

***E. coli* Ribotyping
for Identifying Sources
of Fecal Contamination
in the Salt Ponds of
Martha's Vineyard, MA**

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SUMMARY

Microbial source tracking (MST) often involves using cultures of fecal indicator bacteria isolates from both impacted waters and suspected contamination sources to identify the most significant contaminant source species. For this study, water samples were collected from four salt ponds in Martha's Vineyard, MA, analyzed and brought to the UNH/Jackson Estuarine Lab (JEL). *E. coli* strains were isolated from the water sample cultures and suspected source material and subjected to ribotyping analysis for the purpose source species identification.

The final results from this study are strong indications of significant bird sources in all the ponds and localized dog sources, with Canada geese having particular significance at Farm Pond.

INTRODUCTION

One of the most common issues facing environmental managers concerned with surface water quality is fecal-borne microbial contamination and the threat of diseases to humans who come in contact with contaminated water or shellfish. For purposes of monitoring the sanitary quality of surface waters, fecal coliforms, enterococci and *Escherichia coli* have traditionally served as indicators of water quality for classifying waters to protect public health. However, as untreated sewage from inadequately designed wastewater treatment facilities has been eliminated or reduced in significance, the residual contamination that limits uses of surface waters is typically of unknown origin. Efforts to reduce contamination have often revolved around making a best guess of what potential sources may be significant, conducting extensive sampling programs, eliminating sources and then re-sampling surface waters to see if improvements in water quality have occurred. This process is expensive and oftentimes less fruitful than desired.

Recent adoption of biotechnological techniques for application to water quality issues has spawned a number of approaches to address identification of sources of fecal-borne contamination. These new approaches, often called "microbial source tracking" (MST), have been used successfully for well over 10 years in a number of areas in the U.S. Ribotyping of *E. coli* isolates cultured from target surface waters is one approach that can provide information on a wide range of potential sources of fecal contamination.

Various studies have reported on the use of ribotyping for tracking sources of fecal-borne microbial contaminants. The UNH/JEL lab has conducted well over 30 studies, mostly in NH and ME, but also in MA, VT and NY states. Starting in 2000, these ribotyping studies have been conducted in freshwater watersheds and beaches, and marine and estuarine waters (Jones 2007).

Because ribotyping can provide information on the identity of source species of bacteria found in surface waters, follow-up efforts to identify and eliminate contamination sources can be directed towards those types of sources where the few species responsible for the most significant amounts of contamination can be targeted for management action. Through an iterative process of then finding possible sources of fecal contamination from significant species, ribotyping can be used again to match strains for a given species to specific sources. Thus, the overall effort to improve water

quality can be targeted because the most significant sources actually found in surface waters of concern are directly identified and eliminated. Such an approach also provides significant savings of time and expense compared to traditional approaches.

Water quality problems with microbial contamination have caused several shellfish harvesting areas on Martha's Vineyard to be closed by the Mass. Division of Marine Fisheries, while other places are only open for part of the year, usually during non-summer months. Citizens and shellfish constables are interested in decreasing or eliminating the closure time periods for several salt ponds on the east shore of the island, and some share a concern that birds are the main source of the microbial contamination in the affected salt ponds. Birds have been found to be the most significant sources in several studies in the New England region, including in a recent study of a freshwater pond in New Hampshire where over the vast majority of contaminants were identified as being from Canada geese (Jones 2006). Identification of the source(s) of the contamination from the water samples collected in this study, and in a follow-up study planned for next year, will help direct management activities starting next summer for re-opening these areas to shellfish harvesting. This first round of sampling and analysis will help to test suspicions that Canada geese and cormorants are the most significant sources.

MATERIALS AND METHODS

The study sites were four salt ponds on the east side of Martha's Vineyard. These included Sengekontacket, Farm, Eel and Trapps' ponds. Source samples were also collected from within the pond watersheds. Four source samples were collected on September 5, 2007, one from the Land Bank Property on Farm Pond, two from Sarsons Island in Sengekontacket Pond, and one from the Edgartown Golf Course. These source samples were transported to JEL on the sample day. Another source sample was septic tank effluent from a home on the Boulevard in Edgartown. The septic sample was collected on September 24, 2007, mailed under ice to JEL the same day and received the next day.

