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Detection Of Possible Pathogenicity Of Antibiotic Resistant *Escherichia Coli* Isolated From Urban Playa Lakes And The Feces Of Canada Geese And Resident Waterfowl In Lubbock, Texas

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Abstract

In a collaborative effort to study the effects of migratory and domestic waterfowl on the water quality of urban playa lakes, the Lubbock Christian University Natural Science, Chemistry and Biochemistry departments have isolated *Escherichia coli* from playa lake water and the feces of migratory Canada geese (*Branta canadensis*) and resident waterfowl. In previous studies, it was determined that forty isolated samples from a pool of eighty total isolated samples were resistant to at least one antibiotic. In this study, samples of the antibiotic resistant *E. coli* were further analyzed for the presence of Shiga-like toxin producing genes (stx_1 and stx_2) and the presence of enterohemolysin. Methods for determining the presence stx_1 and stx_2 included isolation of genomic DNA followed by Polymerase Chain Reaction. The PCR product was then sequenced using Beckman Coulter's genomic services. Sequences produced from the shiga toxin primers were compared to known gene sequences, which allowed for positive identification of Shiga toxin-producing *E. coli* (STEC). Methods for determining the presence of enterohemolysin consisted of growing isolates on tryptic soy agar medium supplemented with 5% sheep's blood and, after incubation, observing the plates for hemolysis. *E. coli* producing Shiga-like toxin and enterohemolysin can cause various gastrointestinal complications including hemolytic uremic syndrome (HUS), acute renal failure, and end stage renal disease. In a comparison of the PCR product for the stx_1 gene with the sequence of O157:H7, 2 of 33 samples have a 92% or greater sequence similarity. In the product obtained from amplification of the stx_2 gene, 7 of 38 samples have a 92% or greater sequence similarity. Of the 40 samples, 2 exhibited alpha-hemolysis. The isolated *E. coli* containing the shiga toxin genes of stx_1 or stx_2 and/or enterohemolysin,are potentially pathogenic and may present a health risk to individuals and animals in contact with the affected playa lakes. Urban playa lakes in close proximity to residential areas are often used for recreational activity including fishing and non-motorized boating. Some dog owners allow their pets to swim in the water, and although swimming is prohibited, people have been observed wading. Any water contact could pose exposure to potentially pathogenic *E. coli*.

Keywords: Shiga toxin, *E. coli*, Enterohemolysin

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains are known human pathogens that cause a variety of disease including diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), which can cause kidney failure and is potentially fatal.[1, 2, 3] STEC have historically been responsible for a number of outbreaks. In 1992, The United States had an outbreak of 583 cases across four different States, and of these infections, 41 developed HUS and 4 children died.[4] During 1996, there were more than 17,877 cases of STEC infection in Japan, which included 12 fatalities.[5] More recently, in 2011, 3,800 cases and 54 fatalities in northern Germany and surrounding countries were attributed to

STEC infection.[6, 7] All three outbreaks were due to the consumption of STEC contaminated food products. The most common STEC serotype is O157:H7, and it is this strain that was the causative agent of the 1996 outbreak in Japan and the 1992 outbreak in the U.S. However, 20-50% of all cases annually in the U.S. are due to non-O157 serotypes[8, 10], and such was the case in Germany with O104:H4 as the causative agent.[1] Many outbreaks involving pathogenic *E. coli* infections are associated with ingesting contaminated food[11, 12], raw milk[12], or contaminated vegetables[10]; however, infection also occurs from the ingestion of recreational river or lake water. It is known that fewer than 100 cells constitute an infectious dose[4, 9, 13], thus contact with a body of water that contains STEC is a health concern.[9]

E. coli is defined as STEC whenever at least one type of shiga toxin proteins is expressed in the organism.[1] Shiga toxins are classified in two main categories: Stx1 or Stx2[14, 15] (encoded by genes stx_1 and stx_2 respectively). Shiga toxin is an exotoxin that induces the apoptosis of renal and lung cells; Stx1 is preferential for lung tissue and Stx2 for renal tissue.[13] It is proposed that the cells of the alveoli and glomerular apparatus are rich in Gb3 receptors specific to the respective shiga toxins.[13] The mechanistic action for each type of Shiga toxin is similar. After the STEC colonizes the intestine, the shiga toxin travels in the bloodstream to the target cell. The toxin then binds to a globotriasylceramide (Gb3) receptor on the plasma membrane of the target cell and the receptor-toxin complex is internalized.[2] Through a retrograde process, the protein receptor complex is cleaved and activated in the endoplasmic reticulum, where the now active A1-subunit will enzymatically disrupt cytoplasmic ribosomes. This will ultimately disrupt protein synthesis inducing apoptosis.[13, 16] It has been determined that if *E. coli* contain the stx_2 gene, it is more likely to cause HUS than if it only contains stx_1, thus its presence is of great health concern to humans.[17, 18]

