Identifying Sources of Fecal Coliforms in Lake Macatawa via Antibiotic Resistance Analysis

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INTRODUCTION

Fecal coliforms are bacteria that live in the gastrointestinal tract of warm-blooded animals and are commonly found in human and animal feces. Fecal coliforms can be used as indicator organisms of fecal contamination of water. Fecal coliforms may be organisms that are pathogenic themselves (e.g. *Escheria coli*), but are also often associated with various other pathogenic organisms. Thus, coliform counts are used in federal standards for both recreational and drinking water safety. *E. coli* and *Enterococcus faecalis* are the two most commonly monitored "coliform" species. Both bacterial species are common intestinal organisms, though only *E. coli* is technically considered a fecal coliform. However, *E. fecalis* is often included in coliform counts because it has been found to show a higher correlation with human pathogens than most fecal coliforms (Jin et al. 2004). In the United States federal drinking water quality standards dictate a count of less than 1 coliform/100 ml. Recreational standards are less stringent and therefore may contain no more than 200 coliforms/100 ml, or less depending on the bacterial species used for monitoring. The Ottawa County Health Department monitors the E. coli levels at Dunton Beach on Lake Macatawa. For the past seven years there has been a marked increase in beach closings due to coliform counts that exceed water quality standards (Ottawa County Health Department 2009). The source for the increased coliform levels is yet unknown. There are many possible sources for coliforms in the lake including human waste, waste from livestock runoff, and waste from wild animals. This study aimed to determine sources of fecal coliforms using antibiotic resistance analysis.

Antibiotic resistance analysis is a tool used in many fields to determine the resistance of a given strain of bacteria to an individual or range of antibiotic(s). Certain bacteria are considered “naturally” resistant to certain antibiotics. Bacteria that are naturally resistant to a given antibiotic lack the physical structures or biochemical pathways by which the antibiotic uses as its mode of activation. Other bacteria “acquire” resistance to antibiotics due to exposure to an antibiotic, which promotes the development of resistant strains, usually by acquiring resistant plasmids that contain genes that in some way deactivate a given antibiotic. By testing coliforms from a given known organism against an array of antibiotics, one can determine a specific pattern of antibiotic resistance for that organism. Then, coliforms from an unknown animal source can be tested for antibiotic resistance, and the source of the coliform can be determined by comparing its resistance patterns to those of known coliform sources.

One way of testing antibiotic resistance is by an antibiotic disk assay. In this assay a given bacteria is used to create a “lawn” of bacteria on an agar plate (typically, Mueller Hinton agar is used because it provides a good diffusion medium). To ensure that the relative same amount of bacteria are coated onto each agar plate, a standard of turbidity called the McFarland standard is used and compared to the turbidity of bacterial suspensions to be tested. After a lawn has been created, disks of antibiotic may be placed on the lawn. After incubation the diameter of the ring of inhibition (zone of no bacterial growth) created around a disk can then be translated into an organism’s degree of resistance. Previous studies in other areas of the country have successfully used antibiotic resistance analysis to differentiate between sources of fecal contamination in local waters

(Harwood, Whitlock, and Withington 2000; Whitlock, Jones, and Withington 2002; Wiggins 1996).

The objective of this study was to find the host source (human, wild animal, or domestic animal) of fecal coliforms in the Dunton Park area of Lake Macatawa. The underlying assumption of this study is that humans, wild animals, and domestic animals will each have characteristic resistance patterns to individual antibiotics due to the different manner and types of antibiotics commonly used (or not used) within each group.

METHODS

Sample Collection and Bacterial Isolation

Samples were collected from the following sources: water from Dunton Park Beach close to the Holland wastewater treatment plant effluent (unknown source), wastewater treatment influent ("human" source), feces from multiple ducks ("wild" source), feces from multiple seagulls ("wild" source), feces from a cow treated medicinally with antibiotics ("domestic" source), feces from a cow not treated with antibiotics ("domestic" source), feces from multiple chickens not treated with antibiotics ("domestic" source). Once samples were collected, they were brought to the lab, stored at 3-5°C and plated onto selective media within 8 hours. Samples from animal sources and the wastewater treatment plant were first placed in a saline solution (8.5gNaCl/1000ml H_2O) and then dilutions were performed to create spread plates with multiple isolated bacterial colonies. Membrane filtration was used to isolate colonies from Dunton Park water samples. All samples were plated onto two types of media M-Enterococcus and M-Tec, for isolating *Enterococcus* and *Escheria* species, respectively. Samples on M-Enterococcus were incubated at 37°C for 48 hrs and samples on M-Tec were incubated at 45°C for 24 hrs.

