Development of a biomarker to detect bird fecal waste in environmental waters

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Abstract

Molecular microbial source tracking methods have been developed to identify fecal waste sources entering environmental waters. However, most, such as ribotyping and rep-PCR, rely upon library databases (Hoi et al. [1]; Sikorski et al. [2]). These methods generally are only reliable in a specific geographic region. Also, there is overlap amongst hosts, requiring manipulation of sample isolates to eliminate this segment of the population. Recently, the library independent biomarker method based on toxin genes associated with the diarrheic \textit{E. coli}, has been successful in identifying human, cow or pig waste (Oshiro and Olson [3]; Khatib et al. [4], [5]). Using diarrheic \textit{E. coli} as a model, the temperature-sensitive hemagglutinin, \textit{tsh}, gene associated with avian pathogenic \textit{E. coli} was tested as a biomarker for bird waste, but 46\% of dogs were positive for this trait, causing it to be eliminated. However, sequencing data suggests the possibility to differentiate \textit{tsh} in dogs and birds. Consequently, we selected hemolysin E (\textit{hlyE}) in enterohemorrhagic \textit{E. coli} to test for specificity, geographic and temporal stability, sensitivity, and occurrence within avian populations. Forty-one single or composite fecal samples from birds and 10 environmental water samples were collected, \textit{E. coli} were grown using mTEC media, DNA were extracted and real-time PCR was performed to detect \textit{hlyE} from these samples. \textit{hlyE} was found in 4.5\% of the raw sewage samples (n=22), while none were found in primary (n=3). 13.3\% of the secondary effluent samples (n=30) were positive, but birds were known to be in contact with secondary clarifiers for all of these systems. Twelve human fecal swabs screened were negative for \textit{hlyE}. 8\% of the birds and 20\% of the water samples screened contained \textit{hlyE}. Applying the \textit{hlyE} biomarker to identify avian fecal pollution entering waterways can help to maintain water quality standards.

Keywords: bird, \textit{Escherichia coli}, biomarker, water quality, fecal waste, source tracking method.
1 Introduction

The increasing amounts of contaminants entering waterways not only affect the health of the environment and the public but also coastal regions’ economies as well. In order to maintain acceptable water quality standards, identification of the sources of contamination is very useful to water quality managers and when pollution controls are added, can result in a reduction of waste entering coastal waters. Of major concern to coastal environments is nonpoint source pollution, not all of which can be controlled. For example, wetlands may attract high numbers of birds thus, elevating the levels of fecal coliform and enterococci entering streams or coastal waters. If animal or bird wastes contain pathogens of human importance, then human health risks may result at coastal bathing beaches due to upstream inputs. Since most sources of fecal contamination are of unknown origin and eventually enter environmental waters through rain events, identification of multiple sources becomes even more difficult especially after numerous inputs have converged. By identifying fecal waste sources in the run-off and waterways of watersheds, these contributors can be identified and where possible, strategies to reduce inputs can be implemented.

A variety of molecular methods have been developed to identify the sources of fecal waste in environmental waters. These methods include DNA fingerprinting (Simmons [6]), ribotyping (Hoi et al. [1]), male specific phage genotyping (Hsu et al. [7]), antibiotic resistance (Whitlock et al. [8]) and toxin biomarkers (Oshiro and Olson [3]; Khatib et al. [5]). Some of these methods, ribotyping, DNA fingerprinting and antibiotic resistance, require a library database. The databases appear, to date, to be very location specific requiring the development of new databases for each geographical region. Another aspect of these methodologies is the apparent colonization of multiple hosts by certain bioindicators such as E. coli and male specific phages or methods such as antibiotic resistance testing. Male specific phage genotyping has also been used to indicate fecal contamination. However, this method has been shown to be insensitive since coliphage concentrations are low in many environmental waters (Leclerc et al. [9]).

