Expansion and Evaluation of Texas' Bacterial Source Tracking Program

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Expansion and Evaluation of Texas' Bacterial Source Tracking Program

STATE NONPOINT SOURCE GRANT PROGRAM

TSSWCB PROJECT 13-50

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Table of Contents

List of Figures

List of Tables

List of Acronyms

AMOVA:	Analysis of molecular variance
ARCC:	Average rate of correct classification
ATCC:	American Type Culture Collection
BMP:	Best management practice
bp:	Base pair
BST:	Bacterial source tracking
Cq:	Quantification cycle
CSU:	Carbon source utilization
DMF:	Dimethylformamide
DNA:	Deoxyribonucleic acid
ERIC-PCR:	Enterobacterial repetitive intergenic consensus sequence PCR
HOMOVA:	Homogeneity of molecular variance
KB-ARA:	Kirby-Bauer antibiotic resistance analysis
LB:	Luria–Bertani plates
NMDS:	Nonmetric multidimensional scaling
NPS:	Nonpoint source
NTCs:	Negative controls
OTU:	Operational taxonomic units
PCR:	Polymerase chain reaction
QAPPs:	Quality Assurance Project Plans
qPCR:	Quantitative PCR
R&D:	Research and development
RARCC:	Random average rate of correct classification based on library composition
RCC:	Rate of correct classification
RDP:	Ribosomal Database Project
RP:	RiboPrinting
rRNA:	Ribosomal ribonucleic acid
SARA:	San Antonio River Authority
SCSC:	Texas A&M University Soil and Crop Sciences Department
SOP:	Standard operating procedure
SYBR:	Synergy Brands
TCEQ:	Texas Commission on Environmental Quality
Theta-YC:	Yue-Clayton index
TMDL:	Total maximum daily load
TSSWCB:	Texas State Soil and Water Conservation Board
U/I:	Unidentified
USDA-NASS:	U.S. Department of Agriculture National Agricultural Statistics Program
UTSPH EP:	University of Texas School of Public Health, El Paso
WPP:	Watershed protection plan

Executive Summary

Significant progress was made in expanding and refining the Texas *E. coli* BST Library and updating the template-Standard Operating Procedures. In particular, additional known source isolates were added to address underrepresented wildlife species. Temporal stability evaluations using Leon River known source isolates collected over a 10-year period of time revealed that approximately half of the *E. coli* strains from known sources may change over time even within the same watershed. Similarly, temporal evaluation of *E. coli* water isolates from the Leon River watershed revealed similar temporal variability. Thus, it was not surprising that a fairly high amount of geographical variability was also found for known source isolates. However, source-specific isolates were also identified that have broader geographical distribution and temporal stability, which deserve further attention moving forward with library refinement.

Re-challenge of the Texas *E. coli* BST Library ver. 5-15 with known source isolates from three different watersheds showed an overall increase in identification accuracy and fewer isolates left unidentified compared to previous challenges with an earlier version of the library. Additionally, a preliminary assessment of the library for cosmopolitan and singleton isolates was performed indicating the need to create a new "cosmopolitan" source category and note which water isolates matched singletons in the library in future BST studies and library refinement.

Several published source tracking studies have used *Bacteroidales* PCR assays with favorable results. Although these studies showed tremendous promise for *Bacteroidales* PCR, this same conclusion was not always obtained in other laboratories. In particular, amplification of non-target animal groups has been observed, especially with the *Bacteroidales* PCR for ruminants and less frequently for the human marker assay. To investigate some of these issues the current project included the characterization of PCR amplicons from animal fecal DNA which cross-reacted with the *Bacteroidales* HF183 PCR human marker. DNA sequence analysis of *Bacteroidales* PCR HF183 human marker amplicons from cross-reacting animal fecal samples from previous BST projects revealed identical sequences to human. We further investigated the specificity of the HF183 human marker by analyzing 101 known source animal fecal samples collected under the current project. A total of 11 of 90 (12%) wildlife fecal samples tested positive for the HF183 human marker. These samples were collected from a variety of wildlife species and therefore cross-reactivity is not limited to specific animal species. Again, we found the HF183 amplicon sequences from these cross-reactors to be identical to those obtained from control human fecal and wastewater samples. The extent of this interference is likely case specific and depends on the distribution of the HF183 *Bacteroidales* bacteria in local animal populations. It is recommended that BST studies utilizing *Bacteroidales* PCR include analysis of individual or pooled known source fecal samples. In addition, the use of qPCR is recommended for the analysis of water samples to potentially identify hotspots.

We also evaluated a modified *Bacteroidales* PF163 PCR method to determine if a primer or probe could be developed to specifically identify fecal pollution from feral hogs. Extensive evaluation of a *Bacteroidales* PCR PF163 hog marker assay using modified PCR mastermix and cycling conditions clearly demonstrated that we are able to detect fecal pollution from feral hogs. In contrast to the DNA sequencing results for the HF183 marker, sequence analysis of PF163 amplicons from feral hogs and domestic hogs revealed relatively high sequence heterogeneity.

Additional evaluation and sequence analysis may yield suitable PCR primers or probes for the specific detection of feral hog pollution. Interestingly, several wastewater samples also tested positive for the marker, which will need to be addressed during primer and probe development.

Although we use ERIC-PCR as one of our DNA fingerprinting tools for the construction of the Texas *E. coli* BST library, we know very little about the DNA sequences being amplified. We analyzed DNA sequences of ERIC-PCR amplicons from selected human-specific *E. coli* present in the Texas *E. coli* BST Library to explore the potential development of library-independent PCR targets. A sequence alignment revealed the lack of conserved regions and highly divergent sequences and therefore the development of PCR primers or probes is not currently feasible.

Recently, a poultry-specific molecular assay (LA35) has been developed for assessing poultry litter-specific fecal contamination in environmental samples. Even though the LA35 assay has been shown to be a good candidate marker for assessing poultry-associated fecal contamination, no studies have assessed its performance against fecal and litter samples obtained from Texas watersheds. Hence the objectives of this study were to validate the TaqMan-based LA35 assay using target and non-target fecal and litter samples obtained from Texas and or surrounding states. Overall, results indicate that the poultry marker performed well with the tested Texas samples. Sensitivity (83%) and specificity (99%) for analysis of the Texas samples were very similar to values published for other samples from across the US. As such, this poultry marker is a good candidate for inclusion in the Texas BST toolbox and should be considered for use in future watershed projects where poultry is a potential source of fecal bacteria.

Wildlife sources, such as deer and feral hogs, have been implicated as major contributors of bacterial impairment, but our fundamental knowledge of wildlife gut communities and thus ability to track them as specific contamination sources is lacking. Library-independent means to track deer specifically are hindered by the fact that the most widely accepted ruminant specific marker cannot distinguish between cattle and deer. The ability to distinguish between wildlife and livestock sources is critical to developing best management practices to reduce fecal contamination. This study's objective was to use 454 barcoded pyrosequencing to characterize deer fecal communities in Texas in an effort to evaluate their suitability for development of a deer-specific BST marker. Results suggest that the deer fecal bacterial communities, at least in south and central Texas, were stable over time which bodes well for the potential of a temporal and geographically stable source-specific marker. At least two operational taxonomic units (OUT), OTU_36 and OTU_4560, appeared to be potentially deer-specific with their closest non-deer matches in GenBank being only 95 and 96% similar, respectively, and appear to have potential for further investigation into their suitability as deer-specific BST markers.

Education and outreach on BST continues to be a critical need. To provide greater outreach to water resource managers in Texas, the project team delivered more than 10 presentations and distributed brochures and other materials to at least a dozen venues. The "Layperson" BST Brochure was updated and a new promotional flyer was developed as well. TWRI continued to host and maintain the Texas BST Program website. Website hits since its inception through May 2015 included 3,121 visits from 1,942 unique visitors. More than 20 entities in Texas, and many others nationwide, have received information on BST. As a result of this project, water quality assessment and WPP development was supported in at least four watersheds.

Introduction

Protection of water resources is one of today's most significant environmental challenges. One key component in effectively implementing a pollution abatement program is the identification and assessment of sources of pollution. Proper evaluation of sources is needed to target best management practices (BMPs), develop total maximum daily loads (TMDLs) or watershed protection plans (WPPs) and assess risk to human health. According to the *2010 Texas Integrated Report*, there are over 300 impairments due to excessive bacteria.

Fecal coliform bacteria have extensively been used as an indicator of fecal pollution and the potential presence of other pathogenic microorganisms in water. It has been established that the fecal coliform bacterium *E. coli* is more closely associated with fecal pollution than other fecal coliform bacteria, which may normally reside and multiply in the environment. *E. coli* is a common inhabitant of animal and human intestines and recent studies have shown that isolates from humans and various host animals (e.g., cattle, chickens, and pigs) may differ genetically and phenotypically. Use of genetic and biochemical tests may allow the original host species to be identified and is referred to as bacterial source tracking (BST).

The premise behind BST is that genetic and phenotypic tests can identify bacterial strains that are host specific so that the original host species and source of the fecal contamination can be identified. Often *E. coli* or *Enterococcus* spp. are used as the bacteria targets in BST, as this provides a direct link with water quality standards which are based on these indicators (Parveen, Portier et al. 1999; Dombek, Johnson et al. 2000; Graves, Hagedorn et al. 2002; Field, Chern et al. 2003; Hartel, Summer et al. 2003; Kuntz, Hartel et al. 2003; Stoeckel, Mathes et al. 2004; Harwood, Levine et al. 2005). While there has been some discussion over host specificity and survival of *E. coli* in the environment (Gordon, Bauer et al. 2002), this indicator has the advantage of being known to correlate with the presence of fecal contamination and be useful for human health risk assessments. BST of *E. coli*, thus, has the advantage of direct regulatory significance and availability of standardized culturing techniques for water samples, such as EPA Method 1603 (EPA 2005).

BST is a valuable tool for identifying human and animal sources of fecal pollution. BST has been completed by University of Texas School of Public Health, El Paso (UTSPH EP), formerly with Texas A&M AgriLife Research, for Lake Waco, Belton Lake, San Antonio area, Lake Granbury, Buck Creek, Leon River, and Lampasas River watersheds. The Waco/Belton and Buck Creek studies were funded by the TSSWCB through Clean Water Act §319(h) NPS grants from the U.S. Environmental Protection Agency (EPA) (TSSWCB projects 02-10 and 06-11, respectively) and the Leon and Lampasas project through state general revenue funds (TSSWCB project 10-51); while the San Antonio study and Lake Granbury studies were funded by the Texas Commission on Environmental Quality (TCEQ). In addition, AgriLife Soil and Crop Sciences Department (SCSC) has completed BST projects for the Little Brazos River tributaries and Big Cypress Creek watersheds (TSSWCB projects 09-52 and 09-55, respectively). Additionally, with TSSWCB funding, BST projects have been completed in the Leona River and Attoyac Bayou watersheds to assess water quality impairments (projects 11-50 and 09-10, respectively). A Texas *E. coli* BST Library has been developed based on known source isolates

from the Waco/Belton, San Antonio, Granbury, Buck Creek, Big Cypress, Little Brazos River, Attoyac Bayou, Leon River, Lampasas River, Upper Trinity River and Upper Oyster Creek watersheds. Prior to initiation of this project, the Texas *E. coli* BST Library (ver. 8-12) currently contains 1,669 *E. coli* isolates obtained from 1,455 different domestic sewage, wildlife, livestock and pet fecal samples. While this represented a significant step towards development of a statewide *E. coli* BST library, continued expansion of the library to include additional known source isolates from different Texas watersheds and different animal hosts was still needed. This will allow continued evaluation of the library for geographical stability and the diversity of source specific isolates to identify specific needs for future expansion and refinement of the library. The use of the Texas *E. coli* BST Library will provide for significant cost and time savings for the identification of NPS pollution in the development of TMDLs and WPPs.

A Bacteria TMDL Task Force was jointly established by the TSSWCB and TCEQ in fall 2006. In the Task Force's Report, a strategy to address current and future bacterial TMDLs and Implementation Plans (I-Plans) was outlined, including recommendations for effective use of BST methods in Texas. These included enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR), RiboPrinting (RP), Kirby-Bauer antibiotic resistance analysis (KB-ARA), carbon source utilization (CSU), and *Bacteroidales* PCR. The Task Force recommended using library-independent methods such as *Bacteroidales* PCR for preliminary qualitative analyses and library-dependent methods (e.g., ERIC-PCR and RP) if more quantitative data are required. Further characterization of known source *E. coli* for expansion of the Texas *E. coli* BST Library and continued support of established BST analytical infrastructure will help achieve the recommendations of the Task Force.

The Task Force Report identified certain R&D needs to advance understanding of bacteria. Specifically, 30 types of studies or research needs in 6 categories (including Characterization of Sources and Bacterial Source Tracking) were identified. This list was not exhaustive and no attempt was made to prioritize these activities. As such, there is a need to update, expand and prioritize these BST-related R&D activities.

Lastly, the state of BST science, methodologies, application and confidence has evolved greatly in the past few years. A host of new information is currently available, yet not readily distributed or known to state and federal agency personnel. To address this, the 2012 BST – State of the Science Conference was held. To build on the success of this conference, continued outreach and technology transfer was needed to foster dialogue and collaboration and bring water resource managers up to speed on advances in BST technologies, methodologies, applications and results.

To address these needs, the project goals were to support BST analyses across the State through:

(1) continued staffing and maintenance of analytical infrastructure at public BST labs;
(2) continued development, improvement, and implementation of standardized statewide BST procedures for ERIC-PCR, RiboPrinting, and *Bacteroidales* PCR and coordination among entities conducting BST in Texas to ensure common methodologies are employed;
(3) delivery of information on the BST Program and relevant BMPs to local, state and national audiences;
(4) continued development of the Texas *E. coli* BST Library; and,
(5) further development of source-specific bacteria markers for library independent BST.

Expansion of the Texas *E. coli* BST Library

The previous Texas *E. coli* BST Library version 1-13 contained 1,454 isolates from 1,291 samples from 12 watershed projects: Waco-Belton, San Antonio, Lake Granbury, Buck Creek, Oyster Creek, Trinity River, Attoyac Bayou, Big Cypress Creek, Little Brazos River, Lampasas River, and two projects for the Leon River. This version of the library was based on the collection of 3,342 samples of which 2,519 samples tested positive for *E. coli* and 8,812 isolates were archived. ERIC-PCR was used to screen 6,028 of these isolates for removal of clones and 3,133 isolates were subsequently RiboPrinted. A total of 2,994 isolates from 2,474 samples were selected for local watershed libraries. Self-validation Jackknife screening of these 12 local libraries resulted in the selection of 1,713 isolates from 1,484 samples for further evaluation. After serial Jackknife cross-validation screening, 1,454 isolates from 1,291 samples were incorporated into the Texas *E. coli* BST Library (ver. 1-13). A review of the ver. 1-13 library composition and source representation identified several under-represented sources, especially small mammals. The need for additional sampling of septage, designated cattle (i.e., "dairy" or "beef"), other livestock, and feral hogs was identified as well. Sample collection from small mammals was specifically addressed in the present project (TSSWCB 13-50) and taken into consideration during the planning or completion of four other BST studies (Leona, Birds and Bridges, Arroyo Colorado, and Bacteria Growth and Persistence).

The Leona River watershed project (TSSWCB Project 11-50) was completed shortly after publication of the TSSWCB Project 10-50 report. In that project, 260 samples from various known sources were collected including beef cattle, feral hogs and other non-avian livestock. Self-validated isolates from the Leona study were used to develop the Texas *E. coli* BST Library ver. 6-13 which was subsequently used to identify water isolates from the San Antonio River Authority (SARA) and Guadalupe-Blanco River Authority (GBRA) (see Appendix A for statistics of the ver. 6-13 library).

To specifically address the need for small mammalian wildlife sources under the present project (TSSWCB 13-50), 100 known source fecal samples were collected by Texas A&M Institute of Renewable Natural Resources (IRNR) from the Leon and San Antonio River watersheds. Since these were previously studied watersheds, it also provided an opportunity to explore library temporal stability (see temporal studies below). For the San Antonio watershed study, 75 non-avian wildlife fecal samples were collected including those from deer mouse, opossum, raccoon, rat, skunk, deer and feral hogs. A total of 72 samples were positive for *E. coli* with 358 isolates archived. ERIC-PCR was used to screen 216 of these isolates for removal of clones and 125 isolates were subsequently RiboPrinted and included in the Infrastructure local library. Also included in this local library were *E. coli* isolates from 24 non-avian livestock (goats) and non-avian wildlife (raccoon, skunk, and deer) samples collected from the Leon watershed. All 24 source samples tested positive for *E. coli* with 120 isolates archived. ERIC-PCR was used to screen 72 of these isolates for removal of clones and 31 isolates from the 24 samples were subsequently RiboPrinted and included in the local library. Self-validation Jackknife analysis with seven-way split of source classes was performed for the Infrastructure local library, although it should be noted that the Leon and San Antonio isolates represented only two of seven source classes. Self-validation resulted in the selection of 146 isolates from 90 samples (120 isolates from 70 samples from San Antonio and 24 isolates from 20 samples for Leon).

Additional source isolates were also obtained from three other concurrent BST studies. A total of 24 *E. coli* isolates were obtained from 6 house swallow source samples as part of TSSWCB Project 11-51 *Instream Bacteria Influences from Bird and Bat Habitation of Bridges* ("Birds and Bridges" project). After screening for clones using ERIC-PCR, 11 isolates from the 6 samples were selected for further analysis. No self-validation was performed on this set of isolates because they were all from the same source class (avian wildlife). Small wildlife (including mice, opossum, and rat) and avian wildlife source samples and subsequent *E. coli* isolates were obtained from the Riesel watersheds through the *Bacteria Growth, Persistence, and Source Assessment in Rural Texas Landscapes and Streams* project (TSSWCB 13-56). The ongoing Arroyo Colorado watershed project also made a significant contribution to the development of the current state library. It is noteworthy that the Arroyo Colorado watershed includes coastal and delta areas. Source samples for the project included those from coastal and seabirds (e.g., black and royal terns, pelicans) representing the first samples from these source to be evaluated for expansion of the state library. It should be noted that 116 seabird samples were collected from the Arroyo Colorado watershed, but only 18 samples, or 15%, were positive for *E. coli*.

As of May 2015, a total of 742 source samples have been collected from these additional BST projects. Of these, 448 samples tested positive for *E. coli* with 2,140 isolates archived. ERIC-PCR was used to screen 903 of these isolates for removal of clones and 641 were subsequently RiboPrinted. The local libraries for these five projects included a total of 570 isolates from 448 samples. Self-validation Jackknife analysis (seven-way split) for source class specificity resulted in the selection of 382 isolates from 296 samples. These were added to the previously selected self-validated isolates from the projects included in ver. 1-13 for further evaluation and possible inclusion in the current Texas *E. coli* BST Library ver. 5-15 (see Table 1).

To increase its accuracy and utility, the Texas *E. coli* BST Library, with combined self-validated local watershed libraries as described in Table 1 (2095 isolates) was refined through cross-validation. To remove cosmopolitan (non-specific) *E. coli* source isolates, repetitive Jackknife analyses of the combined self-validated libraries were performed to remove isolates that cross identified between human, domestic animals, and wildlife with the goal of 100% average rate of correct classification (ARCC) using a 3-way split of source classes. After the first Jackknife analysis, 296 isolates were removed leaving 1,799 isolates. Two additional rounds of Jackknife analysis were performed resulting in 1,765 isolates with a 100% ARCC using a 3-way split of source classes and a 91% ARCC using a 7-way split. On average, about 16% of the original self-validated isolates per source class were identified as cosmopolitan strains and removed. The percentage was highest for the pet source class where 33 of the 116 original self-validated isolates (28%) were identified as cosmopolitan and removed. A total of 18% of the isolates were singletons (i.e., unique fingerprints) (Table 2). The Texas *E. coli* BST Library ver. 5-15 contains 1,765 isolates obtained from 1554 individual fecal samples. The ver. 5-15 library composition based on 7- and 3-way source class splits is presented in Figures 1 and 2, respectively. Compared to Texas *E. coli* BST Library ver. 1-13, ver. 5-15 has increased from 0 to 42 isolates from mice, 2 to 15 isolates from rats, and one-and-a-half to twice the number of opossum, skunk, deer, raccoon and feral hog isolates. Therefore, we have made significant progress in addressing identified gaps in animal source representation in the library.

Table 1: Effort for sample collection, fingerprinting, and screening for Texas *E. coli* BST Library ver. 5-15

Watershed	# of total samples collected	# of (+) samples	# of isolates archived	# of isolates ERIC-PCR	# of isolates Ribo-Printed	# of isolates local library	# of samples local library	# of isolates self-validated	# of samples self-validated	# of samples in TXSV 5-15	# of isolates in TXSV 5-15
San Antonio	1013	786	3330	2107	947	932	778	457	403	347	388
Waco-Belton	1143	834	3224	2275	1079	958	813	537	481	442	489
Lake Granbury	74	59	198	173	80	80	59	60	48	39	43
Oyster Creek	355	298	292	286	286	286	286	166	166	130	130
Trinity River	193	130	129	128	128	128	128	67	67	47	47
Buck Creek	60	28	53	53	31	31	28	20	20	13	13
Little Brazos River	75	66	166	63	85	85	66	66	57	51	51
Leon (SCSC)	30	30	146	146	72	72	30	58	27	40	40
Leon (UTSPH)	95	71	323	204	133	132	71	85	60	56	76
Lampasas	118	85	384	244	145	143	83	97	67	59	78
Big Cypress	30	19	73	73	34	34	19	28	16	15	24
Attoyac	156	113	494	113	113	113	113	72	72	57	57
Leona	260	201	900	201	201	201	201	94	94	76	76
Arroyo Colorado	254	99	409	274	144	144	99	75	61	48	59
Infra 2013 Leon	25	24	120	72	31	31	24	26	20	19	24
Infra 2013 SA	75	72	358	216	125	125	72	120	70	67	109
Riesel (avian/ non-avian wildlife only)	56	46	189	116	116	58	46	56	45	44	53
Birds and Bridges (avian wildlife only)	20	6	24	24	24	11	6	11	6	4	8
TOTAL	4032	2967	10812	6768	3774	3564	2922	2095	1780	1554	1765

Table 2. Texas *E. coli* BST Library (ver. 5-15, cross-library validation) composition and rates of correct classification (RCCs) by Jackknife analysis of ERIC-RP composite data sets using an 80% similarity cutoff and 3 and 7-way splits

Source Class	Number of Isolates	Number of Samples	Library Composition and RARCC*	Calculated RCC	RCC to Random Ratio***	Left Unidentified (unique patterns)
HUMAN	**384**	**330**	**22%**	**100**	**4.5**	**6**
DOMESTIC ANIMALS	**532**	**495**	**30%**	**100**	**3.3**	**19**
Pets	83	74	5%	84	16.8	41
Cattle	232	216	13%	93	7.2	11
Avian Livestock	95	88	5%	89	17.8	26
Other Non-Avian Livestock	122	117	7%	94	13.4	15
WILDLIFE	**849**	**729**	**48%**	**100**	**2.1**	**16**
Avian Wildlife	273	250	15%	79	5.3	19
Non-Avian Wildlife	576	479	33%	91	2.8	15
Overall	**1765**	**1554**		**ARCC** ** **= 3-way 100% 7-way 91%**		**18%**

*RARCC, expected random average rate of correct classification based on library composition

**ARCC = average rate of correct classification: the proportion of all identification attempts which were correctly identified to source class for the entire library, which is similar to the mean of the RCCs for all source classes when the number of isolates in each source class is similar

***An RCC/Random Ratio greater than 1.0 indicates that the rate of correct classification is better than random. For example, the rate of correct classification for human is 4.5-fold greater than random chance based on library composition.

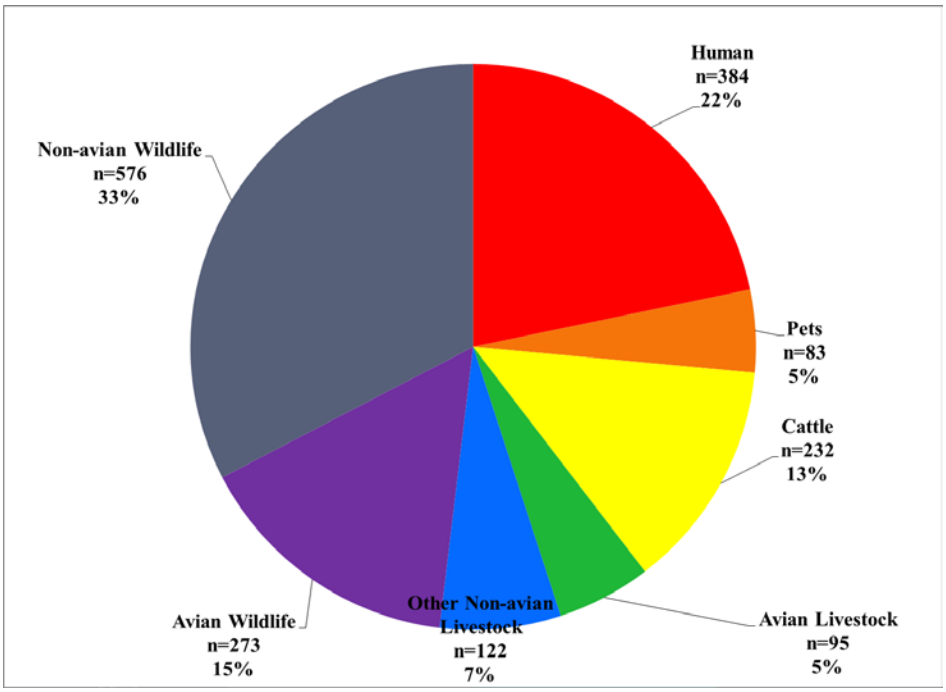


Figure 1. Texas *E. coli* BST Library (ver. 5-15) library composition by 7-way split of source classes (1,765 isolates from 1,554 different fecal source samples).

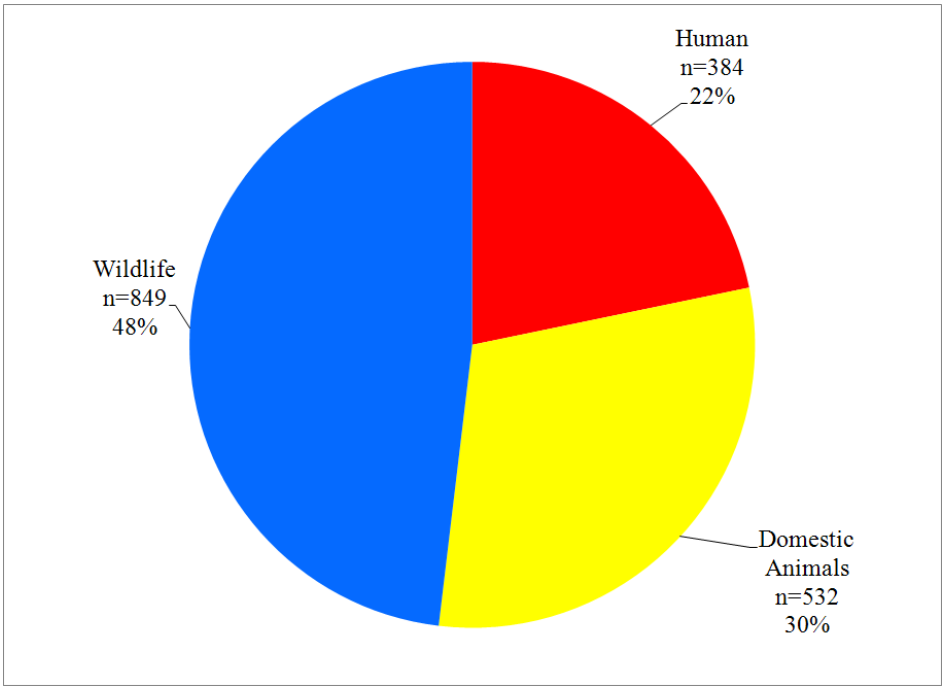


Figure 2. Texas *E. coli* BST Library (ver. 5-15) library composition by 3-way split of source classes (1,765 isolates from 1,554 different fecal source samples).

