Identification of a Poultry Litter Specific Biomarker and Development of a Quantitative Assay—Detailed Report

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ACRONYMS

ATCC	American Type Culture Collection
DNA	deoxyribonucleic acid
MDL	method detection limit
MRCF	Molecular Research Core Facility
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
UV	ultraviolet

Identification of a Poultry Litter Specific Biomarker and Development of a Quantitative Assay—Detailed Report

1 INTRODUCTION

This report details the methods and protocols used to identify a potential poultry litter specific biomarker, confirmation of the specificity of that biomarker for poultry litter (as compared to other fecal material), and the development of a quantitative polymerase chain reaction (qPCR) assay specific to detection of the poultry biomarker in various environmental media. Methods and results of the initial tests to identify potential poultry litter specific biomarkers are discussed in Section 2. Testing of the sensitivity of the potential biomarkers toward fecally contaminated litter and soil samples, and the specificity of the potential biomarkers against other fecal samples and various environmental media is presented in Section 3. The development and validation of an assay to quantify the poultry litter specific biomarker in various environmental media is discussed in Section 4. General methods are presented in Appendix A (e.g., PCR and qPCR methods, TRFLP methods) and methods specific to one experiment are included in the appropriate sections below.

2 IDENTIFICATION OF POTENTIAL POULTRY LITTER SPECIFIC BIOMARKER

The methods utilized to identify a potential poultry litter specific biomarker are discussed in this section. Specifically, the methods and results of microbial community profiling of poultry litter and soils to which the litter had been applied by terminal restriction fragment length polymorphism (T-RFLP) are discussed in Section 2.1. Cloning bacterial DNA from the litter and soil samples and sequencing of the plasmid deoxyribonucleic acid (DNA) containing the terminal restriction fragments (T-RFs) of interest from these clone libraries are presented in Section 2.2. Comparisons of potential biomarker DNA sequences, as compared to published sequences, and development of polymerase chain reaction (PCR) primers specific to these potential biomarkers are discussed in Section 2.3.

2.1 Litter and Soil Community Profiling

2.1.1 Objective

The purpose of the community profiling through the use of T-RFLP was to generate microbial community profiles based on the 16S rRNA gene of all bacteria, *Escherichia coli* strains, and *Bacteroides* spp. present in the poultry litter and the soils where poultry litter has been applied. The T-RFLP profiles were then used to identify common microorganisms present in both litter and soils on which poultry litter was spread.

2.1.2 Methods

Samples. DNA was extracted from two poultry litter samples (five replicates each) and two agricultural soil samples (five replicates each) to which the poultry litter was applied. The poultry litter samples from which DNA was extracted were FAC-01A-1 through 5 and FAC-01B-1 through 5, and the soils samples were LAL3-A-2-1 through 5 and LAL3-B-2-1 through 5. Genomic DNA was extracted from soil and litter samples with Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc). Details of the DNA extraction methodology are presented in Appendix A.

T-RFLP Analysis. T-RFLP was used to generate community profiles targeting *Bacteria* spp., *E. Coli*, and *Bacteroides* spp. Common T-RFs for each PCR primer pair (e.g., universal bacteria, *E.coli* or *Bacteroides spp.*), among all 20 poultry litter and soil replicates, were targeted as potential biomarkers of poultry litter. The general procedure followed for the PCR amplification of *Bacteria* spp., *E.coli*, and *Bacterioides* spp., as well as the TRFLP procedure, are presented in Appendix A.

2.1.3 Results

Common T-RFs observed in the soil and litter samples are presented in Table 1. No *Bacteroides* spp. fragments were identified as being present in both the soil and litter samples. T-RFs that were well-represented in all samples were selected for further development.

2.2 Cloning and Sequencing of Organisms of Interest

2.2.1 Objective

The purpose of the cloning and sequencing was to obtain DNA sequences corresponding to the T-RFs of interest found in both poultry litter and soil upon which poultry litter was applied as shown in Table 1.

2.2.2 Methods

Clone Libraries. Clone libraries were constructed from the original genomic DNA extracted from the soil and litter samples and amplified with either universal bacterial primers 8F-907R, targeting the 16S rRNA genes of Bacteria or the *E. coli* genus specific primers V1SF-V3AR. The TOPO ® Cloning Reaction methods from Invitrogen ™ were followed for clone library construction. Four universal clone libraries were constructed from pooled DNA samples (i.e., 1 μL of genomic DNA extract from each sample was added to the PCR reaction for inclusion into the clones). Four *E. coli* clone libraries were also constructed. Table 2 lists the DNA samples selected for clone libraries based on the abundance of the various potential biomarkers, as evidenced by the TRFLP profiles. Where two samples are indicated in Table 2, the DNA products were combined prior to PCR and cloning.

DNA Sequencing. Purified clone plasmids were subjected to PCR using primers T3 and T7, flanking the insertion site on the vector, to re-amplify the insert; PCR reagents and conditions were previously described. An initial sequencing reaction using 907R was performed on the purified PCR products and a Clustal W alignment (European Molecular Biology Laboratory – European Bioinformatics Institute) was performed. Sequences with greater than 97% similarity were grouped as single phylotype. Additional sequences from one representative of each unique phylotype were sequenced using primers T3, T7, 8F to obtain double coverage of the entire insert. General PCR protocols are presented in Appendix A. Sequencing was performed by Idaho State University, Molecular Research Core Facility (MRCF).

Plasmid DNA was excised from the clones using the QIAprep Spin Miniprep Kit, (Qiagen) and analyzed by T-RFLP (digested with *MspI*) to determine which clones contained the T-RFs of interest (see Table 1). Plasmids containing the T-RFs of interest were PCR amplified and sequenced using the primers T7, T3, 519R, and 338F (reference) for double coverage of the 16S rDNA gene.

Table 1. Common T-RFs found in replicate soil and litter samples.

T-RF[a]	Litter FAC-01A	# Replicates Analyzed	Litter FAC-01B	# Replicates Analyzed	Soil LAL3-A-2	# Replicates Analyzed	Soil LAL3-B-2	# Replicates Analyzed
E.coli* PCR products, digested with *MspI								
496.0	1,2,4,5[b,c]	4	1,2,3,5	5	1,2,4	5	Present in all five	5
498.9	Present in all four [d]	4	Present in all five	5	1,2,4,5	5	Present in all five	5
500.8	Present in all four	4	Present in all five	5	Present in all five	5	Present in all five	5
Universal bacteria PCR products, digested with *MspI*								
80.1	1,2,3,4	4	Present in all five	5	Not present in any sample	5	1,3,4	3
130.9	1,3,4	4	Present in all five	5	3	5	Not present in any sample	3
142.9	Present in all four	4	1,2,3,4	5	1,4	5	1,4	3
147.3	Present in all four	4	Present in all five	5	Present in all five	5	1,4	3
158.9	2,3,4	4	Present in all five	5	2,3,4,5	5	1,4	3
165.0	1,3,4	4	Present in all five	5	1,3,4,5	5	1,4	3
E. coli* PCR products, digested with *HaeIII								
210.7	2,4,5	4	1,3,4,5	4	Not present in any sample	3	2,3	5
227.9	2	4	3,4	4	4,5	3	1,2,4,5	5
Universal bacteria PCR products, digested with *HaeIII*								
62.0	Present in all five	5	Present in all five	5	Present in all five	5	Present in all three	3
229.4	Present in all five	5	Present in all five	5	1,2,3,4	5	Present in all three	3

a: T-RFs of potential biomarkers are indicated in bold typeface.
b: Number indicates the litter or soil replicate sample that the T-RF was identified in.
c: An underlined number indicates that the T-RF represented <1% of community in that replicate.
d: Indicates that this T-RF was detected in all subsamples analyzed within this sample.

Table 2. Samples selected for clone libraries.

Universal Clone Libraries	*E. coli* clone Libraries
FAC-01A-1 and FAC-01A-4	FAC-01A-4
FAC-01B-3 and FAC-01B-4	FAC-01B-4
LAL3-A-2-1 and LAL3-A-2-4	LAL3-A-2-1
LAL3-B-2-1	LAL3-B-2-1

2.2.3 Results

Three of the six T-RFs representing potential biomarkers were found in the universal clone library (i.e., T-RFs 142.9, 147.3, and 158.9). Sequences representing all three *E. coli* T-RFs were found in the *E. coli* clone library. After developing two clone libraries and screening an additional 88 clones with T-RFLP, the target biomarkers with T-RFs of 80.1 and 165 were not found, but the biomarker with T-RF 130.9 was identified. A total of 350 clones were screened. T-RFLP sequence analysis and DNA sequencing of each clone was performed by the MRCF.

2.3 Biomarker Sequence Analysis and PCR Primer Design

2.3.1 Objective

The purpose of the biomarker sequence analysis was to compare the potential biomarker DNA sequences to published 16S rRNA DNA sequences to determine whether these sequences had previously been reported, and whether they were identified as a particular species or associated with a particular host animal. Additionally, these sequences were used to design PCR primers specific to each biomarker.

2.3.2 Methods

Biomarker Sequence Analysis. The three universal and the three *E. coli* sequences corresponding to the T-RFs of interest were compared to the BLAST database (i.e., National Center for Biotechnology Information) to determine closely related organisms to potential biomarkers and sites amenable for the design of PCR primers. PCR primers were designed for the three biomarkers from the universal bacterial library and one of the *E. coli* biomarkers, targeting regions of variability between our sequence and the database sequences of the top 20 matches in the BLAST database. Two of the three *E. coli* sequences did not contain candidate PCR primer locations in that they did not contain variable regions of sequence between the candidate sequence and the most closely related organisms identified in the BLAST search. The clone containing the sequence associated with T-RF 130.9 was not carried forward for PCR primer design as the research group decided to focus on the previously designed PCR primers for the biomarker targets.

The 16S rRNA sequences for each of the clones containing the T-RFs of interest are presented in Figures 1 through 5. The results of the BLAST database searches for the top 10 matching cultured organisms against each clone sequence are presented in Table 3.

PCR Primer Design. PCR primers were designed using the Primer Express v2.0 software (Applied Biosystems). The primers were analyzed for thermodynamic folding problems and compared to the RDPII database (Michigan State University) to determine whether cross-amplification of DNA from other organisms might occur. The results of the analysis of the forward and reverse PCR primer comparison against the RDPII database are presented in the Table 4.

5'-GTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGT
GATCCCCTTTCGGGGGGTGATCGGTGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCCT
GGCTCTGGGACAACCACTGGAAACGGTGGCTAATACCGGATACGACTCACCTCGGCATCGA
GTGTGGGTGGAAAGTTTTTCGGCTGGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGT
AATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAA
GCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCTC
CGAAGAAGCGAAAGTGACGGTAGGAGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGC
GGTGTGTCGCGTCTGCTGTGAAAACTCAGGGCTCAACTCTGAGCTTGCAGTGGGTACGGGCA
CACTAGAGTGCTGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCA
GGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTTACTGACGCTGAGGAGCGAAAGC
ATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGT
GTGGGGTCCATTCCACGGATTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGT
ACGGCCGCAAGGCTAAAACTCAAA-3'

Figure 1. Clone SA19 (i.e., T-RF 158.9) 16S rRNA sequence. Underlined portions represent the location of the SA19F and SA19R PCR primer targets.

5'-TTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCGA
GACGAGGGAGCTTGCTCCCTCCGATCGAGTGGCGGACGGGTGAGTAACACGTAGGCAACCT
GCCCATCAGCCGGGGATAACTGCGGGAAACCGTGGCTAATACCGGATAAACCTTTCCTCCGC
GGGGAGGGGAGTTGAAAGACGGTTTCGGCTGTCGCTGATGGATGGGCCTGCGGCGCATTAG
CTGGTTGGTGGGGTAACGGCCCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC
CGCAATGGACGCAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTA
AAACTCTGTTGTTAAGGAGGAATGACAGGTAGAGTAACTGCTACCTGTGTGACGGTACTTAA
CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA
TCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTCTTTTAAGTCTGATGTGAAAGCCCCC
GGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGA
ATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGGCT
CTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGC
AGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAA-3'

Figure 2. Clone LA39 (i.e., T-RF 130.9) 16S rRNA sequence.

