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Detection of Fecal Contamination using Molecular Methods

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Abstract

This study explores the process of designing a molecular method to detect fecal contamination in the urban watersheds of Rock Island and Moline, Illinois. These urban watersheds are known to contain high ammonia levels and total dissolved solids based on previous studies. Additionally there is concern about the aging combined sewer and storm water system. Therefore, these watersheds are thought to have poor water quality including fecal contamination. Existing molecular methods used for detecting fecal contamination require a considerable amount of resources in the laboratory. Our goal was to design a molecular method that requires fewer resources and can continue to be used in laboratories at Augustana College. We will describe the method we have developed and discuss preliminary results.

Introduction

Water is a finite resource that is necessary for life, and it provides a major pathway for the transportation of various pathogens. Furthermore, water contamination is one of the main causes of health problems in human beings (Azizullah et al 2011). In developing countries, more than 2.2 million people die every year due to drinking unclean water and inadequate sanitation (Azizullah et al 2011). Contaminated water sources are not just limited to developing nations that are trying to establish better infrastructure to support their growing populations. Polluted storm-water, streams, and other water sources are also common in the United States as a result of urban environments. Factors such as

impervious surfaces, combined wastewater and storm water systems, and failing or outdated infrastructure such as leaking septic tanks and sewers contribute to the overall water quality of urban watersheds (Young & Thackston 1999). Another aspect of water quality is the presence of particular indicator organisms such as fecal coliform. They indicate the presence of potential pathogenic organisms including *Cryptosporidium* and *Giardia* (Gaffield et al 2003). Some studies measure the amount of total coliform in a given location, which includes fecal coliform and E. coli. However, fecal coliforms and fecal streptococci may be better indicators of pollution by warm blooded animals (rats, chipmunks, rabbits, cats, dogs) than the total coliform group since they are found within the intestinal tract of these animals (Geldreich et al 1968). The presence of these bacteria in storm water can be due to sources such as domestic pet populations, urban wildlife, cross-connections between storm and sanitary sewers, lack of sanitation, rodent habitation in sewers, sediment accumulation in sewers, and deficient solid waste collection and disposal (Marsalek & Rochfort 2004). This is significant because humans exposed to or that come in to contact with the contaminated water can suffer severe health consequences. The most common pathway for the bacteria to travel in order to induce severe health effects is orally, with water acting as an intermediate. Some health consequences that may result from contact include acute gastrointestinal illnesses, skin rashes, ear and eye discharges, bladder cancer, methemoglobinemia and neural tube defects (Gaffield et al 2003).

Due to these health consequences, many researchers have examined various environmental aspects of fecal coliform such as potential sources, components necessary for its survival, which seasons the bacteria is most prevalent, the significance of heavy rain events, the role of sediment as a substrate to facilitate transportation as well as further health implications associated with fecal coliform (Hathaway et al 2014). In order for fecal coliform to survive in an environment, researchers agree that there are certain components necessary for survival. The survival of indicator organisms and their potential of transferring to storm water rely on soil exposure to sunlight, temperature, and frequency of

rainfall, soil moisture, soil pH, organic matter, and the presence of competing organisms (Geldreich et al 1968). Furthermore, the bacteria can be induced into a dormant state caused by stress factors such as UV exposure, temperature changes, and carbon or energy starvation (Bjergbaek et al 2005). Even when in a dormant state, bacteria can still be easily transported and pose a threat to human health. It is widely supported within the literature that there are links between storms and the outbreak of waterborne disease in human populations (Characklis et al 2005). This suggests that rain events accumulate larger quantities of bacteria and efficiently transport them over a longer range. The heavier the rain, the more widespread the bacteria are dispersed. Rain splash can also have a significant effect in displacing bacteria and transferring pathogens to nearby water sources (Boyer et al 2008). Likewise, the slope of an area can increase the likelihood of this occurring because the slope will then increase the speed at which the water is traveling. Furthermore, sediment has the ability to retain bacteria and storms will displace the sediment causing additional transfer of microbes. Therefore, more fecal coliform and E. coli may be found in highly erosive areas. Coinciding with this idea of erosion, rain events can also influence the yield of indicator organisms. The highest concentrations of fecal coliform often occur during winter, and the lowest are observed during the summer (Hathaway et al 2014). These results are correlated with the amount of precipitation received during each season.