Up to 15 pure cultures of different bacterial strains (which were created from individual, isolated colonies on the spread plates) were created for each sample type (human, Dunton Park water, duck, seagull, cow (possible antibiotic use), cow (no antibiotic use), chicken).

Antibiotic Disk Assay

A McFarland standard was prepared using 99.5mL of 1% H_2SO_4 and 0.5mL $BaCl_2$. Using a sterilized inoculating loop, bacteria from pure cultures were added to test tubes containing saline, using a separate tube for each sample replicate (replicates = various bacterial strains from each sample type). The turbidity of each bacterial suspension was compared to the McFarland standard and the amount of bacteria or saline was adjusted until turbidities matched. A sterile cotton swab was dipped into each bacterial suspension (bacterial replicate) and swabbed across the entire surface of a Mueller-Hinton (MH) agar plate to create a uniform lawn of bacteria across the plate (one MH plate per bacterial suspension). Next, four different types of antibiotic disks were placed on the lawn on each MH plate. A total of eight antibiotics were tested (Ampicillin, Chloramphenicol, Ciprofloxacin, Erythromycin, Penicillin, Streptomycin, Sulfamethoxazole trimethoprin, tetracycline), though not every antibiotic was tested on every strain. Each strain was tested against a minimum of four antibiotics and a maximum of eight antibiotics; strains tested for all eight antibiotics were randomly selected. After placing the antibiotics on MH plates, the plates were incubated for 24 hrs at 37°C. After incubation the diameter of each ring was measured and correlated into sensitive, intermediate, or resistant using Table 1.

Antibiotic	**Resistant (< or =)**	**Intermediate**	**Sensitive (> or =)**
Ampicillin			
E. coli	11	12-13	14
E. faecalis	16		17

Chloramphenicol	12	13-17	18
Ciprofloxacin	15	16-20	21
Erythromycin	13	14-22	23
Penicillin E. coli E. faecalis	 NA 14	 NA	 NA 15
Streptomycin E. coli E. faecalis	 11 6	 12-14 7-9	 15 10
Sulfamethoxazole-Trimethoprin	10	11-15	16
Tetracycline	14	15-18	19

The means and standard deviations of each zone of inhibition were calculated using Excel for interpretation of results.

RESULTS

Of the eight antibiotic disks used in this study, only 4 were usable in the results. Chloramphenicol and Sulfamethoxazole Trimethoprin were not used because all of the replicates were sensitive to the antibiotics. Penicillin was not used because all the replicates were resistant to the antibiotics. Ciprofloxacin was not used in the results, as well as Ampicillin in the cow, duck and chicken results, because enough data was not collected using these antibiotics.

Six to sixteen replicates were made for each sample. From each sample, averages and standard deviations were calculated from the replicates. These averages were then compared to the averages calculated from the Dunton Park sample. Similarities were found in many of the samples. In the following tables, the color code is explained in the legend by the first table. The first value in the cells is the ring of resistance average measured in millimeters. The second value in the cells is that standard deviation for that average.

In the seagull sample, similarities were found in *E. coli* and *E. faecalis* when tested against tetracycline. Both *E. coli* and *E. faeclais* were sensitive to the antibiotic. *E. faecalis* was also similar to Dunton Park when tested against erythromycin. *E. faecalis* had an intermediate resistance to this antibiotic. There were also three averages from the seagull values close to, but not the same as the Dunton Park sample in terms of resistance. These were ampicillin and erythromycin for *E. coli*, as well as streptomycin for *E. faecalis*. The seagull sample was most similar to the Dunton Park sample.

	Seagull		**Dunton Park**	
	E. coli	*E. faecalis*	*E. coli*	*E. faecalis*
Ampicillin	13.3+/-3.2	21+/-0	20.5+/-2.1	
Erythromycin	9.4+/-4.58	18.28+/-7	16.5+/-5.8	20.4+/-10.5
Streptomycin	17+/-6.93	0+/-0	6+/-8.5	8.4+/-14.7
Tetracyline	21.7+/-12.4	25.7+/-6.5	31.17+/-15.7	30.7+/-15.9

Sensitive Intermediate Resistant

In the Human sample collected from BPW, similarities to Dunton Park were found again in both *E. coli* and *E. faecalis* when tested again tetracycline. Both averages were sensitive to the antibiotic. *E. faecalis* was intermediate in resistance, like Dunton Park, when tested by streptomycin. There were also two averages from the human sample close to, but not the same resistance as Dunton Park. *E. faecalis* resistance ring averages were, when tested against erythromycin, close to the Dunton Park averages. *E. coli* averages, when tested against streptomycin, were close to the Dunton Park averages.