5'-AGTTTGATCCTGGCTCAGGACGAACGCTGGCTGCGTGCTTAACACATGCAAGTCGAACGC
TGAAGCCTGGGTGCTTGCATCTGGGTGGATGAGTGGCGAACGGGTGAGTAACACGTGAGTA
ACCTGCCCCTGATTTCGGGATAAGCCCGGGAAACTGGGTCTAATACCGGATACGACCATCTG
CCGCATGGCGGGTGGTGGAAAGTTTTTCGATTGGGGATGGGCTCGCGGCCTATCAGTTTGTT
GGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT
GGGGGAAACCCTGATGCAGCGACGCAGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGC
TTTCAGCAGGGAAGAAGCCTTCGGGTGACGGTACCTGCAGAAGAAGTACCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGTACGAGCGTTGTCCGGAATTATTGGGCGTAAAGA
GCTCGTAGGTGGTTGGTCACGTCTGCTGTGGAAACGCAACGCTTAACGTTGCGCGGGCAGTG
GGTACGGGCTGACTAGAGTGCAGTAGGGGAGTCTGGAATTCCTGGTGTAGCGGTGAAATGC
GCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGACTCTGGGCTGTGACTGACACTGGG
GAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTG
GGCACTAGGTGTGGGGGGCATTCCACGTTCTCCGCGCCGTAGCTAACGCATTAAGTGCCCCG
CCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAG-3'

Figure 3. Clone LA35 (i.e., T-RF 147.5) 16S rRNA sequence. Underlined portions represent the location of the LA35F and LA35R PCR primer targets.

5'-TAAAGGGACTAGTCCTGCAGGTTTAAACGAATTCGCCCTTAGAGTTTGATTCCTGGCTCA
GAGCGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGCCCCGCAAGGGGAGCGGCA
GACGGGTGAGTAACACGTGGGAACGTGCCTTTTGGTTCGGAACAACCCAGGGAAACTTGGG
CTAATACCGGATAAGCCCTAACGGGGAAAGATTTATCGCCAAAAGATCGGCCCGCGTCTGA
TTAGCTAGTTGGTGGGGTAATGGCCCACCAAGGCTACGATCAGTAGCTGGTCTGAGAGGATG
ATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTT
GTAAAGCTCTTTTAGCAGGGAAGATAATGACGGTACCTGCAGAAAAAGCCCCGGCTAACTT
CGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAA
GCGCACGTAGGCGGCTTCTTAAGTCAGGGGTGAAATCCTGGAGCTCAACTCCAGAACTGCCT
TTGATACTGAGGAGCTTGAGTCCGGGGGAGGTGAGTGGAACTGCGAGTGTAGAGGTGAAAT
TCGTAGATATTCGCAAGAACACCAGTGGCGAAGGCGGCTCACTGGCCCGGTACTGACGCTG
AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA
TGGATGCTAGCCGTTGGCGAGCTTGCTCGTCAGTGGCGCAGCTAACGCTTTAAGCATCCCGC
CTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGAAGGGCGAATTCGCGGC
CGCTAAATTCAATTCGCCCTAT-3'

Figure 4. Clone SB37 (i.e., T-RF 142.9) 16S rRNA sequence. Underlined portions represent the location of the SB37F and SB37R PCR primer targets.

5'-GGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTTAATTGAAGAGTTTGATCATGGCTC
AGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGGACGGTAGCACAGAGGAGCTTG
CTCCTTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGGATCTGCCCGATAGAGGGG
GATAACCACTGGAAACGGTGGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCT
TCGGGCCTCTCACTATCGGATGAACCCAGATGGGATTAGCTAGTAGGCGGGGTAATGGCCCA
CCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC
AGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAG
GCGATGTGGTTAATAACCGCATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCG
TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGTATTT
GAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGC
GTAGAGAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTT-3'

Figure 5. Clone SA15 (i.e., T-RF 500.8) 16S rRNA sequence. Underlined portions represent the location of the SA15F and SA15R PCR primer targets.

Table 3. Top 10 closest related cultured organisms as identified in a BLAST search, identity and % similarity to clones containing the T-RFs of interest.

Clone/ T-RF	Top 10 Closest Related Cultured Organisms [Accession number]	Identities	% Similarity
SA19/1 58.9	*Kineococcus radiotolerans* SRS30216 [CP000750]	871/881	98
	Kineococcus-like bacterium AS3630 [AF060693]	870/881	98
	Kineococcus-like bacterium AS3382 [AF060692]	870/881	98
	Kineococcus-like str. SRS30216 [AF247813]	869/881	98
	Kineococcus-like bacterium AS3635 [AF060694]	869/881	98
	Kineosporia sp. CC-YMW-2 [EU008824]	869/883	98
	Kineococcus-like bacterium AS3381 [AF060691]	865/882	98
	K.aurantiacus (IFO 15268)[X77958]	841/853	98
	Kineococcus-like bacterium AS2960 [AF060673]	860/885	97
	Kineococcus-like bacterium AS3041 [AF060680]	857/882	97
LA39/ 130.9	*Salinicoccus sp.* W24 [DQ989633]	840/873	96
	Salinicoccus sp. RM11R [EF675622]	865/918	94
	Salinicoccus roseus 16S [X94559]	864/918	94
	Salinicoccus hispanicus [AY028927]	848/901	94
	Salinicoccus jeotgali strain S2R53-5 [DQ471329]	852/907	93
	Salinicoccus roseus strain 59 [DQ093356]	860/918	93
	Salinicoccus roseus strain Iii10 [DQ093353]	862/921	93
	Salinicoccus kunmingensis strain YIM Y15 [DQ837380]	836/887	94
	Salinicoccus roseus [AF237976]	846/902	93
	Salinicoccus sp. Y22 [EF177689]	817/865	94
LA35/ 147.5	*Brevibacterium* sp. BH [AY577816]	868/892	97
	Brevibacterium sp. CHNDP32 [DQ337537]	868/892	97
	Brevibacterium sp. H15 [AB177640]	868/894	97
	Brevibacterium sp. CNJ737 PL04 [DQ448693]	868/893	97
	Brevibacterium marinum [AM421807]	870/897	96
	Brevibacterium sp. BBH7 [AM158906]	863/887	97
	Brevibacterium sp. J3 [EU099374]	867/894	96
	Brevibacterium casei strain 3Tg [AY468375]	867/894	96
	Brevibacterium casei strain FM1A [AY468365]	868/895	96
	Brevibacterium antiquum strain VKM Ac-2118 [AY243344]	870/898	96
SB37/ 142.9	*Rhodoplanes elegans* [D25311]	810/843	96
	Rhodoplanes sp. TUT3523 [AB250620]	825/865	95
	Rhodoplanes sp. TUT3521 [AB250619]	825/865	95
	Rhodopseudomonas cryptolactis [AB087718]	825/865	95
	Rhodoplanes elegans strain OK5R2b [AF487437]	803/836	96
	Rhodoplanes sp. Wai3R3 [AF077730]	795/829	95
	Rhodoplanes sp. HA17 [AB087717]	822/868	94
	Rhodopseudomonas sp. IL-245 [D15063]	821/871	94
	Bradyrhizobiaceae bacterium KVD-1969-08 [DQ490361]	819/867	94
	Bradyrhizobiaceae bacterium KVD-1921-10 [DQ490360]	819/867	94
SA15/5 00.8	*Pantoea* sp. Ina5 [AM909657]	694/699	99
	Pantoea ananatis strain BD 543 [DQ133545]	694/699	99
	Pantoea ananatis strain Pna 97-1 [DQ777968]	693/699	99
	Pantoea ananatis strain BD 588 [DQ133548]	693/699	99
	Pantoea ananatis strain BD 561 [DQ133546]	693/699	99
	Pantoea ananatis strain JA04 [DQ365569]	683/684	99
	Pantoea agglomerans strain XW123 [AY941838]	693/699	99
	Pantoea ananatis strain BD 315 [AY579212]	692/699	98
	Pantoea agglomerans strain TMPSB-P1 [EU047555]	693/700	99
	Pantoea ananatis strain PD 301 [AY579209]	692/699	98

Table 4. Primer design for potential biomarkers identified during T-RFLP/clone library assessment.

Potential Biomarker Clone Number/ T-RF/Organism[a]	Forward Primer Accession Number of Closest Match in Sequence Database RPDII	Reverse Primer Accession Number of Closest Match in Sequence Database RPDII
Clone SA19 T-RF 158.9 *Kineococcus*	AY919955, AY426452, AF195447, AF513961, AY862810, AM085954, AM182287, AM182297, AM182298, AM182299	Primer sequence did not match any organisms in the database
Clone SB37 T-RF 142.9 *Rhodoplanes*	Primer sequence did not match any organisms in the database	Primer sequence did not match any organisms in the database
Clone LA35 T-RF 147.3 *Brevibacterium* sp.	Primer sequence did not match any organisms in the database	Primer sequence did not match any organisms in the database
Clone SA15 T-RF 500.8 *Pantoea ananatis*	Primer sequence did not match any organisms in the database	AJ010486, DQ221344, AF364845, AF364844, AY528223, AY579209, AY579211, U80196, U80209, AB004758, AB027693, AY530796, AJ629190, AB178169, AB178170, AY898643, AB114622, DQ133548, DQ195524, AB242937, AB242945, AB242946, AB242979 And an additional 30 sequences of *Enterobacter* spp.

a. Organisms were identified in a BLAST search

2.3.3 Results

Only one of the three potential *E. coli* biomarkers had a variable region among the top 20 matches in the BLAST database suitable for design of a PCR primer pair. PCR primers were designed for this variable region and another region that had a mismatch between our sequence and the database sequence in 10 of the top 20 matching sequences. A primer pair was designed that was determined to be specific for T-RF of interest 147.5, a *Brevibacterium* sp., and would have no matches compared to the sequences in the RDPII database. Another primer pair was designed for a *Kineococcus* spp. corresponding to T-RF 158.9, where the forward primer matched the sequences of 10 other organisms in the RDPII database, but the reverse primer was specific to this *Kineococcus* spp. A primer pair was also designed for the *Rhodoplanes* spp. in the BLAST or RDPII databases corresponding to T-RF 142.9.

3 TESTING THE SENSITIVITY AND SPECIFICITY OF THE POTENTIAL POULTRY BIOMARKER TARGETS

The methods utilized to test the sensitivity and specificity of the potential poultry litter specific biomarkers are discussed in this section. Specifically, the methods and results for the testing the PCR primers in the original soil and litter samples used for the T-RFLP are presented in Section 3.1, while the testing of the PCR primers for the biomarkers against closely related organisms in the BLAST database search are presented in Section 3.2. Testing of the PCR primers for the various potential biomarkers against other fecal material is presented in Section 3.3. Cloning and sequencing of PCR amplicons derived from fecal samples that amplified with the *Brevibacterium* sp. specific primers and comparison to the biomarker sequence is presented in Section 3.4 Finally, testing of the PCR primers of the confirmed poultry litter specific biomarker in environmental samples from the potentially poultry litter impacted watershed are presented in Section 3.5.

3.1 Test PCR Primers against Original Soil and Litter Samples

3.1.1 Objective

The objective of this test was to determine if the PCR primers specific for the various biomarkers amplified DNA from the original soil and poultry litter samples used to find the T-RFs of interest (potential biomarker sequences).

3.1.2 Methods

A nested PCR approach was used to increase sensitivity of the PCR assay by first amplifying the DNA with the universal bacterial primers 8F-907R or *E. coli* species-specific primers, purifying the PCR products (QIAquick PCR purification kits, QIAGEN or Sephadex size exclusion chromatography), and then amplifying the DNA with the biomarker-specific primers. PCR amplification was performed as described in Appendix A. PCR products analyzed by gel electrophoresis.