Previous research in our laboratory on a toxin gene biomarker method has been shown to successfully identify sources of cow, pig and human fecal waste in environmental waters (Oshiro and Olson [3]; Khatib et al. [4], [5]). This method does not require a library database, appears to be representative across many geographical regions throughout the United States and provides a simple positive or negative result. The toxin biomarker method uses a unique nucleic acid sequence within a toxin gene from diarrheic Escherichia coli that is specific to a host animal. Thus, based on this methodological approach, we began to develop a biomarker for birds based on the avian pathogenic E. coli. An excellent candidate appeared to be the temperature sensitive hemaglutinin (tsh) gene associated with the colicin V plasmid (Provence and Curtiss [10]). The clinical syndrome is associated with overcrowding of poultry found in commercial operations. We were successful in identifying tsh in fecal material from a variety of birds. However, during our species cross-contamination work,
we found that \textit{tsh} occurred in an unacceptably high percentage (29\%) of dogs. This led us to determine that \textit{tsh} would not be a good toxin to differentiate between bird and dog wastes.

The \textit{tsh} virulence factor had not been unique enough to use as a biomarker, so we chose to test a recently described hemolysin gene, \textit{hly}\textsubscript{E}, which is related to RTX hemolysins associated with some uropathogenic and hemorrhagic \textit{Escherichia coli}. The hemolysin gene is one of the virulence factors that may aid avian \textit{E. coli} in causing infection in the target host. Diarrhea, perihepatitis, and septicemia are some of the diseases caused by these pathogenic \textit{E. coli}. However, the \textit{hly}\textsubscript{E} gene lacks the repeat regions in the toxin domain and does not share similar operon organization of the RTX group (Reingold et al. [11]). This research reports the geographic and temporal stability, specificity, sensitivity, and the occurrence of the \textit{hly}\textsubscript{E} gene within avian populations to determine its adequacy to be used as a biomarker of bird fecal waste in environmental waters.

2 Methods

2.1 Sample collection

A total of forty-one (1g) samples from a variety of bird species in California and Arkansas were collected. A total of ten (500 ml) environmental water samples were collected from creeks or rivers, coastal waters and ponds in Southern California. Upon collection, all samples were stored on ice, shipped overnight, and processed within 8 hours of arrival.

2.2 Bacterial strains

Enterohemorrhagic \textit{E. coli} strain containing the pNSI plasmid was used as positive control for the \textit{hly}\textsubscript{E} gene. The control strain was grown with agitation (150 rpm) overnight in Luria-Bertani (LB) media supplemented with 50 \(\mu\)g/ml kanomycin and then stored at -70\(^\circ\)C in 30\% sterile glycerol.

2.3 DNA collection

A collection of 114 DNA extracts from non-avian fecal samples and various types of wastewater samples was screened to determine the specificity of the \textit{hly}\textsubscript{E} trait in birds. Fifty-nine of these were from fecal samples from humans and a variety of animals. The remaining fifty-five of the DNA extracts were from sewage samples obtained at sewage treatment plants located in California, Ohio, Michigan, and Kentucky.

2.4 Membrane filtration

For each sample, 1 ml of \(10^0\) to \(10^{-2}\) was filtered in triplicate through a 0.45 \(\mu\)m pore-sized (47 mm in diameter) nylon membrane filter (Osmonics) and then placed onto mTEC media (Difco). Plates were incubated at 35 \(\circ\)C for 1.5 hours...
followed by 44.5°C for 20 ± 2 hours overnight (Dufour et al. [12]). After which, yellow colonies were enumerated, followed by flooding with 1 ml phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7 H₂O, 1.4 mM KH₂PO₄ [pH 7.3]). All colonies on each filter were suspended in the buffer using a sterile rubber policeman and pipetted into 1.5 ml tubes. After which, tubes were centrifuged for 10 min at 12,000 x g, the supernatant discarded and pelleted cells kept for DNA extraction.