Genotypic diversity of the Texas *E. coli* BST Library ver. 5-15

To begin assessing the genetic diversity of the Texas *E.coli* BST Library ver. 5-15, the number of genotypes for each source class was calculated one at a time based on its ERIC-RP dendrogram using an 80% similarity cutoff (Table 3). A genotype quotient was also calculated by dividing the number of genotypes by the number of isolates for each source class. Approximately 60% of the genotypes in each source class were represented by singleton isolates. The pet source class appears to be quite diverse with a genotype quotient of 0.69 and 70% of the genotypes represented by singleton isolates. In contrast, the cattle source class appears to be one of the least diverse with a genotype quotient of 0.36 and only 48% of genotypes represented by singleton isolates.

Table 3. Genotypic diversity of the Texas *E. coli* BST Library ver. 5-15

Host Class	# of isolates	# of genotypes	Genotype quotient	# of singletons (% of isolates)	Singletons as % of genotypes	Largest cluster size (% of isolates)
Human	384	164	0.43	98 (26%)	60%	23 (6%)
Pet	83	57	0.69	40 (48%)	70%	4 (5%)
Cattle	232	83	0.36	40 (17%)	48%	39 (17%)
OLA	95	50	0.53	31 (33%)	62%	13 (14%)
OLN	122	46	0.38	25 (20%)	54%	24 (20%)
WA	273	122	0.45	80 (29%)	66%	26 (10%)
WN	576	209	0.36	121 (21%)	58%	32 (6%)

Evaluation of the Texas *E. coli* BST Library

Quality control and reproducibility of DNA fingerprints

Detailed analysis of ERIC-PCR and RiboPrint DNA fingerprint quality control data was performed prior to evaluating the Texas *E. coli* BST Library for temporal and geographical stability. This included assessment of long-term intra- and inter-lab reproducibility for DNA fingerprints generated by the UTSPH and SCSC laboratories. A control strain, *E. coli* QC101 [American Type Culture Collection (ATCC) 51739], is included on every ERIC-PCR gel (1 for every 23 field isolates) and every day the RiboPrinter is run (at least 1 for every 4 batches or 31 isolates). Our standard QC criterion is for *E. coli* QC101 ERIC-PCR and RiboPrint DNA fingerprints to match those from previous sample batches at ≥85% similarity. This is routinely performed only on a project-by-project basis. However, a long-term assessment of reproducibility was needed prior to exploring temporal and geographic stability of the Texas *E. coli* BST Library since it would include analyzing data from numerous individual projects and data generated by the two laboratories.

The ERIC-PCR and RP patterns generated from all studies over the past 12 years were compiled and compared. Intra-lab reproducibility was measured by Jackknife analysis of each fingerprint type to determine the number of unmatched isolates. Inter-lab reproducibility was assessed by using Jackknife analysis of the SCSC data as an "unknown" challenge set against the UTSPH QC101 fingerprints. The comparisons show that both the ERIC-PCR and RP fingerprints are highly reproducible within and between laboratories and projects over time (Table 4).

Table 4. Reproducibility of *E. coli* QC101 quality control strain ERIC-PCR and RP fingerprints

Lab	ERIC-PCR fingerprints (n =)	ERIC-PCR <85%	ERIC-PCR ≥85%	RP fingerprints (n =)	RP <85%	RP ≥85%
UTSPH	558	1 (0.1%)	557 (99%)	618	6 (1%)	612 (99%)
SCSC	154	1 (0.6%)	153 (99%)	71	0 (0%)	71 (100%)
SCSC vs UTSPH	154 and 558	0 (0.0%)	154 (99%)	71 and 618	0 (0%)	71 (100%)

The high reproducibility within and between laboratories and projects over time is partially a product of strict adherence to the template-standard operating procedures (SOPs) adopted by the laboratories. The SOPs are routinely reviewed and updated. Through the current project, several minor updates and edits were made to the template-SOPs. The updated SOPs are included in Appendix B.

Temporal stability of known source *E. coli* isolates

Concerns for library-dependent BST include the geographic and temporal stability of the library. These issues are particularly relevant to Texas BST library that has been developed with *E. coli* isolates from multiple watershed studies collected over several years. As of May 2015, known source isolates have been collected under 17 watershed projects across Texas.

Four of these have covered sections of the Leon River watershed from 2003 to 2013, resulting in 620 self-validated known source isolates (i.e., project specific self-validated local library isolates) (Table 5). It should be noted that while there was overlap, different projects collected samples from different types of animals depending on the focus and concerns of the study. For example, the Waco-Belton study collected known source samples from all seven source classes, whereas only cattle and non-avian wildlife samples were collected during the SCSC Leon River study.

Table 5. Self-validated known source *E.coli* isolates from Leon watershed over time

Time period of sample collection	Project	Human isolates (n =)	Domestic animals isolates (n =)	Wildlife isolates (n =)	Project total (n =)
2003—2004	Waco-Belton	174	143	134	451
2009—2010	SCSC Leon	0	36	22	58
2011—2012	UTSPH Leon	14	27	44	85
2013	Infrastructure Leon	0	13	13	26
	Total	188	219	213	620

The self-validated isolates from each time period were combined by source class (3-way source class split) and compared by project exclusive Jackknife analysis (i.e., isolates could not match others from the same project). There were too few isolates from human sources to provide a reliable evaluation. For the temporal evaluation we determined the number of domestic animal and wildlife *E. coli* isolates that did not match another isolate from a different time period within its respective source class (Table 6). On average, 48% of the domestic animal *E. coli* isolates did not have a match with those from different time periods, while 61% of the wildlife isolates did not have a match.

Table 6. Evaluation of temporal stability using Leon River watershed known source *E. coli* isolates project exclusive Jackknife analyses at ≥80% similarity.

Comparison	% domestic animal isolates without a match (n = # of isolates compared)	% wildlife isolates without a match (n = # of isolates compared)
2003—2004 Waco-Belton vs others	50% (n = 143 vs 76)	62% (n = 134 vs 79)
2009—2010 SCSC Leon vs others	33% (n = 36 vs 183)	45% (n = 22 vs 191)
2011—2012 UTSPH Leon vs others	70% (n = 27 vs 192)	55% (n = 44 vs 169)
2013 Infrastructure Leon vs others	23% (n = 13 vs 206)	46% (n = 13 vs 200)

Overall, results suggest approximately 50% of *E. coli* strains from known sources may change over time even within the same watershed. Changes in *E. coli* strain diversity within animal populations over time is a plausible explanation. However, the number and types of animals sampled in each study varied which may have also influenced the outcome of the comparisons.

Temporal stability of water *E. coli* isolates

As a follow-up to the temporal stability evaluation of known source isolates, a similar evaluation of water *E. coli* isolates was performed. In addition, the number of different *E. coli* ERIC-RP genotypes was also determined. The evaluation of water *E. coli* isolates was undertaken to determine if Leon River water *E. coli* isolates also changed over time. Over 1,000 water *E. coli* isolates have been collected from water stations along the Leon River over a span of nine years under four BST studies (Table 7).

Table 7. Evaluation of temporal stability using Leon River water *E. coli* isolates with project exclusive Jackknife analyses at ≥80% similarity.

Comparison (n = # of isolates)	# of different *E. coli* ERIC-RP genotypes in query set	% of isolates without a match
2003—2004 Waco-Belton (n = 125) vs others (n = 1027)	37	6%
2005 TCEQ (n = 200) vs others (n = 952)	74	22%
2009 – 2010 SCSC Leon (n = 180) vs others (n = 972)	53	12%
2011 – 2012 UTSPH Leon (n = 647) vs others (n = 505)	145	37%

The 1,152 Leon River water isolates represented 273 different *E. coli* ERIC-RP genotypes (80% similarity cutoff). Interestingly, 56 of the genotypes were shared between 2 or more time periods, representing 57% (662) of the water isolates while only 11% (131) of the isolates were singletons. The remaining isolates were members of genotypes unique to their time period.

A similar *E. coli* water isolate comparison was made for the San Antonio River watershed. A total of 370 isolates collected between September, 2013 and February, 2015 from the current SARA study were compared to the 1008 water isolates collected in 2005 under the TCEQ project. When the TCEQ water isolates were treated as the "library," and the SARA isolates were treated as the query set using project exclusive Jackknife analysis (80% similarity cutoff), 21% (71) of the SARA isolates did not have a match.

At first glance it may appear that water *E. coli* isolates exhibit much less temporal variation than known source isolates from the same watershed. However, the differences in numbers of isolates included in the analyses needs to be taken into consideration. For example, when using the 125 Leon River water isolates from the Waco-Belton as the query set and the remaining 1,027 water isolates from other studies as the "library", only 6% of the Waco-Belton isolates did not have a match. In contrast, 37% of the UTSPH Leon water isolates did not have a match with the remaining 505 water isolates from other Leon River studies. Overall, it appears that *E. coli* water isolates exhibit a similar or slightly less temporal variability than known source isolates.

Geographical stability of known source *E. coli* isolates

In an ideal situation, a local watershed library should be developed for library-dependent source tracking methods like ERIC-RP, using a large variety of potential fecal sources collected from the watershed at the same time unknown *E. coli* isolates from water samples are collected. A local library should be representative of the different potential human and animal sources of fecal contamination for the watershed, as well as represent the diversity of *E. coli* populations associated with these different sources. Unfortunately, time and resources infrequently allow for the development of such comprehensive local libraries for every watershed. Because of the limitations of small libraries, including identification accuracy and bias, small local libraries should not be considered as stand-alone libraries. Development of a statewide library by combining and screening the smaller collections of known source isolates may allow acceptable results to be obtained in a timely and cost effective manner. Although temporal stability issues are of some concern with this approach, geographical stability of known source *E. coli* isolates is of overriding importance.

The UTSPH Leon and the Lampasas watershed studies were performed concurrently by the same sampling and analytical personnel and may serve as a temporal constant for evaluation of geographical differences. Project exclusive Jackknife analysis (80% similarity cutoff) was performed using the UTSPH Leon self-validated known source isolates as the query set against the "library" of Lampasas self-validated isolates. Rates of correct classification (RCC) were calculated using a 3-way split of source classes (Table 8). These RCCs were compared to those calculated for the UTSPH Leon known source isolates vs all other self-validated Leon known source isolates from different projects (see Table 5 for composition).

Table 8. Evaluation of geographical stability using Leon and Lampasas River known source *E. coli* isolates using project exclusive Jackknife analyses at ≥80% similarity (RCC=rate of correct classification; U/I-unidentified).

Comparison (n = # of isolates)	Human	Domestic Animals	Wildlife	ARCC
UTSPH Leon (n = 85) vs Lampasas (n = 97)	67% RCC (57% U/I)	6% RCC (33% U/I)	52% RCC (25% U/I)	39% ARCC (33% U/I)
UTSPH Leon (n = 85) vs other Leon (n = 535)	67% RCC (79% U/I)	38% RCC (70% U/I)	81% RCC (52% U/I)	69% ARCC (62% U/I)

Given the results of the Leon River known source *E. coli* temporal stability evaluation, the results of the geographical stability evaluation were not surprising. Leon River domestic animal isolates frequently cross-identified with Lampasas River wildlife isolates resulting in a particularly low RCC for the domestic animal source class. The issue of domestic animal and wildlife isolate cross-identification has been observed in the past and described in earlier reports. Interestingly, the UTSPH Leon source isolates as the query set tested against other Leon River source isolates collected over time as the "library" resulted in a 69% ARCC. However, only 38% of the query isolates matched other Leon River isolates. The highest RCCs were obtained for human isolates, but the identification (matching) rate was only about 30% overall for the two challenge sets. Therefore, it appears that there is a fairly high amount of geographical variability for known source isolates although some source-specific isolates with broader geographical and temporal distribution do indeed occur.

Re-evaluation of library challenges with Lake Granbury, Upper Oyster Creek and Trinity River known source isolates

In previous reports (TSSWCB Projects 08-50 and 08-51, *Increased Analytical Infrastructure and Further Development of a Statewide Bacterial Source Tracking Library*) we conducted preliminary evaluation of geographical stability, and temporal stability to a lesser extent, using data from previous bacterial source tracking projects conducted in different areas of Texas. Previously, "challenges" using known source isolates from studied watersheds were treated as unknowns and identifications were attempted using the TCEQ-TSSWCB Self-Validated library. This represented the first attempt at combining and screening isolates to create a statewide library. We have revisited these evaluations using the current ver. 5-15 of the state library. It should be noted that this provides a very stringent assessment of the library since the challenge isolates can only match library isolates originating from other watersheds. The identification accuracy for the challenge isolates using state libraries were compared to the Jackknife results for the challenge isolates using their local watershed library.

Jackknife analysis (80% similarity cutoff) was performed on the Lake Granbury local library to determine the RCCs using a 3-way split of source classes and 80% similarity cutoff. *E. coli* isolates from individual known source samples were screened to remove clones, but self-validation to remove nonspecific/cosmopolitan isolates was not performed. It should also be noted that smaller libraries tend to have inflated RCCs because small numbers of isolates can represent a relatively large percentage of the library and there are fewer fingerprints that may cause confounding results. As previously described in Figure 10 of the TSSWCB Projects 08-50 and 08-51 final report, the Lake Granbury local library had an ARCC of 76% with 21% of isolates left unidentified using a 3-way split of source classes. These results are presented again here for sake of comparison and the figure has been revised for clarity (Figure 3).

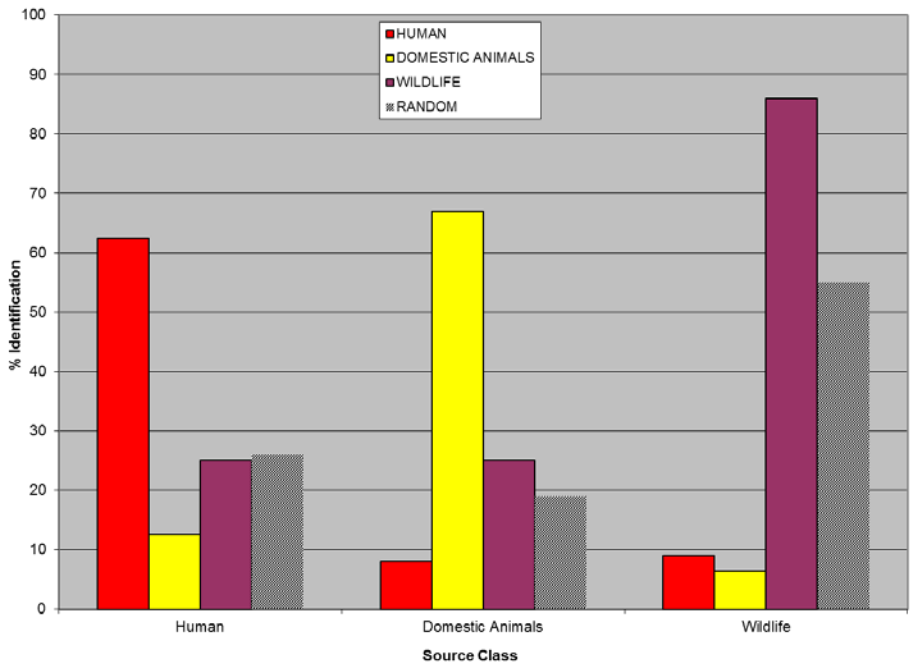


Figure 3. Jackknife analysis of Lake Granbury local library isolates (80 isolates from 59 source samples) using 3-way split of source classes (21% left unidentified).

In the previously reported challenge of the developing state library (TCEQ-TSSWCB self-validated library subset) the ARCC for Lake Granbury known source isolates using 3-way split of source classes was 61% (or ~1.5 times better than random) with 30% of the isolates left unidentified (Figure 11, TSSWCB Projects 08-50 and 08-51 final report). These results are presented again here for sake of comparison and the figure has been revised for clarity (Figure 4). Note that domestic animal isolates are frequently cross-identified as wildlife source.

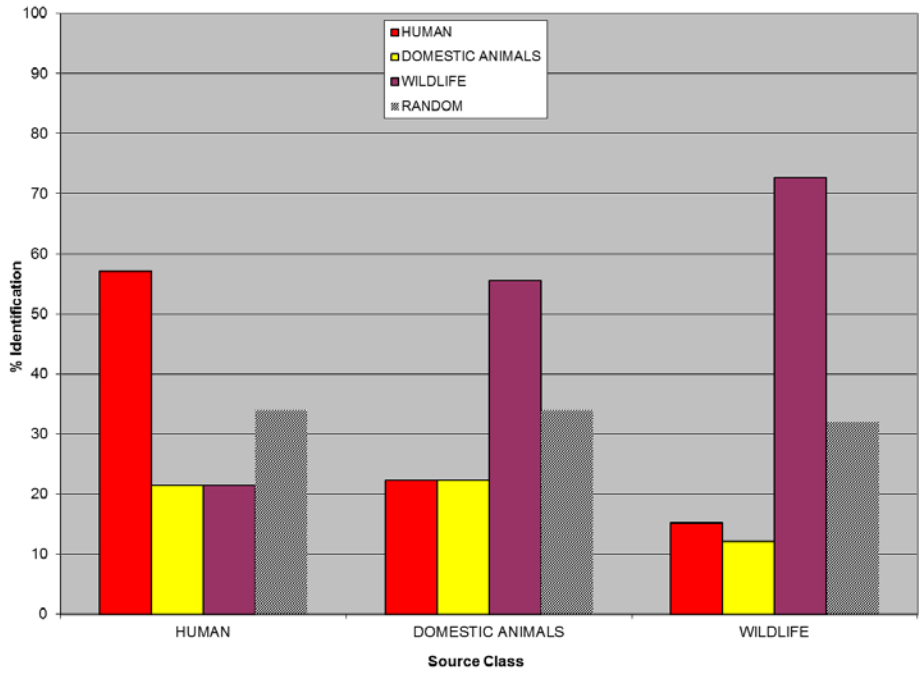


Figure 4. Identification of Lake Granbury local library isolates (80 isolates from 59 source samples) using TCEQ-TSSWCB self-validated library subset and 3-way split of source classes (30% left unidentified).

For the current evaluation, the Texas *E. coli* BST Library ver. 5-15 was used. The 43 self-validated and cross-validated isolates from Lake Granbury were temporarily excluded from the ver. 5-15 for this challenge. This challenge resulted in an ARCC of 58% (or ~1.4 times better than random) with 13% of the isolates left unidentified (Figure 5). While the ARCC was essentially the same as the previous challenge, the previous challenge had 30% of the isolates left unidentified, while the current challenge had only 13%. In the current challenge, there was also 11% more correct matches than before, but this was offset with incorrect identifications for cosmopolitan isolates.

Similarly, the previous challenges with the 286 Upper Oyster Creek and 128 Trinity River known source isolates were repeated using the ver. 5-15. An ARCC of 41% with 21% of the isolates left unidentified was previously reported for the Upper Oyster Creek isolates using the TCEQ-TSSWCB self-validated library and 3-way split of source classes. Challenge of ver. 5-15 resulted in an ARCC of 51% with only 13% of the isolates left unidentified. For the previous challenge of the TCEQ-TSSWCB self-validated library subset with Trinity River known source isolates, an ARCC of 45% with 9% of the isolates left unidentified was reported. Challenge of the ver. 5-15 resulted in an ARCC of 60% with 8% of the isolates left unidentified.

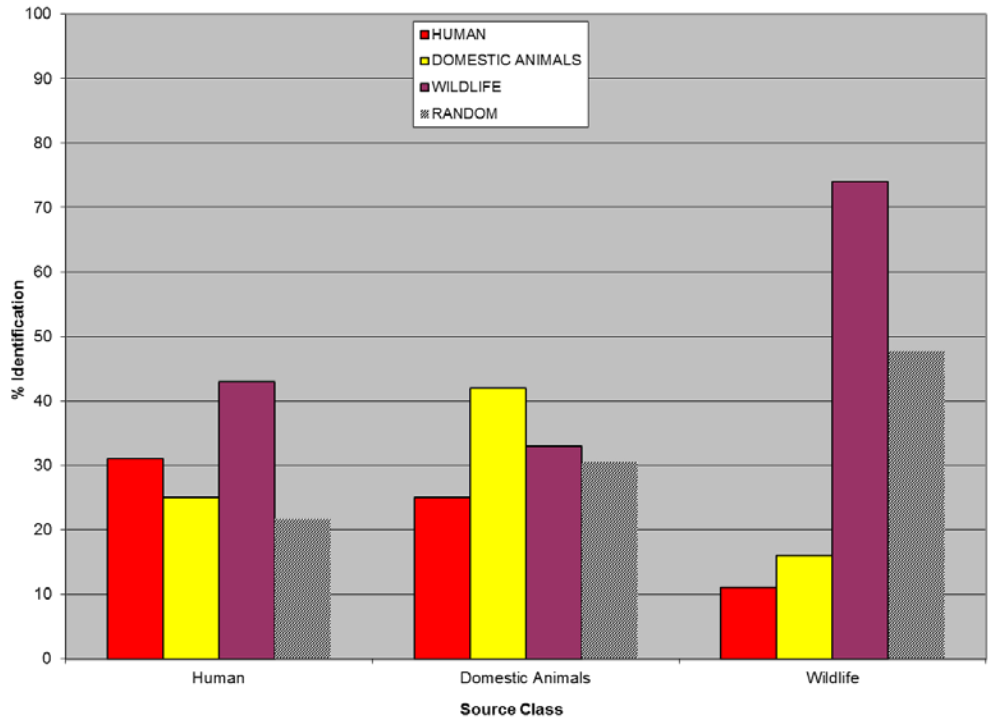


Figure 5. Identification of Lake Granbury local library isolates (80 isolates from 59 source samples) using the Texas *E. coli* BST Library ver. 5-15 (43 Lake Granbury isolates excluded) and a 3-way split of source classes (13% left unidentified).

Of course, the highest ARCCs and lowest percentage of unidentified isolates are obtained when using the Texas *E. coli* BST Library ver. 5-15 including self-validated local library isolates. With this approach ARCCs for the Lake Granbury, Upper Oyster Creek, and Trinity River isolates increased to 69%, 65%, and 64%, respectively; with 10% or less isolates left unidentified.

Cosmopolitan and singleton *E. coli* isolates

Jackknife analysis of the Texas *E. coli* BST Library ver. 5-15 indicates many isolates match back to isolates from their own watershed. Although matching isolates did not come from the same individual known source sample, some may be clonal isolates obtained from the samples collected from the same population of animals. In some cases, these isolates were included in the state library after cross-validation. This is of special concern for isolates which did not undergo a strenuous self-validation screening in their own local library because there were limited isolates from other source classes (or none) to challenge them. As a consequence this has led to some cosmopolitan isolates being included in the state library. Identification of cosmopolitan isolates is an important issue for future refinement of the state library. A preliminary investigation found 70 isolates in the Texas *E. coli* BST Library ver. 5-15 that were matching at greater than even 90% similarity to isolates in another source class (cross identifying) or to those removed during serial Jackknife analyses. These isolates remained in the library due to best matches with isolates from their own watershed. In addition, cross-validation of a library in a watershed exclusive fashion may help identify some of these confounding isolates. Rather than removing these isolates, we may create a new “cosmopolitan” source category.

In addition to cosmopolitan isolates, treatment of singleton isolates also merits further consideration for future refinement of the library. Based on genotype analysis of the library (Table 3), approximately 60% of the library is composed of singleton isolates. Inclusion of singleton isolates helps increase the overall diversity of the library and increases the likelihood of finding matches for water isolates. However, there is a trade-off in including these isolates in the library since their source specificity has not yet been confirmed through cross-validation with other isolates. One approach would be to remove singletons from the library, but that would significantly change the library. Another approach for future studies would be to note which water isolates match library singletons. Regardless of the approaches used to address cosmopolitan and singleton isolates, further refinement of the library will require careful consideration.

Discussion

Significant progress has been made in expanding and refining the Texas *E. coli* BST Library. In particular, additional known source isolates have been added to address underrepresented wildlife species. Temporal and geographical stability evaluations of the library provided useful insight. Temporal stability evaluations using Leon River known source isolates collected over a 10-year period of time revealed that approximately half of the *E. coli* strains from known sources may change over time even within the same watershed. Similarly, temporal evaluation of *E. coli* water isolates from the Leon River watershed revealed similar temporal variability. Therefore, it was not surprising that we also found a fairly high amount of geographical variability for known source isolates. Importantly however, we also identified source-specific isolates that have broader geographical distribution and temporal stability. These isolates deserve further attention as we move forward with library refinement.

Re-challenge of the Texas *E. coli* BST Library ver. 5-15 with known source isolates from three different watersheds showed an overall increase in identification accuracy and fewer isolates left unidentified compared to previous challenges with an earlier version of the library. Lastly, a preliminary assessment of the library for cosmopolitan and singleton isolates was performed. For future BST studies and refinement of the library we may create a new “cosmopolitan” source category and note which water isolates matched singletons in the library.

Utilization of the Texas *E. coli* BST Library

Through the present project, support was provided to several ongoing projects including:

- *BST to Support Adaptive Management of the Arroyo Colorado WPP* (TSSWCB Project 12-10)
- *Development of a WPP for Attoyac Bayou* (TSSWCB Project 09-10)
- *Assessment of Water Quality and Watershed Planning for the Leona River-Bacterial Source Tracking* (TSSWCB Project 11-50)
- *Instream Bacteria Influences from Bird and Bat Habitation of Bridges* (TSSWCB Project 11-51)
- *Leon River Watershed Protection Plan* (TSSWCB Project 06-12)

Efforts continue on *BST to Support Adaptive Management of the Arroyo Colorado WPP*; however, work had been completed on the remaining three projects. BST results supporting *Development of a WPP for Attoyac Bayou* can be found in TWRI Technical Report TR-456, published in May 2014 (http://twri.tamu.edu/media/465202/tr456.pdf). BST results for the Leona River were published in November 2013 (https://www.tsswcb.texas.gov/files/docs/nps-319/projects/11-50-FR-Leona-BST-11-21-13.pdf).

To support Leon River efforts, where significant BST study had been conducted as previously discussed, outreach was conducted to help gather needed stakeholder input to finalize the WPP. TWRI worked with Parsons to arrange a meeting on November 12, 2013 to review draft responses to EPA's comments on the WPP and gather input from the project team and key stakeholders. TWRI and Parsons then worked together to organize a meeting of the Leon WPP Steering Committee on December 18, 2013 to garner input from the broader committee prior to finalizing the response and submitting them to EPA. Parsons led the discussion at this meeting and provided a presentation for the stakeholders summarizing key issues regarding EPA's comments and possible responses to those comments. This ultimately led to the completion of the WPP and resubmission to the EPA for final approval.