3.1.3 Results

The results of the nested PCR with the potential biomarker-specific PCR primers of DNA from the original litter and soil samples are presented in Table 5. In general, it was found that the *Brevibacterium* sp. clone LA35 was amplifiable 100% of the litter samples and in 80% of the soil samples. The *Rhodoplanes* clone SB37 was amplifiable in 83% of the litter samples and in 100% of the soil samples. The *Kineococcus* clone SA19 was amplifiable in 30% of the litter samples and 67% of the soil samples. The *E. coli* clone SA15 was amplifiable in 60% of the litter samples and 90% of the soil samples. These results suggest that the LA35 and SB37 targets may be the most appropriate to continue studying as potential biomarkers.

Table 5. Test of the biomarker-specific PCR analysis on original litter and soil replicates.

	Clone LA35 *Brevibacterium* sp.		Clone SB37 *Rhodoplanes* spp.		Clone SA19 *Kineococcus* spp.		Clone SA15 *E. coli*	
Sample	**% of T-RF Profile**	**Amplified with LA35 Primers?**	**% of T-RF Profile**	**Amplified with SB37 Primers?**	**% of T-RF Profile**	**Amplified with SA19 Primers?**	**% of T-RF Profile**	**Amplified with SA15 Primers?**
Litter Sample Results								
FAC-01-A-1	2.6	yes	2.8	yes	no data[a]	not run	26.4	yes
FAC-01-A-2	2.6	yes	4.6	no	0.8	yes	13	no
FAC-01-A-3	3.4	yes	5.4	yes	1	not run[c]	no data[a]	yes
FAC-01-A-4	3.3	yes	5.9	yes	1.3	not run[c]	23.3	no
FAC-01-A-5	no data[a]	yes	no data[a]	not run[c]	no data [a]	not run[c]	28.5	yes
FAC-01-B-1	3.3	yes	3.6	not run[c]	1.5	not run[c]	33	yes
FAC-01-B-2	4	yes	5.5	yes	1.1	no	43.8	yes
FAC-01-B-3	3.5	yes	6.8	yes	1.3	yes	16.5	yes
FAC-01-B-4	3.2	yes	5.7	not run[c]	1.2	not run[c]	29.1	no
FAC-01-B-5	4.5	yes	8	not run[c]	1.1	not run[c]	48.5	no
Soil Sample Results								
LAL3-A-2-1	7.1	yes	0.8	yes	2.3	not run[c]	6.7	yes
LAL3-A-2-2	12.7	no	not present[b]	yes	3.4	not run[c]	14.3	no
LAL3-A-2-3	9	yes	1	yes	3.6	not run[c]	25.3	yes
LAL3-A-2-4	6.9	yes	0.8	yes	3.2	yes	10.4	yes
LAL3-A-2-5	9.5	no	0.9	yes	3.6	not run[c]	2.5	yes
LAL3-B-2-1	6	yes	0.8	yes	3	yes	18.1	yes
LAL3-B-2-2	no data[a]	yes	no data[a]	yes	no data[a]	yes	6.8	yes
LAL3-B-2-3	not present[b]	yes	not present[b]	yes	3	no	9.1	yes
LAL3-B-2-4	6.3	yes	0.8	yes	3.5	no	2.6	yes
LAL3-B-2-5	no data[a]	yes	no data[a]	yes	no data[a]	yes	7.1	yes

a *No data* indicates that the T-RFLP analysis was not completed on this sample.
b *Not present* indicates that the organism represented by that T-RF was not found in the original analysis (see Section 2.1).
c *Not run* indicates that this sample was not run with PCR.

3.2 Test PCR Primer Set LA35 against a Closely Related Bacterium

3.2.1 Objective

The objective of this test was to determine if the PCR primers for the *Brevibacterium* sp. LA35 potential poultry litter biomarker amplified the same product in *Brevibacterium* sp. CHNDP32 [DQ337537], the second closest related cultured organism and fourth closest overall organism found in the BLAST search.

3.2.2 Methods

16S rDNA of the closely related organism identified in the BLAST search was obtained from Dr. Chee-Sanford from the University of Illinois at Urbana-Champaign. The *Brevibacterium* sp. CHNDP32 was PCR amplified using the LA35 primers, and PCR products were analyzed by gel electrophoresis described in Appendix A.

3.2.3 Results

The *Brevibacterium* sp. biomarkers primers did not amplify the 560 bp potential poultry litter specific *Brevibacterium* sp. PCR product in the *Brevibacterium* sp. CHNDP32.

3.3 Test PCR Primers against Other Fecal Samples from within and outside the Watershed

3.3.1 Objective

The purpose of this test was to determine the specificity of the four potential poultry biomarker targets (i.e., LA35, SB37, SA19 and SA15) against other sources of fecal contamination from within and outside the potentially poultry impacted watershed.

3.3.2 Methods

Fecal samples were collected in duplicate from beef and dairy cattle, swine, geese, ducks, and humans from inside and outside the potentially poultry impacted watershed. Field blank controls were included with each type of fecal sample. The fecal samples were preserved in glycerol and shipped on ice to the laboratory. Genomic DNA extracted from all fecal samples similar to the soil extraction protocol described in Appendix A. Samples were tested for PCR amplification with the four potential biomarkers, and products were analyzed by gel electrophoresis. A nested PCR approach was taken when the fecal samples were first amplified with 8F-907R, cleaned with a 96 well Sephadex cleanup and then taken into the biomarker specific PCR amplification.

3.3.3 Results

The results of the PCR amplification of the fecal samples with the four potential poultry litter biomarkers are presented in Table 6. The *Brevibacterium* sp. clone LA35 was found to amplify only one duck and one goose sample from outside the watershed among the 31 fecal samples tested. The *Brevibacterium* sp. clone LA35 PCR primers did not amplify product in the beef or dairy cattle, swine, or human fecal samples. The other three potential biomarkers exhibited amplification to varying degrees in all the fecal samples tested. Given the abundance of *Brevibacterium* sp. clone LA35 in litter and soil samples and its lack of presence in other fecal samples, this biomarker has been shown to be specific to poultry litter.

Table 6. Results of the PCR amplification of the fecal samples with the four potential poultry litter biomarkers.

Sample	Type of Fecal Sample	Inside or Outside the Watershed	City	Did the Potential Biomarker Sequence PCR Amplify in the Fecal Sample?			
				Clone LA35 *Brevibacterium* sp.	Clone SB37 *Rhodoplanes*	Clone SA19 *Kineococcus*	Clone SA15 *E. coli*
MAN-BC-1-a	Beef Cattle	Outside		No	No	No	No
MAN-BC-1-b		Outside		No	No	No	No
MAN-BC-2-a		Outside		No	Yes	No	No
MAN-BC-2-b		Outside		No	Yes	No	No
MAN-BC-3-a		Outside		No	No	No	No
MAN-BC-3-b		Outside		No	No	No	No
MAN-BC-4-a		Outside		No	No	No	No
MAN-BC-4-b		Outside		No	No	No	No
MAN-BC-5-a		Outside		No	Yes	Yes	No
MAN-BC-5-b		Outside		No	No	No	No
MAN-BC-6-a		Inside		No	Yes, faint[b]	No	No
MAN-BC-6-b		Inside		No	No	No	No
MAN-BC-7-a		Inside		No	Yes	Yes, faint[b]	Yes
MAN-BC-7-b		Inside		No[a]	Yes	Yes	Yes
MAN-BC-8-a		Inside		No	No	Yes	No
MAN-BC-8-b		Inside		No	No	Yes, faint[b]	No
MAN-BC-9-a		Inside		No	No	No	No
MAN-BC-9-b		Inside		No	No	Yes, faint[b]	No
MAN-BC-10-a		Inside		No	Yes, faint[b]	Yes, faint[b]	No
MAN-BC-10-b		Inside		No	Yes	Yes	No
MAN-BC-F-a	Field Blank	Outside		No	Yes	No	No
MAN-DC-1	Dairy Cattle	Outside		No	Yes	Yes, faint[b]	Yes
MAN-DC-2-a		Outside		No	No	No	No
MAN-DC-2-b		Outside		No	No	No	No
MAN-DC-3		Inside		No	Yes	No	No
MAN-DC-3-b		Inside		No	Yes	No	No
MAN-DC-F	Field Blank	Outside		No	No	No	No
MAN-SW-1-a	Swine	Outside		No	Yes	Yes	No
MAN-SW-1-b		Outside		No	Yes	No	No
MAN-SW-2		Inside		No	No	No	No

Table 6. (continued).

Sample	Type of Fecal Sample	Inside or Outside the Watershed	City	Did the Potential Biomarker Sequence PCR Amplify in the Fecal Sample?			
				Clone LA35 *Brevibacterium* sp.	Clone SB37 Unknown Organism	Clone SA19 *Kineococcus*	Clone SA15 *E. coli*
MAN-DK-1-a	Duck	Outside		No	Yes	Yes	Yes
MAN-DK-1-b		Outside		Yes[a]	Yes	Yes	Yes
MAN-DK-2-a		Outside		No	Yes	Yes	Yes
MAN-DK-2-b		Outside		No	Yes	Yes	Yes
MAN-DK-3-a		Inside		No	Yes	Yes	Yes
MAN-DK-3-b		Inside		No	No	Yes	Yes
MAN-DK-4-a		Inside		No	No	No	No
MAN-DK-4-b		Inside		No	No	No	No
MAN-DK-5-a		Inside		No	Yes	No	No
MAN-DK-5-b		Inside		No	No	No	Yes
MAN-DK-F	Field Blank	Inside		No	No	No	No
MAN-GS-1-a	Goose	Outside		Yes, faint[a, b]	Yes	Yes	Yes
MAN-GS-1-b		Outside		No	Yes	Yes, faint[b]	No
MAN-GS-2-a		Outside		No	Yes	No	No
MAN-GS-2-b		Outside		No	No	No	No
MAN-GS-3-a		Inside		No	No	No	No
MAN-GS-3-b		Inside		No	Yes	Yes	Yes
MAN-GS-4-a		Inside		No	Yes	No	No
MAN-GS-4-b		Inside		No	Yes	No	No
MAN-GS-5-a		Outside		No	Yes	Yes	Yes
MAN-GS-5-b		Outside		No	Yes	Yes	Yes
MAN-HM-1	Waste Water Treatment Plant	Outside	Claremore	No	Yes	Yes	Yes
MAN-HM-2		Inside	Siloam Springs	No	Yes	Yes	Yes
MAN-HM-3		Inside	Fayetteville	No	Yes	No	No
MAN-HM-4	Septic System	Outside	Tulsa	No	Yes	Yes	No
MAN-HM-5		Inside	Fayetteville	No	Yes	No	No
MAN-HM-6		Inside	Siloam Springs	No	No	No	No

a: Samples were re-extracted and re-run in duplicate to confirm result.
b: Faint, indicates a very weak band was observed on the gel.

3.4 Sequencing of the Duck and Goose Amplicon Derived from the LA35 Primer Set

3.4.1 Objective

The purpose of the cloning and sequencing of the duck and goose amplicon derived from the LA35 primer set was to determine if these DNA sequences contained variable regions that could be targeted for a more specific set of PCR primers. In addition the sequences from various litter and soil samples were examined for comparison to the *Brevibacterium* sp. clone LA35 sequence.

3.4.2 Method

Several clone libraries were constructed from the duck sample MAN-DK-1-b and the goose sample MAN-GS-1-a by PCR amplification of DNA from these fecal samples with the LA35 primer set, with primers 8F-907R and with 8F-1492R, using the cloning method presented in Appendix A and Section 2.2.2. Extracted plasmids were DNA sequenced using the PCR primers T7, T3, 8F, 907R, or 1492R (as appropriate) by the MRCF. Sequences were compared to the poultry litter *Brevibacterium* sp. biomarker using BioEdit, as previously described, to look for variable regions between the DNA sequences. Litter and soils samples were amplified with only the LA35 primer set.