City infrastructure including stormwater systems and impervious surfaces, as well the housing density of an area are contributing factors to the state of urban water quality. City infrastructure is a particularly large factor in considering water quality. For example, researchers found that the number of indicator bacterial counts in combined sewage overflows (main bacterial source is sanitary sewage) was higher than compared to storm-water (Marsalek & Rochfort 2004). Therefore, combined sewage and stormwater systems negatively affect the overall water quality of urban water resources than a separate stormwater system. The authors of this study additionally conclude that urban wet-weather pollution, the discharge of stormwater, sewers or overflow, is a major source of impairment of water quality in

receiving waters (Marsalek & Rochfort 2004). In addition, leaking or faulty infrastructure also contributes to the accumulation of bacteria such as fecal coliform thereby threatening water quality. Furthermore, the number of people reliant upon these water networks can also contribute to water contamination levels. Young (1999) found a strong correlation between housing density and fecal bacteria densities implying that bacterial loading is associated with septic tank leakage, which can be masked by other pollutants in the watershed. Similarly, Chin (2010) concluded population density is proportional to terrestrial loading intensity because of leaking sewers, pets, and septic tanks.

The significance of these past findings is relevant because the presence of fecal coliform in urban water sources can cause significant health effects in regards to public health. As previously mentioned, there is a number of health issues associated with the exposure to contaminated water sources. Waterborne diseases consist of diarrhea, nausea, gastroenteritis, typhoid, and dysentery (Azizullah et al 2011). These diseases are largely unreported because they are difficult to identify (Gaffield et al 2003). Gaffield (2003) identified the major sources of these health impacts and estimated that the costs of addressing these impacts was between 2.1-13.8 billion dollars. This includes improving drinking water treatment for 33 billion dollars and improving stormwater management at the cost of 9.3 billion dollars (Gaffield et al 2003). Overall, the academic literature regarding fecal coliform within water resources of an urban environment confirm that the transfer of bacteria is dependent on the amount of precipitation and therefore can vary among seasons. Likewise, city infrastructure such as stormwater systems, amount of impervious surfaces, and the housing density of an area are contributing factors of urban water quality. The presence of fecal coliform in urban water sources relates to public human health because it can cause significant health effects.

The cities of Rock Island and Moline, Illinois, consist of more than 13 urban watersheds that drain directly into the Rock or Mississippi Rivers, which are sources of drinking water for both cities. Starting in 2013, the Augustana College Upper Mississippi Center (UMC) partnered with the two cities to

assess the overall health of these watersheds for the first time. For the past two years, these watersheds have been sampled measuring the levels of nitrate, ammonia, phosphorous, total dissolved solids, total suspended solids, and oxygen, yet the watersheds have not been assessed for fecal coliforms. Furthermore, the cities' storm water and sewer systems are aging and deteriorating. This aging infrastructure could be a source of sewage contamination to the surrounding watersheds. In this study, we sought to examine the presence of fecal coliform in the urban watersheds of Rock Island and Moline, Illinois. The method used to answer this question was structured similarly to one proposed by Field (2003), which consists of using PCR (Polymerase Chain Reaction) to amplify species specific genes in fecal coliform (2003). We explored whether the method of extracting filter membranes for PCR analysis is an effective technique for determining the presence of fecal contamination.