The water samples were collected from the four ponds, including two locations in Sengekontacket Pond, on September 4, 2007. No precipitation occurred during the previous two days. The samples were processed for enumerating fecal coliforms and isolating *E. coli* colonies at the Wampanoag Environmental Laboratory in Aquinnah using mFC agar. The mFC plates and the remainders of the water samples were transported on ice to JEL on September 5, 2007. These samples were all re-filtered to insure adequate (10-20) *E. coli* colonies were available for ribotyping. Almost all *E. coli* water sample isolates that were used for ribotyping came from the mFC plates, with extra colonies from the mTEC plates used to augment the total isolates to have 10 to 20 from each sample.

Water samples were decimally diluted to 10^{-2} , while 1.0 g wet weight of each fecal sample was decimally diluted to 10^{-8} . Aliquots (2.5 ml) from the dilution tubes and water sample were filtered through membrane filters (0.45 μm pore size) and the filters placed onto mTEC agar. The agar plates were incubated at 37°C for 2 h then 44.5°C for 22 h. Following urease testing on urea substrate, each plate was inspected and plates giving countable colonies were used for selection of putative *E. coli* strains for ribotyping analysis. The *E. coli* colonies chosen were subject to a battery of biochemical tests to

confirm their identity as *E. coli*. The procedures used for isolating and identifying *E. coli* strains for this study were according to standard lab protocols (Jones, 2002; Jones and Bryant, 2004).

The confirmed *E. coli* isolates were then analyzed for determining ribopatterns. *E. coli* isolates were stored in cryovials at -80°C and re-cultured onto trypticase soy agar (TSA). Cultures on TSA were incubated overnight at room temperature (20°C). Some of the resulting culture was transferred to duplicate cryovials containing fresh glycerol/DMSO cryo-protectant media for long-term storage at -80°C. The culture was then ready for ribotyping.

A RiboPrinter was used to analyze *E. coli* cultures for ribotype determinations. After preparation of the samples, the automated process involved lysing cells and cutting the released DNA into fragments via the restriction enzyme EcoR1. These fragments were separated by size through gel electrophoresis and then transferred to a membrane, where they were hybridized with a DNA probe and mixed with a chemiluminescent agent. The DNA probe targeted 5S, 16S and 23S ribosomal RNA genes. A digitizing camera captured the light emission as image data, from which the system extracted a RiboPrint® pattern. This pattern was initially compared to others in the RiboPrinter database for characterization and identification based on densitometry data. However, our source species identification analysis approach has conformed to other ribotyping studies in using banding patterns as the basis for comparing patterns.

Band Identification

The images were transferred from the RiboPrinter into GelComparII (Applied-Maths) analytical software. The bands in lanes containing the standard were labeled and entered into the memory for optimization of gel pattern images. The densitometry data were processed for band identification. The ribopattern data for each water sample isolate were then selected for identification of source species. The analysis of the Martha's Vineyard salt pond water sample isolates for identification of source species was based first on a local database, then on a regional database (Table 1). The local database consisted of 51 ribopatterns from 3 bird sources and the regional database consisted of 1691 ribopatterns from 35 species and sources.

Data Analysis

All data were analyzed with GelComparII software on a Dell computer, where the source species database was also stored. Similarity indices between the unknown isolates and the known source isolates were determined by using Dice's coincidence index. For this study, 1% band tolerance and 1.5% optimization settings were used. Both of these parameters are used to adjust the ability to differentiate between bands for the degree of accuracy desired, and also to compensate for possible misalignment of homologous bands caused by technical problems.

The source species profile with the best similarity coefficient at a given set of optimization and tolerance settings was accepted as an indication of the possible source species for the water sample isolate. For this study, the predetermined threshold similarity index that was considered to be a minimum value for identifying source species was 90%. Thus, the identification of the source species was considered successful if the value calculated for a given water isolate was equal to or greater than the threshold value;

if the calculated value was below the threshold similarity index, the water sample isolate was considered to be of unknown origin. Further interpretation was conducted based on matching of patterns at slightly lower similarity indices.

The decision for using a 90% similarity index threshold was based on the inter-gel variability within cumulative Dice's coincidence indices determined for *E. coli* positive controls run for every ribotyping study. Thus, identifications reported are almost always less than completely accurate (0% tolerance and 100% similarity). Nonetheless, useful information can be gained to help guide management decisions and resource allocation for pollution source identification and elimination in the Martha's Vineyard area.

Cluster analyses were performed to determine the relationships among isolates from the same source species and the same sites, as well as banding patterns that were identical for different isolates. The cluster analyses were based on the un-weighted pair group method by arithmetic averaging (UPGMA) or the neighbor joining algorithms.

The last step in data analysis is visual inspection of the band matching results. Hard copies of ribotype patterns and similarity coefficients for the unknown and most closely related source species are printed for interpretation. Interpretation and accompanying tabular representations of the data were done using MS Excel on Macintosh computers. The results of identification of source species are summarized according to both the actual and type of source species identified.