3.4.3 Results

The regions of the16S rRNA gene of the duck and goose DNA targeted by the poultry litter specific *Brevibacterium* sp. biomarker PCR primers are identical to our biomarker *Brevibacterium* sp. sequence.

After screening 20 clones from each of the two clone libraries developed (a total of 40 clones) from the duck and the goose fecal samples, only one organism was identified as a *Brevibacterium* sp. using the BLAST database. This sequence was identical to the *Brevibacterium* sp. biomarker sequence obtained from the original soil and litter samples within the 1,200 DNA base pairs sequenced. This data suggests that the *Brevibacterium* sp. clone LA35 is not very prevalent in the goose and duck fecal samples.

```
LA35       428 TGTAAACCGCTTTCAGCAGGGAAGAAGCCTTCGGGTGACGGTACCTGCAGAAGAAGTACC 498
MAN-DK-1-a     ************************************************************
MAN-GS-1-a     ************************************************************
FAC2           ******************************G*AA****************************
FAC3           ******************************G*AA****************************
FAC4           ******************************G*AA****************************
FAC5           ******************************G*AA****************************
LA1            ******************************G*AA****************************
LA2            ******************************G*AA****************************
LA3            ******************************G*AA****************************
LA4            ******************************G*AA****************************
LA5            ******************************G*AA****************************
LB1            ******************************G*AA****************************
LB2            ******************************G*AA****************************
LB4            ******************************G*AA****************************
LB5            ******************************G*AA****************************
SB1            ************************************************************
SB4            ************************************************************
SB5            ************************************************************
SA1            ******************************K*MR****************************
```

Figure 6. *Brevibacterium* sp. sequences from soil, from litter and from fecal samples compared to the biomarker. The numbers indicate the position of the DNA based on E. coli numbering on the 16S rRNA gene. Stars indicate no difference in the bases pairs between the *Brevibacterium* sp. clone LA35 sequence and the target sequence.

3.5 Test for the Poultry Litter Specific Biomarker in Environmental Samples from within the Impacted Watershed

3.5.1 Objective

The purpose of this test was to determine if the *Brevibacterium* sp. biomarker that is specific to poultry litter could be detected in environmental media (poultry litter, soil and water samples) from within a potentially poultry litter impacted watershed.

3.5.2 Methods

Environmental samples were collected from within the potentially impacted watershed and included chicken and turkey litter samples, soil samples from which the litter had been applied, edge of field runoff water samples from the fields to which the litter had been applied, river water to which the runoff samples drained, and lakes down gradient from the previously collected river samples. Additionally, groundwater samples within the potentially impacted watershed were collected and analyzed. Water samples were collected in duplicate 1-L sterile nalgene bottles and shipped on ice to the laboratory, where they were immediately filtered. The filters were frozen at -80°C until DNA extraction. Genomic DNA was extracted as described in Appendix A.

Samples were tested for nested PCR amplification with the universal PCR primers and then the LA35 PCR primers and the products were analyzed by gel electrophoresis as described in Appendix A.

3.5.3 Results

The results of the PCR amplification of the environmental samples for the poultry litter biomarker are presented in Table 7.

Table 7. Results of the PCR amplification of the environmental samples for the poultry litter biomarker.

Sample	Type of Sample	Amplified with LA35 PCR Primers?
FAC1-6-20-06	Litter	Yes
FAC2-6-21-06	Litter	Yes
FAC-03-7-6-06	Litter	Yes
FAC-4-7-12-06	Litter	Yes
FAC-5-7-13-06	Litter	Yes
FAC-6-7-20-06	Litter	No [a]
FAC-7-8-3-06	Litter	Yes
FAC-8-8-15-06	Litter	Yes
FAC-9-8-31-06	Litter	Yes
LAL5-A-2-6-13-06	Soil	No [c]
LAL5-C-2-6-12-06	Soil	Yes
LAL-7-A-2-6-20-06	Soil	Yes
LAL-7-B-2-6-20-06	Soil	Yes, faint[b]

Table 7. (continued).

Sample	Type of Sample	Amplified with LA35 PCR Primers?
LAL-7-C-2-6-19-06	Soil	Yes, faint[b]
LAL10-B-2-6-26-06	Soil	No
LAL10-A-2-6-26-06	Soil	No
LAL10-A-4-6-26-06	Soil	No
LAL8-A-2-6-19-06	Soil	Yes
LAL8-B-2-6-21-06	Soil	No
LAL9-D-2-6-22-06	Soil	No [c]
LAL9-B-2-6-22-06	Soil	Yes
LAL9-A-2-6-22-06	Soil	Yes
LAL7-D-2-6-29-06	Soil	Yes
LAL8-D-2-6-20-06	Soil	Yes
LAL11-C-2-6-28-06	Soil	No [c]
LAL11-D-2-6-28-06	Soil	No
LAL11-A-2-6-29-06	Soil	Yes
LAL11-D-2-Q-6-28-06	Soil	Yes
LAL12-A-2-7-6-06	Soil	No
LAL12-A-2-Q-7-6-06	Soil	Yes
LAL12-C-2-7-7-06	Soil	No
LAL12-D-2-7-7-06	Soil	No [c]
LAL 13-A-2-7-6-06	Soil	Yes
LAL 13-C-2-7-7-06	Soil	No
LAL 13-C-2-Q-7-7-06	Soil	Yes
LAL 13-D-2-7-6-06	Soil	No
EOF-1-6-17-06	Water	No [c]
EOF-Q2-6-17-06	Water	No
EOF-Q1-6-17-06	Water	Yes
EOF-SPREAD073B-6-18-06	Water	Yes
EOF-SPREAD023-6-18-06	Water	Yes
EOF-SPREAD044-6-18-06	Water	No
EOF-SPREAD068-6-18-06	Water	Yes

a: This sample contained a high percentage of soils and very little "litter."
b: Faint indicates that a weak band was visible on the gel.
c: This sample in later experiments tested positive for the biomarker in a qPCR or nested qPCR analysis.

4 OPTIMIZATION AND VALIDATION OF A QUANTITIVE ASSAY FOR THE POULTRY LITTER SPECIFIC *BREVIBACTERIUM* SP. BIOMARKER

4.1 Develop a Quantitative PCR Assay for the *Brevibacterium* sp. LA35 Biomarker

4.1.1 Three short qPCR primer sets for variable regions in the *Brevibacterium* sp. LA35 sequence

4.1.1.1 Objective

The objective of this study was to develop three qPCR primer sets suitable for SYBR Green qPCR assay for quantification of the *Brevibacterium* sp. biomarker in environmental samples.

4.1.1.2 Methods

qPCR Primer Design. Forward qPCR primers were designed using the Primer Express v2.0 software (Applied Biosystems). The primers were analyzed for thermodynamic folding problems and compared to the RDPII database (Michigan State University) and NCBI BLAST database to determine what other organisms they might amplify. The location of the primers on the *Brevibacterium* sp. clone LA35 16S rRNA gene is presented in Figure 7.

5'-AGTTTGATCCTGGCTCAGGACGAACGCTGGCTGCGTGCTTAACACATGCAAGTCGAACGC
TGAAGCCTGGGTGCTTGCATCTGGGTGGATGAGTGGCGAACGGGTGAGTAACACGTGAGTA
ACCTGCCCCTGATTTCGGGATAAGCCCGGGAAACTGGGTCTAATACCGGATACGACCATCTG
CCGCATGGCGGGTGGTGGAAAGTTTTTCGATTGGGGATGGGCTCGCGGCCTATCAGTTTGTT
GGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT
GGGGGAAACCCTGATGCAGCGACGCAGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGC
TTTCAGCAGGGAAGAAGCCTTTCGGGTGACGGTACCTGCAGAAGAAGTACCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGTACGAGCGTTGTCCGGAATTATTGGGCGTAAAGA
GCTCGTAGGTGGTTGGTCACGTCTGCTGTGGAAACGCAACGCTTAACGTTGCGCGGGCAGTG
GGTACGGGCTGACTAGAGTGCAGTAGGGGAGTCTGGAATTCCTGGTGTAGCGGTGAAATGC
GCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGACTCTGGGCTGTGACTGACACTGGG
GAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTG
GGCACTAGGTGTGGGGGGCATTCCACGTTCTCCGCGCCGTAGCTAACGCATTAAGTGCCCCG
CCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAG-3'

Figure 7. *Brevibacterium* sp. clone LA35 16S rRNA sequence. Underlined portions represent the location of the qPCR primer targets (i.e., 422F, 394F, 366F, LA35R). The bold and boxed basepairs represent the variable sequence region compared to other soil and litter samples presented previously.

Standard Curve Development. The qPCR primers specific to the *Brevibacterium* sp. clone LA35 were used to develop a standard curve for each primer set. Biomarker DNA was cloned into a plasmid and was used as the source of the quantitative standards used in the analysis. Plasmid DNA containing the target 16S rRNA gene from the poultry-specific *Brevibacterium* sp. biomarker was purified and quantified fluorometrically. Based on the known size of the plasmid and insert, DNA concentrations were converted to insert copy numbers. A dilution series spanning seven orders of magnitude was generated using known concentrations of each plasmid. Amplification and detection of the DNA was performed using the Bio-Rad Chromo-4 System. The acceptance criterion for the standard curve is a linear R^2 value of greater than 0.995.

qPCR Primer Tests Against Environmental Samples and Fecal Samples. The three qPCR primer sets were tested for amplification in environmental samples and in fecal samples using the methods presented in Appendix A.

4.1.1.3 Results

The standard curves developed for each set of qPCR primer pairs is presented in Figure 8. In general, the efficiency of the reactions was greater than 90% and the R^2 value of the standard curves was acceptable. However, the detection limit was fairly high (i.e., around 300 plasmid copies/µL DNA extraction).

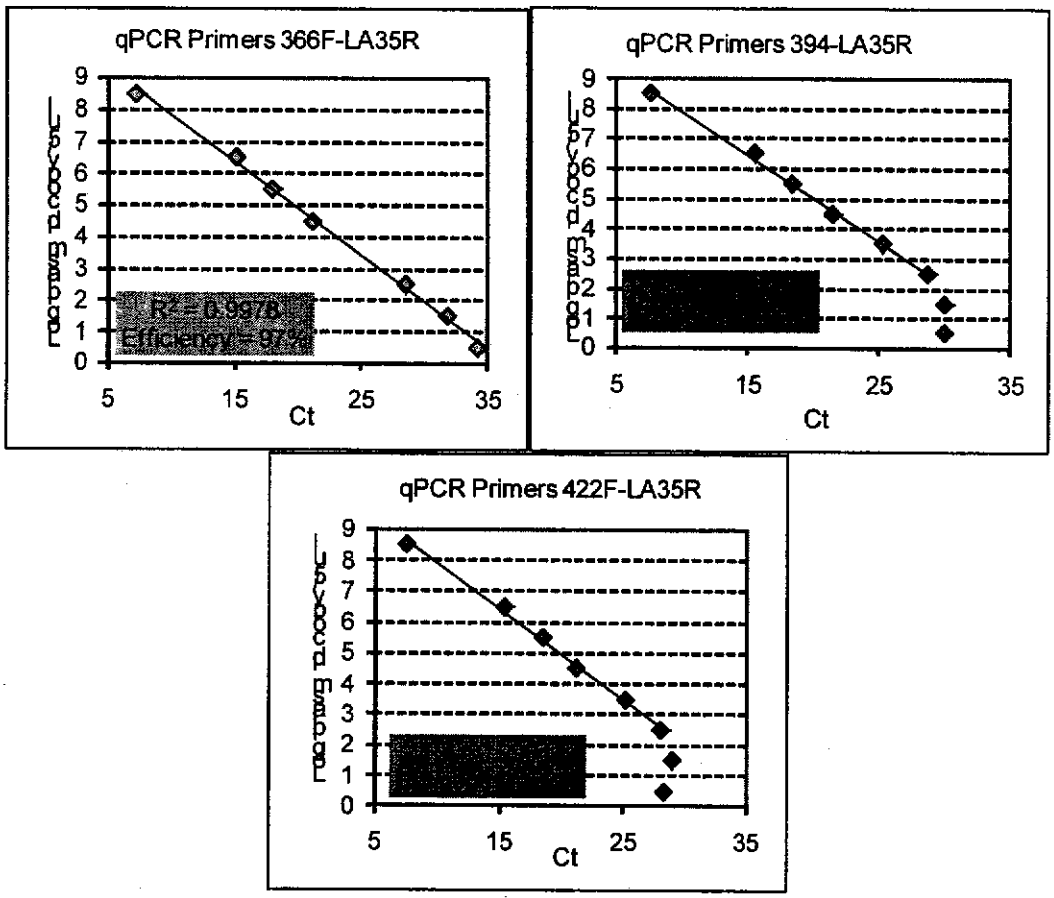

Figure 8. Quantitative PCR standard curves for the short amplicon qPCR primers designed for the *Brevibacterium* sp. clone LA35 biomarker.