Methods

Sample Sites. Water samples were collected from seven urban watersheds within Rock Island and Moline. Three of the watersheds drain into the Mississippi River, and the other four drain into the Rock River. There were three collection sites for each watershed based on the location of the confluence, ravine and headwater sites established by the Augustana College Upper Mississippi Center. A replicate for one watershed was collected to be used for the positive control. Water samples were collected at three different periods during the summer: July 8-9, August 4-5, and July 25, which represented a rain event (significant precipitation within 24 hours of the sampling event). The water samples were collected in sterile 250ml autoclaved Nalgene bottles and immediately placed on ice.

Filter Extraction. The water samples were filtered using disposable 250mL nitrocellulose filters with a 0.45µm pore size. Each water sample was poured into a separate filter with 250mL beakers placed under each filter to collect the supernatant. Once all of the water had dripped through the filter, the filter membrane was cut using a thoracic surgical scalpel with a disposable blade (feather size 24). To prevent contamination, the blade was sterilized using 90% ethanol and a Bunsen burner between filters.

Periodically, some of the filters would become clogged and the remaining unfiltered water was removed using a sterile pipette. After the perimeter of the membrane was detached, sterile forceps were used to fold the membrane and transfer it to a 250mL conical tube. Once all of the filters were collected for each sampling site they were placed in a -80°C freezer until DNA extraction. To prepare the sampling bottles for the next collection, the bottles were rinsed with tap water and then filled with deionized water. Once dry, the bottles were autoclaved.

DNA purification and extraction. A filter membrane was removed from the conical tube with sterile forceps and placed in a sterile petri dish. It was then cut in half using sterile scissors and placed in a new conical tube with glass beads (0.1 – mm-diameter glass beads were used). 15mL of TBS buffer was added to each tube and the samples were then incubated with shaking overnight at room temperature. The next day, the liquid was transferred to a 15mL conical tube and was centrifuged for 1 hour at 6000 rpm. The supernatant was poured out and the pellet resuspended (which spread up the side of the tube) in 600µl of nuclei lysis solution from the Wizard Genomic DNA Purification Kit (Promega Cat. #A1120). Using a P1000 micropipette, the solution was gently pipetted until the cells were resuspended. The samples were incubated at 80°C for 5 minutes to lyse the cells, and then cooled to room temperature. 3µl of RNase Solution was added, and the tube inverted 2-5 times in order to mix the cell lysate. The solution was incubated at 37°C for 60 minutes and then cooled to room temperature. 200µl of Protein Precipitation Solution was added and the solution was then vortexed vigorously at a high speed for 20 seconds. The solution was then incubated on ice for 5 minutes. After incubation, the sample was centrifuged at 13,000 x g for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube and 600µl of room temperature isopropanol was added. The solution was mixed by inversion. After inversion, the sample was centrifuged at 13,000 x g for 3 minutes. Once centrifuged, the supernatant was poured off and the tube was drained on a clean absorbent paper. Then 600µl of room temperature 70% ethanol was added and the tube was inverted several times to wash

the DNA pellet. The sample was then centrifuged at 13,000 x g for 2 minutes and the ethanol was carefully pipetted in order to not disrupt the pellet. After removing the ethanol, the tube was drained on a clean absorbent paper and the pellet air-dried for 15 minutes. The pellet was resuspended in 50µl of DNase free water and was then incubated at 65°C for 1 hour. Occasionally, the solution was mixed by tapping the tube. The solution was then stored at 8°C overnight for PCR. Before setting up the PCR reaction, a spectrophotometer was used to obtain the concentration of the rehydrated DNA.

PCR. The DNA from the *E. coli* controls and from the water samples were amplified using previously designed primers that detect fecal contamination (Bernhard and Field, 2000). The following primer combinations were used: CF193F and Bac708R, HF183F and Bac708R, HF134F and HF654R, and Bac32F and Bac708R (Table 1 & 2). Each 25-µl PCR mixture contained 50 ng DNA, 5 µl of 5X GoTaq® Flexi Buffer (Promega), 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 2 µl of 10 µM forward primer, 2 µl of 10 µM reverse primer, and 0.2 µl of GoTaq® Flexi DNA Polymerase (Promega) . A thermal cycler was used for all reactions with the following conditions: 94°C for 1 min, followed by 35 cycles consisting of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, followed by a final 6-min extension at 72°C. The PCR products were run on a 1% agarose gel.

