Bacteria and Viruses In Freshwater: A Historical Record of Past Pollustion

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INTRODUCTION

Today numerous bacteria, viruses, and other microorganisms occupy fresh water environments. The majority of these microorganisms are beneficial, however, about 10 percent are very harmful. These harmful microorganisms, also known as pathogens, can cause sickness to humans if consumed. Some bacteria can live in large numbers in the intestine of warm and cold-blooded animals. These are called coliform bacteria, which aid with the digestion of food. Fecal coliform bacteria are associated with fecal material of human and animals (Oram 2014). Fecal coliform by themselves are usually not pathogenic. However, they can become pathogenic when infected by bacteriophages. Learning what type of bacteria live in our freshwater water sources can inform us of how polluted the sources are and where contamination is likely coming from. Most importantly, it can help to improve water quality and prevent future contamination and pollution.

This research was conducted on water sources from Kelvin Grove Fountain, Kelvin River, Loch Ness, and a pond near Fort Augustus known as Jenny's Pond. Bacteria from each water source were isolated, gram stained, spore stained, tested using selective and differential media and lastly identified using PCR of 16sRNA sequence. Also viruses were tested using bacteriophage plaque assay. With this knowledge, the level of pollution at each water source will be identified as well as specific bacteria that may be harmful to humans.

PURPOSE

The purpose of this research is to characterize the bacteria of fecal origin, and to attempt detection of bacteriophage in a local watercourse.

METHODS & MATERIALS

Growing Bacteria: Water samples were collected throughout 6 weeks from Kelvin Grove Fountain, Kelvin River, Loch Ness, and Jenny's Pond. Once the water samples were collected, they were diluted and spread onto a nutrient agar and Yeast Extract Peptone agar (YEP) plate. Undiluted, 10^{-1}, and 10^{-2} were the dilutions that were used. One hundred µl of each dilution was put onto the nutrient and YEP plates and spread using a sterilized glass spreader. Dilutions were put in both 22°C and 37°C incubators for about two nights. After two days, bacteria colonies were seen on the diluted nutrient agar plates. Based on unique color and /or size, bacteria were chosen to be grown in their own culture. The plates were then placed into a 22° C or 37°C incubator overnight to grow individual colonies of the bacteria

Gram staining: Once individual colonies were cultivated, they were gram stained. A heat fixed film was prepared by placing a single colony of bacteria onto a microscope slide. A drop of water was added to make the suspension less dense. The microscope slide was then passed through the flames of a Bunsen burner. Once a heat-fixed film was made, they were stained with gram stain solution for 1 minute. The film was then quickly rinsed with water. Next, the slide was covered with iodine solution for 1 minute. The iodine was poured off the slide and the slide was rinsed under running water. Alcohol/acetone mixture was poured onto the slide to decolorize the smear. This was only on the film for 2 to 4 seconds and was quickly rinsed with water. The slide was then counterstained with dilute carbol-fushsin for about 30 seconds. The slide was washed off and blotted dry. After gram staining, the bacteria were examined under 100x oil immersion objective lens.

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METHODS cont'd

***Spore Staining*:**
Heat fixed films were prepared using the isolates that were cultivated. The slides were flooded with 5% aqueous malachite green and gently heated with an alcohol burner until steam rose. This was done 3 to 4 times for about 1 minute. The slides were left to cool down in room temperature for about 10 minutes. Next, the slides were washed off in running water for 30 seconds. The slides were then counterstained with 0.5% aqueous safranin for 30 seconds. They were then washed in running water to remove the aqueous safranin and blotted dry. The slides were examined under 100x magnification. The isolates that were stained green represented bacteria that had spores while the isolates that stained pink did not.

***Selective & Differential Culture Media*:**
Throughout the duration of the 6-week study, different selective and differential bacteria were used to help identify and classify bacteria. The agars that were used were MacConkey (32g MacConkey base, 800ml H_2O), Mannitol Salt (88.8g MSA, 800ml H_2O), Azide Blood (13.2g Azide Agar, 400ml H_2O, 20ml of 5% sheep blood) and Eosin Methylene blue (30g Eosin Methylene blue agar, 800ml H_2O).

Antibiotic Testing:
Antibiotic testing was done on some of the isolates. Bacteria were swabbed onto a nutrient agar plate. The plate was swabbed thoroughly, making sure that there was no spot that was missed. The antibiotic disc was carefully placed onto the agar plate. The plates were then put into the incubator at 22°C or 37° C depending on what temperature the bacteria grew in for 1 day. The bigger the circle around the specific antibiotic the less resistant the bacteria were.

DNA sequencing:

During the 6 weeks, isolates were sent away for sequencing using the 16s rRNA gene. In order to send the gene out for sequencing, several steps were taken. Including DNA prep, master mix and DNA purification. When the sequence returned back to the lab, they were put into NCBI Basic Local Alignment Search Tool (BLAST) to be identified.

Bacteriophage plaque Assay:

Undiluted, 10^{-1}, and 10^{-2} were the dilutions that were used. for the four water samples. Five ml of the filtered water sample and 1 ml of bacteria (grown in 50ml of nutrient broth overnight) was added to 5ml of soft-top agar. The mixture was mixed using a vortex and poured onto a nutrient agar plate. This was done for each dilution. They were then put into a 37°C or 22°C incubator depending on what temperature the bacteria were known to grow in. The agar plates were left in the incubator for 3 to 4 days. If phages were present in the water plaques would show up on the plates.

