Water samples from the To Lich River and Kim Nguu River.

![Figure 2. Total coliform and E. coli pollution.](image)

In the Kim Nguu River, the sample downstream of wastewater treatment discharge demonstrated lower total coliforms and E. coli, although the levels were still higher than those in the Day River, Red River, and Duong River. The levels of total coliforms and E. coli in the Day River were lower than in the Nhue
River, To Lich River, and Kim Nguu River. It is likely that loading from human activity in the Day River watershed is lower than in the urban areas. While groundwater samples taken from near to the Day River were negative for *E. coli*, a relatively higher level of total coliforms (13 and 265 CFU/ml) was detected. The pollution levels based on FIB were lower in the Red River and Duong River compared with those in the other rivers.

3.3. Characterization of fecal sources in different rivers in Hanoi

The MST method was applied to evaluate the sources of fecal pollution in different rivers. Human-specific (HF183, crAssphage), pig-specific, and ruminant-specific markers were quantified by quantitative PCR, with the results shown in Figure 3. All host-specific markers were widely detected in the water environment. All markers were detected in the Nhue River, indicating loading from multiple fecal sources. The concentration of crAssphage was always higher than that of HF183. HF183, crAssphage, and ruminant-specific markers were detected from groundwater samples near the Nhue River, suggesting that the groundwater in that area is vulnerable to fecal pollution.

The To Lich River and Kim Nguu River are used for urban drainage, collecting domestic wastewater in Hanoi. It is a reasonable finding that human-specific markers were exclusively detected from these drainage channels. One sample was collected from the vicinity of a discharge point of a wastewater treatment plant in Kim Nguu River. A comparison of HF183 and crAssphage abundance before and after the wastewater effluent discharge point demonstrated that the log reduction value of HF183 was 1.9 log₁₀ and the concentration of crAssphage was below the LOQ after the discharge point, indicating that wastewater treatment is effective for the removal of human fecal pollution.

In the Day River, the concentration of pig and ruminant markers was generally higher than human-specific markers. This finding was consistent with the fact that there are many livestock farms in the the Day River.

![Figure 3](image-url)  
*Figure 3. The abundance of target markers in rivers and groundwater. Open plots denote the values corresponding to LOQ/2 (RW: river water, GW: groundwater).*
Figure 4. Relationship between HF183 and crAssphage markers in rivers.

River watershed. All markers were below the LOQ for groundwater samples taken from near the Day River, suggesting that fecal pollution is not a serious problem for the groundwater in this area. Fecal marker concentrations were relatively low in the Duong River and Red River, probably due to the large dilution effect. Ruminant-specific markers showed the highest concentration, followed by pig-specific markers and crAssphage. Although HF183 was below the LOQ, crAssphage were detected.

Figure 4 shows the relationship between HF183 and crAssphage markers in the water environment. Samples with concentrations of both HF183 and crAssphage that were below the LOQ were removed from the analysis. HF183 and crAssphage showed a high correlation coefficient ($r = 0.96$), indicating that the novel crAssphage marker is useful for detecting human fecal pollution. For most of the samples, the concentration of crAssphage was 6.1 times higher than that of HF183. This result indicates that the crAssphage marker is more sensitive than HF183 for detecting human fecal pollution. In Thailand, it was shown that crAssphage demonstrated a better performance compared with that of the HF183 assay [9][10]. The present study shows that the crAssphage marker is also applicable in Hanoi, Vietnam.

4. Conclusion

- Four fecal markers (HF183, crAssphage, Pig-2-Bac, and BacR) were applicable for MST, while chicken- and duck-specific markers demonstrated both low specificity and accuracy. The novel human-specific marker, crAssphage, showed a high correlation with Bacteroidales HF183.
- Urban rivers in Hanoi were characterized by human-specific markers, while pig-specific markers were dominant in rural river samples (Day River).
- The crAssphage marker is generally more sensitive than HF183, indicating that crAssphage is more useful for detecting human fecal pollution.
- Water polluted by feces is used for irrigation and aquaculture in Vietnam and can therefore pose a potential risk to human health. The results of MST are useful when considering effective countermeasures for water pollution in the river water environment in Hanoi.
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