After extensive testing, these primers were not carried forward for full testing in environmental samples for one or all of the following reasons:

- Forward primers 366F and 394F demonstrated non-specific amplification in environmental samples as evidenced by multiple peaks present in SYBR green melt curves,
- Limited to no amplification of qPCR products were observed in some samples that had previously tested positive with the LA35F and LA35R primers, this may be due to secondary structure effects of the 16S rRNA limiting access of the short primers to the annealing sites,

- Forward primer 422F was the most robust of the three primer sets in terms of reproducibility of amplification in environmental samples, however when tested against fecal samples, this primer set showed amplification of products from several fecal samples by nested PCR.

4.1.2 Optimization of the LA35 long qPCR products

4.1.2.1 Objective

The purpose of this study was to determine if the original PCR primers LA35F and LA35R could work as a SYBR Green qPCR primer set. If amplification was observed, further studies were designed to determine the standard curve and the detection limit for the extraction and quantification protocol.

4.1.2.2 Method

qPCR Assay Conditions. LA35 PCR primers developed in Section 2 and validated for specificity to poultry litter in Section 3 were applied as qPCR primers using SYBR green chemistry. Plasmids containing the LA35 DNA sequence were used to create a standard curve, to determine a method detection limit (MDL) for positive control plasmids, and to determine the efficiency of the reaction. The qPCR methods used for this analysis are presented in Appendix A.

Standard Curve Development. The standard curves for the LA35 qPCR primers were developed similar that presented in Section 4.1.1.2.

Method Detection Limits Experiment. Detection limits of the assay in environmental samples were determined by spiking soil and water samples with LA35 contained in *E. coli* clone plasmids. Controls were run to determine the average number of plasmids present in the *E. coli* cells used as plasmid carriers. Additionally, nanopure water and a composite water sample from the watershed were spiked with the *E. coli* containing the biomarker sequence and were filtered according to the standard filtering methods. Cloning was performed as previously described in Section 2.2.2; however, PCR products amplified with the LA35 specific primers were cloned into the *E. coli*. Genomic DNA was extracted from the spiked samples using the Bio 101 FastDNA SPIN Kit for Soil, DNA extraction kit (Qbiogene, Inc). A graphic depicting the MDL protocol is presented in Figure 9.

4.1.2.3 Results

The SYBR green qPCR standard curve is presented in Figure 10. Additionally, the plasmid MDL and reaction efficiency are presented in the figure. Efficiency of the qPCR reaction was determined by equation 1.

$$\text{Efficiency} = -1 + 10 \wedge (-1/\text{slope}) \qquad (1)$$

To improve the SYBR green melt curves, DMSO was added to the qPCR mastermix to allow for more accurate determination of the non-specific amplification of the biomarker. The effect of DMSO on the melt curves is presented in Figure 11.

The results of the minimum detection limit test in spiked environmental samples are presented in Table 8.

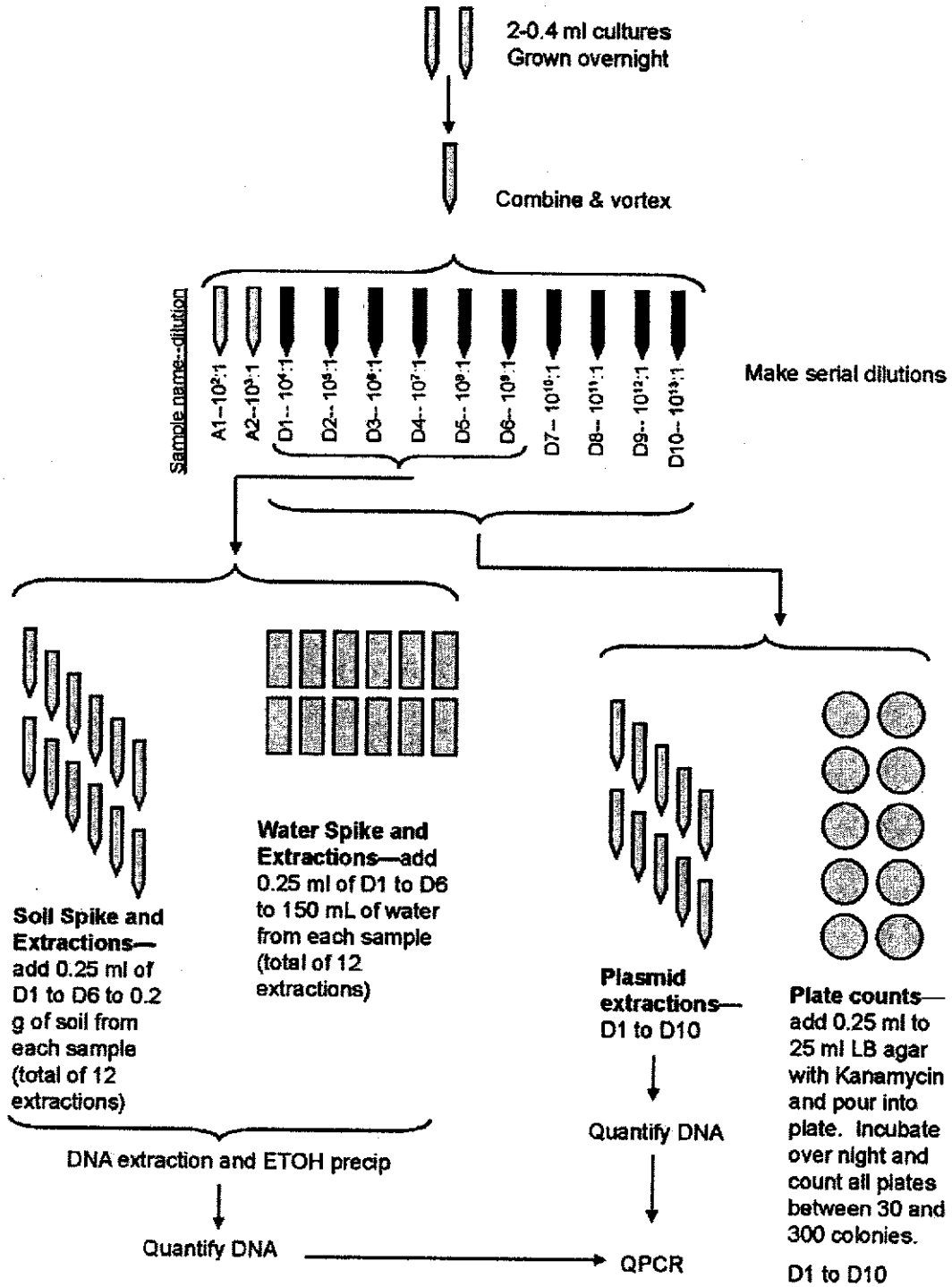

Figure 9. Outline of MDL protocol.

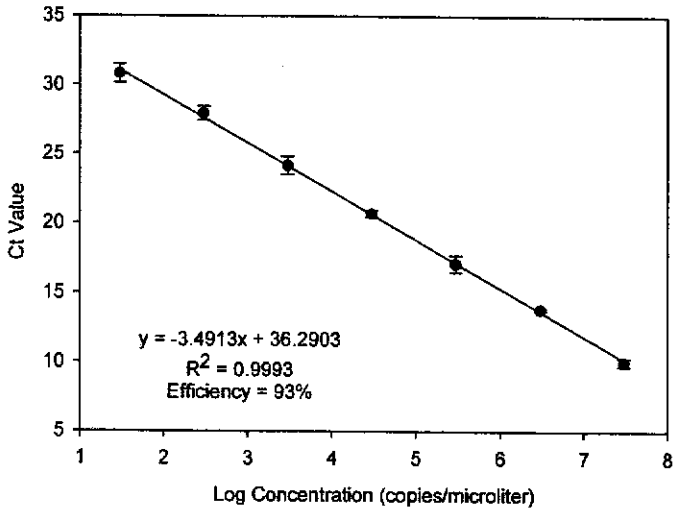

Figure 10. SYBR green qPCR standard curve of the poultry litter specific primers against plasmid DNA containing the *Brevibacterium* sp. biomarker DNA. Error bars indicate the standard deviation of cycle thresholds of triplicate samples.

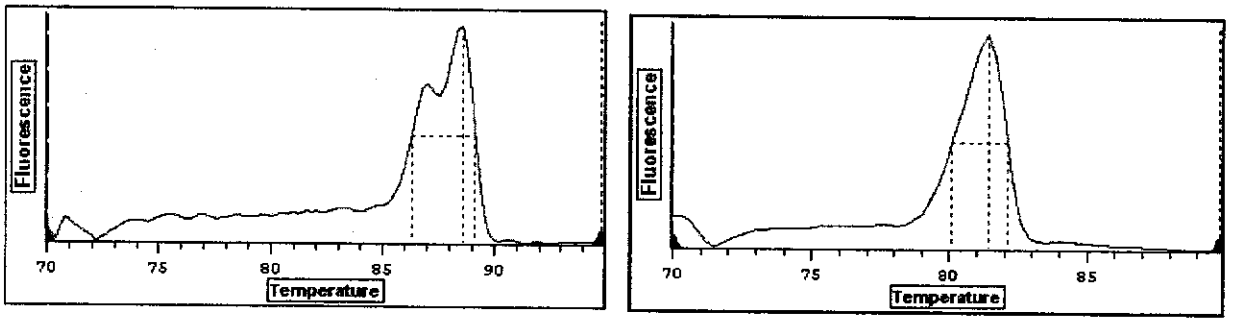

Figure 11. Effect of DMSO on SYBR green melt curves after amplification with LA35 primer set.

Table 8. Detection limit of the qPCR assay for a poultry litter specific *Brevibacterium* sp. in soils and water.

Sample Type	Minimum Detection Limit	Units
Plasmid DNA (standard)	6	copies/µL DNA extraction
Nanopure Water	18	cells/L
Composite Water Sample	78	cells/L
LAL11D-2Q-6-28-06 (soil) with sepharose cleanup	73	cells/g

4.2 Test qPCR Specificity to Distinguish among *Brevibacterium* sp.

4.2.1 Objective

The purpose of this test was to determine if the qPCR assay is specific enough to distinguish between the poultry litter biomarker and the closely related (but not identical) *Brevibacterium casei* 16S rRNA gene. Additionally, the phylogeny of the *Brevibacterium* sp. clone LA35 as compared to other cultured *Brevibacterium* sp. was determined.

4.2.2 Methods

ATCC Culture DNA Extraction. A *Brevibacterium casei* culture was ordered from American Type Culture Collection (ATCC) and the DNA was extracted with the standard protocol. The extracted DNA was then tested for amplification with the qPCR method previously described.