	Human		**Dunton Park**	
	E. coli	*E. faecalis*	*E. coli*	*E. faecalis*
Ampicillin	5.7+/-9.8	33.5+/-3.5	20.5+/-2.1	
Erythromycin	6.75+/-5.7	23.8+/-5.07	16.5+/-5.8	20.4+/-10.5
Streptomycin	12.3+/-10.69	8+/-5.3	6+/-8.5	8.4+/-14.7
Tetracyline	24+/-7.7	28.4+/-2.3	31.17+/-15.7	30.7+/-15.9

In the sample from the cow that could have received

	Cow with Antibiotic		**Dunton Park**	
	E. coli	*E. faecalis*	*E. coli*	*E. faecalis*
Erythromycin	16+/-2.63	18+/-5.27	16.5+/-5.8	20.4+/-10.5
Streptomycin		12+/-3.1	6+/-8.5	8.4+/-14.7
Tetracyline	30+/-2.38	11+/-11.33	31.17+/-15.7	30.7+/-15.9

	Cow without Antibiotic *E. faecalis*	Dunton Park *E. faecalis*
Erythromycin	16.7+/-13	20.4+/-10.5
Streptomycin	0+/-0	8.4+/-14.7
Tetracyline	19.3+/-15.97	30.7+/-15.9

antibiotics, there were similarities in both *E. coli* and *E. faecalis* when testing them against erythromycin. Both were intermediate in terms of resistance ring diameter. There was also a similarity in *E. coli* when tested against tetracycline and compared to Dunton Park. *E. faeclais*, when tested against streptomycin, was close to the Dunton Park sample. In the cow sample that did not contain antibiotics, *E. faecalis* was similar to Dunton Park when tested against erythromycin as well as tetracycline. Streptomycin showed some similarity as well, but the averages were not classified as the same resistance.

In the duck and chicken samples, there were no similarities found when comparing the different classifications of resistance. However, when tested against streptomycin, both samples were close to the Dunton Park sample in terms of ring of resistance diameter.

	Duck *E. faecalis*	Dunton Park *E. faecalis*
Erythromycin	31+/-3	20.4+/-10
Streptomycin	0+/-0	8.4+/-14.7
Tetracyline	15+/-15	30.7+/-15.9

	Chicken *E. faecalis*	Dunton Park *E. faecalis*
Erythromycin	9.2+/-9.4	20.4+/-10.5
Streptomycin	6+/-13.4	8.4+/-14.7
Tetracyline	7.6+/-10.2	30.7+/-15.9

Even though the data seems to point to humans, seagulls, and cows as being the main sources of fecal coliforms in Lake Macatawa, no conclusions can be made. The standard deviations for these data are usually half of the actual average and sometime are more than the average. Therefore, it is obvious that the data is not statistically significant. A guess can be made as to what group contributes the most feces to Lake Macatawa, but it cannot be proven.

DISCUSSION

Seagulls, humans, and cows have been the suggested source of *E. Coli* in Lake Macatawa according to this experiment. Although, the experiment does not have enough

evidence to conclude that these are, in fact, the main sources. There is reason to believe that the process used in this experiment can produce reliable results with further in-depth testing. Antibiotic resistance analysis is time consuming which makes it difficult to create dependable results. It takes time to make agar plates, incubate bacteria, produce pure cultures of specific bacteria, and test a variety of antibiotics on all samples. At minimum, it takes 6 days to complete this process. Resources, funds, and time can help produce dependable results. This project used about 500 agar plates and only tested a few samples. This experiment needs to be magnified immensely for a future study to be carried out. Not only will there need to be more agar plates, but more samples will need to be collected and tested. If there are more samples to test, there will be a better representation of each population which will make the results more reliable.

It costs about one dollar per culture using antibiotic resistance analysis where as DNA testing costs about sixty to one hundred dollars per culture. Antibiotic resistance analysis seems to cost less money, but DNA testing takes much less time to receive dependable results. Both processes have proven to provide dependable results with enough people, time, and money backing the experiment. This trial cannot conclude that it would be more cost effective than DNA testing because it does require resources and time to find results that will be representative of each population. Antibiotic resistance analysis will work with more time allotted.

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