Table 1: Primers used in this study

Primer	Sequence (5'-3')
Bac32F	AACGCTAGCTACAGGCTT
Bac708R	CAATCGGAGTTCTTCGTG
CF193F	TATGAAAGCTCCGGCC
HF183F	ATCATGAGTTCACATGTCCG
HF134F	GCCGTCTACTCTTGGCC
HF654R	CCTGCCTCTACTGTACTC

The pairings are as follows: CF193F and Bac708R, HF183F and Bac708R, HF134F and HF654R, and Bac32F and Bac708R. The sequences were obtained from a previously cited study (Bernard & Field 2000).

Table 2: Primer combinations used in this study

Primer Pair	Host Specificity
CF193F-Bac708R	Cow
HF183F-Bac708R	Human
HF134F-HF654R	Human Negative Control
Bac32F-Bac708R	General Fecal Coliform

Host specific genetic markers (Bernard & Field 2000).

Results

Figure 1 is a positive control of E. coli DNA. The master mix (MM) lane contains the PCR master mix with an absence of gDNA, and therefore is a negative control. In examining this lane, there is no amplification indicating that there is no contamination in the lab. Lane 1 is the combination of Bac708R and CF193 primers. E. coli is present in this lane. Lane 2 is the primer combination of Bac708R and HF183F. Since this is the human specific primer combination E. coli is not present in this lane. Lane 3 contains HF134F and HF654R primers. E. coli is not present in this lane because the combination of these primers is a negative control. Lane 4 contains Bac32F and Bac708R. E. coli is present in this lane.

Figure 2 is the amplification of DNA from filter 13R. As in Figure 1, the MM lane is a negative control. Lane 5 contains Bac708R and CF193F primers. The absence of an amplified gene indicates that the fecal contamination at the ravine site of watershed 13 is not from a cow. Lane 6 is the Bac708R and HF183F primer combination. Since there is an absence of gene amplification in this lane, fecal contamination at the ravine site of watershed 13 is not human. Lane 7 contains the negative control primers HF134F and HF654R. As expected, there is no amplification present. Lane 8 is the combination of Bac32F and Bac708R. Amplification is present in this lane, and therefore fecal contamination is present in the ravine site of watershed 13. Furthermore, the fecal contamination is not due to humans or cows.