Development/Evaluation of Source-Specific Bacterial Markers for Library-Independent BST

Bacteroidales PCR marker specificity

Several published source tracking studies have used *Bacteroidales* PCR assays with favorable results. Although these studies showed tremendous promise for *Bacteroidales* PCR, this same conclusion was not always obtained in other laboratories. In particular, amplification of non-target animal groups has been observed, especially with the *Bacteroidales* PCR for ruminants and less frequently for the human marker assay.

To investigate some of these issues, the current project included the characterization of PCR amplicons from animal fecal DNA which cross-reacted with the *Bacteroidales* HF183 PCR human marker. We also evaluated a modified *Bacteroidales* PF163 PCR method to determine if a primer or probe could be developed to specifically identify fecal pollution from feral hogs.

Methods

Water samples were processed and analyzed for *Bacteroidales* markers per SOP (Appendix B). The QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) was used to extract DNA from approximately 200 mg of each fecal sample. Extracted DNA was stored at −80°C until analyzed by PCR. *Bacteroidales* markers were amplified using the following primer sets: GenBac general *Bacteroidales* marker BAC32F (5′-AACGCTAGCTACAGGCTT-3′) and BAC708R (5′-CAA TCG GAG TTC TTC GTG-3′) (Bernhard and Field, 2000); HF183 human marker HF183F (5′-ATC ATG AGT TCA CAT GTC CCG-3′) and BAC708R (Bernhard and Field, 2000b); PF163 hog marker PF163F (5′-GCGGATTAATACCGTATGA-3′) and BAC708R (Dick et al., 2005); CF128 ruminant marker CF128F (5′-CCAACYTTCCCGWTACTC-3′) and BAC708R (Bernhard and Field, 2000b). Amplification was performed per SOP (Appendix B) with the following exceptions. DNA template volumes were 5 µL for water samples and 1 µL for fecal samples. For the GenBac marker water samples were analyzed using 35 cycles of PCR while fecal samples were amplified using 30 cycles. For all other markers water samples were amplified using 40 cycles of fecal samples were amplified using 35 cycles. The annealing temperature was 53°C for the GenBac and PF163 markers, 58°C for the CF128 marker and 60°C for the HF183 marker. In some cases, PCR was performed using a SYBR Green mastermix (Life Technologies, Grand Island, NY) for real-time PCR and high-resolution melt analysis. Amplification was performed using a RotorGene 6000 thermal cycler (Qiagen, Valencia, CA). PCR products were purified using the QIAquick PCR purification kit or the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). Commercial DNA sequencing was performed (SeqWright, Houston, TX) and DNA sequences were analyzed using Kodon (Applied Maths, Houston, TX) and GenBank BLAST (Basic Local Alignment Search Tool) searches (Altschul, Madden et al., 1997).

Specificity of the HF183 *Bacteroidales* human PCR marker

Specificity for *Bacteroidales* PCR human, ruminant and hog markers is very high based on prior studies (Field, Chern et al. 2003; Gawler, Beecher et al. 2007; Gourmelon, Caprais et al. 2007; Lamendella, Domingo et al. 2007; Lamendella, Santo Domingo et al. 2009) and results from our laboratory (Di Giovanni, Truesdale et al. 2009). Collective results from these studies revealed the human HF183 marker was detected in 149/174 (86% sensitivity) of human fecal samples tested,

and cross-reactivity was reported for only 16/513 (i.e., 97% specificity) of non-target fecal samples from livestock, wildlife, and pets. Despite these promising results, some unusual circumstances have been encountered. For example, in our studies, we have encountered very low numbers of known source fecal samples from badger, porcupine, whitetailed deer, coyote, raccoon and rabbit that cross-reacted with the *Bacteroidales* HF183 human PCR marker. In order for this technology to become effective, this cross-reactivity must be addressed.

Buck Creek watershed sanitary surveys suggested the primary cause of impairment was wildlife (Gregory, Barrella et al., 2012; Farnleitner, Reischer et al., 2011). Preliminary observation supported this suggestion since there was very limited direct human influence in most of the watershed. Water samples and known source samples were collected and tested using standardized PCR for *Bacteroidales* and other BST tools. As expected, the *Bacteroidales* PCR revealed that the prominent source of pollution was wildlife. We found that most of the pollution was originating from ruminants and feral hogs; but, there were a small percentage of water samples that tested positive for the HF183 human marker. Analysis by our laboratory revealed that the HF183 human marker was detected in a few badger and porcupine fecal samples collected from a remote site with very limited human contact. Water samples from that site also frequently tested positive for the HF183 human marker (Gregory, Barrella et al., 2012; Farnleitner, Reischer et al., 2011).

This type of phenomenon was also observed during the Lake Granbury BST study (Di Giovanni, Truesdale et al., 2009). As we observed with Buck Creek, the HF183 marker cross-reacted with a few wildlife fecal samples from coyote, raccoon, deer, and rabbit (Di Giovanni, Truesdale et al., 2009; Farnleitner, Reischer et al., 2011). The HF183 human marker data obtained from the Buck Creek and Lake Granbury studies indicated that there needs to be a clearer understanding of marker specificity.

Cross-reactivity of animal fecal samples for the HF183 human marker was based on the presence of appropriate sized PCR products. However, the DNA sequence of generated PCR products was not determined. If DNA sequences of cross-reacting samples and human source control samples differ, it may be possible to distinguish them. Further, sequence differences could also be targeted for development of new PCR primers or probes for increased specificity. To further investigate HF183 human marker specificity, we examined 93 water and known source fecal samples from 3 different locations that tested positive for the HF183 marker (Table 9). DNA sequence analysis revealed that all HF183 cross-reacting animal fecal samples and all HF183 positive water samples had sequences identical to those obtained from control human fecal and wastewater samples.

Table 9. Water and known source fecal samples used for evaluation of *Bacteroidales* HF183 human marker specificity.

Source	# of samples	Location
Water	35	Buck Creek
Badger	2	Buck Creek
Porcupine	3	Buck Creek
Water	19	Lake Granbury
Deer	1	Lake Granbury
Rabbit	5	Lake Granbury
Coyote	3	Lake Granbury
Raccoon	1	Lake Granbury
Wastewater Effluent (Human)	2	Lake Granbury
Septage (Human)	14	Lake Granbury
Septage (Human)	8	West Virginia
Total	93	

To further investigate the specificity of the HF183 human marker, 101 known source animal fecal samples collected under the current project were analyzed for the presence of the GenBac and HF183 human *Bacteroidales* markers (Table 10). The GenBac marker is for general *Bacteroidales* and should amplify for all samples unless the sample is degraded or there is carryover of PCR inhibitory substances from the sample.

Table 10. TSSWCB Project 13-50 known source fecal samples used for evaluation of *Bacteroidales* PCR HF183 human marker specificity.

Animal	# of samples	Positive for GenBac marker	Positive for HF183 human marker
Cattle	1	1/1	0/1
Deer	18	18/18	0/18
Deer Mouse	13	12/13	1/13
Dog	1	1/1	0/1
Goat	13	13/13	3/13
Cotton Rat	1	1/1	0/1
Mule	1	1/1	0/1
Opossum	12	11/12	2/12
Owl	1	1/1	1/1
Rabbit (domestic)	1	1/1	0/1
Raccoon	16	9/16	2/16
Skunk	7	5/7	0/7
Feral Hog	16	16/16	2/16
Total	101	90	11

A total of 90 of the 101 animal fecal samples tested positive for the GenBac marker. In particular, only 9 of 16 raccoon samples tested positive. Raccoon fecal samples were found to be difficult to amplify in the Lake Granbury project as well (Di Giovanni, Truesdale et al., 2009)

indicating a possible PCR inhibition problem for raccoon feces. A total of 11 of 90 (12%) samples which successfully amplified for the GenBac marker also tested positive for the HF183 human marker, including one or more samples from deer mice, goats, opossums, owl, raccoons and feral hogs. As found in our previous work, DNA sequences of PCR amplicons from these animals samples revealed that they were identical to sequences obtained from human source samples. This could lead to false positives when testing for human fecal pollution in watersheds. The extent of this interference is likely case specific and depends on the distribution of the HF183 *Bacteroidales* bacteria in local animal populations. It is recommended that BST studies utilizing *Bacteroidales* PCR include analysis of individual or pooled known source fecal samples. In addition, the use of quantitative PCR (qPCR) is recommended for the analysis of water samples to potentially identify hotspots.

Evaluation of a *Bacteroidales* PCR assay for detection of feral hog fecal pollution

According to the Texas Department of Agriculture, Texas has the largest feral hog population in the United States with nearly 2 million hogs inhabiting various regions of the state (LaFlure, 2009). There are few inhibiting factors to slow population growth and distribution. Feral hogs prefer to inhabit bottomlands such as rivers, creeks, and drainages. The main concern with feral hogs is that they compete directly with livestock, game, and nongame wildlife species for food. However, most of the damage caused by feral hogs is due to indirect destruction of habitat and agriculture. Damage to agricultural crops, fields, livestock feeding, and watering facilities, is caused by excessive rooting and trampling for food. These hogs also destabilize wetland areas, springs, creeks, and tanks by excessive rooting and wallowing (Taylor, 2003). It is estimated that about $51.7 million in damage every year is due to the feral hogs (Weber, 2009). However, land damage is not the only issue of concern. Due to the fact that feral hogs prefer bottomlands, there is concern that natural waters are becoming contaminated with feces from feral hogs, which poses a potential risk to human health (Zeiler, 2007).

Although a limited number of samples were analyzed, we have previously determined that feral hog feces may test positive for the *Bacteroidales* PF163 hog marker (Lamendella, Santo Domingo, et al., 2009). Due to the potential impacts of feral hogs on water quality in Texas, a *Bacteroidales* PCR method to differentiate domestic and feral hogs would be useful. However, this poses a challenge because feral hogs are domestic hogs that have adapted to the wild. Therefore, the gut of each is the same and the only difference between the two is diet.

To evaluate the ability of a *Bacteroidales* PF163 PCR assay to detect feral hog feces, fecal samples and environmental water samples (n =121) were analyzed for the presence of the marker and amplicon DNA sequence analysis (Table 11). Feral and domestic hog fecal samples and archived Buck Creek and Lake Granbury project water samples were included in the evaluation. Samples collected from Sinton, Texas came from the Welder Wildlife Refuge which is a wildlife management and conservation refuge and cattle operation. Domestic hog fecal samples or fecal DNA from West Virginia and Sierra Blanca, Texas were collected from known sources and sent to us by colleagues.

Table 11. Water and known source fecal samples used for evaluation of *Bacteroidales* PCR PF163 marker for the detection of feral hog fecal pollution.

Location	No. Feral Hog	No. Domestic Hog	No. Water Samples	No. Treated Sewage
Buck Creek	22	3	7	0
Lake Granbury	7	6	3	3
Sinton, TX	18	0	0	0
Las Cruces, NM	0	4	0	0
Lampasas River	11	0	0	0
Leon River	26	0	0	0
Sierra Blanca, TX	0	1	0	0
West Virginia	0	10	0	0
Total	84	24	10	3

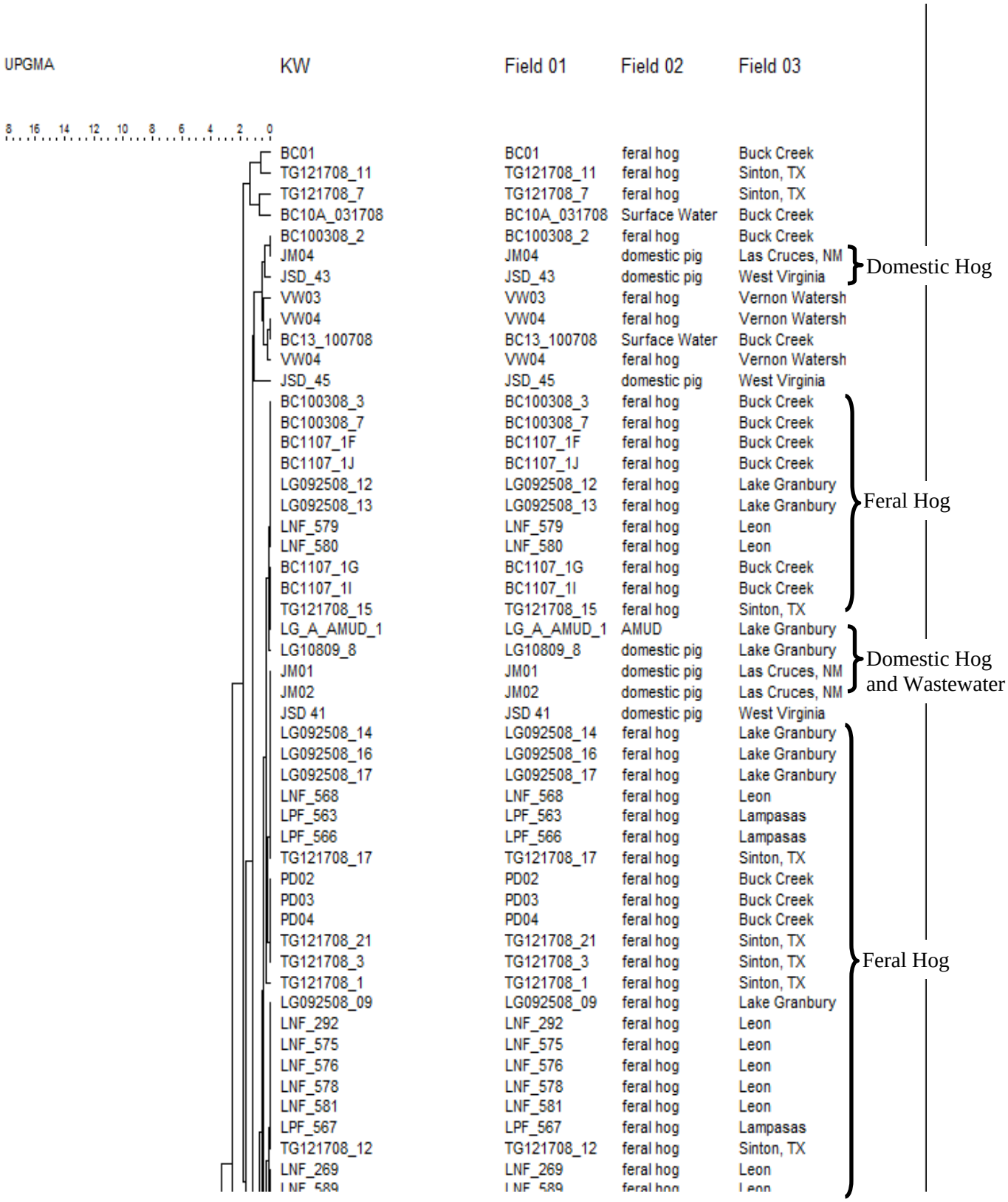
Samples were analyzed for the presence of the *Bacteroidales* general GenBac, PF163 hog, HF183 human and CF128 ruminant markers (Table 12). A SYBR Green real-time PCR assay was also used for the detection of the PF163 marker. All samples tested positive for the GenBac marker. As expected, all 24 domestic hog samples tested positive for the PF163 marker. More importantly, 82 of 84 (98%) feral hog fecal samples tested positive for the PF163 marker using conventional and/or SYBR Green PCR. Previously analyzed environmental water samples re-tested positive for the PF163 marker, including three treated wastewater samples from Lake Granbury. All three treated wastewater samples also tested positive for the HF183 human marker. Only 1 of 42 (2%) domestic and feral hog samples tested positive for the HF183 human marker (also see the previous section on HF183 cross-reactivity). Almost half (25 of 55) of the domestic and feral hog samples tested positive for the CF128 ruminant marker, confirming our previous report of animal cross-reactivity for this marker.

Table 12. Water and known source fecal sample *Bacteroidales* PCR results for evaluation of the PF163 marker.

Sample	Positive for GenBac marker	Positive for PF163 marker	Positive for PF163 marker (SYBR)	Positive for HF183 human marker	Positive for CF128 ruminant marker
Domestic Hog (Buck Creek)	100% (3/3)	100% (3/3)	100% (3/3)	0% (0/3)	33% (1/3)
Domestic Hog (NM)	100% (4/4)	100% (4/4)	100% (4/4)	0% (0/4)	50% (2/4)
Domestic Hog (WV)	NT**	100% (10/10)	100% (10/10)	NT**	NT**
Domestic Hog (Lake Granbury)	100% (6/6)	100 % (6/6)	100 % (6/6)	0% (0/6)	83% (5/6)
Domestic Hog (Sierra Blanca)	100% (1/1)	100% (1/1)	100% (1/1)	NT**	NT**
Feral Hog (Buck Creek)	100% (22/22)	95% (21/22)	95% (21/22)	5% (1/22)	14% (3/22)
Feral Hog (Sinton)	100% (18/18)	100% (18/18)	100% (18/18)	NT**	53% (8/15)*
Feral Hog (Lake Granbury)	100% (7/7)	100% (7/7)	100% (7/7)	0% (0/7)	86% (6/7)
Feral Hog (Lampasas)	100% (11/11)	NT**	91% (10/11)	NT**	NT**
Feral Hog (Leon)	100% (26/26)	NT**	100% (26/26)	NT**	NT**
Surface Water (Buck Creek)	100% (7/7)	100% (7/7)	100% (7/7)	43% (3/7)	100% (7/7)
Surface Water (Lake Granbury)	100% (3/3)	100% (3/3)	100% (3/3)	0% (0/3)	33% (1/3)
Treated Sewage (Lake Granbury)	100 % (3/3)	100% (3/3)	100% (3/3)	100% (3/3)	33% (1/3)

*Three samples were not tested
** NT, not tested

In contrast to the DNA sequencing results for the HF183 marker, sequence analysis of PF163 amplicons from feral hogs and domestic hogs revealed relatively high sequence heterogeneity (Fig. 6). Samples frequently tended to group together based on geographic location. Some but not all surface water samples and treated sewage samples clustered with known fecal samples. Interestingly, many of the Buck Creek and Lake Granbury surface water samples sub-clustered separately from hog sequences. Possible explanations include environmental selection of hog-derived PF163 *Bacteroidales* bacteria or possible cross-reactivity with other fecal sources. Additional analysis of these sequences in sequences available in GenBank will be needed to determine if there are possible targets for refinement of PCR primers or probe development.


UPGMA
KW
Field 01
Field 02
Field 03
8 16 14 12 10 8 6 4 2 0
BC01 BC01 feral hog Buck Creek
TG121708_11 TG121708_11 feral hog Sinton, TX
TG121708_7 TG121708_7 feral hog Sinton, TX
BC10A_031708 BC10A_031708 Surface Water Buck Creek
BC100308_2 BC100308_2 feral hog Buck Creek
JM04 JM04 domestic pig Las Cruces, NM
JSD_43 JSD_43 domestic pig West Virginia
Domestic Hog
VW03 VW03 feral hog Vernon Watersh
VW04 VW04 feral hog Vernon Watersh
BC13_100708 BC13_100708 Surface Water Buck Creek
VW04 VW04 feral hog Vernon Watersh
JSD_45 JSD_45 domestic pig West Virginia
BC100308_3 BC100308_3 feral hog Buck Creek
BC100308_7 BC100308_7 feral hog Buck Creek
BC1107_1F BC1107_1F feral hog Buck Creek
BC1107_1J BC1107_1J feral hog Buck Creek
LG092508_12 LG092508_12 feral hog Lake Granbury
LG092508_13 LG092508_13 feral hog Lake Granbury
Feral Hog
LNF_579 LNF_579 feral hog Leon
LNF_580 LNF_580 feral hog Leon
BC1107_1G BC1107_1G feral hog Buck Creek
BC1107_1I BC1107_1I feral hog Buck Creek
TG121708_15 TG121708_15 feral hog Sinton, TX
LG_A_AMUD_1 LG_A_AMUD_1 AMUD Lake Granbury
LG10809_8 LG10809_8 domestic pig Lake Granbury
JM01 JM01 domestic pig Las Cruces, NM
JM02 JM02 domestic pig Las Cruces, NM
Domestic Hog and Wastewater
JSD 41 JSD 41 domestic pig West Virginia
LG092508_14 LG092508_14 feral hog Lake Granbury
LG092508_16 LG092508_16 feral hog Lake Granbury
LG092508_17 LG092508_17 feral hog Lake Granbury
LNF_568 LNF_568 feral hog Leon
LPF_563 LPF_563 feral hog Lampasas
LPF_566 LPF_566 feral hog Lampasas
TG121708_17 TG121708_17 feral hog Sinton, TX
PD02 PD02 feral hog Buck Creek
PD03 PD03 feral hog Buck Creek
PD04 PD04 feral hog Buck Creek
TG121708_21 TG121708_21 feral hog Sinton, TX
TG121708_3 TG121708_3 feral hog Sinton, TX
TG121708_1 TG121708_1 feral hog Sinton, TX
LG092508_09 LG092508_09 feral hog Lake Granbury
LNF_292 LNF_292 feral hog Leon
LNF_575 LNF_575 feral hog Leon
LNF_576 LNF_576 feral hog Leon
LNF_578 LNF_578 feral hog Leon
LNF_581 LNF_581 feral hog Leon
LPF_567 LPF_567 feral hog Lampasas
TG121708_12 TG121708_12 feral hog Sinton, TX
LNF_269 LNF_269 feral hog Leon
Feral Hog

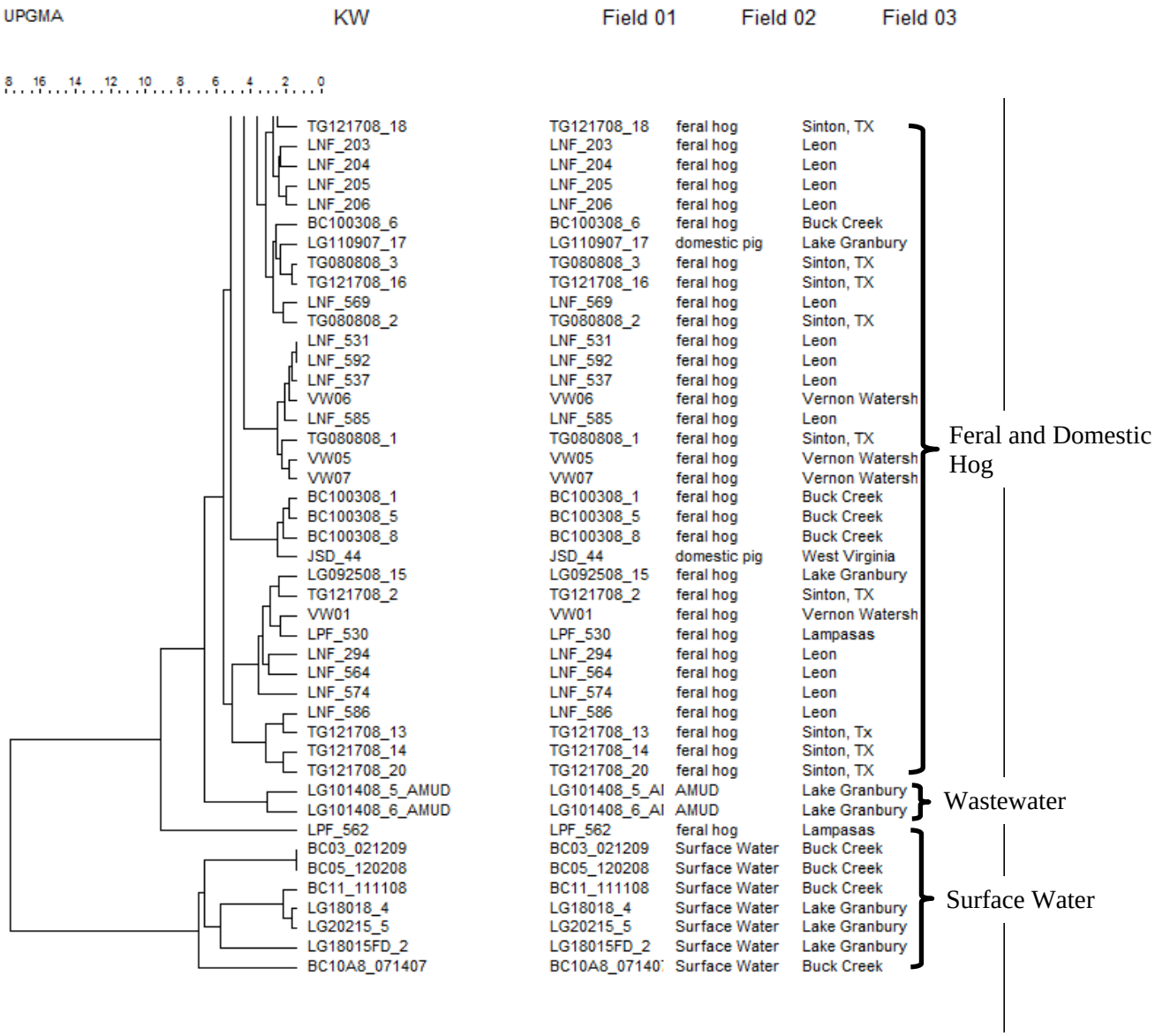


Figure 6. Comparison of PF163 *Bacteroidales* hog marker DNA sequences from domestic and feral hog fecal samples and environmental water samples from Texas watersheds. Scale is nucleotide differences per 100 bases.

Discussion

DNA sequence analysis of *Bacteroidales* PCR HF183 human marker amplicons from cross-reacting animal fecal samples from previous BST projects revealed identical sequences to human. We further investigated the specificity of the HF183 human marker by analyzing 101 known source animal fecal samples collected under the current project. A total of 11 of 90 (12%) wildlife fecal samples tested positive for the HF183 human marker. These samples were collected from a variety of wildlife species and therefore cross-reactivity is not limited to specific animal species. Again, we found the HF183 amplicon sequences from these cross-reactors to be

identical to those obtained from control human fecal and wastewater samples. The extent of this interference is likely case specific and depends on the distribution of the HF183 *Bacteroidales* bacteria in local animal populations. It is recommended that BST studies utilizing *Bacteroidales* PCR include analysis of individual or pooled known source fecal samples. In addition, the use of qPCR is recommended for the analysis of water samples to potentially identify hotspots.

Extensive evaluation of a *Bacteroidales* PCR PF163 hog marker assay using modified PCR mastermix and cycling conditions clearly demonstrated that we are able to detect fecal pollution from feral hogs. In contrast to the DNA sequencing results for the HF183 marker, sequence analysis of PF163 amplicons from feral hogs and domestic hogs revealed relatively high sequence heterogeneity. Additional evaluation and sequence analysis may yield suitable PCR primers or probes for the specific detection of feral hog pollution. Interestingly, several wastewater samples also tested positive for the marker, and this will need to be addressed as well during primer and probe development.

ERIC-PCR amplicon characterization

Although we use ERIC-PCR as one of our DNA fingerprinting tools for the construction of the Texas *E. coli* BST library, we know very little about the DNA sequences being amplified. The goal of this work was to analyze DNA sequences of ERIC-PCR amplicons from selected human-specific *E. coli* present in the Texas *E. coli* BST Library to explore the potential development of library-independent PCR targets capable of differentiating human and animal derived *E. coli*.