Table 1. Regional and local Martha's Vineyard source species databases.

Species	REGIONAL			MARTHA'S VINEYARD		
	# Samples	# Ribotypes	# Unique Ribotypes	# Samples	# Ribotypes	# Unique Ribotypes
Alpaca	1	3	2	-	-	-
Buffalo	2	10	8	-	-	-
Cat	7	44	21	-	-	-
Chicken	5	33	25	-	-	-
Cormorant	7	34	20	1	20	10
Cow	11	89	68	-	-	-
Coyote	10	41	31	-	-	-
Deer	44	170	104	-	-	-
Dog	24	163	84	-	-	-
Duck	8	21	14	-	-	-
Fox	19	75	53	-	-	-
Goat	2	10	8	-	-	-
Goose	21	115	79	2	16	6
Horse	14	65	54	-	-	-
Human	8	115	54	-	-	-
Landfill Trash	4	20	20	-	-	-
Mouse	1	3	2	-	-	-
Muskrat	5	32	17	-	-	-
Otter	3	14	9	-	-	-
Oxen	1	10	4	-	-	-
Pig	1	16	5	-	-	-
Pigeon	2	7	4	-	-	-
Rabbit	5	30	24	-	-	-
Raccoon	31	79	61	-	-	-
Robin	1	4	2	-	-	-
Seagull	31	165	105	-	-	-
Septage	5	32	23	-	-	-
Sheep	2	8	5	-	-	-
Skunk	1	6	4	-	-	-
Sparrow	1	4	3	-	-	-
Starling	1	3	1	-	-	-
Unidentified Avian†	2	20	14	1	15	9
Unidentified Wildlife‡	6	45	31	-	-	-
Wastewater	36	188	165	-	-	-
Wild Turkey	3	17	13	-	-	-
Totals	325	1691	1137	4	51	25

†**Unidentified Avian Samples:** Goose or Duck; Unidentified Avian: Martha's Vineyard 9/5/07

‡**Unidentified Wildlife Samples:** Raccoon or dog; Fox or coyote; Unknown.

RESULTS

The water samples collected from the salt ponds were enumerated for fecal coliforms prior to being sent to JEL. No attempt to count colonies from the culture plates sent from the Aquinnah lab was made at JEL because of the holding time effects on the colony coloration. However, all mFC plates yielded *E. coli* isolates for ribotyping. Most of the source species feces samples also yielded useful *E. coli* isolates, with the exception of two samples (Table 2). The cormorant sample from Eel Pond had no culturable *E. coli* colonies, and the septage sample had many putative *E. coli* isolates from several analyses that all turned out to be other bacterial species. The single septage isolate that was

ribotyped confirmed this, as it turned out to be *Citrobacter freundii*, a related species, based on identification by the RiboPrinter database. The single isolate from the Trapps Pond goose sample was *E. coli* and used in the local database for identifying sources. The “mix/avian” sample was a sample from beach sand that had at least two different types of (bird) feces mixed together.

Table 2. Source species types, locations and *E. coli* concentrations.

Sample ID	Local Species	Sample Date	Sample location	Concentration FC / g ww	Concentration Ec / g ww
GO1	Goose	9/5/07	Trapps Pond	1.11E+01	1.11E+01
GO2	Goose	9/5/07	Farm Pond	2.37E+08	2.37E+08
CO1	Cormorant	9/5/07	Eel Pond	<1.11E+01*	<1.11E+01
CO2	Cormorant	9/5/07	Sarson's Island	4.78E+04	4.78E+04
Mix/Avian	Mixed Avian Feces	9/5/07	Sarson's Island	6.78E+05	6.67E+05
Septage01	Septage†	9/24/07	Edgartown	5.22E+04	2.22E+03

*no *E. coli* isolated from this sample

†All septage colonies counted as *E. coli* based on mTEC/urea test failed the biochemical tests.

Ribotyping Success

Not all water samples yielded the targeted 10-20 *E. coli* colonies (Table 3) because of low concentrations in the water samples, or other closely related species interfering with colonies on plates. Most of the colonies chosen as putative *E. coli* isolates from the Sengekontaktet and Trapps pond samples turned out to be other bacterial species, resulting in only 4 and 2 isolates, respectively, for ribotyping. All of the confirmed *E. coli* isolates that were ribotyped gave useable ribopatterns for analysis, resulting in a total of 48 water sample ribopatterns and 51 source species patterns. As expected, the variability of the water sample ribopatterns was greater than that for the source species patterns. For water samples, 67% (32/48) of the patterns were unique, while 49% (25/51) of the source species patterns were unique (Table 3).