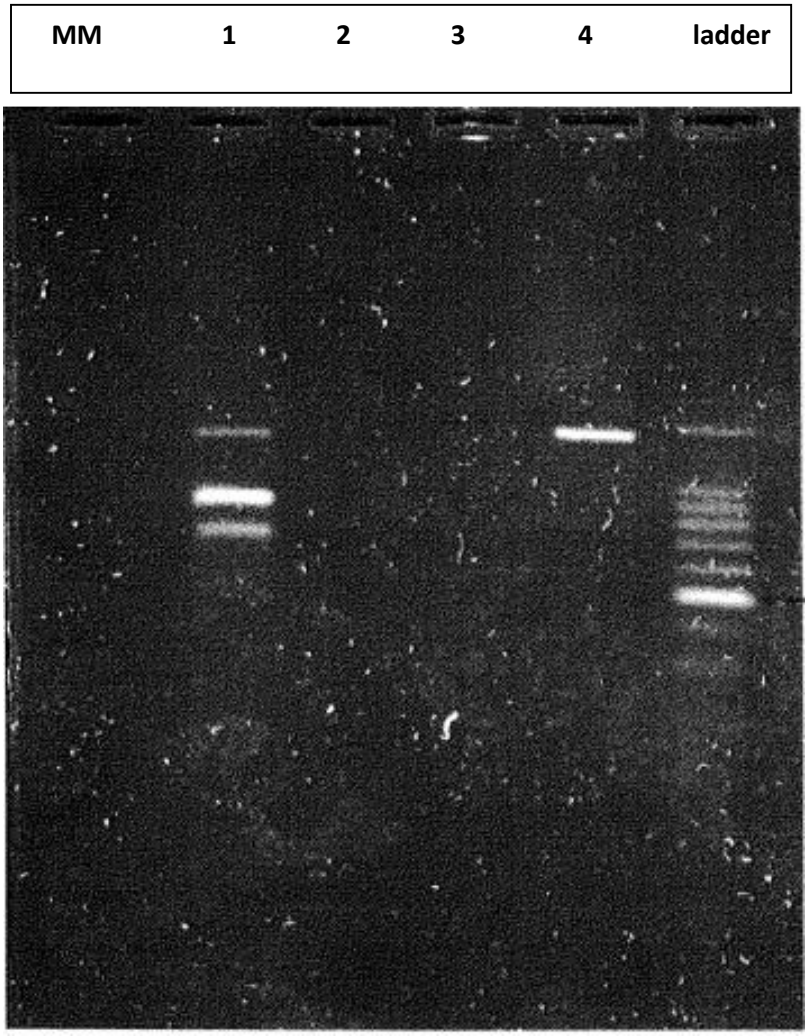

Figure 1: Specific amplification of E. coli DNA using Bacteroides (Bac) primers.