Methods

A band matching comparison of human isolates in the Texas *E. coli* BST Library ver. 3-12 was performed. Several of the isolates that matched four or more other human source isolates were chosen for further analysis. Band matching comparison for these isolates was performed to identify amplicons for sequence analysis (Figure 7).

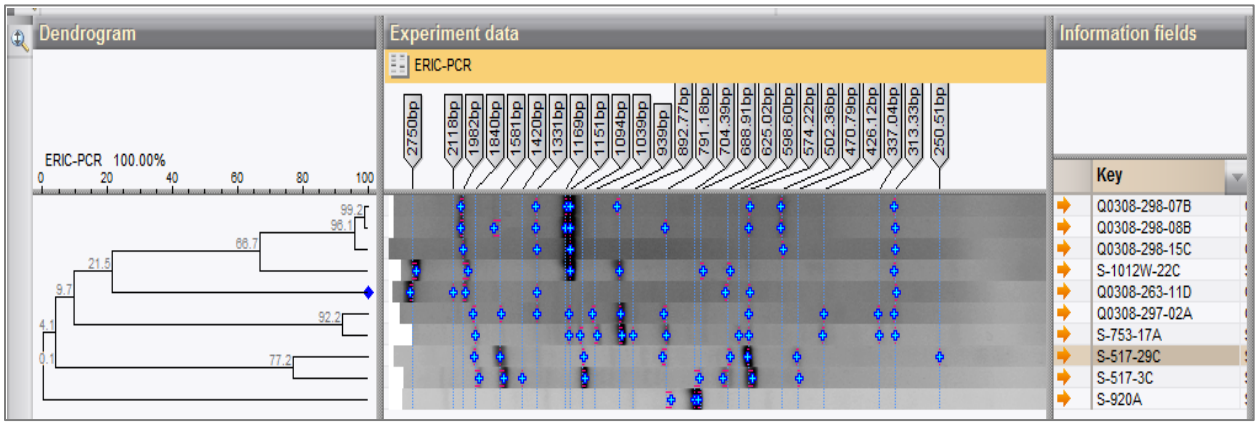


Figure 7. Band matching comparison of ERIC-PCR fingerprints of selected *E. coli* human source isolates from the Texas *E. coli* BST Library ver. 3-12.

Fresh cultures were prepared for the isolates and ERIC-PCR performed per SOP (Appendix B). Agarose gel slices containing ERIC-PCR amplicon bands of interest were excised and transferred into sterile 1.5-ml Eppendorf tubes and DNA was purified with the QIAquick Gel

Extraction kit (Qiagen, Valencia, CA). The purified fragments were ligated with the pCR 2.1-TOPO vector from the TOPO TA cloning kit (Invitrogen, Grand Island, NY) and transformed into chemically competent One Shot *E. coli* cells. The transformants were plated on Luria–Bertani (LB) (BD, Franklin Lakes, NJ) plates containing 50 µg/mL of kanamycin and 40 mg/mL of X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) in dimethylformamide (DMF). Three white, 2 white/blue colonies and 2 blue colonies were picked for each signature band. Clones were screened with an M13 PCR protocol flanking the pCR 2.1-TOPO plasmid cloning site. Earlier attempts were made using the ERIC-PCR primers but were unsuccessful due to background DNA from the One Shot *E. coli* host cells. Clones were screened using the M13 Forward (-20) primer (5' GTA AAA CGA CGG CCA G) and the M13 Reverse primer (5' CAG GAA ACA GCT ATG AC). A 10 µL tip was used to pick part of an individual colony and was resuspended in 5 µL of molecular grade water. Then a 50µL reaction containing 1X PCR Buffer with 1.5 mM Mg (final) (ABI, Foster City, CA), 200 µM each of dNTP (GE Healthcare Biosciences, Piscataway, NJ), 200nM M13 Primers forward and reverse (Invitrogen, Grand Island, NY), 1.5 µg/µl bovine serum albumin, 2.5 units AmpliTaq Gold (ABI, Foster City, CA), and 5µL of cell suspension (described above). Amplification was conducted in an DNA Engine Dyad thermal cycler (BioRad, Hercules, CA) under the following conditions: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min with a final extension at 72°C for 10 min. Amplification products were stored at -20°C until analyzed by agarose gel electrophoresis. Commercial DNA sequencing was performed (SeqWright, Houston, TX) and DNA sequences were analyzed using Kodon (Applied Maths, Houston, TX) and GenBank BLAST (Basic Local Alignment Search Tool) searches (Altschul, Madden et al., 1997).

ERIC-PCR amplicon sequence analysis

DNA sequence analysis was successful for 10 of 13 ERIC-PCR fragments. A BLAST search of the sequences revealed that there were no GenBank sequences which were 100% identical to the sequences we obtained. However, the sequences were 93-95% similar to *Escherichia coli* W sequences confirming specificity for *E. coli*. A sequence alignment revealed the lack of conserved regions and highly divergent sequences (Fig. 8). The development of PCR primers or probes to ERIC-PCR sequences is not feasible due to the lack of conserved regions suitable as targets.

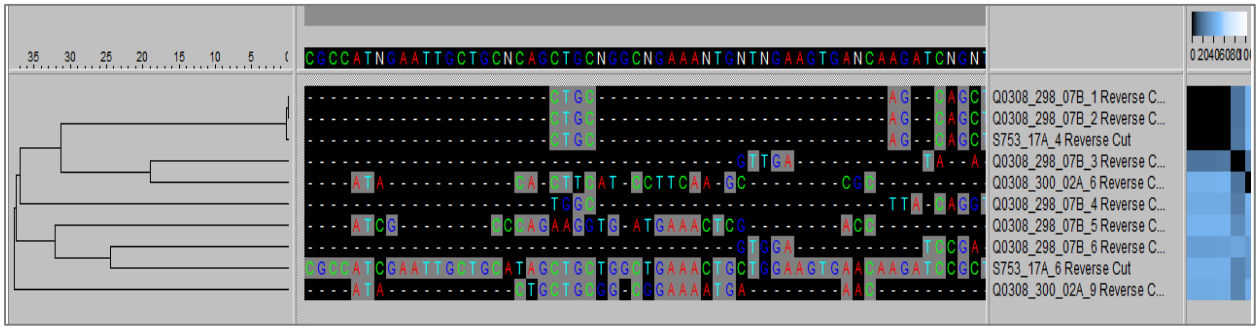


Figure 8. Multiple DNA sequence alignment (partial) and cluster analysis of ERIC-PCR amplicons from selected *E. coli* human source isolates.

Discussion

Although we use ERIC-PCR as one of our DNA fingerprinting tools for the construction of the Texas *E. coli* BST library, we know very little about the DNA sequences being amplified. We analyzed DNA sequences of ERIC-PCR amplicons from selected human-specific *E. coli* present in the Texas *E. coli* BST Library to explore the potential development of library-independent PCR targets. A sequence alignment revealed the lack of conserved regions and highly divergent sequences and therefore the development of PCR primers or probes is not currently feasible.

Finally, although there are many benefits of library-independent BST, in some cases library-dependent *E. coli* BST, or a combination of approaches, may provide the most accurate and useful results. We still have more to learn about the sensitivity and specificity of published library-independent BST tools. Moving forward with probe based (e.g., TaqMan) quantitative PCR assays seems a logical direction. However, most of the published probe-based assays have not been as widely applied to field samples as the conventional PCR assays and questions regarding their sensitivity and specificity exist. While quantification of BST markers is highly desirable, additional sensitivity and specificity evaluations are needed. Fortunately, as a starting point, we have a collection of archived fecal DNA for the side-by-side comparison of current assays with new assays selected from the literature.

Evaluation of a Poultry BST Marker for Addition to the Texas BST Toolbox

Introduction

Recently, a poultry-specific molecular assay (LA35) has been developed for assessing poultry litter-specific fecal contamination in environmental samples (Weidhaas et al., 2011; Weidhaas and Lipscomb, 2013). So far, this assay has been used to detect and quantify poultry litter-specific contamination in environmental waters and fecal samples originating from various watersheds across several states including Oklahoma, Georgia, West Virginia, Delaware, Utah, Arkansas and Florida. The assay targets a 571-bp region of the 16S rRNA gene in *Brevibacterium* spp. which makes it a good candidate for use as a poultry-specific marker since the *Brevibacterium* spp. are abundant in poultry litter and feces and 16S rRNA genes possess relatively low mutation rates and are present in multiple operons thus increasing the template DNA levels available for detection especially at low target concentrations (Sadowsky and Whitman, 2011).

So far, two LA35 qPCR-based assays have been developed for assessing poultry-specific contamination in environmental samples. One involves a SYBR green-based approach (Weidhaas et al., 2011) and the other involves a more specific TaqMan-based approach (Weidhaas and Lipscomb, 2013). Primers and probe used for both assays are shown in Table 13.

Table 13. Primers and probe used for the LA35 PCR and qPCR assays.

LA35 TaqMan Assay	Sequence (Weidhaas and Lipscomb, 2013)
Primers	
LA35F	5' ACC GGA TAC GAC CAT CTGC-3'
LA35R	5' TCC CCA GTG TCA GTC ACA GC 3'
Probe	
LA35P	5'-FAM-CAG CAG GGA AGA AGC CTT CGG GTG ACG GTA-TAMRA1-3'

In published research, the SYBR green assay has been tested against numerous target samples (17 soiled litter and 40 chicken fecal samples) as well as various non-target samples (116 non-target samples). Non-target samples included composites of samples from different individuals collected from different states (turkey, beef cattle, dairy cattle, swine, duck, geese, human septage, WWTP influent, WWTP effluent) (Weidhaas et al., 2011). This assay showed 76% sensitivity to target samples and 93.1% specificity to composite and individual non-target samples. In addition, the SYBR green qPCR reaction had an efficiency of 93% and r^2 of 0.99. The LA35 marker in fecal-contaminated poultry litter samples was found to be correlated with culturable enterococci but not to *E. coli* concentrations.

Similarly, the TaqMan-based assay has also been tested against numerous target and non-target samples (28 poultry litter/fecal samples and 126 non-target samples). Non-target samples tested included composite samples of: beef cattle pats (n=3), dairy cattle pats (n=8), rooster scats (n=3), goose scats (n=9), sheep scats (n=6), dog scats (n=4), goat scats (n=2), horse dropping piles (n=2), horse manure pile (n=multiple animals), elk scats (n=5), skunk scats (n=3), wolf scats (n=4), bison scats (n=4), bobcat scats (n=3), fisher scats (n=3), white-tailed deer (n=3), red fox scat (n=4), black bear scat (n=4), grey fox scant (n=2), raccoon scat (n=4), red-tailed hawk scat (n=3), great horned owl scat (n=3), screech owl scat (n=3), wild turkey scats (n=10), and domestic turkey scat (n=1). The TaqMan assay showed an almost similar sensitivity to the SYBR green assay (76%). However, it had a much higher specificity (100%). Overall, the TaqMan qPCR reaction had an efficiency of 102% and r^2 of 0.99.

Even though the LA35 assay has been shown to be a good candidate marker for assessing poultry-associated fecal contamination, no studies have assessed its performance against fecal and litter samples obtained from Texas watersheds. Hence the objectives of this study were to validate the TaqMan-based LA35 assay using target and non-target fecal and litter samples obtained from Texas and or surrounding states. Standard curves for relative quantification of unknown fecal DNA were created using positive controls (plasmid containing the target gene). The average amplification efficiency (E), r^2 and slope obtained from two standard curves were used to generate a calibration equation for relative quantification of unknown DNA samples.

Material and Methods

Sample Collection

A total of 58 target samples (poultry litter and fecal samples) were collected from farms in the Waco, Old Hearne Road, Lufkin, Nacogdoches, and College Station, Texas areas (Table 14). Poultry (layer) manure samples were also collected from Pike County, AR. Non-target fecal

samples originated from various projects and locations including Leona River, Attoyac Bayou, Leon River, Plum Creek and Lake Somerville (Table 15). Specifically, samples were obtained from various animals including cows, horses, goats, feral hogs, deer and coyotes.

Table 14. Target fecal/litter/manure samples tested against the LA35 marker.

Sample Type/Origin	# of Samples	Positive hits
Poultry Feces	**2**	
TAMU poultry farm	2*	2/2
Poultry Litter	**56**	
Lufkin	6	6/6
Nacogdoches	26	16/26
Old Hearne Road	6	6/6
Pike County, AR	11	11/11
TAMU poultry farm	1	1/1
Waco area	6	6/6
Grand Total	**58**	**48/58**

*Sample was a composite of ~ten different individuals

DNA Extraction and qPCR analysis

Approximately 0.3 g of sample (fecal or poultry litter) was weighed into a MoBio Power bead tube (MoBio PowerSoil, Carlsbad, CA) and DNA extraction was conducted using the MoBio PowerSoil kit (MoBio PowerSoil, Carlsbad, CA) according to manufacturer's instructions. Extracted DNA was stored at -20 °C pending qPCR analysis. An extraction blank (tube containing no fecal DNA sample) was included with each DNA extraction batch. Total mass of extracted DNA was quantified using a NanoDrop (Thermo Scientific, Wilmington, DE), and it ranged from 5 ng/µl to 40 ng/µl for most of the samples.

The qPCR analysis for the LA35 assay was conducted in simplex format (no internal amplification control) using the TaqMan-based chemistry described in Weidhaas et al. (2013) (Table 13). Environmental MasterMix (Life Technologies, Grand Island, NY) was used for all qPCR reactions. Simplex reactions consisted of 0.5 mg/mL bovine serum albumin (Sigma Aldrich, St. Louis, MO), 0.4 µM of each forward and reverse primers (IDT Technologies, Coralville, IA), 0.4 µM FAM labeled TaqMan® probe (Life Technologies, Grand Island, NY), 5 µL of template DNA, and volume was brought up to 25 µL using nuclease-free H_2O. For standard curve reactions, 5 µL of linearized plasmid constructs containing the LA35 gene with concentrations ranging from $10^1 – 10^6$ copies per reaction were used as a template. Plasmid DNA for calibration curve reactions were ordered from IDT Technologies, Coralville, IA.

All qPCR reactions were performed in triplicate using a Mastercyler RealPlex^2 (Eppendorf, Hauppauge, NY). The thermal cycling profile for the qPCR reactions were 2 min at 50°C, 15 min at 95°C followed by 45 cycles of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C for the plate read. A minimum on 3 negative controls (NTCs) were included with each 96-well plate run. Quantification cycle (Cq) values from standard curves and unknown samples were exported to excel and used for further analysis.

Table 15. Non-target fecal samples tested against the LA35 marker

Site/Animal	# of Samples Extracted	Positive Hits
Attoyac Bayou	**19**	
Black cow	1	0/1
Chicken, yard	1	0/1
Coyote	3	0/3
Deer	7	0/7
Duck	1	0/1
Feral hog	3	0/3
Goat	1	0/1
Goose	1	0/1
Quail	1	0/1
Leon River	**3**	
Cow, dairy	3	0/3
Leona River	**89**	
Black Angus-calf	1	0/1
Black Angus-cow	1	0/1
Black Angus-heifer	1	1/1
Bobcat	1	0/1
Brown duck	1	0/1
Cat	3	0/3
Chicken, yard	9	0/9
Cow, beef	4	0/4
Coyote	3	0/3
Deer	6	0/6
Dog	7	0/7
Duck	2	0/2
Eurasian collared dove	1	0/1
Feral hog	4	0/4
Fox	7	0/7
Goat	5	0/5
Grackle	3	0/3
Guinea	1	0/1
Horse	6	0/6
Mule	1	0/1
Peacock	1	0/1
Raccoon	5	0/5
Roadrunner	1	0/1
Sheep	7	0/7
Turkey	6	0/6
White duck	2	0/2
Plum Creek	**3**	
Feral hog	3	0/3
(Other) Texas	**5**	
Cattle egret	5	0/5
Grand Total	**119**	**1/119**

Results

QA/QC and performance metrics

A master calibration curve equation for relative quantification of unknown samples (Table 16) was generated using average Cq values from two standard curves. The calibration curve was subsequently used to estimate gene copies in unknown fecal samples. Average performance metrics for the master standard curve for the r^2, slope and efficiency were 98.1, 3.39 and 97.24 respectively (Table 16). A total of 24 NTCs and 17 extraction blanks (each run in triplicate) were used to assess potential contamination by extraneous DNA, and none contained Cq values below 40 Cq.

Table 16. Performance metrics for calibration equation used for relative quantification of unknown target and non-target samples.

Assay	Target	Equation	*E*	R^2
LA35	16S rRNA gene of the *Brevibacterium* sp	Y= 42.1 – 3.39X	0.97	0.98

Amplification of target and non-target samples

In total, 48 of the 58 (83%) tested poultry fecal and poultry litter samples amplified positively with the LA35 assay (Table 14). Gene copy numbers ranged from 10^4-10^6 copies per 5 µl of DNA for poultry fecal samples and 10^1-10^4 per 5 µl of DNA for poultry litter samples. Most notably, a large percentage of the target samples that did not amplify with the LA35 marker were from one particular site (Nacogdoches), suggesting that perhaps site-specific litter characteristics might have inhibited marker amplification.

Out of the 119 non-target samples, only one sample consistently amplified with LA35 assay (99% specificity). This was from a beef cattle sample collected during the Leona project (3/3 technical replicates tested positive; 3.91 x 10^2 average gene copies/ 5 µl of DNA). There was limited amplification (2 of the 3 technical replicates) for one additional duck sample (3.07 x 10^1 average gene copies per 5 µl of DNA).

Overall, these results indicate that the poultry marker performed well with the tested Texas samples. Sensitivity (83%) and specificity (99%) for analysis of the Texas samples were very similar to values published for other samples from across the U.S. (sensitivity = 78% and specificity = 100%). This poultry marker is a good candidate for inclusion in the Texas BST toolbox and should be considered for use in future watershed projects where poultry is a potential source of fecal bacteria.

Microbial characterization of deer fecal communities in Texas and potential for development of a deer-specific BST marker

Introduction

Wildlife sources, such as deer and feral hogs, have been implicated as major contributors of bacterial impairment, but our fundamental knowledge of wildlife gut communities and thus ability to track them as specific contamination sources is lacking. Library-independent means to track deer specifically are hindered by the fact that the most widely accepted ruminant specific marker, CF128F, cannot distinguish between cattle and deer (Bernhard and Field, 2000). The ability to distinguish between wildlife and livestock sources is critical to developing best

management practices to reduce fecal contamination. The objective of this study was to use 454 barcoded pyrosequencing to characterize deer fecal communities in Texas in an effort to evaluate their suitability for development of a deer-specific BST marker.

Materials and Methods

Sample Collection

Deer fecal pellets were obtained directly from the lower section of the large intestine at time of field dressing of recently killed animals. Samples were collected at the Welder Wildlife Refuge Foundation, near Sinton Texas, in both 2008 and 2009 during annual youth hunts. Welder staff members assisted in weighing the animals prior to field dressing and approximated the age of the animals. Leon samples from Comanche County, TX were collected by stakeholders in the watershed during the winter of 2009. Samples were kept on ice during handling and transport to College Station and stored at -80°C.

Bacterial Tag-Encoded Amplicon Pyrosequencing

Bacterial community DNA was extracted in triplicate from each sample using a Power Soil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA) per manufacturer's instructions. DNA samples were purified using illustra MicroSpin™ G-25 Columns (GE Healthcare Biosciences, Pittsburg, PA) and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Extracts were stored at -20°C for downstream applications. Community DNA was submitted to the Research and Testing Laboratory (Lubbock, TX) for tag-pyrosequencing. Samples were amplified using primers 27F and 519R and sequenced using Roche titanium chemistry (Acosta-Martinez et al., 2008).

Sequence Analysis and Community Comparisons

Sequence libraries were analyzed using a combination of The Ribosomal Database Project (RDP) (Cole et al., 2009) (accessed 17 May 2011) and MOTHUR (version 1.18.1) (Schloss et al., 2009). Using MOTHUR, sequencing primers and tags were removed, the database was quality checked, and chimeras removed prior to downstream processing. The RDP pipeline was used to assign taxonomic identities to the quality-screened, final 454 sequence data. Each sequence was classified down to the genus level, but if an organism could not be classified with at least 80% confidence in RDP, it was named Unclassified at the previous scientific classification level.

The dist.seqs function in MOTHUR was used to create distance matrices and then assign sequences to operational taxonomic units (OTUs, 97% similarity). Diversity estimates were calculated including Shannon's and Simpson's diversity indices and Chao I richness estimates. For community comparisons, the samples were grouped into three treatments by location (Welder and Leon) and year (2008 or 2009).

Phylogenetic structure of the libraries was assessed using the Yue-Clayton index (Theta-YC) as this approach utilizes both incidence and relative abundance of OTUs and thus is not sensitive to sample size. Parsimony test, analysis of molecular variance (AMOVA), and homogeneity of molecular variance (HOMOVA) were conducted using the Theta-YC similarities. Parsimony, AMOVA, and HOMOVA tests with P-values <0.05 were considered to be significant (Schloss, 2008). Relative abundance of OTUs across each sample was also used to generate nonmetric

multidimensional scaling (NMDS) plots using PAST (version 2.05) (Hammer et al., 2001). Graphs were generated using Sigma Plot 11.0. Nearest-neighbor joining trees were created in MEGA (version 5.10) to showcase overlapping and the most abundant OTUs using representative sequences from the deer fecal communities and their closest GenBank hits (Altschul et al., 1990).

Results

Deer physical characteristics

The physical characteristics of the deer samples collected are summarized in Table 17. Detailed information was available for the Welder samples from both 2008 and 2009, but was not available for the Leon samples. The Welder Wildlife refuge is home to large herds of deer and the samples collected varied in age and weight for both years.

Table 17. Deer physical descriptions.

Site	Sample ID	Sex	Age (yrs)	Live Weight (kg)
Welder 2008	81	Male	4.5	47
	84	Female	5.5	42
	87	Female	7.5	46
	88	Female	1.5	23
Welder 2009	91	Female	4.5	44
	92	Female	3.5	44
	93	Female	4.5	48
	95	Male	1.5	38
Leon	L1	N/A	N/A	N/A
	L2	N/A	N/A	N/A
	L3	N/A	N/A	N/A

Community composition, diversity, and estimated richness

A total of 32,163 amplicon sequences were utilized in the analysis with an average sequence library of 2,923 ± 610bp (mean ± sd) (Table 18). The sequence libraries ranged in size from 1,948 sequences in sample 91 to 3825 sequences in sample 81. The samples contained between 525 OTUs in sample 93 and 1559 OTUs in sample 92. Chao richness estimates suggest that the sequencing efforts captured approximately half of the diversity within the samples and additional sequencing would most likely yield additional OTUs in each sample. Shannon and Simpson diversity index values suggest similar diversity across the samples except for sample 93 which was the least diverse of all the samples.

Table 18. Summary of sequence library size, OTUs, and diversity and richness estimates.

Sample	Sequence Library Size	# of OTUs	Chao I Richness Estimate	Shannon H'	Simpson D
81	3825	1388	2595	6.63	0.99
84	2304	1168	2893	6.59	0.99
87	2825	1358	3092	6.73	0.99
88	3262	1298	2916	6.55	0.99
91	1948	903	2014	6.30	0.99
92	3508	1559	3593	6.78	0.99
93	2047	525	1269	4.97	0.97
95	3368	1145	2301	6.17	0.99
L1	3198	1327	3029	6.51	0.99
L2	2752	1019	1929	6.33	0.99
L3	3126	1148	2317	6.42	0.99
Overall	32163	8956	-	-	-

Community Structure

The parsimony test showed no significant difference in the community structure overall between the Welder samples in 2008 and 2009 and the Leon samples (P=0.457). Similarly, the AMOVA also showed no significant differences in the three communities (P=0.129). The test for HOMOVA did show significant differences between the three communities (P=0.007). Pairwise comparisons showed significant differences between Welder 2009 and Leon samples (P<0.001) but no significant difference between Welder 2008 and 2009 (P=0.135) and Leon and Welder 2008 (P=0.054). NMDS plots of all 11 samples show communities grouping across one axis but not necessarily by location or time (Figure 9). However, the three Leon samples grouped together more prominently than did the Welder samples from either year. The Yue-Clayton similarity values were very high (ranging from 0.81 to 0.991) indicating that the bacterial communities in all of the samples were very similar (Yue-Clayton estimator is scored on a 0 to 1 scale with 0 representing complete dissimilarity and 1 representing complete similarity) (Table 19).

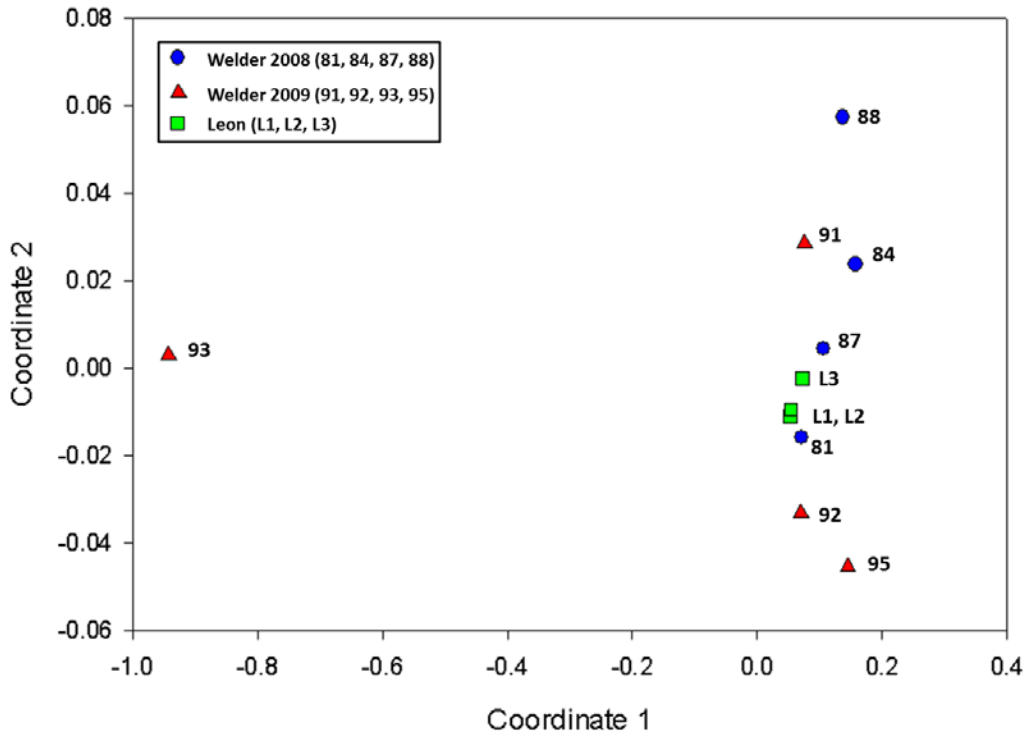


Figure 9. NMDS plot (Bray-Curtis) of 11 deer bacterial fecal communities based on relative abundance of all OTUs (97% similarity).

Table 19. Yue-Clayton Similarities based on OTUs (97% similarity) of all 11 deer bacterial fecal communities.