Hemolysin (also known as enterohemolysin in hemolytic strains of STEC[5]) is another factor that contributes to virulence.[14, 19] Hemolysin is not present in all pathogenic strains of *E. coli*, however its presence can cause harmful complications. Strains of *E. coli* that contain at least one of the shiga toxins and hemolysin are termed as Enterohemorrhagic *Escherichia coli* (EHEC).[7] Hemolysin was expressed in the causative organism of the outbreaks in Japan and the United States mentioned previously.[4, 7] The mechanistic action of enterohemolysin begins by secretion from the EHEC in an outer membrane vesicle (OMV). The OMV-hemolysin complex can then target a red blood cell in which it will create a pore of approximately 2 nm in diameter in the cell membrane, lysing the erythrocyte.[20, 21] Alternatively, the OMV-hemolysin complex can be internalized by host microvascular endothelial and intestinal epithelial cells. Once in the cell, the hemolysin is separated from the OMV and targets the mitochondria. The membrane potential of the mitochondria is disrupted, impairing ATP production, which ultimately begins a pathway resulting in apoptosis.[22]

The natural reservoir for STEC/EHEC is the intestinal tract of cattle[11] and other organisms that have a ruminant digestive system.[23] It is proposed that these animals have a low concentration of Gb3 receptors on the surface of renal and lung tissue and are therefore unaffected by the cytotoxigenic effects of Shiga toxin.[13]Additionally, it has been shown that pigeons, gulls[13, 24, 25], and of particular interest, Canada Geese (*Branta canadensis*) and Mallard Ducks (*Anas platyrhynchos*)[26], can be vectors of pathogenic *E. coli*. It is postulated that these birds eat from sources which are contaminated with bovine fecal matter, and thus become carriers of STEC.[25] Birds can then deposit these organisms in bodies of water or in areas which run off into waters used for recreational purposes.[26]

In a collaborative effort to study the effects of migratory and domestic waterfowl on the water quality of local urban playa lakes, the Lubbock Christian University Natural and Physical Sciences departments have isolated *E. coli* from playa lake water and the feces of migratory Canada geese and resident waterfowl over a three year period. Previous studies with the ecology of the playa lakes, and particularly the *E. coli* within and around the lakes, has shown that the *E. coli* is deposited by the migratory and domestic waterfowl on the bank soil, and is subsequently washed into the lake by rain and water run off.[27] Temperature and precipitation have also been found to be factors that effect the bacterial counts found in the lake.[27] Additionally, it was determined that forty of the isolated *E. coli* samples were resistant to at least one clinically used antibiotic including ampicillin, tetracycline and streptomycin.[28, 29]

In the present study, the antibiotic resistant samples were genetically analyzed to determine the presence of stx_1 and stx_2 genes, and were analyzed to determine the expression of enterohemolysin. If these virulence factors are present in the samples, then the *E. coli* found in the playa lakes of Lubbock is potentially pathogenic, and could possibly be highly virulent. Urban playa lakes are often used for recreational activity and are in close proximity to residential areas. The shallow urban playa lakes in Lubbock, Texas collect rainwater seasonally. Recreational water has recently been the topic for recent study in the transmittance of pathogenic *E. coli*.[9, 26] The presence of antibiotic resistant STEC would be of significant clinical importance for the city of Lubbock, Texas.

2. Materials and Methods

2.1 Detection Of Stx_1 And Stx_2

Previously isolated strains of *E. coli* from the feces of Canada Geese, domestic waterfowl, and from playa lake water samples are stored in glycerol stock suspensions at -80 °C. The 40 samples that had previously shown resistance to at least one antibiotic[28, 29] were transferred to tryptic soy agar (TSA) medium and were then isolated into pure culture. The cultures were transferred to Luria HiVeg Broth, incubated overnight at 35 °C, and 1 mL of culture was centrifuged to a cell pellet for DNA extraction using UltraClean Microbial DNA Isolation Kit. DNA was stored at -20 °C.