Clone LA35 Phylogenetic Tree Resconstruction. The phylogeny of the LA35 clone was reconstructed using the following methods. The clone sequences were assembled and aligned with BioEdit v. 7.0.5.3 and sequences were checked for chimeras with the Ribosomal Database Project II, Chimera Check program and Bellerophon. The 16S rRNA sequences of the closest neighbors to the clone sequences were downloaded for inclusion in the phylogenic analysis. Multiple sequence alignments were constructed with Clustal W alignment tool and manually aligned in BioEdit. The bootstraps (1,000 resamplings), maximum likelihood and distance matrix analysis (Kimura), and the reconstruction of the phylogenetic trees (FITCH) were performed with the Phylip 3.65 package and in particular the programs SEQBOOT, DNAML, DNADIST, FITCH, CONSENSE, and RETREE. The reconstructed phylogenetic tree was visualized with PhyloDraw V. 0.8 (Graphics Application Lab, Pusan National University). The reconstructed phylogenetic tree for the *Brevibacterium* sp. is presented in Figure 12.

4.2.3 Results

Our qPCR primers amplified the *Brevibacterium casei* 16S rRNA gene. As shown in Figure 12, we are able to distinguish *B. casei* from the LA35 sequence by the SYBR green melt curves. The reconstructed phylogenetic tree of the *Brevibacterium* sp. clone LA35 are presented in Figure 13.

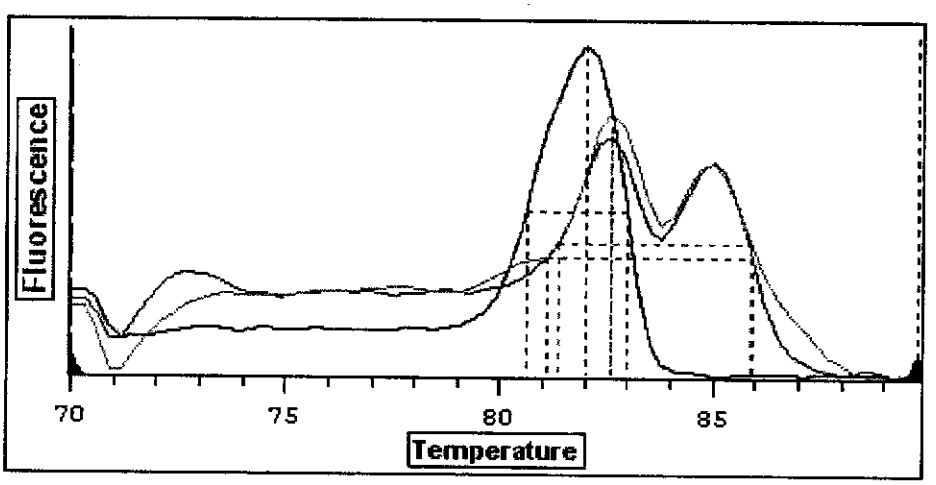

Figure 12. SYBR green melting curve profile of the LA35 16S rRNA sequence (blue) and that of *Brevibacterium casei* [ATCC 35513] (red and green).

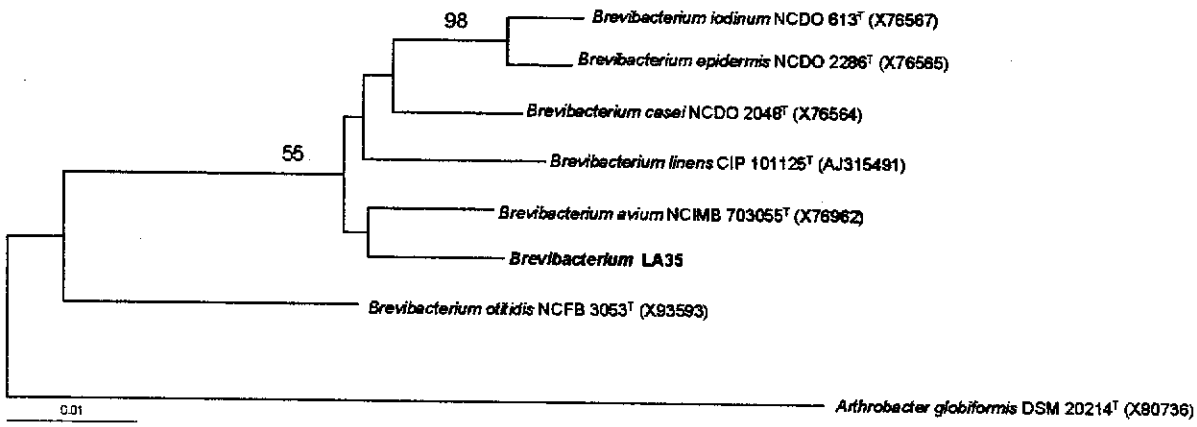

Figure 13. Reconstructed phylogentic tree of the *Brevibacterium* sp. Numbers at the nodes represent bootstrap values (i.e., the number of times this organism was found in this position relative to other organisms in 1,000 resamplings of the data). Bootstraps less than 50% are not shown. The closest cultured organisms as reported in an NCBI Blast search are reported. The distance bar represents a 1% sequence divergence determined by measuring the lengths of the horizontal lines connecting two species.

4.3 Test for the Poultry Litter Specific Biomarker in Environmental Samples from within the Impacted Watershed by the qPCR Assay

4.3.1 Objective

The purpose of this test was to determine if the qPCR assay could detect the poultry litter specific biomarker in environmental samples from within the potentially impacted watershed.

4.3.2 Method

Genomic DNA was extracted from various environmental media (poultry litter, soil and water samples) using methods described in Appendix A. Samples were selected that contained varying levels of fecal indicator bacteria, which was used to gauge the expected biomarker concentration (Table 9). Using methods previously described, DNA was subjected to a diagnostic PCR to verify that the samples did not contain contaminants that might inhibit qPCR and to determine the appropriate sample volume to use for the qPCR assay. DNA was then analyzed by the qPCR assay for the poultry litter specific biomarker. Any sample not amplifying in the qPCR assay was tested by a nested qPCR assay, where universal bacterial primers were first used to amplify the 16S rRNA gene, and then these PCR products were analyzed by the qPCR assay.

4.3.3 Results

The results of the qPCR and nested qPCR assay are presented in Table 9. In general the biomarker was quantifiable in samples containing a high concentration of fecal indicator bacteria and was less prevalent in samples with decreasing concentrations of fecal indicator bacteria. These results indicate that the biomarker can be amplified or detected in a variety of environmental samples from the watershed with relative accuracy as compared to fecal indicator bacterial levels.

Table 9. Results of the test for the LA35 biomarker in environmental samples with the qPCR assay.

Sample ID	Matrix	Expected Biomarker Concentration	DNA (ng/L or ng/g)[a]	qPCR Poultry Specific Biomarker (copies/µL water or g soil or g litter)[a]			qPCR Matrix Spike Amplified?[b]	Nested qPCR Amplified?[b]	Biomarker Melt Peak Identified?	Other Melt Peaks Observed?
EOF-spr-010-5-9-06	Water	High	1.7	1.05E+07	±	1.70E+06	Yes	N/A	Yes	No
EOF-spr-17A-01-5-1-06	Water	High	72.5	2.48E+06	±	4.71E+05	Yes	N/A	Yes	Yes
EOF-spr-023-6-18-06	Water	High	4.3	1.11E+05	±	2.49E+03	Yes	N/A	Yes	No
EOF-spread-073B-6-18-06	Water	High	19.2	1.92E+06	±	4.42E+04	Yes	N/A	Yes	No
LAL16-SPR2-7-18-06	Water	High	-1.0	BDL			Yes	No	N/A	N/A
LAL16C-2-7-18-06	Soil	High	14.5	N/A			No, Inhibited	N/A, Inhibited	N/A	N/A
LAL11C-2-6-28-06	Soil	High	73.2	Present			Yes	Yes	Yes	No
HFS16-BF1-01-6-15-06	Water	Medium	6.8	4.00E+03	±	1.60E+03	Yes	N/A	Yes	No
SALspr-6-28-06	Water	Medium	-0.6	5.82E+02	±	1.56E+02	Yes	N/A	Yes	No
LAL15-SP2-7-11-06	Water	Medium	5.0	2.89E+03	±	7.69E+02	Yes	N/A	Yes	No
RS-PRICEcrk-01-4-29-06	Water	Medium	4.7	3.45E+05	±	1.43E+05	Yes	N/A	Yes	No
RS-574-BIO	Water	Medium	6.7	1.80E+05	±	6.09E+04	Yes	N/A	Yes	No
Lk04-0-01-5-16-06	Water	Low	6.8	3.69E+03	±	3.24E+03	Yes	N/A	Yes	No
HFS28A-BF1-01-6-15-06	Water	Low	-0.7	2.48E+03	±	1.28E+03	Yes	N/A	Yes	Yes
Rs-1-01-8-8-06	Water	Low	7.0	3.19E+04	±	6.75E+03	Yes	N/A	Yes	Yes
FAC-01A-1	Litter	High	33.7	2.18E+09	±	3.53E+08	Yes	N/A	Yes	No
FAC-01A-2	Litter	High	4.7	2.47E+08	±	3.22E+07	Yes	N/A	Yes	No
FAC-01A-3	Litter	High	-0.5	2.67E+07	±	2.69E+06	Yes	N/A	Yes	No
FAC-01A-4	Litter	High	3.4	1.49E+08	±	1.10E+07	Yes	N/A	Yes	No

Table 9. (continued).

Sample ID	Matrix	Expected Biomarker Concentration	DNA (ng/L or ng/g)	qPCR Poultry Specific Biomarker (copies/µL water or g soil or g litter) [a]			qPCR Matrix Spike Amplified? [b]	Nested qPCR Amplified? [b]	Biomarker Melt Peak Identified?	Other Melt Peaks Observed?
FAC-01A-5	Litter	High	4.1	5.67E+08	±	3.75E+07	Yes	N/A	Yes	No
FAC-01B-1	Litter	High	94.5	3.94E+09	±	6.28E+08	Yes	N/A	Yes	No
FAC-01B-2	Litter	High	40.5	2.66E+09	±	7.57E+08	Yes	N/A	Yes	No
FAC-01B-3	Litter	High	34.5	4.75E+06	±	4.23E+06	Yes	N/A	Yes	No
FAC-01B-4	Litter	High	117.1	5.99E+09	±	1.74E+09	Yes	N/A	Yes	No
LAL8-A-2-6-19-06	Soil	High	22.34	7.00E+03	±	4.43E+02	Yes	N/A	Yes	No
LAL16B-2-7-18-06	Soil	High	28.94	2.91E+05	±	1.95E+04	Yes	N/A	Yes	No
RS-901-BIO	Water	Low	1.3	BDL			Yes	No	N/A	N/A
LAL16-GW2-7-18-06	Water	None	2.0	BDL			Yes	No	N/A	N/A
CollinsWell#1-7-7-06	Water	None	4.0	BDL			Yes	No	N/A	N/A
66783-7-26-06	Water	None	0.8	BDL			Yes	No	N/A	N/A
LK-01-0-01-8-9-06	Water	None	5.2	BDL			Yes	No	N/A	N/A
Hester-498-8-10-06	Water	None	2.9	BDL			Yes	No	N/A	N/A

a. Concentration in water is given in ng/L; concentration in soil is in ng/g.

b. N/A, not applicable. The sample was not run with the nested qPCR assay and/or the biomarker melt peak was not identified because none was detected in the qPCR sample run. Inhibited indicates that the sample did not amplify with qPCR even after a sepharose cleanup was performed and the sample was diluted to a lower DNA concentration.

4.4 Determine the Effect of Dilution on Quantification of the LA35 Biomarker in Soil and Water Samples by the qPCR Assay

4.4.1 Objective

This test was conducted to determine a concentration for poultry litter in an environmental sample below which the biomarker is likely not to be detected in environmental samples (i.e., simulate runoff conditions in the lab and test for the presence of biomarker at increasingly dilute samples).