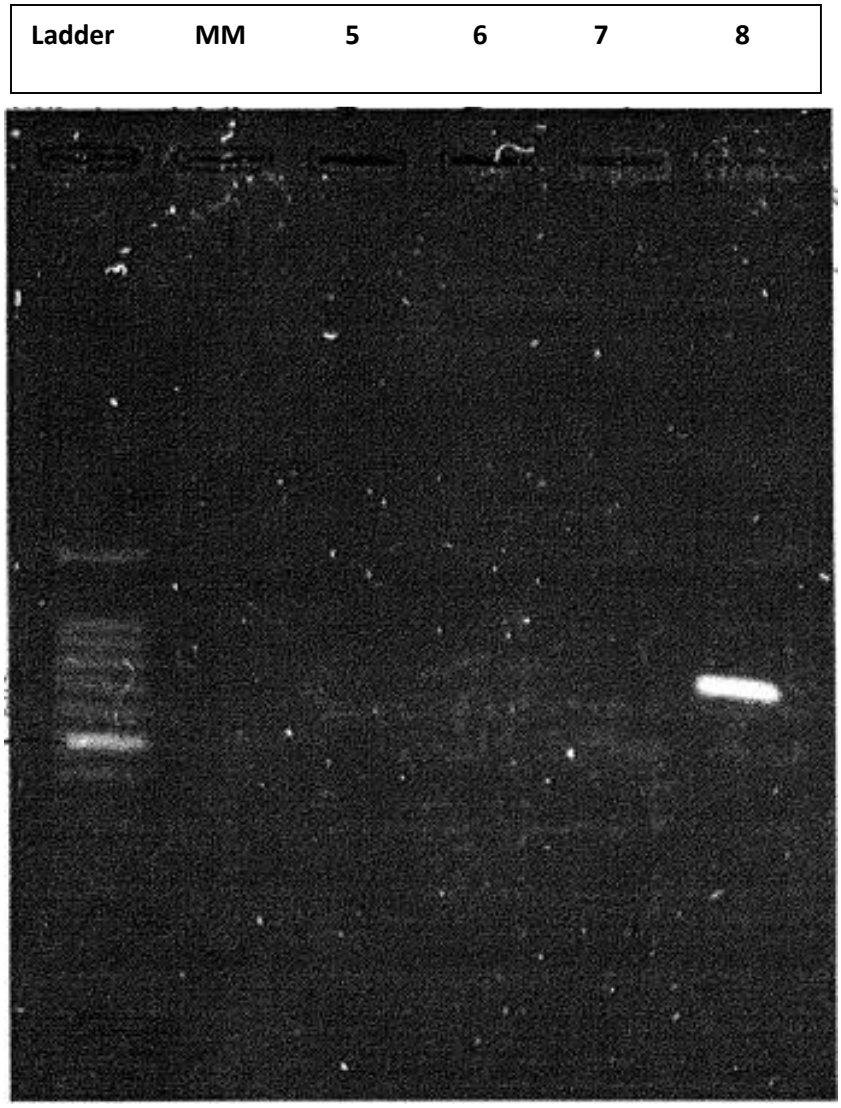

Figure 2: Specific amplification of fecal coliform DNA using Bac primers from filter 13R.

Discussion

Our preliminary results indicate fecal contamination is present at the ravine site of watershed 13 during the time of sampling, June 25. Human and cow fecal coliform was absent at the site, therefore we can conclude the source of contamination is likely due to pets or wildlife. The methods used for this study were developed in order to provide an inexpensive and quicker alternative to more expensive molecular methods and relatively slower microbiology methods, in which researchers are exposed to potentially harmful bacteria. We determined our modified molecular method is a viable option in terms

of expenses. We spend approximately $25 per sample, which includes the sample collection through the PCR reaction. In regards to efficiency, this is more difficult to determine due to a few challenges we had faced.

One of the main challenges we faced was removing the bacteria from the filter for DNA extraction. This delay was eventually addressed by using a bead beating method, which then improved the efficiency of our molecular method. A significant limiting factor of this technique is the average time to filter the water samples, approximately 3.5 hours until the filter drips dry as well as an additional 10 minutes per sample for pipetting clogged filters. This is partially due to the water samples containing many particles filtering through a membrane with a 0.45µm pore size. Another important factor to note, is many large labs use specialized vacuums unique to a glass filter apparatus which helps pull the water through the filter membrane (Field & Bernhard 2003). Since we modified the molecular method by using individual disposable filters, we relied solely on gravity to draw the water through the membrane. A hand pump as well as a water vacuum were both attempted as a means to reduce the amount of time spent filtering the water samples, yet neither option proved effective. Overall, once the samples are filtered the remaining protocol is a standard PCR procedure and is therefore relatively quick. Therefore, despite these challenges, our technique remained an inexpensive alternative to a molecular method for detecting fecal contamination.

The presence of fecal contamination in watershed 13 is significant because fecal coliform is an indicator bacteria representing poor water quality. Furthermore, the presence of fecal coliform suggests that more harmful pathogens are also present including human carcinogens and microorganisms such as *Cryptosporidium* and *Giardia* (Characklis et al 2005). The illnesses associated with urban runoff due to these pathogens include acute gastrointestinal illnesses, skin rashes, ear and eye discharges, bladder cancer, methemoglobinemia and neural tube defects (Gaffield et al 2003). Granted, the filter from watershed 13 is only our preliminary results, but these local watersheds are located near schools, in the

backyards of subdivisions, and drain in the rivers in which the cities receive their drinking water. Due to the presence of the indicator organism, the chance of exposure to a more harmful pathogen is likely. Granted, the use of chlorination for disinfecting waste water will decrease fecal indicator organisms, but not *Cryptosporidium* oocysts (Lalancette et al 2004). Therefore, since fecal contamination is present in watershed 13, it is possible that more harmful bacteria are also present in these waters and may compromise the drinking water regardless of water treatment plants. Overall, more PCR results will help determine the state of water quality in Rock Island and Moline, Illinois.

In conclusion, our preliminary results indicate fecal contamination is present at the ravine site of watershed 13 and we can confirm this contamination is not from humans or cows. For future studies we recommend using a wider variety of specific fecal contamination PCR primers in order to detect the source of contamination. Since our preliminary results indicate fecal contamination is present, a follow-up study would be beneficial to determine the abundance of fecal coliform. This would further help assess the threatened water quality of Rock Island and Moline, Illinois.

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