Sample	81	84	87	88	91	92	93	95	L1	L2	L3
81											
84	0.9722										
87	0.9216	0.9096									
88	0.9667	0.9459	0.9210								
91	0.9510	0.8937	0.9536	0.9608							
92	0.9198	0.9718	0.9565	0.9751	0.9658						
93	0.9825	0.9892	0.9718	0.9844	0.9841	0.9808					
95	0.8732	0.8761	0.9662	0.9851	0.9689	0.9771	0.9906				
L1	0.9500	0.9718	0.9545	0.9605	0.9502	0.9402	0.9793	0.9714			
L2	0.9112	0.8266	0.9188	0.9568	0.9391	0.9321	0.9685	0.9698	0.8989		
L3	0.9477	0.9140	0.9449	0.9576	0.9356	0.9455	0.9637	0.9753	0.9026	0.8212	

Phylogenetic Classifications

The entire sequence database (32,163 sequences) was identified to their highest potential taxonomic level using RDP classifier. To account for variable numbers in each sequence library, relative abundances of each particular taxonomic level were calculated. At the order level,

Clostridiales and *Bacteroidales* dominated all eleven of the samples accounting for 90 to 97% of the total community (Figure 10). *Clostridiales* ranged from 56 to 93% of the community composition across samples while *Bacteroidales* ranged from 4 to 41% (Figure 10).

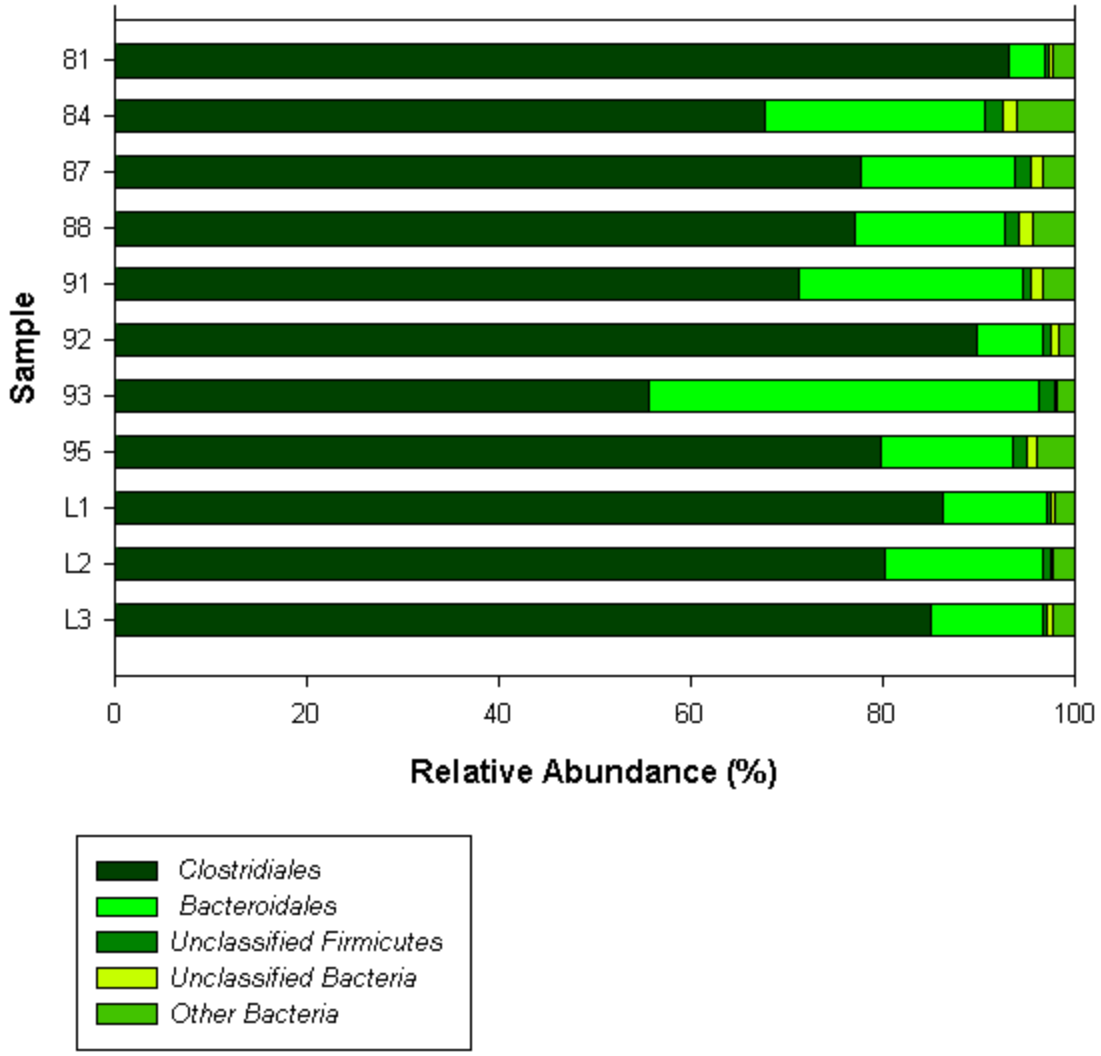


Figure 10. Bacterial composition across all 11 deer fecal samples at the order level.

At the family level, abundances for major taxonomic levels are shown as a heat map relative to the average with hierarchical clustering by site (Figure 11). The sites did not appear to cluster at the family level by particular location or year. Again, Welder sample 93 appeared to be the most distant of the group due to an enrichment of *Bacteroidales* members and a depletion of *Ruminococcaceae* (Figure 11).

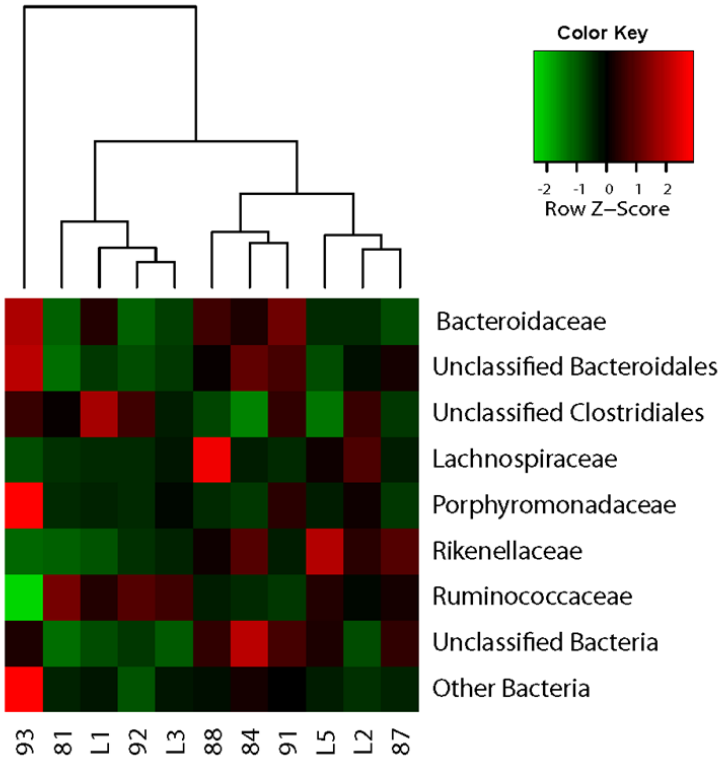


Figure 11. Heatmap depicting family-level taxonomic relative abundance across all 11 deer fecal communities. Abundances for each taxonomic group (row) were scaled relative to the mean across all samples and depicted by color (red=above average, green= below average).

Abundant and Overlapping OTUs and GenBank Hits

Of the 8,956 total overall OTUs, 2 were seen in all 11 communities. Referencing the RDP classifier data, these OTUs were identified as members of the *Ruminococcaceae* and *Veillonellaceae* families. Representative sequences from these OTUs were chosen and a neighbor joining tree was created using their best GenBank hits (Figure 12). The five most abundant OTUs represented 12% of the total OTUs across samples and included members of the *Rikenellaceae*, *Porphyromonadaceae*, *Bacteroidales* (Unclassified), and *Ruminococcaceae* families (Figure 13). The most commonly shared and abundant OTUs along with their top GenBank hit and maximum identity are summarized in Table 20. *Ruminococcaceae* family members dominated both the common and most abundant OTUs in the dataset (Table 20). All but one (OTU_2593) of the OTUs top hits were to uncultured bacterial clones originating from fecal communities. The top GenBank hits for the *Ruminococcaceae* family OTUs were to dairy cattle, humans, and a miniature gazelle.

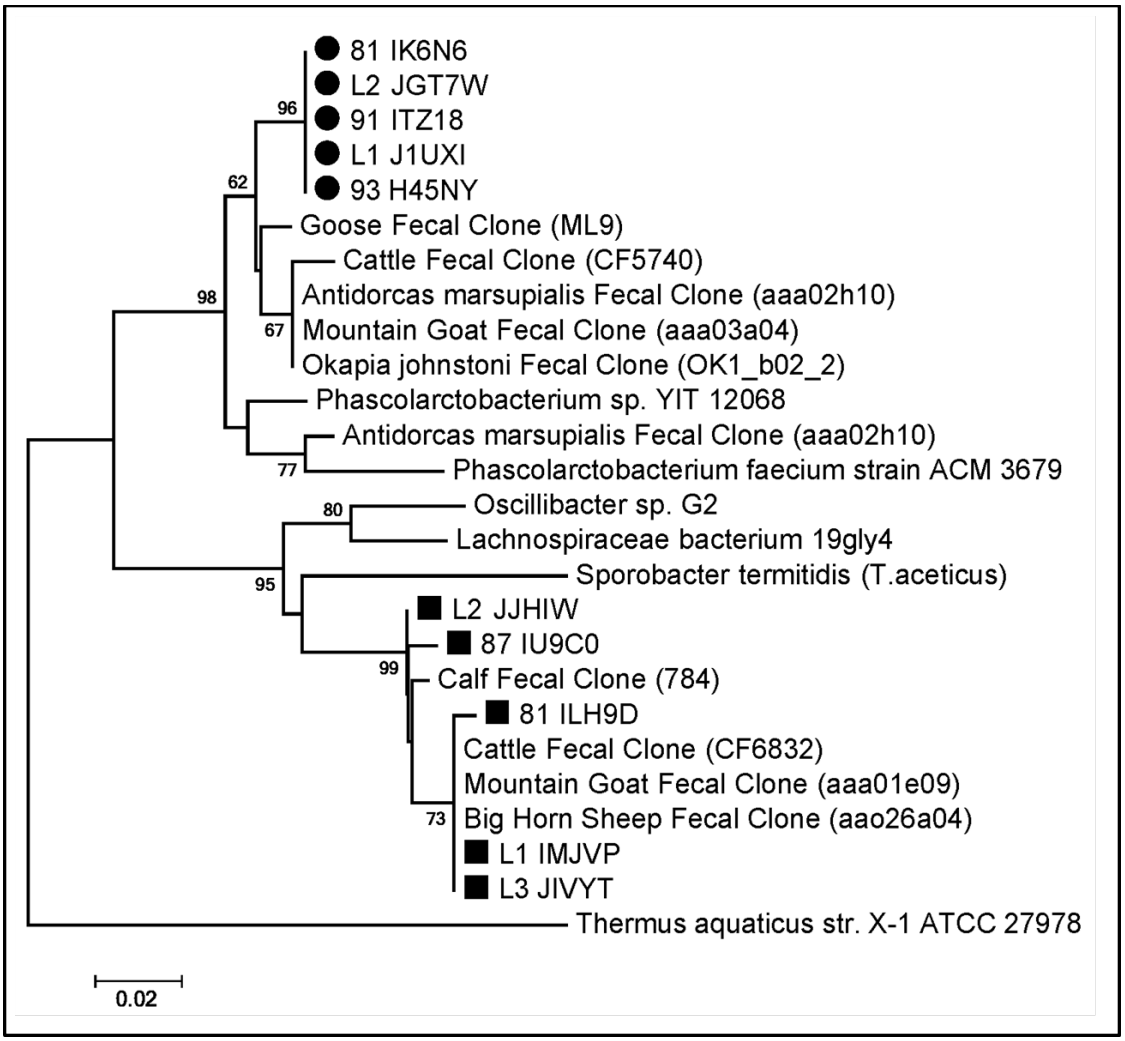


Figure 12. Neighbor-joining phylogenetic tree of the two OTUs represented in all 11 of the deer fecal communities and their top GenBank hits. Deer fecal community sequences representing the two OTUs are indicated with either a black circle representing *Ruminococcaceae* or a black square representing *Veillonellaceae* followed by their site name and five digit individual sequence identity code.

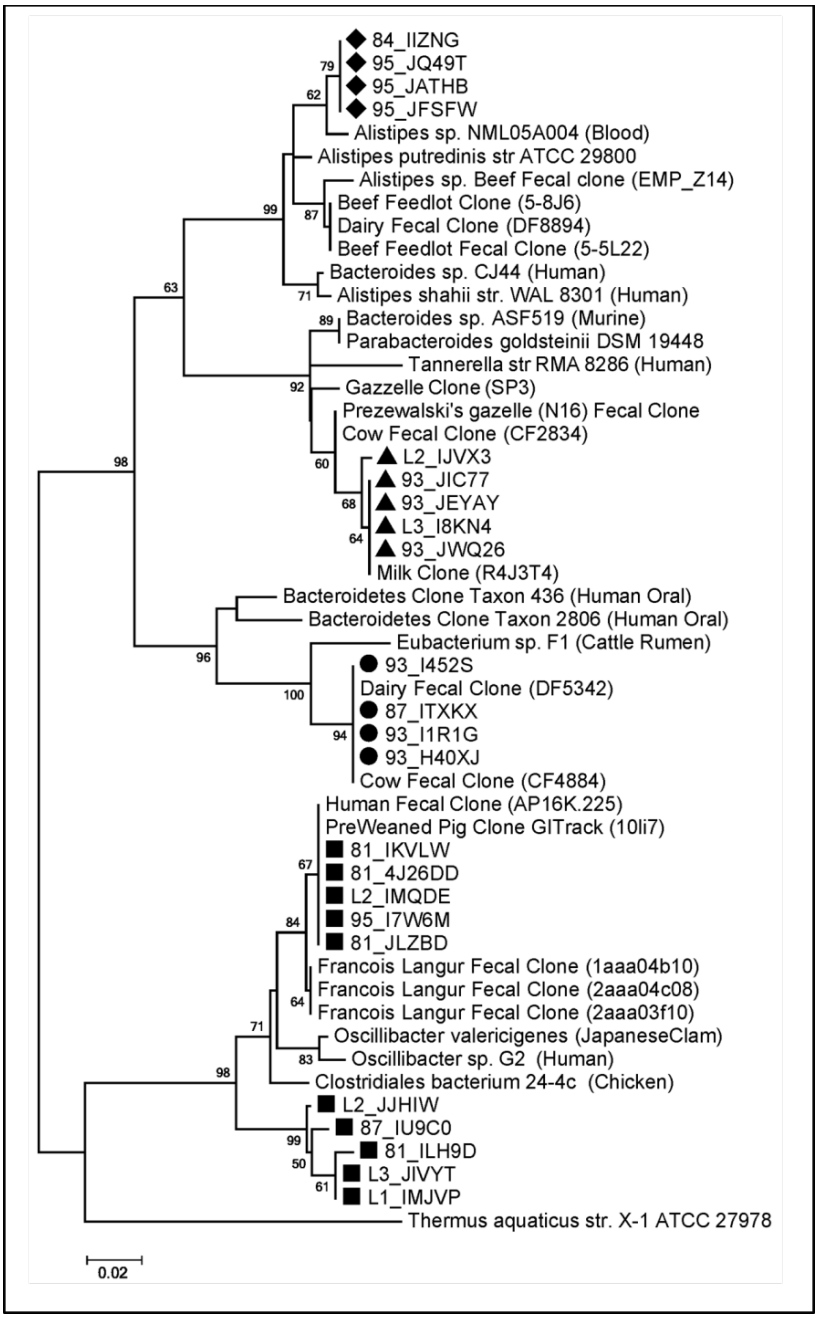


Figure 13. Neighbor-joining phylogenetic tree of the five most abundant OTUs found within the deer fecal communities collectively and their top GenBank hits. Deer fecal community sequences representing the five OTUs are indicated with a black circle representing *Bacteroidales,* a black square representing *Ruminococcaceae,* black triangle representing *Porphyromonadaceae,* and black diamond representing *Rikenellaceae* followed by their site name and five digit individual sequence identity code.

Table 20. The five most shared and abundant OTUs found in the deer fecal communities. The top GenBank hit with the maximum identity is listed along with a description of the hit and its maximum percentage identity with the OTU.

OTU	OTU Taxonomy	Top GenBank Hit Accession#	Top GenBank HIT Description (16s rRNA gene)	GenBank Hit Source	GenBank Max Identity
OTU_36[1]	Veillonellaceae	EU778779.1	Uncultured bacterium clone SBSD_aaa02h10_1	Springbok antelope feces	95%
OTU_111[1, 3]	Ruminococcaceae	GU611449	Uncultured bacterium clone DF3272	dairy cow feces	98%
OTU_207[2]	Ruminococcaceae	EU468955	Uncultured bacterium clone SP2_h05	Speke's gazelle feces	97%
OTU_2512[3]	Ruminococcaceae	FJ651134	Uncultured Firmicutes bacterium clone OB_425	human feces	98%
OTU_2593[2]	Clostridiales	JX109040	Uncultured bacterium clone MID39_30977	dairy cow uterus	98%
OTU_3135[3]	Rikenellaceae	GU617071	Uncultured bacterium clone DF8894	dairy cow feces	97%
OTU_3604[3]	*Bacteroidales*	GU613519	Uncultured bacterium clone DF5342	dairy cow feces	97%
OTU_3630[3]	Porphyromonadaceae	EU469137	Uncultured bacterium clone SP3_a11	bighorn sheep feces	97%
OTU_4560[2]	Ruminococcaceae	GU604899	Uncultured bacterium clone CF4911	cow feces	96%

[1]OTUs common in all 11 samples
[2]OTUs common in 10 of 11 samples
[3]Five most abundant OTUs

Discussion

This survey aimed to better characterize deer fecal communities as a first step toward potentially developing deer-specific BST markers. The bacterial communities were characterized using an OTU approach and then further classified taxonomically. Chao I richness estimates showed that larger sequence libraries, approximately double, from each sample would be necessary to capture the breadth of diversity across the fecal communities (Table 18). Shannon and Simpson diversity indices were consistent across the samples with the exception of Welder sample 93 which was the least diverse of all the samples and proved to be somewhat of an outlier.

Hypothesis testing was utilized to examine overall community structure and ask whether the communities were significantly different than would be expected by chance. The samples were grouped into three treatment categories for testing, Welder 08, Welder 09, and Leon. The global parsimony test showed no significant differences between the three treatments. AMOVA is a non-parametric analog of a traditional analysis of variance and tests the hypothesis that genetic diversity within two populations is not significantly different from that which would result from pooling the two populations. This test also showed no significant difference between the communities. Finally, HOMOVA is a non-parametric analog of Bartlett's test for homogeneity of variance. There was a significant difference between variance across the groups. Using the Yue-Clayton distance measure, the central communities from all three groups were statistically the same, but the Leon samples displayed greater variance within samples. Diversity within individual groups was greater than between them. The significant difference in variances between samples likely masked any potential power to see differences in the overall communities (Schloss, 2008; Schloss et al., 2009). For ease of interpretation, the OTUs were converted to a relative abundance per sample basis and were plotted using NMDS (Figure 9). All of the communities lined up on one axis except for Welder sample 93. There was little clustering of samples across years.

The phylogenetic analysis yielded much lower perceived taxonomic diversity than the OTU-based analysis, but could have been expected. The OTU-based analysis does not depend on a pre-defined taxonomy, and since many of the sequences could not be classified down to the genus level in RDP with confidence, they were left at the family level or higher. Many of the individual OTUs classified to the same families. The communities were dominated by two phyla, *Firmicutes* and *Bacteroidetes*. These phyla have previously been shown to constitute the majority of gut-associated bacteria in other mammals (Durso et al., 2010; Ley et al., 2006; Shanks et al., 2011) and each of the overlapping and abundant OTUs fell into these two phyla. *Proteobacteria*, including *E. coli*, averaged 0.5% across all 11 samples. The relative abundances of the family level taxonomic classification did not appear to cluster by site location or year (Figure 11). The Welder, 93, sample was the least diverse and exhibited a shift from the *Firmicutes* into the *Bacteroidetes* overall as seen in the depletion of *Ruminococcaceae*. Previous studies have shown diet and geographical location cause shifts in gut and fecal microbial populations (Ley et al., 2008; Shanks et al., 2011). The deer communities examined in this study would seem to fit the description of being geographically distant, approximately 350 miles apart and are in two completely different ecoregions of the state - the Western Gulf Coastal Plains ecoregion at the Welder Wildlife Refuge versus the Cross Timbers ecoregion where the Leon River is located. But surprisingly at the family-level taxonomy none of the samples tended to cluster by location or by year. Both the OTU analysis and taxonomic classifications suggest the deer fecal communities in these two parts of Texas are similar and stable over time.

Two OTUs were shared across all 11 samples and were classified as *Ruminococcaceae* and *Veillonellaceae*. An additional 3 OTUs occurred in 10 of the 11 samples, two of which were also *Ruminococcaceae* and the other *Clostridiales* (Unclassified). A majority of the OTUs overall were singletons (56%). Eckburg et al. (2005) noted a similar trend assessing diversity of the human intestinal microbial flora where 60% of the genera were recovered only once. Further, the five most abundant OTUs only represented 12% of the total. The overlap or percentage of abundant OTUs overlapping at either Welder or Leon also did not represent over 10% of the total

OTUs. The five most abundant taxa were classified as *Ruminococcaceae*, *Veillonellaceae*, *Rikenellaceae*, *Porphyromonadaceae*, and *Bacteroidales* (Unclassified). The top GenBank hits for representative sequences from all of the OTUs were from fecal communities, except for the dairy cow uterus hit. The top GenBank hit for the *Veillonellaceae* OTU_36 was to feces from Springbok antelope which is a ruminant like deer and cattle. The GenBank maximum identity to all of the common and abundant OTUs was less than 100% indicating uniqueness in the database. The two strongest candidates for potential marker development are OTU_36 and OTU_4560. The *Veillonellaceae* OTU_36 has the lowest identity match (95%) and was common across all of the samples and the *Ruminococcaceae* OTU_4560 also has a low maximum identity (96%) and was found in 10 of the 11 samples.

Conclusions

The goal of this project was to utilize 454 pyrosequencing to better characterize deer bacterial fecal communities in Texas with the aim of finding organisms that were common across geographic regions and time in order to serve as a starting point for future research toward development of a deer-specific BST marker. The microbial communities were not significantly different from an overall OTU (97% cutoff) standpoint and did not cluster by site or year, suggesting that the deer fecal bacterial communities, at least in south and central Texas, were stable over time which bodes well for the potential of a temporal and geographically stable source-specific marker. At least two of these OTUs, OTU_36 and OTU_4560, appear to be potentially deer-specific with their closest non-deer matches in GenBank being only 95 and 96% similar, respectively, and appear to have potential for further investigation into their suitability as deer-specific BST markers.

Outreach

An important component of the project was the delivery of BST and BMP informational materials describing the state of the science, applicability, usefulness, and analytical capabilities of State-supported BST laboratories to water resource professionals across the state and nation. To accomplish this, the project team delivered materials electronically via the web, orally via presentations at meetings and conferences, and in print via development and distribution of brochures and flyers.

TWRI has hosted and maintained the website http://texasbst.tamu.edu/ since September 2010 in order to disseminate educational materials, project updates, science updates, notify readers about educational opportunities, and other outreach efforts. Website hits since its inception through June 2015 included 3,121 visits from 1,942 unique visitors. Of these visitors, 65% were from Texas while the remainder was from out-of-state. Direct visits (e.g. the address was typed directly into the search bar) accounted for 41% of visits, while 13% were referred from twri.tamu.edu. Typical flow visitors took through the website is shown in Figure 14.

Resources on BST were provided by participation in conferences, workshops, and seminars and by developing and disseminating informational materials. In August 2013, UTSPHEP presented "*Waterborne Pathogen Research to Support Water Quality Regulations*" to EPA Region 6 and other participants of the EPA webinar. TWRI distributed educational brochures to participants of the 2013 Texas Watershed Planning Short Course. TWRI delivered a seminar on the use of BST in watershed planning efforts to students in the Texas A&M Water Management and Hydrological Sciences Program course on Developing and Implementing Watershed Plans (WMHS-685) in September 2013. UTSPH EP personnel presented the following talk and poster, respectively, at the Rio Grande Branch of the American Society of Microbiology meeting held February 2014 in El Paso: "*The Development and Use of the Texas E. coli Bacterial source Tracking Library in Identifying Sources of Fecal Pollution in Texas Watersheds*" and "*Identification of Potentially Pathogenic Microorganisms from Unexpected Wildlife Source*."

TWRI presented: "*Bacterial Source Tracking in Texas: A Retrospective Assessment of a Decade of Use in the Lone Star State*" at the WEF/WEAT meeting in April 2014; "*The Impact of Background Loadings: An Assessment of Contributions Using Edge-of-Field Studies and Watershed Scale Bacterial Source Tracking in Texas*" at the 2014 Water Microbiology Conference in May 2014; "*Review of Bacterial Source Tracking in Texas*" in July 2014 at the Texas Watershed Roundtable in Waco; and "*What're the sources of bacteria in your watershed? They may not be what you expect*" at the 2015 Waste to Worth Conference in March 2015. TWRI and SCSC also presented on Microbial Source Tracking at the HGAC CWI Workshop in July 2014 in Houston. SCSC presented on "*Use of bacterial source tracking to characterize fecal source contributions in watersheds*" at the Soil in the City Conference in July 2014 in Chicago, and "*Resiliency of E. coli and Enterococci in poultry litter, and subsequent efficiency of poultry markers, through wetting and drying cycles*" at the ASA/CSSA/SSSA Meetings in November 2014.

Figure 14: User flow through BST website (http://texasbst.tamu.edu/).

The "Layperson" BST Brochure developed under project 10-50 was updated and a new promotional flyer was developed for distribution at the 2015 Texas Environmental Trade Fair. The updated BST brochure and flyer can be found in Appendix C. TWRI, UTSPH EP, and AgriLife SCSC participated in the 2015 Environmental Trade Fair and Conference in Austin in order to provide resources on BST (Figure 15). TWRI, TSSWCB, SCSC, and UTSPH EP discussed hosting another *BST – State of the Science Conference* as was held in 2012. However, it was determined that for now, no follow-up conference will be held.

Figure 15. Terry Gentry, George DiGiovanni, and Kevin Wagner manning booth at the 2015 Texas Environmental Trade Fair.