4.4.2 Methods

Litter samples FAC2 and FAC8 (see Table 10) were each carried through the entire dilution procedure. A fraction of the original sample (0.25 g) was diluted into 50 mL of nanopure water and vortexed. Half of this dilution was diluted into 25 mL of nanopure water and vortexed. These diluted samples were then vortexed again, and a concentrated to 25 mL of diluted sample. The samples were centrifuged to pellet cells and litter and resuspended. DNA extraction and PCR were performed as described in Appendix A. Those samples not amplifying with qPCR were subject to nested qPCR to increase the sensitivity of the assay.

FAC2 was one of the original litter samples used to find LA35. Litter sample FAC8 is a turkey litter sample. Both FAC2 and FAC8 previously tested positive for the poultry litter specific biomarker by the qPCR assay. An outline of the protocol is presented in Figure 14.

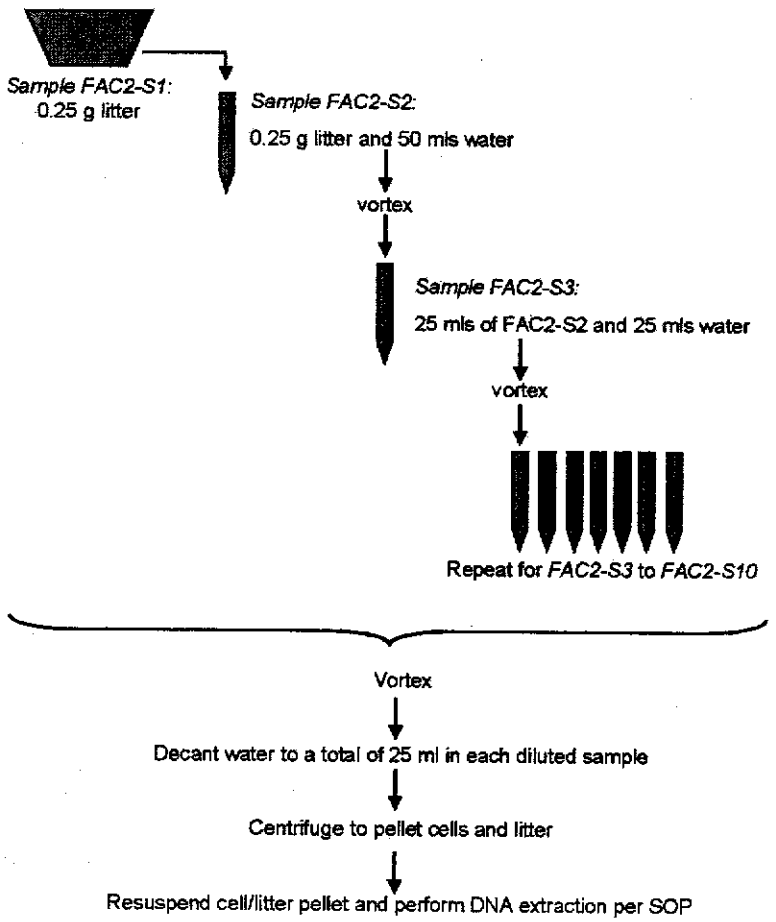

Figure 14. Outline of "dilution limit" protocol using litter sample FAC2 as the example.

4.4.3 Results

LA35 was amplifiable in samples down to 0.1 mg of litter per L. The concentration of LA35 in the litter samples themselves was greater than $2*10^8$ copies/g of litter. There was a very strong correlation between litter concentration (grams of litter per L) and the concentration of LA35 (copies of LA35 per gram of litter), as indicated by the R^2 values of 0.97 and 0.99 shown in Figure 15. These R^2 values indicate that the DNA extraction and *Brevibacterium* sp. quantification methods are relatively precise. Table 10 shows the results if the analysis.

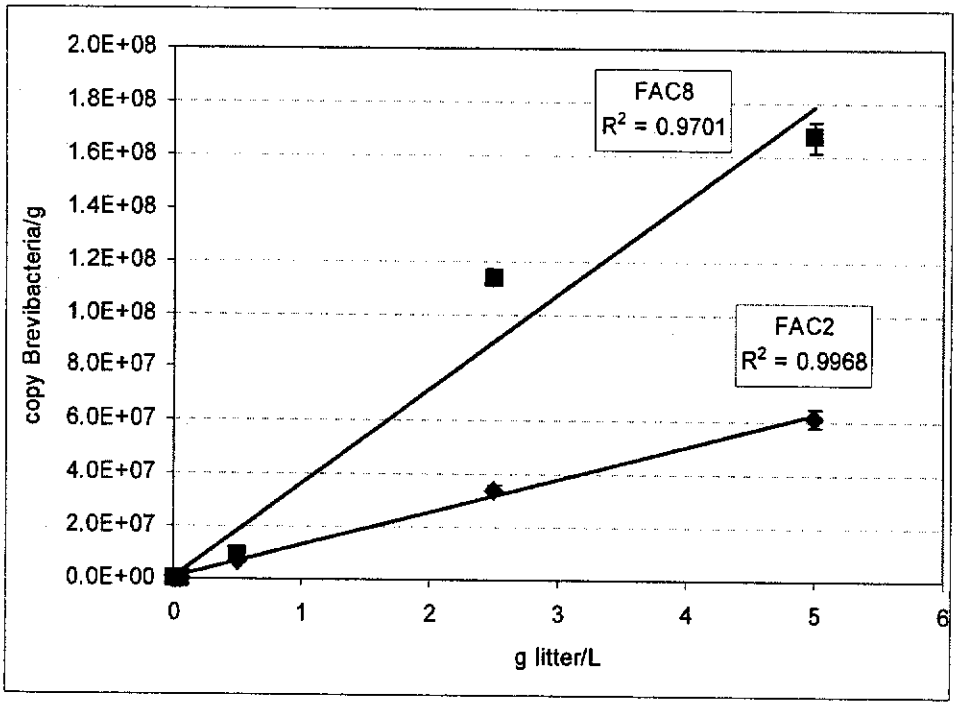

Figure 15. Copies of *Brevibacterium* sp. poultry litter biomarker per gram of soil versus grams of soils per litter in the extractions.

Table 10. Results of the PCR, qPCR, and nested qPCR for *Brevibacterium* sp. in litter.

Sample	"Litter" Concentration^	PCR Bacteria#	Nested qPCR*	*Brevibacterium* sp. 16S rRNA (copy/L water or g litter)
FAC2-S1	NA	+	NA	2.32E+08 ± 8.88E+06
FAC2-S2	5,000 mg/L	+	NA	6.14E+07 ± 3.37E+06
FAC2-S3	2,500 mg/L	+	NA	3.42E+07 ± 1.61E+06
FAC2-S4	500 mg/L	+	NA	6.86E+06 ± 8.27E+05
FAC2-S5	50 mg/L	-	NA	3.54E+04 ± 4.84E+03
FAC2-S6	10 mg/L	-	NA	1.90E+04 ± 4.79E+03

Table 10. (continued).

Sample	"Litter" Concentration^	PCR Bacteria#	Nested qPCR*	*Brevibacterium* sp. 16S rRNA (copy/L water or g litter)
FAC2-S7	5 mg/L	-	NA	1.01E+04 ± 9.35E+03
FAC2-S8	1 mg/L	-	NA	Present, not quantifiable
FAC2-S9	0.1 mg/L	-	+	Present, not quantifiable
FAC2-S10	0.01 mg/L	-	-	Not detected
FAC8-S1	NA	+	NA	2.56E+08 ± 2.49E+07
FAC8-S2	5,000 mg/L	+	NA	1.67E+08 ± 5.88E+06
FAC8-S3	2,500 mg/L	+	NA	1.14E+08 ± 1.56E+05
FAC8-S4	500 mg/L	+	NA	9.05E+06 ± 8.69E+05
FAC8-S5	50 mg/L	-	NA	7.48E+03 ± 2.53E+03
FAC8-S6	10 mg/L	-	NA	7.59E+04 ± 2.97E+04
FAC8-S7	5 mg/L	-	NA	3.20E+04 ± 2.23E+04
FAC8-S8	1 mg/L	-	+	Present, not quantifiable
FAC8-S9	0.1 mg/L	-	-	Not detected
FAC8-S10	0.01 mg/L	-	-	Not detected

^ NA indicates that this was the original 0.25 g undiluted litter sample from which the dilutions were made.

"+" indicates that PCR products were observed after gel electrophoresis, indicating that amplifiable products were obtained from the sample. "-" indicates that PCR products were not observed by gel electrophoresis and UV visualization. Note that the detection limits of the gel electrophoresis method are much higher than the qPCR method detection limits.

* "+" indicates that the *Brevibacterium* sp. were detected with the nested qPCR protocol. "NA" indicates that the samples were not tested with the nested qPCR protocol.

5 SUMMARY OF DETECTIONS REPORTED TO DATE FOR POULTRY LITTER BIOMARKER

A summary of the presence or absence of the *Brevibacterium* sp. LA35 biomarker in all samples analyzed to date is included in Table 11.

Table 11. Summary of *Brevibacterium* sp. LA35-specific PCR and qPCR results in DNA extracted from fecal and environmental samples.

Type of Sample	Number Analyzed	Number Positive	% Containing LA35 Sequence	Assay	Section	Note
Previously reported data						
Original soil and litter samples	20	18	90.0	PCR	3.1	
Fecal Samples	57	2	3.5	Nested PCR	3.3	LA35 sequences were present in one duck and one goose sample from outside the watershed
Litter samples	9	8	88.9	Nested PCR	3.5	One of the litter samples (FAC-06) was taken from a poultry house without a concrete floor and likely contained a high percentage of soil.
Soil samples	27	14	51.9	Nested PCR	3.5	
Edge of field water samples	7	4	57.1	Nested PCR	3.5	
qPCR Data to December 2007						
Soil samples	38	34	89	qPCR		
Water samples	64	33	52	qPCR		
Litter samples	10	10	100	qPCR	N/A	
Groundwater samples	14	2	14	qPCR		

6 REFERENCES

Bernhard, A.E. and K.G. Field, 2000, "Identification of nonpoint sources of fecal pollution in coastal waters byes using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes," *Applied and Environmental Microbiology*. **66**(4): p. 1,587-1,594.

Tsen, H.Yes., C.K. Lin, and W.R. Chi, 1998, "Development and use of 16S rRNA gene targeted PCR primers for the identification of Escherichia coli cells in water," *Journal of Applied Microbiology*. **85**(3): p. 554-560.

Appendix A

General Methods

Soil and Litter Sample DNA Extraction. Genomic DNA was extracted from soil and liter samples with Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc) following the manufacturer's instructions. Typically 0.25 g of soil or litter were used in each extraction. The final mass of sample extracted was recorded in a lab notebook.

Water Sample DNA Extraction. Water samples were filtered through a Supor-200, 0.2 μM filter and frozen at -80°C. Filters were then shattered with sterile glass beads and vortexed vigorously for 15 minutes to remove solids and cells from the filters. The cell suspension was removed from the centrifuge tubes by pipette and placed in a 2 mL bead beating tube from the Bio101 Fast®Spin® DNA extraction kits. The cells were centrifuged at 20,000 x g for 10 minutes, and the supernatant was decanted. Genomic DNA was then extracted using the Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc). The extracted DNA was quantified using a Nanodrop® UV-Vis Spectrophotometer method and recorded.

Universal PCR Assay Conditions. Polymerase chain reaction (PCR) was used to amplify approximately 900 bp of the 16S rRNA genes from *Bacteria*. Each 25μL PCR reaction included 0.4 mg mL^{-1} molecular-grade Bovine Serum Albumin (BSA) (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5μM of both the forward (8F) and reverse (907R) primer (Invitrogen), 1U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1μL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocylcer using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C (1 minute), 55°C (45 seconds), and 72°C (2 minute). A final extension at 72°C for 7 minutes was performed and the PCR products were held at 4°C. PCR products were examined in a 1.2% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions. The PCR primers used in this study are presented in Table A-1.