Each DNA sample was quantified using the Qubit 2.0® fluorometer dsDNA BR Assay. Polymerase Chain Reaction (PCR) for amplification of stx_1 and stx_2 genes was performed in 50 µL solution for each sample, which included 50 ng of DNA, 4% dimethyl sulfoxide (DMSO), 0.5 µM of forward and reverse primers listed in Table 1, 25 µL of iProof HF Master Mix manufactured by Bio-Rad, and PCR grade water. This solution was placed on the thermocycler and the DNA was initially denatured for 3 minutes at 98 °C. Thirty-five cycles were then performed which included these conditions: denaturation for 10 seconds at 98 °C, annealing for 45 seconds at 61 °C, extension for 1 minute at 72 °C. Concluding the 35 cycles, final extension was allowed for 5 minutes at 72 °C. PCR product was stored at -20 °C.

PCR product was sequenced by Beckman Coulter Genomics. Sequences were then compared using the online BLAST function on the website of the National Center for Biotechnology Information (NCBI). Sequences given from PCR were compared to known pathogenic strains of *E. coli* with a 92% sequence similarity between the samples as determination of positive identification of the gene as used in other studies.[30]

Table 1. Primers used for Polymerase Chain Reaction

Primer	Sequence	Target gene	GenBank Accession Number
stx_1-F	5'-TCAGGGGACCACATCGGTAT-3'	stx_1	AB083043.1
stx_1-R	5'-TCCGGAAGCACATTGCTGAT-3'		
stx_2-F	5'-ATGTGGCCGGGTTCGTTAAT-3'	stx_2	AB048226.1
stx_2-R	5'-CCGGCGTCATCGTATACACA-3'		

2.2 Detection Of Enterohemolysin

The 40 antibiotic resistant strains stored at -80 °C were also grown on TSA medium. The positive control strain that expresses enterohemolysin, ATCC 35218, was acquired from University Medical Center in Lubbock, Texas. Each sample was transferred to a blood agar medium (TSA supplemented with 5% sheep erythrocytes). The cultures were incubated at 36 °C for 24 hours and removed to observe hemolysis.[31, 32, 33]

3. Results

3.1 Sequencing Of Stx_1 And Stx_2

In a comparison of the PCR product for the stx_1 gene with the sequence of O157:H7, 2 of 33 samples have a 92% or greater sequence similarity. In the product obtained from amplification of the stx_2 gene, 7 of 38 samples have a 92% or greater sequence similarity. These results are shown in Table 2. Figure 1 depicts the relationship of sample Dm7-

352 with O157:H7 as an example of sequence comparison that is a 99% match. This analysis does not definitively determine that these antibiotic resistant samples produce shiga toxin; however, it does give a genetic relationship to a pathogenic strain of *E. coli*. As indicated in the introduction, there are other non-O157 serotypes of pathogenic *E. coli*; however, these were not compared in this study.

Table 2. Samples with a 92% or greater sequence similarity with O157:H7

Sample	Gene	Sequence Similarity (%)	Source of Isolate
G4-64	stx1	97	Canada Geese
Dm7-352	stx1	99	Domestic Geese
G14A-95	stx2	94	Water
DG2-329	stx2	92	Domestic Geese
DG7-333	stx2	94	Domestic Geese
DG8-334	stx2	98	Domestic Geese
DR9-344	stx2	98	Domestic Geese
DR10-345	stx2	95	Domestic Geese
DG24-358	stx2	95	Domestic Geese

Figure 1. A portion of the sequence comparison of sample Dm7-352

3.2 Enterohemolysin Assay

Of the 40 samples, 2 exhibited alpha-hemolysis. These two samples were JB2-129 and Dm11-355. These samples were isolated from different locations and different sample types: Dm11-355 from the feces of a domestic goose and JB2-129 from water of the playa lake.[28, 29] Dm11-355 is resistant to ampicillin, whereas JB2-129 is resistant to ampicillin, tetracycline, and streptomycin.[28, 29] Figure 2 depicts both of the hemolytic samples along with the non-hemolytic samples of DW1-321 and Dm12-356.