Information on BST was delivered to a wide audience in Texas including faculty and staff from Texas Tech University, the US Environmental Protection Agency, Texas A&M AgriLife Research, Texas A&M AgriLife Extension Service, Houston-Galveston Area Council, Texas Parks and Wildlife Department, Texas Institute for Applied Environmental Research, USDA Natural Resources Conservation Service, Nueces River Authority, Texas Commission on Environmental Quality, Texas State Soil and Water Conservation Board, Texas A&M Forest Service, Tarrant Regional Water District, Guadalupe-Blanco River Authority, North Central Texas Council of Governments, University of Texas School of Public Health – Houston, City of Houston, East Texas Baptist University, San Antonio River Authority, and Guadalupe-Blanco River Authority, and Texas A&M University-Galveston (Dr. Brinkmeyer).

In addition to presenting at the aforementioned outreach events, TWRI coordinated the Southwestern Stream Restoration Conference in San Antonio during May 2013 in order to educate others on reducing pollutant contributions, including bacteria, to streams. This successful conference was attended by approximately 230 participants. In conjunction with this conference, TWRI facilitated a Riparian Vegetation Workshop which was attended by 50 participants.

In recognition of the team's efforts, the Bacterial Source Tracking Team received the 2014 Texas A&M University College of Agriculture and Life Sciences Dean's Outstanding Achievement Award for Interdisciplinary Research.

Through the project, a stratified random sampling scheme was also implemented to assess barriers to BMP adoption using a target population of beef cattle producers who completed the 2012 Census of Agriculture. The USDA-NASS Texas Field Office assisted with the sampling survey by tracking response/non-response to returned survey materials and handling the logistics of sending and receiving survey materials. Survey materials included an introductory postcard, the first survey packet with cover letter and survey instrument, a reminder postcard, and a second survey packet with cover letter, and survey instrument. This information supported assessment of barriers to BMP adoption in conjunction with TSSWCB Project #12-08. Data collection ceased on November 1, 2013. Twenty postcards and/or survey packets were returned undeliverable, 16 individuals reported they had sold all of their cattle, and 46 individuals indicated they did not wish to participate in the study. This yielded a frame error of 4.8% and reduced the total sample to 1,618 beef cattle producers. A total of 90 surveys (5.6%) were completed online and 687 (42.5%) were completed on paper and mailed back to the student researcher for a total response rate of 48.1%. Results of this survey were recently published in the Journal of Agriculture and Environmental Sciences (http://jaesnet.com/journals/jaes/Vol_4_No_1_June_2015/21.pdf).

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Weidhaas, J. L., T. W. Macbeth, et al. (2010). Identification of a Brevibacterium marker gene specific to poultry litter and development of a quantitative PCR assay. *J. Appl. Microbiol.* 109:334-347.

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Appendix A

Texas *E. coli* BST Library (ver. 6-13, cross-library validation) composition and rates of correct classification (RCCs) by Jackknife analysis of ERIC-RP composite data sets using an 80% similarity cutoff and 3 and 7-way splits

Source Class	Number of Isolates	Number of Samples	Library Composition & Expected Random Rate of Correct Classification	Calculated Rate of Correct Classification (RCC)	RCC to Random Ratio***	Left Unidentified (unique patterns)
HUMAN	**364**	**315**	**24%**	**100**	**4.2**	**22**
DOMESTIC ANIMALS	**531**	**474**	**35%**	**100**	**2.9**	**19**
Pets	86	76	6%	83	13.8	40
Cattle	237	207	16%	93	5.8	11
Avian Livestock	96	83	6%	89	14.8	25
Other Non-Avian Livestock	112	108	7%	90	12.9	14
WILDLIFE	**629**	**569**	**41%**	**100**	**2.4**	**19**
Avian Wildlife	239	221	16%	85	5.3	21
Non-Avian Wildlife	390	348	26%	92	3.5	17
Overall	**1524**	**1358**		**ARCC** ** **= 100% 92%**		**20%**

*RARCC, expected random average rate of correct classification

**ARCC = average rate of correct classification: the proportion of all identification attempts which were correctly identified to source class for the entire library, which is similar to the mean of the RCCs for all source classes when the number of isolates in each source class is similar

***An RCC/Random Ratio greater than 1.0 indicates that the rate of correct classification is better than random. For example, the rate of correct classification for human is 4.0-fold greater than random chance.

Appendix B

Template SOP #TXBST-01:	Collection of Fecal Samples for Bacterial Source Tracking
Template SOP #TXBST-02:	Cultivation of *E. coli* from Water Samples and Pre-Processing for Isolation and Bacterial Source Tracking
Template SOP #TXBST-03:	Archival of *E. coli* Isolates
Template SOP #TXBST-04:	DNA Fingerprinting of *E. coli* Isolates Using Enterobacterial Repetitive Intergenic Consensus Sequence (ERIC)-PCR
Template SOP #TXBST-05:	DNA Fingerprinting of *E. coli* Isolates Using the Dupont RiboPrinter® System
Template SOP #TXBST-06:	Preprocessing of Water Samples for *Bacteroidales* PCR
Template SOP #TXBST-07:	Bacterial Source Tracking (BST) Analysis Of Water Samples Using *Bacteroidales* PCR

COLLECTION OF FECAL SAMPLES FOR BACTERIAL SOURCE TRACKING

June 5, 2015

Elizabeth Casarez
University of Texas-Houston School of Public Health El Paso Regional Campus

APPROVED:

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University of Texas-Houston
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Soil & Aquatic Microbiology Laboratory
Texas A&M AgriLife Research

Annual Reviewer			
Date			

1.0. PURPOSE AND APPLICABILITY

The purpose of this Standard Operating Procedure (SOP) is to establish a uniform procedure for the collection and transport of fecal samples to the laboratory for subsequent isolation of *E. coli* for Bacterial Source Tracking (BST) analyses.

2.0. SUMMARY OF THE METHOD

Fresh fecal, sewage, or septage samples are collected, placed at 4°C, and shipped/transported to the appropriate BST laboratory as soon as possible.

3.0. HEALTH AND SAFETY WARNINGS

Fecal, sewage, or septage samples may contain pathogenic microorganisms. The sampler should treat all such samples as though each contained a chemical and/or a biological agent that could cause illness. The sampler should wear protective gloves and handle containers with care. The sampler should exercise special caution to avoid environmental hazards such as animals (e.g., snakes), extreme climatic conditions, and automobiles (if collecting a sample near a major road).

4.0. INTERFERENCES

Possible issues include the collection of old, unidentifiable, or contaminated samples. Only fresh fecal samples of known origin should be collected. Samples should be carefully collected to avoid contamination from the surrounding environment (soil, etc.). Specific suggestions for avoiding these interferences are provided in the procedures section of this SOP.

5.0. PERSONNEL QUALIFICATIONS

This SOP is written for persons with a thorough knowledge of field sampling procedures and a basic understanding of microbiological procedures, especially aseptic technique.

6.0. EQUIPMENT AND SUPPLIES

6.1 Sterile fecal tubes (Sarstedt, cat# 80.734.311) or similar containers
6.2 Sterile spatulas, or similar, for collection of samples
6.3 Sterile plastic loops (optional)
6.4 Sterile scalpels (optional)
6.5 Sterile bottles (optional; for wastewater collection)
6.6 Whirl-Pak bags, or similar
6.7 Cooler with ice or blue ice for transport of samples
6.8 Refrigerator (~4°C)

7.0. PROCEDURAL STEPS

7.1. Only fresh fecal samples of known origin should be collected. Specifically, fecal samples should be obtained in one of five ways:
   a. Collected from animals visually observed defecating.
   b. Collected from trapped animals.
   c. Collected from intestines of animals legally harvested.
   d. Collected from intestines of animals recently killed by cars (within 24 hours).

e. Human (wastewater) samples collected from individual septic tanks, composite septic samples from pump trucks, from wastewater treatment plant influent (for plants with secondary disinfection or lagoon treatment), or from lagoon treatment effluents.

7.2. Samples should be carefully collected to avoid contamination. Samples on the ground should be collected with a sterile spatula, or similar device, while avoiding collection of material in contact with soil or other possible sources of contamination. Intestinal samples should be collected from animals by using sterile loops inserted anally or by cutting into the intestine using a sterile scalpel. Wastewater samples can initially be collected with sterile bottles or other suitable device and then transferred to the fecal tubes described below.

7.3. Each fecal sample should be placed in a new, sterile fecal tube, or similar container. Tubes should be filled approximately ¾ full (can provide less material for smaller animals).

7.4. Samples should be placed in a cooler on ice and/or refrigerated (~4°C) following collection.

7.5. At the time of sampling, record detailed information on the tube regarding the sample including:
a. Sampling date
b. Sampling time
c. Animal species
d. Sample location (e.g., GPS coordinates [preferred] or town, city, and/or county)
e. Sample collector's name/initials
f. Any other pertinent information, e.g. sex of animal or any other easily obtainable information such as beef cattle versus dairy cattle

7.6. Notify the appropriate lab via email or phone as soon as possible (prior to or immediately following sample collection) with an estimated number of samples that will be shipped and the expected date of shipment. This will allow the lab to make appropriate preparations to process the samples immediately upon arrival. BST Laboratory contact information is below:

UTSPH-EP
Elizabeth Casarez; elizabeth.a.casarez@uth.tmc.edu; 915 747-8076
George Di Giovanni; george.d.digiovanni@uth.tmc.edu; 915 747-8509
Joy Archuleta-Truesdale; joy.a.truesdale@uth.tmc.edu; 915-747-6868

SAML
Heidi Mjelde; hmjelde@ag.tamu.edu; 979-845-5604
Terry Gentry; tgentry@ag.tamu.edu; 979-845-5323

7.7. Samples should be shipped (at 4ºC) as soon as possible (within 3 days) to the appropriate lab (addresses below). Ship samples (and COCs) in insulated coolers (marked on outside to indicate that contents are perishable) with sufficient ice packs to maintain ~4°C. 'Blue-ice' or freezer blocks should be used to keep the samples cool, but not frozen during transport. Samples should be placed in secondary containment such as large Whirl-Pak or zip-top bags. Shipping addresses for BST Laboratories are:

UTSPH-EP
George Di Giovanni
UT-Houston School of Public Health
Biology Building B224
500 W. University
El Paso, TX 79968
915-747-8509

SAML
Terry Gentry
Texas A&M University
Soil & Crop Sciences; Heep Center 539
370 Olsen Blvd
College Station, TX 77843
979-845-5604

7.8. Notification of shipment should be sent to the appropriate lab via email or phone (see contact info above) no later than the day of overnight shipping. Notification should include tracking number and contact person for confirmation upon receipt of samples.

8.0. QUALITY ASSURANCE AND QUALITY CONTROL

Care should be exercised to avoid the interferences listed in section 4.0. Any potential issues for the BST Laboratory to consider should be noted on the COC form. Following collection, samples should be maintained at ~4°C and transported/shipped to the BST Laboratory as soon as possible in order to minimize changes in microbial composition of the samples.

9.0. REFERENCES

Casarez, E. A., S. D. Pillai, J. B. Mott, M. Vargas, K. E. Dean and G. D. Di Giovanni. 2007. Direct comparison of four bacterial source tracking methods and use of composite data sets. *J. Appl. Microbiol*. 103:350-364.

Di Giovanni, G. D., E. A. Casarez, T. J. Gentry, E. C. Martin, L. Gregory, and K. Wagner. 2013. Support analytical infrastructure and further development of a statewide bacterial source tracking library. TR-448. Texas Water Resources Institute, College Station, TX.

10.0. REVISION HISTORY

Revision	Date	Responsible Person	Description of Change
1	June 2015	Elizabeth Casarez	Initial Release

CULTIVATION OF *E. COLI* FROM WATER SAMPLES AND PRE-PROCESSING FOR ISOLATION AND BACTERIAL SOURCE TRACKING

June 5, 2015

Elizabeth Casarez
University of Texas-Houston School of Public Health El Paso Regional Campus

APPROVED:

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Author Date

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Di Giovanni Laboratory
University of Texas-Houston
School of Public Health El Paso Regional Campus

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Director Date
Soil & Aquatic Microbiology Laboratory
Texas A&M AgriLife Research

Annual Reviewer			
Date			

1.0. PURPOSE AND APPLICABILITY

The purpose of this Standard Operating Procedure (SOP) is to establish a uniform procedure for the initial growth and pre-processing of *E. coli* from water samples for transport to Bacterial Source Tracking Laboratories (BST) for further *E. coli* isolation and characterization for BST analyses.

2.0. SUMMARY OF THE METHOD

Water samples are processed with EPA Method 1603 to grow and enumerate *E. coli* on solid media. Plates containing *E. coli* colonies are then transported/shipped to a BST Laboratory for subsequent analyses.

3.0. HEALTH AND SAFETY WARNINGS

Water samples may contain pathogenic microorganisms. The analyst should treat all such samples as though each contained a chemical and/or a biological agent that could cause illness. The analyst should wear protective gloves and handle containers with care.

4.0. INTERFERENCES

Turbid waters may clog membrane filters before the desired volume of sample can be processed. If this occurs, filter as much water as possible (up to the desired volume) and record the amount of water filtered on bag/tube that the filter is placed into and also on the chain-of-custody form.

5.0. PERSONNEL QUALIFICATIONS

This SOP is written for persons with a thorough knowledge of laboratory and microbiological procedures, especially aseptic technique.

6.0. EQUIPMENT AND SUPPLIES

6.1 Pipettes, sterile, plastic, of appropriate volume
6.2 Sterile graduated cylinders, 100 mL, covered with aluminum foil.
6.3 Sterile membrane filtration units (funnel), plastic, wrapped with aluminum foil. Sterile membrane filtration units (funnel), plastic, in individual autoclave bag or covered with aluminum foil.
6.4 Electric vacuum pump, as a vacuum source
6.5 Filter flask, vacuum, usually 1.0 L, with appropriate tubing
6.6 Forceps, straight or curved, with smooth tips to handle filters without damage
6.7 Ethanol, wide-mouth container, for flame-sterilizing forceps
6.8 Whirl-Pak® bags or equivalent
6.9 Autoclave or steam sterilizer capable of achieving 121°C [15 lb pressure per square inch (PSI) for 15 minutes
6.10 Burner for sterilizing loops and needles
6.11 Modified mTEC agar plates
6.12 Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 µm pore size (Cat#HAWG047S6)
6.13 Incubator maintained at 35°C ± 0.5°C

6.14 Waterbath maintained at 44.5°C ± 0.2°C
6.15 Filter paper
6.16 Marker
6.17 Parafilm
6.18 Cooler with ice or blue ice for transport of samples
6.19 Refrigerator (~4°C)

7.0. PROCEDURAL STEPS

7.1. Follow the EPA Method 1603 Modified mTEC procedure (EPA-821-R-09-007; http://water.epa.gov/scitech/methods/cwa/bioindicators/upload/method_1603.pdf).

7.2. After 22 +/- 2 hour incubation at 44.5°C, red or magenta colonies are considered 'typical' *E. coli*.

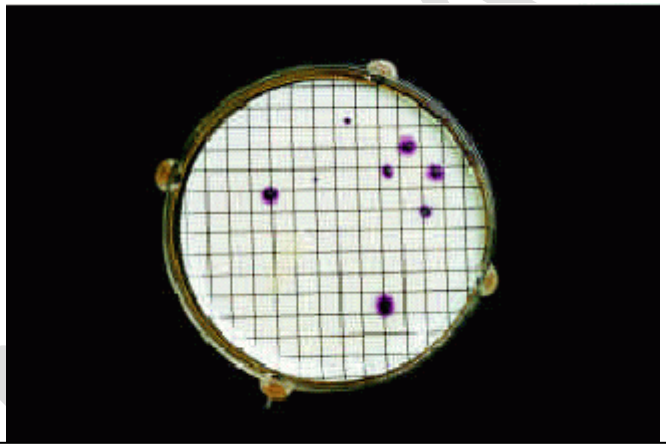

Figure 1. *E. coli* colonies on modified mTEC agar are red to magenta.

7.3. Using a black Sharpie or similar marker, mark *E. coli* colonies with a 'dot' on the back of the plate. This helps to ensure that colonies which grew during the incubation period, as opposed to during shipping or storage, are subsequently isolated. If the colonies were counted, please also write the total number of counted colonies on the back of each plate.

7.4. After incubation and counting, immediately store plates at 4ºC 'media-side up' (i.e., upside down), so condensation does not fall onto the filter during storage.

7.5. The plates should be shipped as soon as possible (preferably the day after filtration, but no later than three days following filtration) to the BST Laboratory (addresses below) via overnight delivery.

7.6. In preparation for shipment, each plate should be sealed with Parafilm around the edge to protect the cultures from contamination during transit. Dilution

series for each sample should subsequently be grouped together and placed in secondary containers such as large Whirl-Pak or zip-top bags.

7.7. 'Blue-ice' or freezer blocks should be used to keep the plates cool (~4ºC), but not frozen during transport. Do not use dry ice for shipment as this will freeze the media and cultures.

7.8. Ship plates (and COCs) in insulated coolers with sufficient ice packs to maintain ~4°C to:

UTSPH-EP
George Di Giovanni
UT-Houston School of Public Health
Biology Building B224
500 W. University
El Paso, TX 79968
915-747-8509

SAML
Terry Gentry
Texas A&M University
Soil & Crop Sciences; Heep Center 539
370 Olsen Blvd
College Station, TX 77843
979-845-5604

7.9. Notification of shipment should be sent to the appropriate lab via email or phone (see contact info below) no later than the day of overnight shipping. Notification should include the *E. coli* count datasheet (if available), shipment tracking number, and direct contact person for confirmation upon receipt of samples.

UTSPH-EP
Elizabeth Casarez; elizabeth.a.casarez@uth.tmc.edu; 915 747-8076
George Di Giovanni; george.d.digiovanni@uth.tmc.edu; 915 747-8509
Joy Archuleta-Truesdale; joy.a.truesdale@uth.tmc.edu; 915-747-6868

SAML
Heidi Mjelde; hmjelde@ag.tamu.edu; 979-845-5604
Terry Gentry; tgentry@ag.tamu.edu; 979-845-5323

8.0. QUALITY ASSURANCE AND QUALITY CONTROL

A method blank (sterile water or phosphate-buffered saline (PBS)) is processed with each batch of samples.

9.0. REFERENCES

Casarez, E. A., S. D. Pillai, J. B. Mott, M. Vargas, K. E. Dean and G. D. Di Giovanni. 2007. Direct comparison of four bacterial source tracking methods and use of composite data sets. *J. Appl. Microbiol*. 103:350-364.

Di Giovanni, G. D., E. A. Casarez, T. J. Gentry, E. C. Martin, L. Gregory, and K. Wagner. 2013. Support analytical infrastructure and further development of a statewide bacterial source tracking library. TR-448. Texas Water Resources Institute, College Station, TX.

USEPA. 2009. Method 1603: *Escherichia coli* (*E. coli*) in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC). EPA-821-R-09-007. December 2009.

10.0. REVISION HISTORY

Revision	Date	Responsible Person	Description of Change
1	June 2015	Elizabeth Casarez	Initial Release

ARCHIVAL OF *E. COLI* ISOLATES

June 5, 2015

Elizabeth Casarez
University of Texas-Houston School of Public Health El Paso Regional Campus

APPROVED:

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Soil & Aquatic Microbiology Laboratory
Texas A&M AgriLife Research

Annual Reviewer			
Date			

1.0. PURPOSE AND APPLICABILITY

The purpose of this Standard Operating Procedure (SOP) is to establish a uniform procedure for the isolation and archival of *E. coli* isolates for further Bacterial Source Tracking (BST) analyses.

2.0. SUMMARY OF THE METHOD

Individual *E. coli* colonies are selected from EPA Method 1603 plates, streaked onto nutrient agar-MUG plates for verification and placed into glycerol stocks for archival at -80°C.

3.0. HEALTH AND SAFETY WARNINGS

Environmental *E. coli* isolates may be pathogenic. Water samples may contain pathogenic microorganisms. All handling of cultures will be performed using a Class II biological safety cabinet to minimize the exposure of laboratory personnel to pathogens. Analysts should wear appropriate personal protective equipment (e.g., gloves). Analysts should wear eye protection and exercise caution when using UV light to examine plates.

4.0. INTERFERENCES

It is important that the isolates be streaked to purity in order to prevent issues arising from co-cultures. The archived isolates should be from colonies which have been plated for purity several times and lab personnel are confident that purity has been achieved.

5.0. PERSONNEL QUALIFICATIONS

This SOP is written for persons with a thorough knowledge of laboratory and microbiological procedures, especially aseptic technique.

6.0. EQUIPMENT AND SUPPLIES

6.1 Sterile, plastic inoculating loops or needles; alternatively a wire loop or needle can be used if sterilized between transfers
6.2 Nutrient agar-MUG plates
6.3 Brain Heart Infusion (BHI) agar plates
6.4 Longwave UV lamp
6.5 Sterile cryovials (2 ml)
6.6 Liquid nitrogen in dewar vessel
6.7 Sterile, tryptone soy broth (TSB) containing 20% reagent grade glycerol
6.8 Vortex
6.9 Pipette and sterile tips (1 ml)
6.10 Incubator (~35°C)
6.11 Freezer (-80°C)
6.12 Class II biosafety cabinet

7.0. PROCEDURAL STEPS

7.1 Select a presumptive *E. coli* colony from the EPA Method 1603 plates used to process tested water samples. Streak cells from the colony onto a NA-MUG plate. Depending upon the number of isolates desired, repeat the process streaking each isolate onto an individual NA-MUG plate.

7.2 Incubate NA-MUG plates at ~35°C for ~24 hr.

7.3 Examine the plates using a long-wave handheld UV lamp. *E. coli* colonies will fluoresce.

7.4 If a culture is verified as *E. coli* in the above step and appears to be pure based on uniform appearance on the NA-MUG plate, select a well-isolated colony using a bacteriological loop and streak onto a Brain Heart Infusion (BHI) agar plate. Incubate the plate at ~35°C for ~24 hr. If the culture is not pure, continue streaking individual colonies onto NA-MUG until purity is achieved.

7.5 Select a well-isolated colony using a bacteriological loop and transfer the colony to a labeled, sterile cryovial containing 1 mL of tryptone soy broth (TSB) with 20% reagent grade glycerol.

7.6. Once the colony has been transferred to the cryovial, firmly cap the cryovial and verify that the cells have been resuspended by vortexing for several seconds.

7.7. Plunge the cyrovial into liquid nitrogen until frozen. Immediately transfer to a cryostorage box and place in -80°C freezer. Cultures may be stored for several years under these conditions.

7.8. To recover cultures from frozen storage, remove the cultures from the freezer and place the cryovials in a freezer block. Do not allow cultures to thaw. Using a bacteriological loop, scrape the topmost portion of the culture and transfer to growth medium, being careful not to contaminate the top or inside of the vial. Invert and incubate plates at 35 to 37°C for 20 to 24 hr. Reclose the cryovial before the contents thaw and return it to the -80°C freezer.

8.0. QUALITY ASSURANCE AND QUALITY CONTROL

A positive control (*E. coli* QC101) is processed with each batch of samples.

9.0. REFERENCES

Casarez, E. A., S. D. Pillai, J. B. Mott, M. Vargas, K. E. Dean and G. D. Di Giovanni. 2007. Direct comparison of four bacterial source tracking methods and use of composite data sets. *J. Appl. Microbiol*. 103:350-364.

Di Giovanni, G. D., E. A. Casarez, T. J. Gentry, E. C. Martin, L. Gregory, and K. Wagner. 2013. Support analytical infrastructure and further development of a statewide bacterial source tracking library. TR-448. Texas Water Resources Institute, College Station, TX.

USEPA. 2009. Method 1603: *Escherichia coli* (*E. coli*) in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC). EPA-821-R-09-007. December 2009.

10.0. REVISION HISTORY

Revision	Date	Responsible Person	Description of Change
1	June 2015	Elizabeth Casarez	Initial Release

DNA FINGERPRINTING OF *E. COLI* ISOLATES USING ENTEROBACTERIAL REPETITIVE INTERGENIC CONSENSUS SEQUENCE (ERIC)-PCR

June 5, 2015

Joy Truesdale
University of Texas-Houston School of Public Health El Paso Regional Campus

APPROVED:

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Author Date

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Di Giovanni Laboratory
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Director Date
Soil & Aquatic Microbiology Laboratory
Texas A&M AgriLife Research

Annual Reviewer			
Date			

PURPOSE AND APPLICABILITY

The purpose of this Standard Operating Procedure (SOP) is to establish a uniform procedure for DNA fingerprinting *E. coli* isolates using enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR.

1.0. SUMMARY OF THE METHOD

Cell suspensions from individual *E. coli* isolates are DNA fingerprinted using ERIC-PCR. Following PCR, amplicons are analyzed using gel electrophoresis to generate the DNA fingerprint which will ultimately be used for further Bacterial Source Tracking (BST) analysis.

2.0. HEALTH AND SAFETY WARNINGS

Environmental *E. coli* isolates may be pathogenic. Water samples may contain pathogenic microorganisms. All handling of live cultures will be performed using a Class II biological safety cabinet to minimize the exposure of laboratory personnel to pathogens. Ethidium bromide is a mutagen and should be handled with care. Analysts should wear appropriate personal protective equipment (e.g., gloves). Analysts should wear eye protection and exercise caution when using UV light to image gels.

3.0. INTERFERENCES

It is important that the isolates be streaked to purity in order to prevent issues arising from co-cultures. The tested isolates should be from colonies which have been plated for purity several times and lab personnel are confident that purity has been achieved.

4.0. PERSONNEL QUALIFICATIONS

This SOP is written for persons with a thorough knowledge of laboratory and microbiological procedures, especially aseptic technique, PCR, and gel electrophoresis.

5.0. EQUIPMENT AND SUPPLIES

5.1 Sterile, plastic inoculating loops
5.2 Sterile, 1.5 ml microfuge tubes
5.3 Sterile, molecular-grade water
5.4 Vortex
5.5 *E. coli* QC101 cell-suspension
5.6 Thermal cycler
5.7 Pipettes and sterile tips (5-1000 µl)
5.8 PCR master mix (recipe below)
5.9 Agarose
5.10 1X Tris/Borate/EDTA (TBE)
5.11 Microwave
5.12 Electrophoresis system with recirculating pump
5.13 Ziploc bags
5.14 Freezer (-20°C)

5.15 6X ERIC-PCR loading buffer
   a. 25 mg bromphenol blue (0.25%)
   b. 1.5 g ficoll 400 (15%)
   c. Add molecular grade water to 10 mL
   d. Divide into 1 mL aliquots and freeze
   e. The aliquot currently being used can be stored in the cold room or at 4°C
5.16 100 bp ladder (0.33 µg/10 µL) (1500 µL final, enough for 150 lanes)
   a. 200 µL Roche DNA Marker XIV (Cat. #1721933) 0.25 µg/µL 100 bp ladder
   b. 300 µL 6X ERIC-PCR loading buffer
   c. 150 µL 10X PCR buffer
   d. 850 µL molecular grade water
   e. Store in cold room
5.17 Ethidium bromide stain (0.5 µg/mL)
   a. 1250 mL 1X TBE
   b. 62.5 µL ethidium bromide (Sigma, 10 mg/mL)
   c. Store covered at room temp,
   d. Can use up to 5 times by adding 10 µL ethidium bromide each additional use
5.18 ERIC-PCR blank;
   a. 100 µL 10X PCR buffer
   b. 200 µL 6X ERIC-PCR loading buffer
   c. 900 µL molecular grade water
   d. Store in cold room or at 4°C
5.19 Class II biosafety cabinet
5.20 PCR plates
5.21 Platform shaker
5.22 Gel imager
5.23 Cold room (~4°C)

6.0. PROCEDURAL STEPS

6.1. Select isolated colonies from overnight cultures of *E. coli* isolates on Brain-Heart Infusion (BHI) plates.

6.2. Transfer colonies using a 1 µL loop to a sterile microfuge tube containing 100 µL of sterile molecular grade water; vortex briefly to suspend cells.

