

2016

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Recommended Citation

Moritzky, Savannah A.; Frost, Victoria; and Heard, Matthew (2016) "Determination of phylo-group diversity of *Escherichia coli* in environmental sand samples collected from a South Carolina beach," *The Winthrop McNair Research Bulletin*: Vol. 2, Article 8.

Available at: <https://digitalcommons.winthrop.edu/wmrb/vol2/iss1/8>

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Determination of phylo-group diversity of *Escherichia coli* in environmental sand samples collected from a South Carolina beach

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ABSTRACT

Escherichia coli (*E. coli*) is a common bacterial species that can persist in many environments found around the world. One environment where it can be found that is of particular concern is an oceanic beach, where it can serve as an indicator of both fecal and microbial pollution. While the majority of strains of *E. coli* are non-pathogenic to humans, some phylo-groups are associated with virulent strains and could cause disease. Therefore, it is of critical concern that we determine where this *E. coli* is coming from and whether it is potentially harmful to human health and well-being. In this study, we attempted to answer this question by using a newly developed molecular technique, which allows us to identify which phylo-groups environmental isolates of *E. coli* belong to. Classification into phylo-groups can help infer the source of the pollution. For this analysis, we collected sand samples from Folly Beach, SC, which is one of the most visited beaches in the Southeastern US. In our analysis, we identified environmental isolates of *E. coli* that differ from the lab strain and belong to two distinct phylo-groups including phylo-group A, which is likely from human fecal contamination and phylo-group B1, which is likely from a domesticated and/or wild animal source. The same molecular technique was altered to test for virulence factors of *E. coli*, and all isolates showed a band corresponding to a virulence factor, but further analysis is needed to determine the validity of this technique. Collectively, our findings indicate that multiple types of *E. coli* are able to persist in these environments and that more research is needed to determine whether these strains are of public health concern.

INTRODUCTION

Escherichia coli (*E. coli*) is a rod-shaped, gram negative bacterium that is very common in the intestines of animals such as humans and birds. The prevalence of *E. coli* make them a common subject for studies such as genetic tests, and much is known about the genome of *E. coli*. Most strains are non-pathogenic, but some pathogenic strains can cause illnesses if ingested. *E. coli* have also been shown to survive outside of their host in many different ecosystems (Halliday et al 2012). Because *E. coli* can be found in so many different environments, they show a vast genetic diversity that can be used to classify them into different groups, which can later be used to determine the original source of the bacteria.

E. coli are also important because they are classified as Fecal Indicator Bacteria (FIB) species, which means that when they are present in an ecosystem, they may indicate signs of

pollution or the presence of other pathogens. This distinction is important because it means that agencies such as the EPA who assess pollution levels can do one test for *E. coli* in a sample and can assume that if *E. coli* are present that other unwanted pathogens may be there as well. Since FIBs have been found to persist in beach sand, it makes the testing of beach sand samples a relevant way to determine whether other unwanted bacteria could be present in places with a large amount of human contact.

E. coli are generally unable to persist in oceanic beach sand and must come from an outside source. Therefore, once the presence of *E. coli* is detected, questions arise about its origin. One way to determine the origin of the bacteria is through a method called multi-plex PCR. Clermont et al. created a type of multi-plex PCR that places *E. coli* into separate phylo-groups, which can be analyzed to determine the potential origin of the *E. coli* (2013). Knowledge

of the origin of the *E. coli* contamination is important for a variety of reasons including preventing it from occurring again. For example, many seagulls in Portugal have been found to harbor multi-drug resistant *E. coli* and are a target of public health concern (Simoes et al 2010). By determining their most likely causes, the people and animals of the beach can be better protected, and the amount present can hopefully be reduced.

Another important consideration is whether or not the *E. coli* that are present in oceanic beach sand are virulent and can cause clinical disease in humans. Each phylo-group also has distinct characteristics including the presence of virulence factors and so determining which phylo-group they originate from can indicate how concerning these pathogens are. For example, in phylo-group B2 and D, strains have been determined to be more likely to cause “extra-intestinal infection” than those in group A or B1 (Clermont et al 2013). Phylo-groups B2 and D have also been determined to have more virulence factors than the others (Johnson and Stell 2000). By analyzing certain virulence factors such as the Shigella-like toxins and attachment proteins, the pathogenicity of the strains of *E. coli* found on the beach can be analyzed and precautions can be taken to prevent these pathogens from growing more abundant on the beach sand.

In this study, we collected samples of beach sand from Folly Beach, South Carolina, to determine the origins of *E. coli* and whether or not the samples are showed signs of virulence. To do this, we used the Clermont method of multi-plex PCR to place the *E. coli* samples into phylo-groups and to determine what animals they could have potentially originated from. The same DNA samples were also used to determine whether or not virulence factors were present. Collectively, this approach allows us to determine the origin of *E. coli* in this ecosystem and whether it is of substantial public health concern.