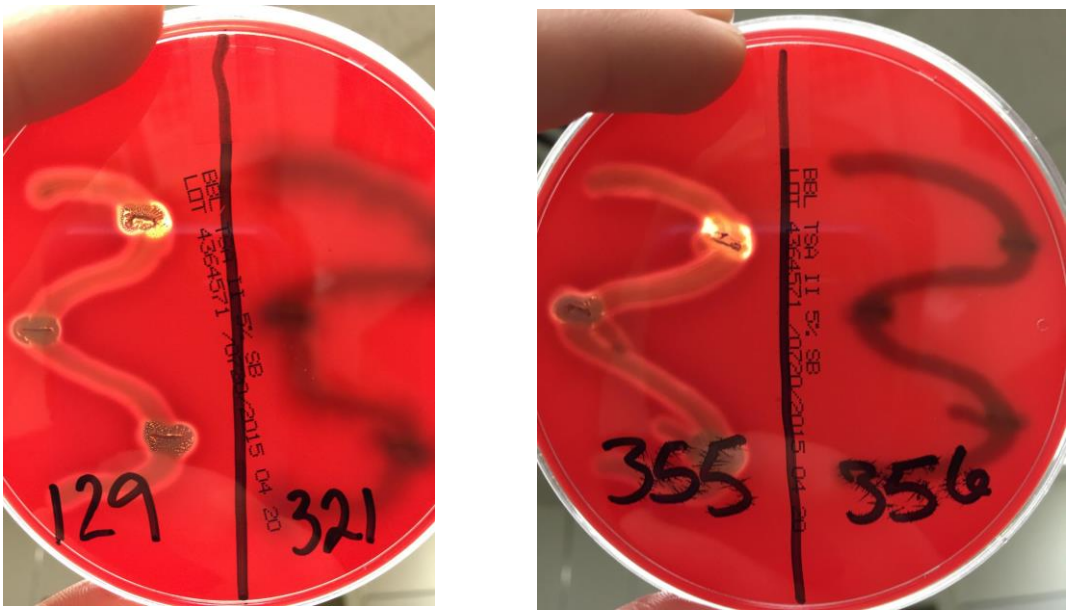

Figure 2. Alpha hemolytic samples contrasted with non-hemolytic samples

4. Discussion

The local playa lakes are natural bodies of water found typically within city parks. It has been shown that *E. coli* counts are high in the bank soil and in the sedimentation of the playa lakes during the winter months. It is postulated that the settling into the soil allows for the *E. coli* to survive below freezing temperatures.[34] These lakes are enjoyed by the community of Lubbock both aesthetically and recreationally. Although entering the lakes is against city law, residents often fish and bring animals to the parks to enjoy the playas, which increases the possibility of human contact with the water and soil of the lakes. Additionally, the playa lakes predominantly receive water through rains in the spring, and these areas are ill equipped for large amounts of rainfall. It is not unusual for these lakes to flood, covering a large area of the park and neighboring roadways in water. The lakes are often situated in residential areas, thus increasing human contact with the lakes. Because of these factors, the presence of antibiotic resistant and pathogenic *E. coli* in the lakes and on the banks is of concern to residents in Lubbock that have direct or indirect contact with the playas.

The results of the PCR product sequencing indicates a genetic relationship between two of the stx_1 samples and seven of the stx_2 samples with O157:H7, and these nine samples can be classified as STEC strains. Furthermore, two of the samples have been shown to express enterohemolysin; however, neither of these samples expressed Stx1 or Stx2 and cannot be classified as EHEC. In conclusion, there are antibiotic resistant strains of *E. coli* isolated from

Lubbock playa lakes that could be potential pathogens. Therefore, this is a public health concern for the residents of Lubbock, Texas.

Additionally, the nature of genetic recombination among bacterium of the same species provides further importance to this study. Because *E. coli* can transfer genetic material, the presence of antibiotic resistant genes or virulent genes could easily be transferred to harmless strains of *E. coli*.[35] In turn, this would make the harmless strains possibly pathogenic.

Further study must be conducted in order to understand the nature of the *E. coli* found within the playa lakes. This includes genetic analysis to determine the identity of the genes responsible for antibiotic resistance of these organisms, and by what mechanism the *E. coli* are resistant. This analysis will provide more information on how the organisms acquired the resistance to commonly used antibiotics. Additionally, genetic studies can be done to determine the rate of gene transfer between microorganisms within the playa lakes. Up to this study, only the presence of *E. coli* within the lakes has been the focus, however additional study will be done to assess for other harmful enterics such as *Shigella* and *Salmonella* spp. Finally, and more immediately, the entire library of isolates of *E. coli* acquired by the Lubbock Christian University Natural and Physical Science Departments, not just the antibiotic resistant strains, will be cultured on blood agar medium to determine the hemolytic activity.

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