Table 3. Speciation and ribotyping success.

Site/Species	Sample date	# Isolates from mFC + mTEC	# ID as <i>E.coli</i> from Biochems	# Isolates Ribotyped	# Usable Ribotypes	# Unique patterns (no clones)
WATER SAMPLES						
1-Sengehontachett	9/4/07	23	4	4	4	4
2-Trapps Pond	9/4/07	13	2	2	2	2
3-Eel Pond	9/4/07	15	11	11	11	8
4-Sengehontachett Edge	9/4/07	20	10	10	10	8
5-Farm Pond	9/4/07	24	22	21	21	10
TOTAL		95	49	48	48	32
SOURCE SAMPLES						
Goose-GO1	9/5/07	1	1	1	1	1
Goose-GO2	9/5/07	20	15	15	15	5
Cormorant-CO1	9/5/07	0	0	0	0	-
Cormorant-CO2	9/5/07	20	20	20	20	10
Mixed Avian Feces	9/5/07	20	15	15	15	9
Septage-01	9/24/07	14	0	1	0	-
TOTAL		75	51	52	51	25

Source Species Identification

Overall, 77% of the water isolates were successfully identified, with 65% identified using only the local database. This percentage of identifications is considered highly successful, given that most similar studies in New England have resulted in 50-60% identifications (Jones 2007). The identified source species consisted mainly of bird species at all the sites (Table 4). 100% of the identified *E. coli* isolates from the Sengekontacket, Trapps and Farm pond samples were from bird sources, while five of six isolates from Eel pond were as well. At the Sengekontacket “Edge” site, three of seven isolates were from bird sources. The only non-bird source of any significance was dogs at the Sengekontacket “Edge” site, where three isolates appeared to be of dog origin. For both Sengekontacket sites combined, 71% of the isolates were identified, with 43% from birds, 21% from dogs and 7% from livestock.

The ‘mixed avian’ source was identified at all five sites, with cormorant and sea gulls identified at 2 sites, and the other source species identified at only one site. Canada geese were identified at Farm Pond, where they were by far the most prevalent identified source species, at 76% of the total isolates analyzed.

Table 4. Martha's Vineyard salt pond *E. coli* source identification

Site ID	# of isolates	Isolates identified		LOCAL DATABASE			REGIONAL DATABASE		
				mixed avian	goose	cormorant	sea gull	red fox	mixed livestock
1-Seng	4	3	75%	2			1		
2-Trapps Pond	2	2	100%	1			1		
3-Eel Pond	11	6	55%	5				1	
4-Seng Edge	10	7	70%	2		1			1
5-Farm Pond	21	19	90%	2	16	1			3
Total Sengehontachett	14	10	71%	4	0	1	1		1
TOTAL	48	37	77%	12	16	2	2	1	1
		local database ID:	63%						

A useful way of considering identified source species is to group them into types of sources that would require unique management actions. The UNH/JEL lab reports results according to the following types:

- humans, pets, livestock, birds, wild animals

The first three are related to human activities and thus can be directly managed. Management strategies for bird and wild animal sources are less direct. For this study, no human, and insignificant livestock and animal sources were indicated (Table 5). Birds were overwhelmingly the most significant sources, constituting 69% of all isolates, and 87% of all identified isolates from all pond sites. Birds were also the most significant source type at the two Sengekontacket Pond sites, constituting 43% of all isolates and 60% of identified isolates. However, pets were also significant, with 30% of the identified isolates as dog sources, or half the percentage of the bird sources.

Other Observations

Use of the local database for initial analyses is the best method for identifying source species at a given study area because identification is based on isolate patterns from the same place and time. However, the large regional database at UNH/JEL, made up of source species patterns for isolates collected from around the New England region over the past few years, also includes many more species and patterns for all species. For patterns containing relatively few (8-9) DNA bands that represent the low end of variability, identical patterns can be shared among several different source species. This complicates identification of sources because no single source can be identified for water sample isolates that match these ‘mixed source’ patterns. Analysis of the isolates from this study with the regional database did indicate the possibility of a few different sources, and also resulted in some isolates matching to the ‘mixed source’ patterns. This was true for Eel Pond and the Sengekontacket ‘Edge’ sites, where the water sample patterns included some that were considered ‘mixed source’. However, the patterns from Farm Pond were still mostly from Canada geese, and the identifiable patterns for Sengekontacket and Trapps ponds were from birds. Overall, this study again illustrates the value of local database patterns for accurate identification of source species.