6.3. Prepare sufficient PCR Master Mix for samples, including one blank per 10 samples to account for volume loss due to repeat pipetting. Prepare Master Mix for each sample as noted below. One full PCR batch on the thermal cycler 48 well-plate will have 46 samples, *E. coli* QC101, and a no-template control.

ERIC-PCR Master Mix – 24 samples + 2 blanks, prepare X 2 for full 48-well plate

MASTER MIX	Amt (µL)	Final Calc	Final Units
Molecular Grade Water	**819**		
10X PCR buffer I w Mg (Life Technologies)	**130**	1	X (1.5 mM)
20 mM dNTP (GE Healthcare)	**13**	200	µM each
ERIC Primer Mix*	**130**	600	nM each
BSA (30 mg/ml)	**65**	1.5	µg/µL
AmpliTaqGold (Life Technologies)	**13**	2.5	Units/rxn

*ERIC1R 5' ATGTAAGCTCCTGGGGATTCAC;
ERIC2 5' AAGTAAGTGACTGGGGTGAGCG

6.4. Dispense 45 µl of Master Mix for each sample into the appropriate well of PCR plate.

6.5. Briefly vortex cell suspensions, then add 5 µl of each cell suspension to the appropriate PCR well.

6.6. Carefully seal plate using an adhesive PCR cover.

6.7. Load the plate into the thermal cycler and run under the "ERIC-PCR" program with the following cycling conditions:
   a. Initial denaturation at 95°C for 10 min
   b. 35 Cycles:
      i. Denaturation at 94°C for 30 sec
      ii. Annealing at 52°C for 1 min
      iii. Extension at 72°C for 5 min
   c. Final Extension at 72°C for 10 min

6.8. Store completed reactions at -20°C until analyzed by gel electrophoresis.

6.9. Prepare a 250 mL, 2% agarose gel using a 500 mL bottle. Add 250 mL of 1 X Tris/Borate/EDTA (TBE) buffer and 5.0 g agarose. Microwave until agarose is fully dissolved, tighten cap and let cool 1 to 2 minutes, then pour agarose into casting tray with 30-tooth, 1 mm thick comb.

6.10. Allow gel to solidify for approximately 30 minutes on the bench, then without removing comb place in Ziploc bag and solidify overnight in the refrigerator. The next day carefully remove comb, transfer to gel tank containing pre-cooled 1X TBE buffer. Replace TBE in gel tank after it has been used twice.

6.11. Remove PCR reactions from freezer. Add 10 µL of 6X ERIC-PCR Loading Buffer to each PCR well and mix with pipette tip.

6.12. Load the gel in the cold room as follows (max. of 23 samples + QC101 + NTC per gel):
   a. Load 10 µl of 100 bp ladder (0.33 µg) into the first lane
   b. Load 10 µl of sample ERIC-PCR reactions into next 6 lanes
   c. Load 10 µl of 100 bp ladder (0.33 µg)
   d. Load 10 µl of sample ERIC-PCR reactions into next 6 lanes
   e. Load 10 µl of 100 bp ladder (0.33 µg)
   f. Load 10 µl of sample ERIC-PCR reactions into next 6 lanes
   g. Load 10 µl of 100 bp ladder (0.33 µg)
   h. Load 10 µl of sample ERIC-PCR reactions into next 5 lanes
   i. Load PCR Batch *E. coli* QC101 and NTC into next 2 lanes
   j. Load 10 µl of 100 bp ladder (0.33 µg)

   If running a gel with fewer samples, follow steps above until last sample, followed by *E. coli* QC101, NTC and ladder, then load ERIC-PCR Blank into remaining lanes on gel.

6.13. Start electrophoresis power supply set at 100 volts, run for 1 hour.

6.14. Stop power supply, set time to “000”, set voltage to 200 ,start circulating pump at setting #2, and run for 4 hours.

6.15. After electrophoresis, stain gel in Ethidium Bromide Stain for 20 minutes with rocking on a platform shaker (save stain, see Step 6.17d).

6.16. Destain gel for 10 minutes in 1X TBE buffer. Save destaining solution. Discard after three uses.

6.17. Follow Gel Imager SOP for image capture. Save digital photograph as a TIFF file (default) and print a hardcopy for notebook.

7.0. QUALITY ASSURANCE AND QUALITY CONTROL

A method blank (sterile water; “no template control”) and positive control (*E. coli* QC101) is processed with each batch of samples.

8.0. REFERENCES

Casarez, E. A., S. D. Pillai, J. B. Mott, M. Vargas, K. E. Dean and G. D. Di Giovanni. 2007. Direct comparison of four bacterial source tracking methods and use of composite data sets. *J. Appl. Microbiol*. 103:350-364.

Di Giovanni, G. D., E. A. Casarez, T. J. Gentry, E. C. Martin, L. Gregory, and K. Wagner. 2013. Support analytical infrastructure and further development of a statewide bacterial source tracking library. TR-448. Texas Water Resources Institute, College Station, TX.

9.0. REVISION HISTORY

Revision	Date	Responsible Person	Description of Change
1	June 2015	Joy Truesdale	Initial Release

DNA FINGERPRINTING OF *E. COLI* ISOLATES USING THE DUPONT RIBOPRINTER® SYSTEM

June 5, 2015

Elizabeth Casarez
University of Texas-Houston School of Public Health El Paso Regional Campus

APPROVED:

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Author Date

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Director Date
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Director Date
Soil & Aquatic Microbiology Laboratory
Texas A&M AgriLife Research

Annual Reviewer			
Date			

1.0. PURPOSE AND APPLICABILITY

The purpose of this Standard Operating Procedure (SOP) is to establish a uniform procedure for DNA fingerprinting *E. coli* isolates using the DuPont RiboPrinter® System.

2.0. SUMMARY OF THE METHOD

Cell suspensions from individual *E. coli* isolates are DNA fingerprinted using the DuPont RiboPrinter® System. The DuPont™ RiboPrinter® System automates restriction fragment length polymorphism (RFLP) analysis and targets the rRNA-coding region of the bacterial genome. Restriction enzymes cut bacterial DNA into fragments that are processed to form a characteristic banding pattern or "fingerprint." The system captures an image of the banding pattern and digitizes it as a RiboPrint™ pattern. This pattern is ultimately compared to a reference database of patterns for further Bacterial Source Tracking (BST) analysis.

3.0. HEALTH AND SAFETY WARNINGS

Environmental *E. coli* isolates may be pathogenic. All handling of live cultures will be performed using a Class II biological safety cabinet to minimize the exposure of laboratory personnel to pathogens. Analysts should wear appropriate personal protective equipment (e.g., gloves).

4.0. INTERFERENCES

It is important that the tested isolates be streaked to purity in order to prevent issues arising from co-cultures. The tested isolates should be from colonies which have been plated for purity several times and lab personnel are confident that purity has been achieved.

5.0. PERSONNEL QUALIFICATIONS

This SOP is written for persons with a thorough knowledge of laboratory and microbiological procedures, especially aseptic technique.

6.0. EQUIPMENT AND SUPPLIES

6.1 DuPont RiboPrinter® System and consumables
6.2 *Hin*dIII restriction enzyme 50 U/µL working stock (*Hin*d III (NEB Cat. #R0104M) is prepared in a Sarstedt 500-µL microfuge tube (Cat. #72730-005) as follows. 50 U/µL: 26.5 µL *Hin*d III and 26.5 µL of NEB 10X Buffer 2.1.
6.3 BHI agar plates
6.4 Incubator (37°C)
6.5 Sterile, plastic inoculating loops or needles
6.6 Sterile, microcentrifuge tubes
6.7 Pipette and sterile pipette tips
6.8 Surface disinfecting solution (e.g., 10% bleach or 70% ethanol)
6.9 Class II biosafety cabinet

7.0. PROCEDURAL STEPS

7.1. Storing and Handling Disposables

a. Check lot expiration date on each label for details & rotate stock to optimize use.

b. Heating membrane and probe (MP) Base. After storage and the temperature changes that occur during shipment, the oxygen in the buffer loaded in the MP base may need to be removed before use. This is called degassing and is accomplished if needed by heating the base pack overnight in your incubator. To degas buffer:

1. Place enough MP base packs for the next day's production in their storage pouches in an incubator set at 37°C.

2. Allow the base pack to degas for 16 to 24 hours prior to loading in the characterization unit. You may do this while you are incubating samples, since the base packs are sealed in their pouches. This procedure allows you to start a batch immediately at the beginning of the next shift.

3. If you do not use the heated base packs, you can return them to storage and reuse them. These base packs should be heated again before reuse since temperature cycling affects oxygen content in the buffer.

c. Preparing Lysing Agent (for *Staphylococcus* and lactic-acid bacteria only). Lysing agent (A and B) is shipped frozen and must be stored at -20°C. Lysing agent must be thawed before use. This only takes about 5 minutes. If the lysing agent will not be used again for more than 2 hours, the material should be returned to the freezer. Lysing agent can be re-frozen several times with no effect on performance.

7.2. Sample Preparation Procedures

a. Incubate and Inspect the Samples

1. Use BHI agar plates prepared within the last 30 days. Do not use plates that appear dry or dehydrated. Such plates can cause problems when you attempt to "pick" the colonies for use in the RiboPrinter® system.

2. Using a pure isolated colony as the source, streak BHI agar plates heavily in the upper portion of the plate to create a lawn. Streak the remainder of the plate lightly to create single colonies.

3. Follow standard laboratory techniques. Heat plates for 18 to 30 hours in a humidified incubator at 37 °C.

b. Transfer Sample Buffer to Intermediate Tubes

1. Locate the 250 mL twist-top bottle of sample buffer supplied in Pack # 1. Install the twist cap.

2. Transfer about 5 mL of buffer to a sterilized disposable 15 mL intermediate working tube.

c. Add sample buffer to microcentrifuge tubes

1. Place a sterile 0.65 mL microfuge tube in each of the eight holes in the lower row of the sample preparation rack.

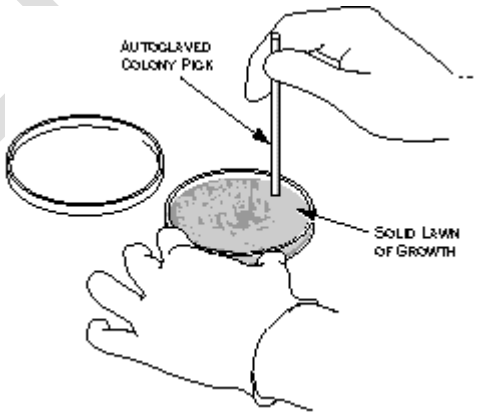
2. For Gram negative samples (including *E. coli*), add 200 µL of sample buffer from the intermediate tube.

   For Gram positive samples (e.g. *S. aureus* and *L. innocua* QC strains), add 40 µL of sample buffer.

3. Close the lids on the tubes.

d. Harvest the Samples

1. Using autoclaved colony picks and making certain not to gouge the agar, carefully place the pick into one of the single colonies or the lawn. You need a sample area at least equal to that of the bottom of the colony pick. In most cases you will need to harvest from the lawn area of the plate. If you are working with large colonies, a single colony will be adequate.



2. For Gram negative samples (e.g. *E. coli*), perform 1 pick placed into 200 µL of sample buffer.

CAUTION! Do not try to use the same pick twice on a plate. You need to harvest only enough sample to cover the bottom surface of the pick. Make sure the end of the pick is flat, if not, use a different pick.

CAUTION! Do not overload the harvesting pick. Collect only enough sample to cover the base of the pick. Over sampling will cause inaccurate results. Over sampling is a particular problem with *Staphylococcus*.

e. Mix the Samples

WARNING! *Perform sample preparation using a Class 2 biological safety cabinet since aerosols may be formed during mixing of the samples.*

1. Making certain not to touch the sample end of the pick, place the pick into one of the filled sample tubes.

2. While holding the tube with the open end facing away from you, carefully attach pick to hand-held mixer. The fit of the pick in the coupling will be loose.

   WARNING! *Do not turn on the mixer unless the pick is inside the sample tube and below the surface of the liquid. Turning the unit on at other times will cause the sample to aerosolize and may cause contamination.*

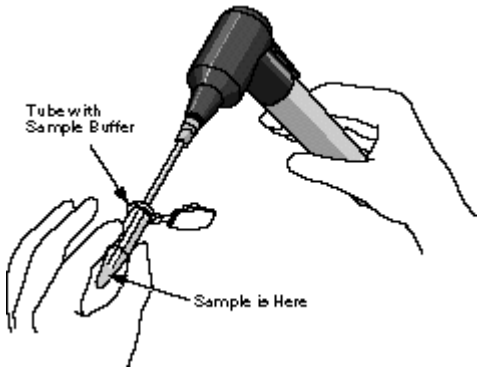
3. Press the ON lever on the mixer for about 5 seconds.

4. Release the lever and carefully remove the colony pick. The sample liquid should appear turbid.

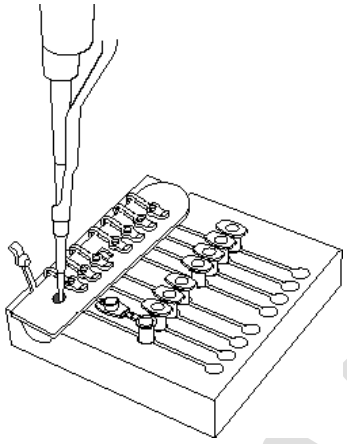
5. For **Gram positive samples only**, (e.g. *Staphylococcus* and *Listeria*) locate a new colony pick and repeat steps for harvesting and mixing samples, adding a second sample to the original tube. Discard used picks in biowaste bag.

6. Cap the sample tube.

7. Move the tube to the top row of the sample preparation rack. This indicates that the tube is filled.



7.3. Transfer the Samples to the Sample Carrier



a. Open the lid covering the first well of the sample carrier.

b. Using a 100 µL pipetter, pipette 30 µL of sample from the microcentrifuge tube into the well.

c. Close the lid cover for the well.

d. Repeat for remaining samples using a new pipet tip for each sample.

CAUTION! *Transfer the sample carrier to the Heat Treatment Station within 2 hours. If you wait longer than 2 hours, you will have to discard the sample carrier and begin again for this batch.*

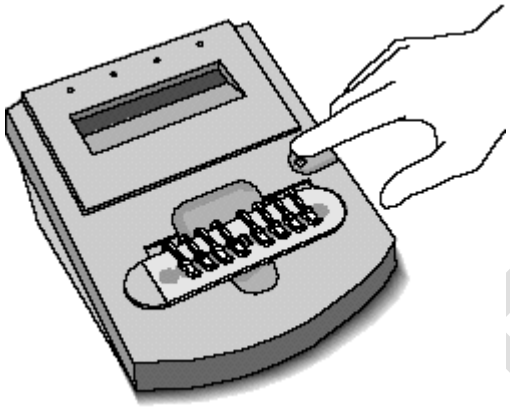
e. Lightly wipe down the outer surfaces of the sample carrier with a lab wipe wetted with surface disinfectant (10% bleach or 70% alcohol).

f. Write down the name or code you use to identify the sample and the well number in the sample carrier for each sample using a sample log sheet.

7.4. Place the Sample Carrier in the Heat Treatment Station and Process the Sample Carrier

a. Place the sample carrier into the Heat Treatment Station. The display on the Heat Treatment Station will show **Insert**, if power is available. If the display is blank, make certain that the power cord on the back of the station is properly connected.

After you insert the carrier, the display shows **Press Button.**



b. Press the button on the Heat Treatment Station.

The display shows **Warm up** and counts down from **10** while the station is warming up. The actual warm up cycle varies with the condition of the room and the heat treatment station. Normal time is about 4 minutes.

When the station reaches operating temperature, the display changes to **Heat** and counts down from **13.** This represents each minute of heat treatment.

The indicator message changes to **Cool.** The display counts down from **9**, indicating the minutes remaining in the cooling cycle**.** If necessary, you can remove the carrier as soon as the **Cool** message appears.

c. The heat treatment step is finished when the display shows **READY** and counts down from **90.** The display will flash and an audible beep will sound three times. The alarm will then beep once every 10 minutes until the sample is removed or 90 minutes elapses.

Caution! *The heat-treated samples must be used within the 90-minute period at room temperature or they must be discarded. The heat-treated samples may be stored at this point (prior to adding Lysis Agents, if required) for 1 week at 4 °C, or for several months at -70 °C.*

7.5. Add the Lysing Agents (for *Staphylococcus* and lactic-acid bacteria only)

a. Using a 10-µL pipetter and new tips for each addition, add 5 µL of Lysing Agents A and B to each sample. Note: this step is omitted for *E. coli* as it has no effect on ribopatterns. Lysing Agents were specifically developed for *Staphylococcus* and Lactic-Acid bacteria samples.

Caution! *This step must be performed just prior (within 10 minutes) of loading the samples into the RiboPrinter and starting the run.*

7.6. Creating and Loading a Batch

a. There are three options under the Operations menu for creating standard batches:
   - *Eco*RI batches (VCA)
   - *Pst*I batches (VCB)
   - *Pvu*II batches (VCC)

   You can also create special batches:
   - Restriction Enzyme Flexibility batches
   - **Substitute Enzyme batches (including *Hin*d III)**

b. From the Instrument Control Base Window:

1. Move the pointer to Operations and click with the mouse button. The Operations menu appears.

2. Move the pointer to Create Substitute Enzyme Batch and click with the mouse button.

3. Use the View menu to remove any optional items you do not wish to fill in. The system requires at least Sample Type and RiboGroup Library information for each sample. You cannot remove these options. The **Clear** option de-selects the **Use Default ID Libraries.** You will have to enter a DuPont ID and Custom ID library name for all samples. These become required fields and the system will make you enter data before you can save the information in this window.

   CAUTION! *If you change the display after you have entered information, you will lose all the information in the window. The window will redraw with a new blank display showing the items you have selected.*

4. To enter information about the sample, click on the **View** button with the mouse button, then click on **Sample Items**. Click on the options you want to display.

5. Enter your initials and any comment you want to record about the batch.

6. Select the lot number fields and record for all reagents.

CAUTION! *All fields must be completed or the system will not let you start processing the batch.*

7. For each well in the sample carrier, choose the type (Sample or Control [QC Number]) from the Sample Type field. The system defaults to Sample.

8. Once you define the Sample Type as Sample, type in the name you actually want to use. This information will appear as Sample Label in the Data Analysis software screens.

9. You can change the RiboGroup library name if needed. Do this by clicking on the button next to the field with the mouse button. A pop up menu appears listing your choices. If you want to add a new library name, move the pointer to the line and click with the mouse button to get a cursor, then type in the new library name. Once you have saved this file, the new name will be added to the pop up list for future use. Do **NOT** change the DuPont ID field. If you select one of the QC strains, the system automatically enters QC in the DuPont ID and RiboGroup Library fields. Do not change these names. If you wish, you may enter a name for the Custom ID library.

10. Repeat for the other seven samples.

11. Click on Save and Submit Batch to Instrument.

7.7. Loading Disposables

a. Follow the screen prompts to load disposables and check the DNA Prep Waste. The icons on the window will flash red to tell you to remove and load an item. The screen prompts you about which Separation and Transfer chamber to use for the membrane and gel cassette. The LDD Pipette will move to physically block you from placing samples in the wrong chamber.

CAUTION! *Do not try to move the pipette manually. You will cause the system to lose the step count. This can result in the loss of batch data. If the pipette is blocking the S/T chamber that you are instructed to use, STOP. Call Customer Support.*

CAUTION! *Do not load disposables until you are prompted by the system. If you try to load them earlier, the alarm will sound as long as the doors are open. If you do load disposables ahead of time, the MP Base will be moved to the wrong position and you will not be able to begin processing the batch. You will not be able to move the MP base manually.*

b. Check the DNA Preparation Waste Container

1. The DNA Prep waste container must be visually checked before every batch. If the container looks nearly full (about 1 inch from the top), remove the container, unscrew the cap and empty into the liquid biohazard waste.

   WARNING! *Do not tip the DNA Preparation waste container when you remove it.*

   WARNING! *Do not unscrew the cap from the DNA Preparation waste container if the fluid level has risen into the cap. First pour the excess waste liquid into the liquid biohazard waste.*

   WARNING! *When replacing container make sure that the cap is properly threaded in place. If the cap is only partially threaded, it can snag the pipette during operation.*

c. Load the Sample Carrier

1. Place the sealed carrier into the labeled slot on the far right of the characterization unit.

2. Push the sample carrier down firmly until it snaps into place.

   CAUTION! *Place the rounded edge of the sample carrier on your right as you view the characterization unit. Position the carrier this way to insure correct identification of the sample wells.*

d. Load the DNA Prep Carrier

1. Remove the DNA Prep carrier from the refrigerator.

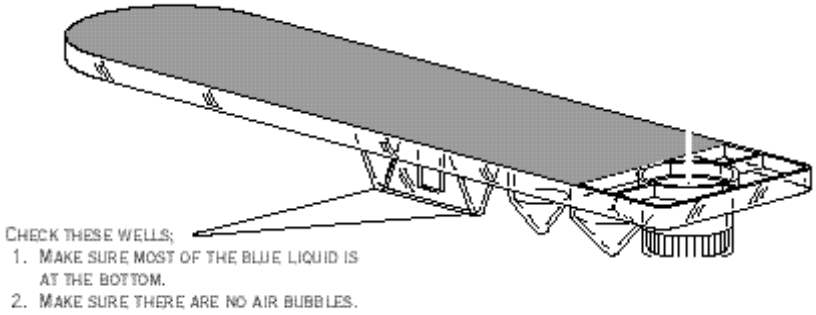
2. Check the wells in the carrier. If most of the liquid appears to be in the bottom of the wells and there are no bubbles, go to step 3. Otherwise **lightly tap the side of the carrier a few times with your finger to release any material that has adhered to the lid.**

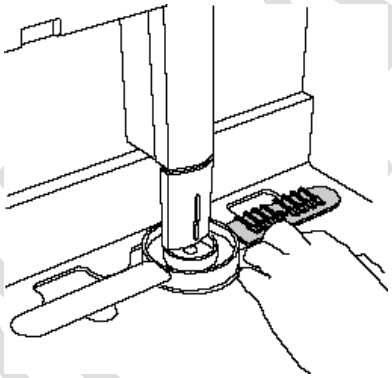
3. **CAUTION!** Do not tap the carrier briskly. This may cause the marker to degrade which can create inaccurate results.

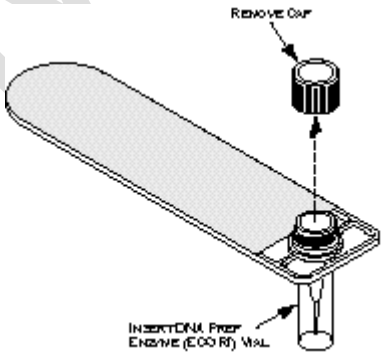
4. Remove a vial of DNA Prep Enzyme (*Hin*d III or *Eco*R I) from the freezer. ***Hin*d III (NEB Cat. #R0104M) is prepared in a Sarstedt 500-µL microfuge tube (Cat. #72730-005) as a 50 U/µL working stock as follows.**

   <u>50 U/µL:</u> 26.5 µL *Hin*d III and 26.5 µL of NEB 10X Buffer 2

During addition of the Buffer, mix enzyme and buffer to homogeneity by stirring with the micropipette tip.





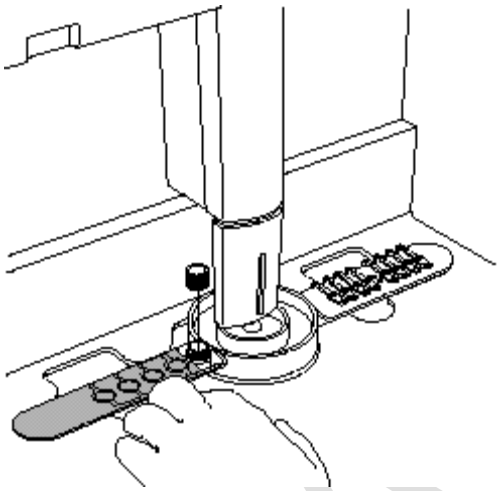


5. Remove the cap from the Enzyme vial.

6. Insert the vial into the carrier.

7. Place the DNA Prep carrier into the slot labeled **Reagent** to the left of the sample carrier slot.

8. Push the DNA Prep carrier down firmly until it snaps into place.

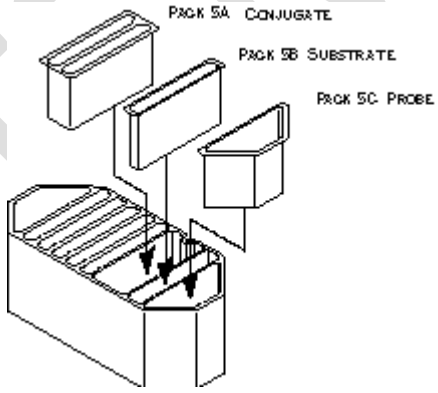


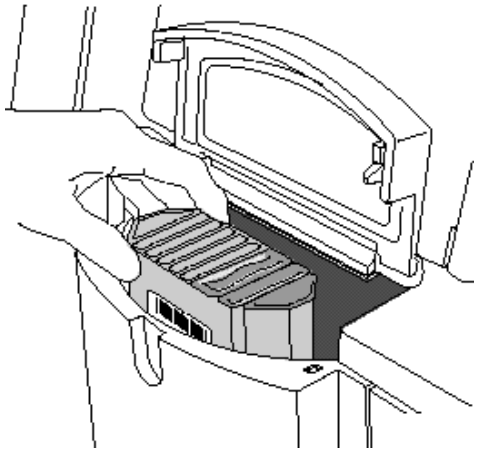
e. Load the MP Base and Carousel

1. Unpack the disposables.

2. Remove the MP base (Pack 5) from the incubator and the Conjugate (Pack 5A), Substrate (Pack 5B), and Probe (Pack 5C) from the refrigerator.

3. Remove each insert from its pouch. Tap the powdered reagent packs gently to bring all powder to the bottom of the packs. Place reagent packs in the MP base and load the base in the carousel.