MATERIALS AND METHODS

We collected sand samples from Folly Beach, South Carolina using sterile conical flasks on three sections of the beach: the dunes,

the intertidal zone, and the subtidal zone. All of these samples were put onto coli plates to determine that *E. coli* was present. The plates that showed the most fluorescence were recorded, and those samples were grown on Levine eosin methylene blue (EMB) agar plates (Neogen 2011).

From the original sand samples, a total of 7.0 grams of sand were added to 35 mL of water in a sterile conical flask. The flask was shaken for 2 minutes and allowed to settle for 30 seconds before 50.0 μ L of water from the flask was pipetted onto an EMB plate and incubated at 37°C. The plates were examined after 24 and 48 hours and single, metallic *E. coli* colonies were re-isolated onto fresh EMB plates until only metallic colonies were growing on the plates. The isolated metallic colonies from each sample were re-inoculated into a broth at 37 °C.

The broth was diluted to the McFarland Standard (1.5×10^8 CFU/mL) and the DNA was extracted using the Qigen miniprep kit. We performed multi-plex PCR using different primers for each test, and each PCR gel was compared to a 100 bp ladder. The environmental strain’s DNA was also compared to a lab strain of *E. coli*, K-12, which is isolated from the human gut. The DNA used in both the phylo-grouping and virulence factor reactions were the same environmental strains isolated.

Phylo-grouping

The sets of primers for the multi-plex PCR are the following: *ChuA1b*, *ChuA2*, *yjaA1b*, *yjaA2b*, TspE4C2.1b, TspE4C2.2b, *Acexf*, and *ArpA1r*. Each primer was used in the amount of 20 pmol (Clermont et al 2013). 1.0 μ L of all of the primers except *Acexf* and *ArpA1r* were added to 10 μ L of DNA and 5.0 μ L of deionized water. For *Acexf* and *ArpA1r* 2.0 μ L was added to the 10 μ L of DNA and other primers along with 5 μ L of deionized water.

A T100 Thermal Cycler was used for the following cycles:

| Temperature (°C) | Cycle Time | Number of Cycles Repeated |
|------------------|------------|---------------------------|
| 94 | 4 minutes | N/A |
| 94 | 5 seconds | 30 cycles |
| 59 | 20 seconds | |
| 72 | 5 minutes | |
| 4 | ∞ | N/A |

Table 1: The cycles used for the phylo-group PCR method.

A 2.0% agarose gel was run at 60 volts for 90 minutes. The bands were compared to Clermont et al. to determine in which clades the *E. coli* strains belong (2013).

Virulence factors

Using the same DNA samples, a virulence factor multi-plex PCR was also performed using the genes *stx1*, *stx2* and *eaec*. Each primer was diluted to 2.0 μM (SOURCE).

1.0 μL of all of the primers were added to 10 μL of DNA and 4.0 μL of deionized water.

A T100 Thermal Cycler was used for the following cycles:

| Temperature (°C) | Cycle Time | Number of Cycles Repeated |
|------------------|------------|---------------------------|
| 95 | 5 min | N/A |
| 95 | 30 sec | 35 cycles |
| 60 | 30 sec | |
| 72 | 90 sec | |
| 72 | 5 min | N/A |
| 4 | ∞ | N/A |

Table 2: The cycles used for the virulence factor PCR method.

A 1.5% agarose gel was run at 90 volts for 185 minutes. The bands were then

compared to the results of Yang et al 2014 to determine if any virulence factors were present.

RESULTS

In the sample collection, we found large amounts of *E. coli* and were able to isolate the following samples:

| Sample Lane | Isolated Region | Time Isolated |
|-------------|------------------------------------|---------------|
| 1 | Human intestines (lab strain, K12) | N/A |
| 2 | Dunes | June 2014 |
| 3 | Dunes | June 2014 |
| 4 | Dunes | March 2015 |
| 5 | Intertidal zone | April 2015 |

Table 3: The sample lanes and the time and location where each sample was found. The same samples were used for both experiments, so this table can be referenced to determine the location of the environmental strain for both gels shown (figure 1 and 2).

The multi-plex PCR reaction for the phylo-groups was compared to Clermont et al to determine to which phylo-group each sample corresponds (2013) (see figure 1). The virulence factor PCR was compared to Yang et al to determine whether or not the virulence factors tested for were present in the sample DNA.