2.5 DNA extraction

Total DNA was extracted using a modified version of the phenol chloroform method (Tsai and Olson [13]). The addition of Tris-HCl-saturated phenol to the DNA after the freeze-thaw cycles was omitted. An additional wash step was added after the isopropanol precipitation of the DNA. The DNA was washed in 200 µl of 75% ethanol (reagent grade), kept at 20°C for 1 h, and processed as described in the method. The extracted DNA without further purification was stored at 50°C until PCR analysis.

2.6 Real time PCR

The specificity of primers and probe (Table 1) was determined by screening all sequences contained in GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST (Altschul et al. [14]). The taqman probe was 5’-labeled with FAM and quenched using TAMRA (Applied Biosystems, Foster City, CA.). Amplification of the hlyE gene was performed using 100 nM of labeled probe and 10 µl of DNA sample extract for each 25 µl reaction. Amplification occurred with an initial start at 95°C for 2 min and a final extension at 72°C for 1 min. Forty-five PCR cycles (95°C for 30 s and 59°C for 60 s) were carried out in a RotorGene 3000 (Corbett Research, Portlake, AUS.).

Table 1: Primer and probe for hlyE detection.

<table>
<thead>
<tr>
<th>Targeted Region</th>
<th>Primer/Probe (5’-3’)</th>
<th>Target Size</th>
<th>Genbank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>226-246</td>
<td>AGTACTCTCAATCGGCATCCA</td>
<td>141 bp</td>
<td>AF052225</td>
</tr>
<tr>
<td>347-366</td>
<td>ATGAAAGATAGGCTGTCAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>282-313</td>
<td>FAM-AGCCAAGATAGATACTTCGAGGCGACACAAGT-TAMRA</td>
<td>32 bp</td>
<td></td>
</tr>
</tbody>
</table>

3 Results and discussion

This research reports on our laboratory’s approach to developing a biomarker to detect bird waste in river and coastal waters. When our research group develops a biomarker to identify waste for a specific classification of host, we test for the sensitivity of the method. The approach must be able to detect low numbers of the biomarker in environmental sources. Our method was able to detect hemolysin E, hlyE, at 1 attogram (ag) or 13 copies of target DNA using real time
PCR. For DNA extracts of environmental samples, a positive result for the trait was based on a gene frequency of > 13 copies or >1 ag of target DNA. Further, the specificity, frequency, geographical occurrence and temporal stability need to be determined before a biomarker is suitable for use by utilities or regulatory agencies.

Table 2: Number of the type of samples and mean copy number of hlyE / 5 µl of DNA screened using real time PCR.

<table>
<thead>
<tr>
<th>Type of Sample Screened</th>
<th>Number screened</th>
<th>Mean copy No. of hlyE / 5 µl of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BIRDS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crested wood partridge</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Kookaburra</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Helmeted guinea fowl</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cout/Ruddy Turnstone/Teals</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Rhea</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Emu</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Scarlet Ibis/Spoonbill</td>
<td>&gt;1</td>
<td>0</td>
</tr>
<tr>
<td>Macaw</td>
<td>&gt;4</td>
<td>0</td>
</tr>
<tr>
<td>Caique</td>
<td>&gt;1</td>
<td>25</td>
</tr>
<tr>
<td>Motmot</td>
<td>&gt;1</td>
<td>9</td>
</tr>
<tr>
<td>Aracari</td>
<td>&gt;2</td>
<td>0</td>
</tr>
<tr>
<td>Oropendola</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hawk/Great Horned Owl</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Kiskadee flycatcher/cardinal</td>
<td>&gt;1</td>
<td>0</td>
</tr>
<tr>
<td>Amazon Parrot (yellow-headed)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Black crow</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chicken/turkey (yellow-headed)</td>
<td>&gt;102</td>
<td>5.74 x 10^6</td>
</tr>
<tr>
<td>Seagull</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Ducks/Geese/Swan</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td><strong>HUMAN</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fecal Swab</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Raw Influent</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>Primary Sewage</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Secondary Unchlorinated Effluent</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td><strong>COWS</strong></td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td><strong>CATS</strong></td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><strong>DOGS</strong></td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td><strong>PIGS</strong></td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td><strong>SHEEP</strong></td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td><strong>AGOUTI</strong></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>GOAT</strong></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>HORSES</strong></td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
Fecal wastes from a variety of animals were screened for the presence of hlyE to establish specificity (Table 2). Results in this study showed the hlyE gene to be specific to bird fecal waste as this trait was absent in all other fecal samples tested to date. These results also showed that the hlyE trait was present in 4.5% of the 22 raw sewage samples and 13.3% of the 30 secondary effluent samples screened. However, ducks and other aquatic waterfowl frequently fed on secondary clarifiers where our samples were taken. These positive samples, shown in figure 1, had very low copy numbers of hlyE (approximately an average of 20 copies per $10^6$ E. coli screened). None of the primary sewage samples were positive for hlyE.