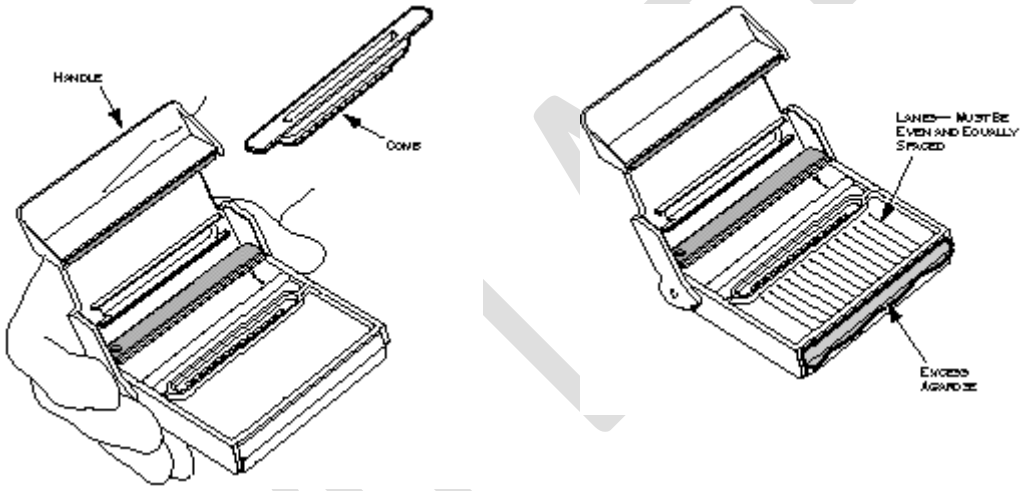




CAUTION! *Push each insert firmly into place. If part of the insert extends above the top of the base, it could catch on the bottom of the deck and cause a system error. You could lose one or more batches as a result. Each insert is keyed by shape and cannot be inserted incorrectly.*

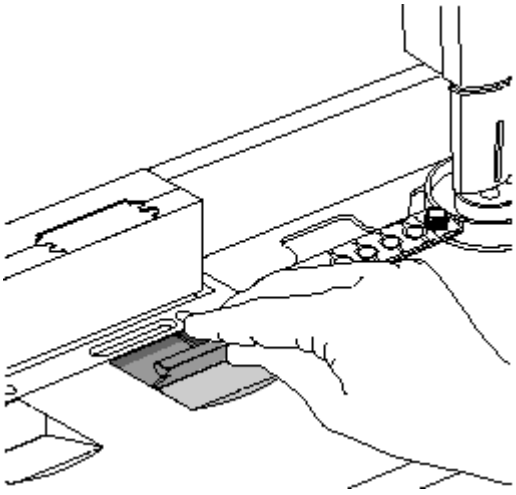
f. Load the Gel Cassette

1. Remove the gel cassette from its package.
2. Grasp one end of the rubber comb and gently pull the comb from the cassette.
3. Unfold the handle of the cassette towards you until the handle snaps into place.
4. Check the front edge of the gel cassette and the lanes of the gel.



Warning! *If the cassette shows a build up of excess gel on the front edge, or if you notice any shrinkage of the gel away from the cassette or bubbles, record the lot number and call Customer Support. Use a new cassette for this run.*

5. Insert the gel cassette into the slot labeled **Gel Bay.** The RiboPrinter® system will prevent the insertion of the cassette into the incorrect slot by blocking one slot with the LDD Pipette.

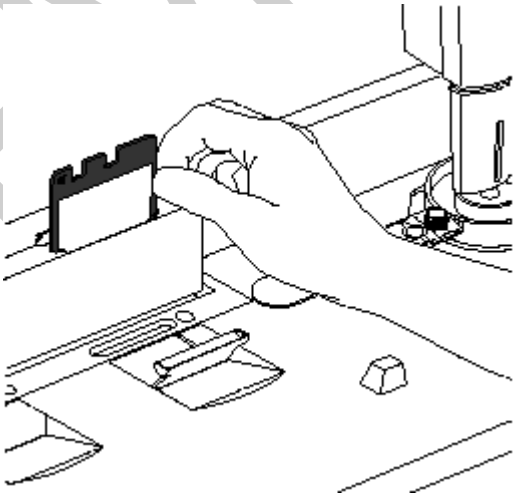


6. Press the cassette forward firmly until it snaps into place.

g. Load the Membrane

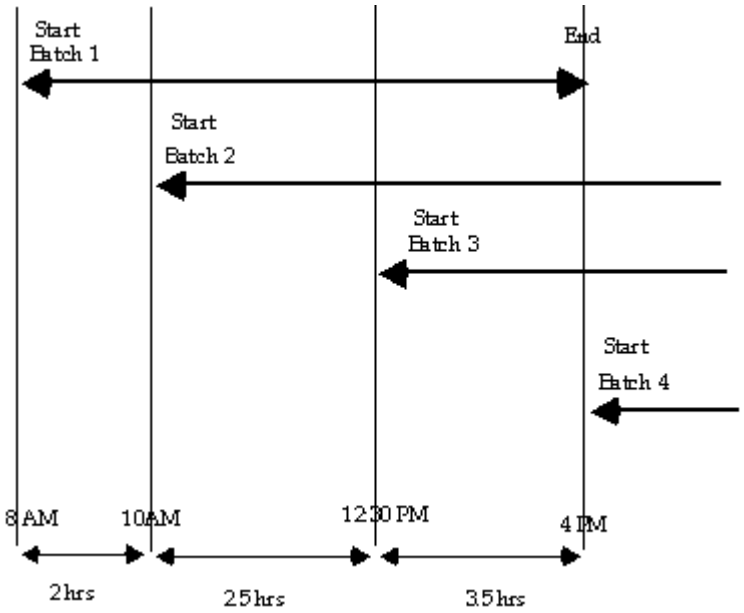
1. Grasp the membrane and carefully drop it into the front slot and flip the metal bracket against the back of the membrane.

CAUTION! *You can insert the membrane backwards. This will cause an alarm that prevents the sample from being processed until the error is corrected. Always make certain that the two large slots are on top and that the square hole on the side faces your left as you insert the membrane.*



h. Close all doors and the instrument will begin sample processing.

i. Load the Next Batch. The RiboPrinter® microbial characterization system lets you load up to four VCA batches in an eight hour period. Other batches may take longer



to process. The chart below shows the approximate loading times for each batch in a work shift using only the VCA protocol.

1. You can now use the **Create Batch** option to set up a new pending batch.

2. When you complete the information window and click on **Start Normal Batch** option, the window displays a message telling you when you can load the next batch.

7.8. Batch Report

After image processing is completed, the system automatically runs a series of analysis functions and generates a Batch Information Report. This task does not require any action on the part of the operator. Reports are automatically saved to the hard disk of the computer and sent to the printer.

8.0. QUALITY ASSURANCE AND QUALITY CONTROL

A positive control (*E. coli* QC101) is processed each day the RiboPrinter is run.

9.0. REFERENCES

Casarez, E. A., S. D. Pillai, J. B. Mott, M. Vargas, K. E. Dean and G. D. Di Giovanni. 2007. Direct comparison of four bacterial source tracking methods and use of composite data sets. *J. Appl. Microbiol*. 103:350-364.

Di Giovanni, G. D., E. A. Casarez, T. J. Gentry, E. C. Martin, L. Gregory, and K. Wagner. 2013. Support analytical infrastructure and further development of a statewide bacterial source tracking library. TR-448. Texas Water Resources Institute, College Station, TX.

DuPont. 2013. DUPONT™ RIBOPRINTER® SYSTEM. DuPont, Wilmington, DE.

10.0. REVISION HISTORY

Revision	Date	Responsible Person	Description of Change
1	June 2015	Elizabeth Casarez	Initial Release

PREPROCESSING OF WATER SAMPLES FOR *BACTEROIDALES* PCR

June 5, 2015

Joy Truesdale
University of Texas-Houston School of Public Health El Paso Regional Campus

APPROVED:

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Author Date

_____ _____
Director Date
Di Giovanni Laboratory
University of Texas-Houston
School of Public Health El Paso Regional Campus

_____ _____
Director Date
Soil & Aquatic Microbiology Laboratory
Texas A&M AgriLife Research

Annual Reviewer			
Date			

PURPOSE AND APPLICABILITY

The purpose of this Standard Operating Procedure (SOP) is to establish a uniform procedure for the initial processing of water samples for archival at -80°C in preparation for future Bacterial Source Tracking analyses targeting library-independent DNA markers such as those for source-specific bacteria within the order *Bacteroidales*.

1.0. SUMMARY OF THE METHOD

Water samples are passed through 0.2 µm-pore size membrane filters to collect microbial biomass. Filters, with attached biomass, are then immersed in a lysis buffer solution and frozen until future analysis.

2.0. HEALTH AND SAFETY WARNINGS

Environmental water samples may contain pathogenic microorganisms. The analyst should treat all sources of wastewater as though each contained a chemical and/or a biological agent that could cause illness. The analyst should wear protective gloves and handle containers with care.

3.0. INTERFERENCES

Turbid waters may clog membrane filters before the desired volume of sample can be processed. If this occurs, filter as much water as possible (up to the desired volume) and record the amount of water filtered on bag/tube that the filter is placed into and also on the chain-of-custody form.

4.0. PERSONNEL QUALIFICATIONS

This SOP is written for persons with a basic knowledge of laboratory and microbiological procedures.

5.0. EQUIPMENT AND SUPPLIES

5.1 Pipets (sterile), T.D. bacteriological, plastic, of appropriate volume
5.2 Sterile membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper
5.3 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source (In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used)
5.4 Filter flask, vacuum, usually 1 L, with appropriate tubing
5.5 Filter manifold to hold a number of filter bases (optional)
5.6 Flask for safety trap/filter placed between the filter flask and the vacuum source
5.7 Forceps, straight or curved, with smooth tips to handle filters without damage
5.8 Ethanol, methanol or isopropanol in a small, wide-mouth container, and cigarette lighter for flame-sterilizing forceps
5.9 Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops
5.10 Supor membrane filters, 0.2 µm pore size, sterile, white, 47 mm diameter (VWR cat # 28147-979)

5.11 Sterile, 15 ml polypropylene centrifuge tubes, Whirl-Pak® bags, or equivalent
5.12 Guanidine isothiocyanate (GITC) lysis buffer:
100 ml of GITC lysis buffer
50 ml reagent grade (deionized) water
59.08 g guanidine isothiocyanate (VWR # 100514-046; 5 M final)
3.7 g EDTA [pH 8.0] (VWR # VW1474-01; 100 mM final)
0.5 g Sarkosyl (VWR # 200026-724; 0.5% final)
Adjust to pH 8.0 with NaOH (approx. 0.4 g of pellets) to dissolve EDTA and heat with vigorous stirring to dissolve guanidine
Bring up to 100 ml total volume with reagent grade (deionized) water
Autoclave and store at room temp
5.13 Freezer (-80°C)

6.0. PROCEDURAL STEPS

6.1 Within six hours of sample collection, water samples (100 ml) are filtered through 0.2 µm pore size Supor-200 filters.
6.2 Discard filtrate and place the filter into a pre-labeled sterile 15 ml tube (or bag) using ethanol-flamed forceps and aseptic technique. If 100 ml of water cannot be filtered, record the volume filtered on the 15 ml tube and chain of custody form.
6.3 Add 500 µl of guanidine isothiocyanate (GITC) lysis buffer to each 15 ml tube with filter.
6.4 Store samples at -80°C.

7.0. QUALITY ASSURANCE AND QUALITY CONTROL

A method blank (sterile water or phosphate-buffered saline (PBS)) is processed with each batch of samples.

8.0. REFERENCES

Bernhard, A.E. and Field, K.G. (2000) A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. *Appl Environ Microbiol* 66(10), 4571-4574.

9.0. REVISION HISTORY

Revision	Date	Responsible Person	Description of Change
1	June 2015	Joy Truesdale	Initial Release

BACTERIAL SOURCE TRACKING (BST) ANALYSIS OF WATER SAMPLES USING *BACTEROIDALES* PCR

June 5, 2015

Joy Truesdale
University of Texas-Houston School of Public Health El Paso Regional Campus

APPROVED:

_____ _____
Author Date

_____ _____
Director Date
Di Giovanni Laboratory
University of Texas-Houston
School of Public Health El Paso Regional Campus

_____ _____
Director Date
Soil & Aquatic Microbiology Laboratory
Texas A&M AgriLife Research

Annual Reviewer			
Date			

1.0. PURPOSE AND APPLICABILITY

The purpose of this Standard Operating Procedure (SOP) is to establish a uniform procedure for the Bacterial Source Tracking (BST) analyses of water samples targeting library-independent DNA markers including those for source-specific bacteria within the order *Bacteroidales*.

2.0. SUMMARY OF THE METHOD

DNA is extracted from microbial biomass collected from a water sample. Source specific markers are amplified using the polymerase chain reaction (PCR). The presence or absence of specific markers is determined by visualizing the PCR products using gel electrophoresis.

3.0. HEALTH AND SAFETY WARNINGS

Environmental water samples may contain pathogenic microorganisms. The analyst should treat all sources of wastewater as though each contained a chemical and/or a biological agent that could cause illness. The analyst should wear protective gloves, lab coat, and handle containers with care. Ethidium bromide is a mutagen and should be handled with care. Analysts should wear appropriate personal protective equipment (e.g., gloves). Analysts should wear eye protection and exercise caution when using UV light to image gels.

4.0. INTERFERENCES

Environmental waters may contain substances which inhibit PCR. In some cases, it may be necessary to further purify or dilute the sample prior to PCR analysis.

5.0. PERSONNEL QUALIFICATIONS

This SOP is written for persons with a thorough knowledge of laboratory and microbiological procedures, especially aseptic technique, PCR, and gel electrophoresis.

6.0. EQUIPMENT AND SUPPLIES

6.1 Qiagen QIAamp DNA mini kit
6.2 Ethanol (100%)
6.3 Pipettes and sterile pipet tips
6.4 Slide warmer (or similar)
6.6 Centrifuge
6.7 PCR plates, strips, or tubes
6.8 Thermal cycler
6.9 PCR master mix (recipe below)
6.10 Agarose
6.11 1X Tris/Borate/EDTA (TBE)
6.12 Microwave
6.13 Electrophoresis system with recirculating pump
6.14 Ethidium bromide
6.15 6X PCR loading buffer

a. 25 mg bromphenol blue (0.25%)
b. 1.5 g ficoll 400 (15%)
c. Add molecular grade water to 10 mL
d. Divide into 1 mL aliquots and freeze
e. The aliquot currently being used can be stored in the cold room

6.16. 50 bp ladder (0.33 µg/10 µL) (1500 µL final, enough for 150 lanes)
a. 200 µL Roche DNA Marker XIII (Cat. #1172193301) 0.25 µg/µL 50 bp ladder
b. 300 µL 6X PCR loading buffer
c. 150 µL 10X PCR buffer
d. 850 µL molecular grade water
e. Store in cold room

6.18 Water bath (70°C)
6.19 Freezer (-20°C)
6.20 Gel imager

7.0. PROCEDURAL STEPS

7.1. DNA EXTRACTION

1. DNA is extracted from the water concentrates using QIAamp DNA mini kit. Turn on the slide warmer and set to maximum. Preheat a microfuge tube rack and 0.01X TE buffer pH 8.0 for elution and a 70°C water bath.

2. Thaw samples and transfer filters (using flame sterilized forceps) and GITC buffer (using a serological pipet) to Ziploc bags.

3. Add 500 µl of Buffer AL to each filter and massage filter through Ziploc bag for approximately 1 minute.

4. Incubate in a 70°C water bath for 10 minutes by placing between two racks and making sure the zip seal is not submerged under water.

5. Squeegee lysis buffer to corner of the bags using a serological pipet.

6. Transfer lysate from Ziploc bags to a 2.0 ml microfuge tube.

7. Add 500 µl of 100% ethanol and pulse vortex mix for 15 sec. Centrifuge at high speed (14K rpm) for 1 minute to pellet debris and remove droplets from cap.

8. Transfer half of the sample lysate (600 to 750 µl) to a labeled QIAamp column placed in a Qiagen collection tube. Avoid debris pellet while transferring lysate. Centrifuge at 14K rpm, with brake, for 1 minute. If necessary, at each step wipe off any buffer from outside of column with a lab tissue before placing into a new collection tube.

9. Place column in a new collection tube and repeat Step 8 with remaining sample.

10. Place column in new collection tube and add 500 µl of AW1 wash buffer. Centrifuge as above and place column in a new collection tube.

11. Add 500 µl of AW2 wash buffer and centrifuge as above, then repeat once more. Place column in a clean collection tube and centrifuge as above to remove all traces of AW2 buffer.

12. Place in a clean collection tube in the heated rack on the slide warmer. Add 100 µl of 70 - 80 °C 0.01X TE buffer pH 8.0 and let incubate at 70 - 80 °C for 5 minutes with columns capped.

13. Immediately centrifuge at 14K rpm for 3 minutes and transfer the filtrate containing the eluted DNA to a labeled 0.65 ml tube. Store at -80 °C until analyzed by PCR. Keep the remainder of the unused aliquot of 0.01X TE to use as a no template control for the PCR.

7.3. PCR AMPLIFICATION

1. Prepare sufficient PCR Master Mix for samples and controls, as well as one blank per 10 samples to account for volume loss due to repeat pipetting.

Bacteroidales PCR Master Mix – per sample

MASTER MIX	Amt (µL)	Final Calc	Final Units
Molecular Grade Water	**30.2**		
10X PCR buffer I w Mg (Life Technologies)	**5**	1	X
MgCl2 (25 mM) (Life Technologies)	**1**	0.5 (2.0 final)	mM
each dGTP, dCTP, dATP (33 mM mix) (GE Healthcare)	**0.3**	200	µM each
dUTP (100 mM) (GE Healthcare)	**0.2**	400	µM
Bacteroidales Primer Mix*	**5**	200	nM each
BSA (30 mg/mL)	**2.5**	1.5	µg/µL
AmpliTaq Gold (Life Technologies)	**0.5**	2.5	Units/rxn
Uracil DNA glycosylase (UNG; Life Technologies)	**0.25**	0.5	Units/rxn

*See marker specific mastermix

***Bacteroidales* Marker Sequences**

Marker Name	Sequence	Reference
**General Marker	BAC708R 5'CAATCGGAGTTCTTCGTG	Bernhard and Field, 2000
General Marker (GenBac)	Bac32F 5' AACGCTAGCTACAGGCTT	Bernhard and Field, 2000
Human Marker	HF183F 5' ATCATGAGTTCACATGTCCG	Bernhard and Field, 2000b
Hog/Pig Marker	PG163F 5' GCGGATTAATACCGTATGA	Dick et al., 2005
Ruminant Marker	CF128F 5' CCAACYTTCCCGWTACTC	Bernhard and Field, 2000b

**This primer will be used in all reactions

2. Dispense 45 µl of Master Mix for each sample into the appropriate well of PCR plate.

3. Briefly vortex DNA extracts, quick spin, then add 5 µl to the appropriate PCR well.

4. Carefully seal plate using an adhesive PCR cover.

5. Load the plate into the thermal cycler and run under the appropriate *Bacteroidales* program with the following cycling conditions:
    a. UNG digestion 50°C for 10 min
    b. Initial denaturation at 95°C for 10 min
    c. Cycling conditions (see table for number of cycles and annealing temperature):
        i. Denaturation at 95°C for 30 sec
        ii.

Assay	Number of Cycles	Annealing Temperature (°C)	Template Volumes
GenBac – fecal	30	53	1 ul
GenBac – water	35	53	5 ul
Human – fecal	35	60	1 ul
Human – water	40	60	5 ul
Hog – fecal	35	53	1 ul
Hog – water	40	53	5 ul
Ruminant – fecal	35	58	1 ul
Ruminant – water	40	58	5 ul

        iii. Extension at 72°C for 1 min
        iv. Final Extension at 72°C for 10 min

6. Store completed reactions at -20°C until analyzed by gel electrophoresis.

7.4 GEL ELECTROPHORESIS

1. Prepare a 200 mL, 2% agarose gel using a 500 mL bottle. Add 200 mL of 1 X TBE buffer and 4.0 g agarose. Microwave until agarose is fully dissolved, add 10 µl of ethidium bromide (10 mg/ml), tighten cap, swirl to mix and let cool 1-2 minutes.

2. Pour agarose into casting tray with one or two 30-tooth, 0.75 mm thick combs.

3. Allow gel to solidify for 30-60 minutes on the bench, remove comb(s), and place in gel tank with TBE buffer. Discard TBE in gel tank after it has been used twice.

4. Mix 10 µl of PCR product with 2 µl of 6X Loading Buffer.

5. Load gel, starting with 10 µl of 50 bp ladder in the first lane, followed by 12 µl of each sample with Loading Buffer, and 10 µl of 50 bp ladder after the last sample.

6. Start electrophoresis power supply set at 100 volts, run for 1.5 hours.

7. Follow Gel Imager SOP for image capture. Save digital photograph as an 8-bit TIFF file with no scaling and print a hardcopy for notebook.

8.0. QUALITY ASSURANCE AND QUALITY CONTROL

A method blank (no-template control) is processed with each batch of samples.

9.0. REFERENCES

Bernhard, A. E., and K. G. Field. (2000). Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. *Appl Environ Microbiol* 66:1587-1594.

Bernhard, A.E. and Field, K.G. (2000b) A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. *Appl Environ Microbiol* 66(10), 4571-4574.

Dick, L. K., Bernhard, A. E., Brodeur, T. J., Santo Domingo, J. W., Simpson, J. M., Walters, S. P., et al. (2005). Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal genetic markers for fecal source identification. *Appl Environ Microbiol, 71*(6), 3184-3191.

10.0. REVISION HISTORY

Revision	Date	Responsible Person	Description of Change
1	June 2015	Joy Truesdale	Initial Release

Appendix C

texasbst.tamu.edu

LOOKING TO IDENTIFY SOURCES OF *E.COLI*?

Submit water samples for bacterial source tracking

Bacterial source tracking (BST) is a valuable tool that can identify, and also rule-out, significant sources of *E. coli* pollution in a watershed. Using DNA fingerprints and bacterial markers, fecal pollution sources are identified by comparing *E. coli* DNA to those in a statewide library of known sources.

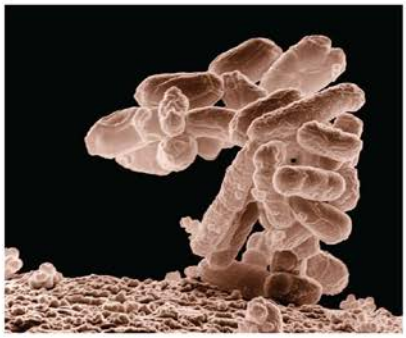
Bacterial source tracking can:

- Identify primary sources of *E. coli*
- Illustrate the relative abundance of *E. coli* from identified sources
- Determine the presence or absence of major watershed sources
- Inform watershed management decisions
- Allow resources to be used wisely and focused where pollutant reductions are needed most

We provide:

- Timely results
- Thorough quality assurance and control standards
- Easy to understand presentation and interpretation of results

For more than a decade, the Texas BST Library has successfully identified sources of *E. coli* in dozens of watersheds across Texas.



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Texas Water Resources Institute
make every drop count

UTHealth | School of Public Health
The University of Texas Health Science Center at Houston

Texas A&M AgriLife
RESEARCH | EXTENSION

AgriLIFE RESEARCH & EXTENSION
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BST

Bacterial Source Tracking

UTHealth School of Public Health

TSSWCB

Texas Water Resources Institute

AgriLIFE RESEARCH & EXTENSION
Texas A&M System

Developed by the Texas Water Resources Institute and funded through a State General Revenue nonpoint source grant from the Texas State Soil and Water Conservation Board

Cover photo: © Lynn McBride EM-111

Learn why Bacterial Source Tracking is the foremost tool for identifying sources of fecal pollution

texasbst.tamu.edu

The Need

There are 273 bacterially impaired water bodies in Texas. Identifying and assessing sources of these bacteria — *E. coli*, *Enterococcus*, fecal coliforms — is critical to properly determining risk to water recreation, developing effective watershed restoration strategies such as watershed protection plans (WPP) and total maximum daily loads (TMDL), and efficiently targeting management measures.

Options for Bacterial Source Identification and Advantages of BST

Bacterial sources are extensive and include all birds, wildlife, livestock, pets and humans. Source surveys, computer models and bacterial source tracking (BST) are the primary methods for identifying predominant bacterial sources in impaired waters. BST offers distinct advantages over computer modeling and source surveys. Source surveys, which estimate the numbers and distributions of animals and humans in a watershed, are not capable of assessing most wildlife species or how bacteria from sources are transported to the impaired waters. Computer modeling addresses the issues with transport; however, because wildlife populations are rarely known, models are unable to adequately assess contributions from wildlife. BST is able to evaluate wildlife, along with other major sources, and the impacts of transport because BST uses instream water samples for its assessment. However, best results are generally achieved when used in conjunction with the other methods.

Proper evaluation of nonpoint sources is needed to accurately assess risk to water recreation, target best management practices, and develop effective watershed restoration strategies.

BST Technologies

The premise behind BST is that DNA fingerprinting and other molecular targets can identify bacterial strains specific to each animal species since each species has different diets and digestive system conditions that select for distinct strains of bacteria. This distinction allows the original source of the fecal contamination to be identified.

Numerous BST methods are available. Based on a multi-year study initiated in 2002, Texas selected the two-method approach using ERIC-PCR and RiboPrinting, as this approach was found to be the most accurate and cost-effective. Because it provides a direct link with water quality standards, *E. coli* is used as the target bacteria. Using these methods required development of a DNA fingerprint library.

About the Texas BST Library

The Texas BST Library currently contains more than 1,500 *E. coli* isolates obtained from more than 1,300 different domestic sewage, wildlife, livestock and pet fecal samples. These isolates, which represent more than 50 animal subclasses, were selected after screening several thousand isolates from more than a dozen different studies throughout Texas.

Currently, Dr. George Di Giovanni at the University of Texas School of Public Health – El Paso Regional Campus and Dr. Terry Gentry at the Texas A&M University Soil and Aquatic Microbiology Laboratory oversee and maintain the Texas BST Library, continuously testing its accuracy and refining its composition.

BST accuracy is best when identifying broad source categories and decreases as the sources are more specifically identified. As such, 3-way (wildlife, human, domestic animals), 5-way (non-avian wildlife, avian wildlife, pets, livestock, human), and/or 7-way (non-avian wildlife, avian wildlife, pets, cattle, other non-avian livestock, avian livestock, human) categorizations are typically reported (Fig. 1).

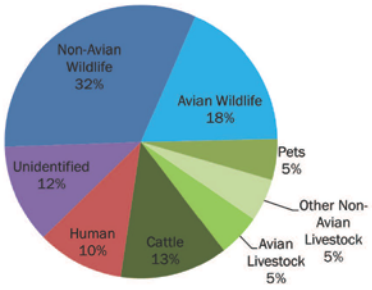

Figure 1. Average 7-way categorization based on 7 studies conducted in Texas

Moving Forward

The technologies used for BST are continuously evolving and improving. In addition to expansion and refinement of the Texas BST Library, library-independent molecular methods are being explored, as recommended by the Bacterial Total Maximum Daily Load Task Force. Library-independent tools provide an alternative for those waters impaired due to *Enterococcus* or fecal coliforms. They can also be successfully used in combination with library-dependent methods as demonstrated by the Buck Creek and Lake Granbury BST projects.

For future WPPs and TMDLs, a "toolbox" approach is recommended. The assessment phase should include targeted monitoring of suspected pollution sources, use of library-independent and -dependent methods to identify the presence of domestic sewage pollution and screening of water isolates from the new watershed against the existing library to determine the need for collecting local source samples and expanding the library.

BST has been tremendously helpful in identifying significant bacterial sources throughout Texas. The state BST laboratories can provide guidance and assistance with performing BST for watersheds. For more information, visit texasbst.tamu.edu.