RESULTS

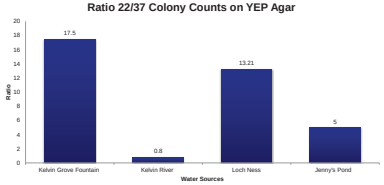

Figure 1. *Figure 1 represents the amount of colonies that were found in each YEP plate at both 22° C and 37C° . They are measured in colony forming units per milliliter (cfu/ml). These numbers were determined from the undiluted sample.*

Colonies from all assays were counted for the 5 locations, which include Kelvin Grove Fountain, Kelvin River, Loch Ness, Cyprus Duck Pond and Jenny's Pond. The ratio of 22° C/37°C was calculated and showed that Kelvin Grove water Fountain and Loch Ness were not polluted water sources. However, the Kelvin River, Cyprus Duck pond and Jenny's pond are polluted according to the ratio calculated. The Kelvin Grove Fountain had the highest ratio, which was 17.5. Loch Ness also had a high ratio of 13.21. The Kelvin River had the lowest ratio, which was 0.8. Jenny's Pond had a low ratio of 5.

Sample #	Species	Location	Gram Stain	Spores	Shape	Pathogenic
1	Bacillus pumilus	Kelvin G. Fountain	+	Yes	Bacillus/ Rod	Yes
2	Aeromonas aquatic	Kelvin G. Fountain	-	No	Bacillus/ Rod	Yes
3	Corynebacterium sp.	Kelvin G. Fountain	+	No	Bacillus/ Rod	Yes
4	Aeromonas salmonicida	Kelvin G. Fountain	-	No	Bacillus/ Rod	Yes
5	Bacillus licheniformis	Kelvin G. Fountain	+	Yes	Bacillus/ Rod	No
6	Bacillus licheniformis	Shower head	+	Yes	Bacillus/ Rod	No
7	Unknown	Water-Fountain	+	No	Cocci	No
9	Bacillus infantis	Kelvin R.	+	Yes	Bacillus/ Rod	No
10	Lysinibacillus sphaericus	Kelvin R.	+	Yes	Bacillus/ Rod	Yes
11	Wautersiella falsenii	Kelvin R.	-	No	Cocci	No
12	Shewanella putrefaciens	Kelvin R.	-	No	Bacillus/ Rod	No
13	Bacillus aerius	Kelvin R.	+	No	Bacillus/ Rod	No
14	Chromobacterium violaceum	Kelvin R.	-	No	Bacillus/ Rod	No
15	Bacillus pocheonensis	Kelvin R.	+	No	Bacillus/ Rod	No
16	Janthinobacterium sp.	Jenny's Pond	-	No	Bacillus/ Rod	No
17	Pseudomonas fluorescens	Jenny's Pond	-	No	Bacillus/ Rod	No
18	Bacillus amyloliquefaciens Sp.	Jenny's Pond	+	Yes	Bacillus/ Rod	No
19	Unknown	Loch Ness	-	No	Bacillus/ Rod	No
20	Unknown	Loch Ness	-	No	Bacillus/ Rod	No
21	Unknown	Kelvin R.	-	No	Bacillus/ Rod	No
22	Unknown	Kelvin R.	+	Yes	Bacillus/ Rod	Yes
23	Bacillus subtilis	Kelvin R.	+	Yes	Cocci	Yes
24	Bacillus sp	Kelvin R.	+	Yes	Bacillus	Yes

Table 1. *Table 1 shows a total of 24 bacteria that were sampled and their different characteristics.*

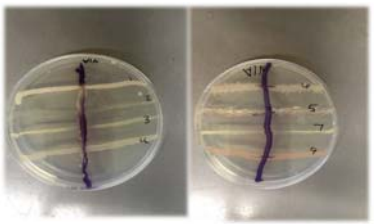

Figure 2. *Antibiotic testing of Chromobacterium violaceum on bacteria samples. Sample 3 and 7 show the bacteria not growing the closer they get to Chromobacterium violaceum. This indicates that they are susceptible to the antibiotic*

DISCUSSION

When comparing the 4 samples that were taken, it was shown that Loch Ness and the Kelvin Grove Fountain were considered unpolluted. However, Jenny's Pond and the Kelvin River were very polluted. The Kelvin River was shown to be the most polluted out of all the water sources with a ratio of 0.8. In order to find how polluted the water sources were, the amount of colonies found on each YEP plate at both 22° C and 37° C was counted and converted to colony forming units per milliliter (cfu/ml). If the ratio was less than 10, the water was considered polluted. However, if the ratio was greater than 10, the water was consider unpolluted. .

The Kelvin River was also the only water source that formed plaques during the bacteriophage assay. However, it only formed 13 plaques with the *E. coli* DS902 and not chosen bacteria found from the Kelvin River. This shows that there are phages in the water that can attack certain bacteria, however not the bacteria that were chosen. It also shows that there is a historical record of past pollution because viruses need hosts to survive and fecal coliforms such as *E. coli* are most likely their host. Even though plaques did not form using the other water sources, there is reason to believe that there are viruses in Jenny's Pond due to the fact that it is highly polluted.

One bacterium found in the Kelvin River that was interesting was *Chromobacterium violaceum*. It is a gram-negative rod that is found in the soil and water of subtropical areas (J. Lee, 1999). What makes this bacterium interesting is that it produces a natural antibiotic known as violacein. Violacein may be useful for treatments of colon and other types of cancer (Kumar, 2012). *Chromobacterium violaceum* was tested as an antibiotic source for the other isolates that were identified in this research. Sample 3, Corynebacterium *spp* were susceptible to the antibiotic as well as sample 7, which is unidentified.

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