Figure 1: hlyE standards and DNA extracts from raw influent and secondary effluent using real time PCR.

Using real time PCR the frequency of hlyE was 8% for bird fecal wastes screened. In table 2, the different types of birds screened for the presence or absence of hlyE in bird fecal waste can also be seen. The majority of samples were from individual birds and not composite samples from a group of birds. The frequency of occurrence in bird waste in this study is low compared to the study by Reingold et al. [11], where the hlyE trait was found to be present in 1 of 4 or 25% of avian E. coli strains tested. However, the 1999 study was on E. coli isolates from birds with a variety clinical symptom, whereas in our current work fecal samples were taken from healthy birds. Thus, the lower frequency of hlyE is not surprising. Another explanation could be that the trait’s occurrence is more common amongst flocks of animals, as opposed to single bird fecal samples. Furthermore, the vast majority of birds tested were from the wild or zoos. Since all the fecal material was fresh and processed immediately upon
collection, the lack of hlyE was not due to differential die-off of E. coli strains carrying this trait. More composite samples of bird waste from high-density flocks or commercial operations may increase the frequency of hlyE detection amongst bird waste.

The frequency of this gene in a variety of creeks and rivers entering coastal waters as well as ponds frequented by ducks and geese is higher (20%) than in bird waste. Water samples may have a higher rate of occurrence of this trait because waste from many different birds has commingled in the aquatic environment or our hlyE dual labelled markers cross react with the trait contributed from waste from a different animal. Although in this study we observed no evidence of cross reactivity for this trait in the fecal wastes from different animals.

To date, this study shows that the hlyE gene is present in at least two different states in the United States: one in the western region, California, and the other in the central region, Arkansas. This trait has also been found to occur in avian E. coli isolated from chickens in Georgia (Reingold et al. [11]). To determine the geographical distribution of this biomarker many different regions must be sampled.

In developing the cow, pig and human biomarkers in our laboratory, the stability of each of these biomarkers had also been assessed through repeat sampling over time (Oshiro and Olson [3]; Khatib et al. [4], [5]). Results for each of these biomarkers showed that the traits were temporally stable over a period of at least 7 years and certainly have been known in the literature for one to two decades (Gyles [15]). Thus, we assume that the hlyE gene carried in E. coli will also be temporally stable.

Identification of bird waste in environmental waters can benefit water quality managers. As more wetlands are constructed, greater numbers of birds will be attracted to these areas and levels of fecal coliforms entering streams or coastal waters may increase. The use of the hlyE gene as a biomarker of avian fecal waste in environmental waters could greatly aid in the identification of the group of fecal coliforms contributed by birds. However, for the method to be viable greater number of birds carrying the trait must be identified. This study provides a simple positive or negative method to differentiate avian fecal waste entering streams or coastal waters, which can help to sustain the growing population along coastal regions and its economy.

References