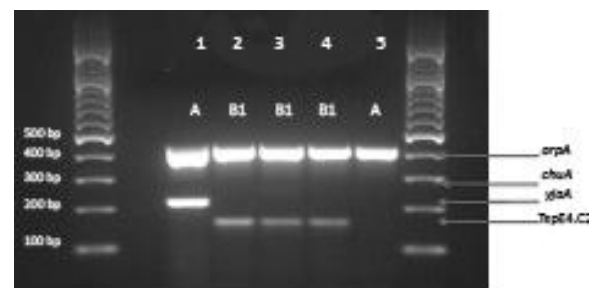


Figure 1: The phylo-group PCR results run on a 2.0% gel. The lane numbers are referenced in table 2. Lane 1 contains bands that are around 350 and 220 bp; lanes 2, 3, and 4 all contain bands that are

approximately 350 bp and 180 bp; lane 5 contains a band that is approximately 350 bp. The gene that each band size corresponds to is also shown on the right.

The K12 in the first lane (figure 1) has been sorted into phylo-group A. Phylo-group A has been known to be associated with humans, so K12, which is associated with the human gut should be sorted there (Carlos et al 2010). This acts as a sort of positive control for the experiment. The DNA found in lane 5 that came from the intertidal zone in April (figure 1) was also found to belong to phylo-group A. The DNA in lanes 2-4 (figure 1) has been sorted into phylo-group B1, which is most closely associated with wild and domestic animals (Carlos et al 2010).

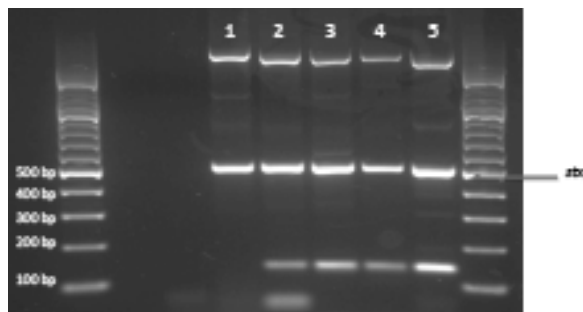


Figure 2: The virulence factor PCR results run on a 1.5% gel. The lane numbers are referenced in table 2. There are bands in lanes 1-4 that are over 2000 bp and a band that is approximately 500 bp. Lanes 2-4 also have a band that is approximately 150 bp, and lane 2 has one that is around 50 bp.

The multi-plex PCR analysis demonstrated that there was a presence of a virulence factor around 500 bp in all of the samples (figure 2). This virulence factor is the gene *stx₂*, which may correspond to the *stx₂* gene.

DISCUSSION

In our study, we found *E. coli* from two different phylo-groups (A, B1) in the sand at Folly Beach, SC (Fig. 1). These findings are of concern because samples from phylo-group A could indicate potential signs of human fecal pollution and those from phylo-group B1 are most closely associated with the fecal waste of

wild and domestic animals (Carlos et al 2010). Since seagulls have been a known source of *E. coli* on oceanic beaches, this is most likely the source of this contamination of the dunes (Simoes et al 2010).

We also found virulence factors in all samples (Fig. 2). This virulence factor identified in our study results from expression of the gene *stx₂*, which is a Shiga-like toxin (Yang et al 2014). However, whether or not if the *stx₂* gene is present in the *E. coli*, there should be little concern by the public about this finding. The *stx₂* gene codes for a Shiga-like toxin that can cause bloody diarrhea and other unwanted symptoms, but without the *eae* gene co-occurring, it is unlikely to cause any harm to the host. This is because the *stx₂* gene is the toxin, but the *eae* gene is the one that codes for the protein that allows it to stick to the membrane before the toxin is released to the cell (Yang et al 2014). Interestingly, the *stx₂* gene was found in the K12 laboratory strain isolated. Generally, the K12 strain does not possess this gene; so further analysis must be done to conclude why this band is being expressed.

An important limitation of this study was that we had a small number samples of *E. coli* with which we could work and that we only collected samples from one beach. Therefore, in future research, we would collect more sand samples from a variety of beaches to use to isolate different strains of *E. coli*. In addition, we would also like to look for signs of antibiotic resistance that may be emerging in these environments. Previous research has shown that phylo-groups B1 and D have been found to harbor many extended-spectrum β -lactamase producing (ESBL) *E. coli* in seagulls (Simoes et al 2010). Here, we found bacteria in phylo-group B1, so further testing of this DNA for signs of antibiotic resistance would be important.

CONCLUSION

From the samples we collected, there were isolates from two different phylo-groups present. Phylo-group A, which usually pertains to humans, and phylo-group B1, which is usually associated with wild and domesticated animals. Along with this, a virulence factor that

codes for the Shiga-like toxin gene, *stx₂*, was found in all of the samples including the K12 strain. The presence of fecal indicator bacteria in beach sand that originates from humans could be a cause for concern that could be linked to some sort of pollution of our beaches by an outside source such as a sewage overflow. The virulence factors found should not be of concern to the public because there were bands that were not co-occurring with those from the *eae* gene, which is necessary to make them pathogenic. Further research is needed to better understand where these bacteria are coming from, and whether or not they pose an imminent threat to beach goers.

community by monitoring sanitary sewage. *App and Environ Micro.* 80(1): 1633-1638.

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