Table 5. Source species types identified for all ponds and Sengekontacket Pond

Source type	Isolates	% Total	% of ID'd
ALL POND SAMPLES			
HUMAN	0	0%	0%
PETS	3	6%	8%
LIVESTOCK	1	2%	3%
WILD ANIMALS	1	2%	3%
BIRDS	32	67%	86%
mixed avian	12	25%	32%
geese	16	33%	43%
cormorant	2	4%	5%
sea gull	2	4%	5%
IDENTIFIED	37	77%	100%
UNKNOWN	10	21%	
TOTAL	48	100%	
SENGEKONTACKET (2 sites)			
HUMAN	0	0%	0%
PETS	3	21%	30%
LIVESTOCK	1	7%	10%
WILD ANIMALS	0	0%	0%
BIRDS	6	43%	60%
mixed avian	4	29%	40%
cormorant	1	7%	10%
geese	0	0%	0%
sea gull	1	7%	10%
IDENTIFIED	10	71%	100%
UNKNOWN	4	29%	
TOTAL	14	100%	

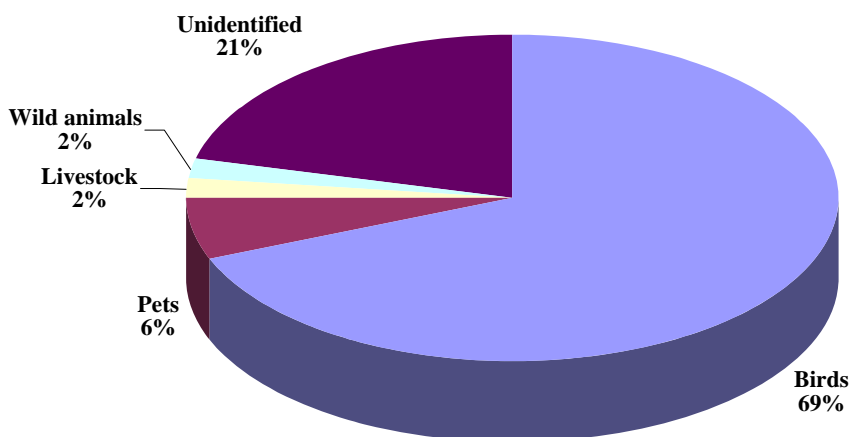


Figure 1. The relative significance of identified source species in all 4 ponds.

CONCLUSIONS

This study was conducted to help identify the most significant sources of bacterial contamination in the targeted ponds. 67% of the total *E. coli* isolates matched to source species patterns from bird feces samples collected from the local area at the same time water samples were collected. This suggests that the suspected local sources, cormorants, Canada geese and other birds, are significant sources of contamination for the water in all the ponds. Canada geese were by far the dominant contamination source at Farm Pond, where *E. coli* isolates from the water samples matched well to the unique geese patterns found in isolates from the analyzed feces sample.

Other observations are also important. There was no evidence of human-borne contamination, even when the isolate patterns were analyzed using the large, regional database. The local septage sample did not yield useable *E. coli* isolates. This has been observed previously in other studies around the region, where the septage sample contains high levels of bacteria but it has not been possible to isolate *E. coli* out of the mixed culture. In any future study, use of *E. coli* isolates from local septage samples will help to more accurately answer the question of the potential significance of human contamination sources. In addition, it appears that dogs may be contributing to the water quality problems in the ponds, at least in some areas. This is also a relatively common observation, and is evidence supporting management of dog waste near the shoreline.

Overall, the results from this study are strong indications of significant bird sources and localized dog sources, with Canada geese having particular significance at Farm Pond.

REFERENCES

Jones, S.H. 2007. Optimized Use of *Escherichia coli* Ribotyping for Identifying Pollution Sources in New Hampshire's Coastal Waters. Final Report. . New Hampshire Department of Environmental Services, Concord, NH.

Jones. S.H. 2006. Freshwater Beach Total Maximum Daily Load Microbial Source Tracking Study. Final Report. New Hampshire Department of Environmental Services, Concord, NH.

Jones, S.H. 2002. QA Plan for the Jackson Estuarine Laboratory Microbiology Lab. USEPA approved: 2002.

Jones, S.H. and T. Bryant. 2004. Standard procedure for detection of total coliforms, fecal coliforms, *Escherichia coli* and enterococci from environmental samples. Jackson Estuarine Laboratory, University of New Hampshire, Durham, NH.