Clone plasmid DNA PCR Assay Conditions. PCR was used to amplify the variable length of the vector insert in the clones. The PCR reaction master mix and thermocycler conditions were similar to the universal PCR mastermix and thermocycler conditions. The PCR primers used in this study are presented in Table A-1.

***Bacterioides* spp. PCR Assay Conditions.** PCR was used to amplify 621 bp of the 16S rRNA genes from *Bacterioides-Prevotella*. Each 25μL PCR reaction included 0.4 mg mL^{-1} molecular-grade BSA (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 1μM of both the forward and reverse primer (Invitrogen), 1.25 U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1μL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocylcer using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C (1 minute), 55°C (45 seconds), and 72°C (2 minute). A final extension at 72°C for 7 minutes was performed and the PCR products were held at 4°C. PCR products were examined in a 1.2% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions. The PCR primers used in this study are presented in Table A-1.

***Escherichia coli* PCR Assay Conditions.** PCR was used to amplify 449 bp of the 16S rRNA genes from *Escherichia coli*. Each 25μL PCR reaction included 0.4 mg mL^{-1} molecular-grade BSA (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5μM of both the forward and reverse primer (Invitrogen), 1.25 U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1μL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocylcer using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C (1 minute), 55°C (45 seconds), and 72°C (2 minute). A final extension at 72°C for 7 minutes was performed and the PCR products were held at 4°C. PCR products were examined in a 1.2% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions. The PCR primers used in this study are presented in Table A-1.

Table A-1. PCR and qPCR primer used in these studies.

Primer pairs	Target	5' to 3' sequence	Amplicon length (bp)	Assay	Reference
8F 907R 1492R	Bacteria	aga gtt tga tcc tgg ctc ag ccg tca att cct ttg agt tt ggc tac ctt gtt acg act t	~900 ~1490	PCR	
Bac32F Bac708R	*Bacteroides-Prevotella*	aac gct agc tac agg ctt caa tcg gag ttc ttc gtg	621	PCR	Bernhard & Field, AEM, 2000, 66(4):1587-1594
V1SF V3AR	*Escherichia coli*	aat tga aga gtt tga tca tg ctc tac gca ttt cac cgc tac	449	PCR	Tsen, et al. J. Appl. Microbiol. 1998, 85(3):554-560
LA35F LA35R	LA35 *Brevibacterium* sp.	acc gga tac gac cat ctg c tcc cca gtg tca gtc aca gc	569	PCR and qPCR	This study
SA19F SA19R	SA19 *Kineococcus*	tac gac tca cct cgg cat c act cta gtg tgc ccg tac cc	460	PCR	This study
SB37F SB37R	SB37 *Rhodoplanes*	aac gtg cct ttt ggt tcg gct cct cag tat caa agg cag	483	PCR	This study
SA15F SA15R	SA15 *Pantoea ananatis*	cga tgt ggt taa taa ccg cat aag cct gcc agt ttc aaa tac	200	PCR	This study
422F LA35R	*Brevibacterium* sp. biomarker	ggg cag tgg gta cgg gct tcc cca gtg tca gtc aca gc	132	qPCR	This study
366F LA35R	*Brevibacterium* sp. biomarker	gag ctc gta ggt ggt tgg tc tcc cca gtg tca gtc aca gc	187	qPCR	This study
394F LA35R	*Brevibacterium* sp. biomarker	tgt gga aac gca acg ctt aac tcc cca gtg tca gtc aca gc	185	qPCR	This study
T3 T7	Clone plasmid inserts	att aac cct cac taa agg ga taa tac gac tca cgta tag gg	Variable	Sequencing	Topo ® TA cloning manual (Invitrogen)

LA35 PCR Assay Conditions. PCR was used to amplify 531 bp of the 16S rRNA gene from the *Brevibacterium* sp. biomarker. Each 25µL PCR reaction included 0.4 mg mL^{-1} molecular-grade BSA (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5µM of both the forward and reverse primer (Invitrogen), 1 U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1µL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocylcer using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C (1 minute), 60°C (45 seconds), and 72°C (2 minute). A final extension at 72°C for 7 minutes was performed and the PCR products were held at 4°C. PCR products were examined in a 1.2% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions. The PCR primers used in this study are presented in Table A-1.

SA19 PCR Assay Conditions. PCR was used to amplify 460 bp of the 16S rRNA genes from *Kineococcus* spp.. Each 25µL PCR reaction included 0.4 mg mL^{-1} molecular-grade BSA (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5µM of both the forward and reverse primer (Invitrogen), 1 U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1µL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocylcer using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C (1 minute), 60°C (45 seconds), and 72°C (2 minute). A final extension at 72°C for 7 minutes was performed and the PCR products were held at 4°C. PCR products were examined in a 1.2% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions. The PCR primers used in this study are presented in Table A-1.

SA15 PCR Assay Conditions. PCR was used to amplify 200 bp of the 16S rRNA genes from *Pantoea ananatis* (*E. coli*). Each 25µL PCR reaction included 0.4 mg mL^{-1} molecular-grade BSA (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5µM of both the forward and reverse primer (Invitrogen), 1 U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1µL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocylcer using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C (1 minute), 60°C (45 seconds), and 72°C (2 minute). A final extension at 72°C for 7 minutes was performed and the PCR products were held at 4°C. PCR products were examined in a 1.2% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions. The PCR primers used in this study are presented in Table A-1.

SB37 PCR Assay Conditions. PCR was used to amplify 483 bp of the 16S rRNA genes from bacteria. Each 25µL PCR reaction included 0.4 mg mL^{-1} molecular-grade BSA (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5µM of both the forward and reverse primer (Invitrogen), 1 U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1µL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocylcer using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C (1 minute), 60°C (45 seconds), and 72°C (2 minute). A final extension at 72°C for 7 minutes was performed and the PCR products were held at 4°C. PCR products were examined in a 1.2% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions. The PCR primers used in this study are presented in Table A-1.

LA35 qPCR Assay Conditions (LA35F-LA35R). Quantitative PCR (qPCR) was used to amplify 530 bp of the 16S rRNA gene from bacteria. DNA samples were diluted to final concentrations of 3 ng/5µL DNA. Each 25µL qPCR reaction included: 1X SYBR Green Master Mix (Roche), 0.5 µM of both the forward and reverse primer (Invitrogen), 5 % DMSO, 5 µL of diluted sample DNA, and molecular-grade water (Promega). Amplification was performed in triplicate on a Biorad Chromo4 thermocylcer using the following conditions: 50°C for 2 minutes, 95°C for 15 minutes, 45 cycles of 95°C (30 seconds), 60°C (30 seconds), and 72°C (30 seconds) with a plate read. The 45 cycles is followed by a final extension at 50°C for 5 minutes. Immediately following the final extension is a melting curve from 70°C to 90°C, by 0.1 degree increments, holding for 5 seconds with a plate read.

LA35 qPCR Assay Conditions (366F-LA35R). qPCR was used to amplify 187 bp of the 16S rRNA genes from bacteria. DNA samples were diluted to final concentrations of 15 ng/5µL DNA. Each 25µL qPCR reaction included 1X SYBR Green Master Mix (Roche), 0.5 µM of both the forward and reverse primer (Invitrogen), 5 µL of diluted sample DNA, and molecular-grade water (Promega). Amplification was performed in triplicate on a Biorad Chromo4 thermocylcer using the following conditions: 50°C for 2 minutes, 95°C for 15 minutes, 40 cycles of 95°C (30 seconds) then 60°C (60 seconds) with a plate read. The 40 cycles is followed a melting curve from 60°C to 90°C, by 0.1 degree increments, holding for 5 seconds with a plate read.

LA35 qPCR Assay Conditions (394F-LA35R). qPCR was used to amplify 485 bp of the 16S rRNA genes from bacteria. DNA samples were diluted to final concentrations of 15 ng/5µL DNA. Each 25µL qPCR reaction included 1X SYBR Green Master Mix (Roche), 0.5 µM of both the forward and reverse primer (Invitrogen), 5 µL of diluted sample DNA, and molecular-grade water (Promega). Amplification was performed in triplicate on a Biorad Chromo4 thermocylcer using the following conditions: 50°C for 2 minutes, 95°C for 15 minutes, 40 cycles of 95°C (30 seconds) then 60°C (60 seconds) with a plate read. The 40 cycles is followed a melting curve from 60°C to 90°C, by 0.1 degree increments, holding for 5 seconds with a plate read.

LA35 qPCR Assay Conditions (422F-LA35R). qPCR was used to amplify 132 bp of the 16S rRNA genes from bacteria. DNA samples were diluted to final concentrations of 15 ng/5µL DNA. Each 25µL qPCR reaction included 1X SYBR Green Master Mix (Roche), 0.5 µM of both the forward and reverse primer (Invitrogen), 5 µL of diluted sample DNA, and molecular-grade water (Promega). Amplification was performed in triplicate on a Biorad Chromo4 thermocylcer using the following conditions: 50°C for 2 minutes, 95°C for 15 minutes, 40 cycles of 95°C (30 seconds) then 60°C (60 seconds) with a plate read. The 40 cycles is followed a melting curve from 60°C to 90°C, by 0.1 degree increments, holding for 5 seconds with a plate read.

TRFLP Analysis. Extracted genomic DNA and/or clone DNA was PCR amplified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM) labeled universal bacterial primers 8F-907R, with *E.coli* genus specific primers (Tsen, et al. 1998), and *Bacteroides* genus specific primers (Bernhard and Field, 2000). All PCR primers targeted the 16S rRNA gene. Triplicate PCR products were generated from each DNA extraction, combined and purified using QIAquick PCR purification Kits (Qiagen). Approximately 200 ng each of PCR product was digested at 37°C for 6 hours with each *Aci*I, *Msp*I, and *Hae*III restriction enzymes (20µ/µL) (New England BioLabs). The digested fragments were purified using ethanol precipitation. Samples were denatured by heating to 95° C for 3 minutes followed by cooling to 4°C.

Sepharose Cleanup. DNA was cleaned to remove contaminants inhibitory to PCR reactions by size-exclusion chromatography. Sepharose CL-4B (Sigma-Aldrich) was resuspended in Tris-HCL and sterilized by autoclave at 121°C for at least 20 minutes. Micro-bio spin columns (Bio-Rad Laboratories) were packed with 1 mL of Sepharose CL-4B through centrifugation. Sepharose columns were then washed twice with Tris-HCl buffer (pH 8) and 50 to 150 ul of sample was added. Sepharose cleaned DNA was concentrated with ethanol precipitation and re-eluted in 100µL sterile water.

Sephadex Cleanup. Universal PCR products were cleaned (e.g., excess primers, dNTPs, and proteins were removed) by size exclusion chromatography. The Sephadex G-50 superfine (Sigma-Aldrich), was placed in a 96 well centrifuge plate (MultiScreen HTS, Millipore, 0.45um Hydrophil Low Protein Binding Durapore membrane) and swelled with 300 ul of Tris-HCl at pH 8.0 for at least 3 hours at room temperature. After swelling, excess buffer was removed and the columns were packed by centrifugation at 500 x g for 5 minutes. PCR products (25 to 50 ul) were added to the columns and eluted at 500 x g for 3 to 5 minutes.

Nested PCR. The first PCR reaction in all nested PCR reactions was with the 8F-907R universal bacterial primers. PCR products were examined in a 1.2% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions. Amplified PCR products were cleaned either with the 96 well Sephadex column method or individually with the QIAGEN QIAquick PCR purification kit. The second PCR reaction in the nested PCR was as specified for the experiment (e.g., primers, mastermix and thermocycler conditions specific to the test).