IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF OKLAHOMA

STATE OF OKLAHOMA,	)	
	)	
Plaintiff,	)	
	)	
v.	)	**Case No. 05-cv-329-GKF(PJC)**
	)	
TYSON FOODS, INC., et al.,	)	
	)	
Defendants.	)	

DECLARATION OF MICHEAL J. SADOWSKY, Ph.D.

I, Michael J. Sadowsky, Ph.D., hereby declare as follows:

1. I am a Distinguished McKnight University Professor in the Department of Soil, Water and Climate, and The BioTechnology Institute at University of Minnesota, St. Paul, and I am also the current co-director of the Microbial and Plant Genomics Institute, the Director of the Microbial Ecology Minors Graduate Program, and co-Director of Graduate Studies of the Soil Science Graduate Program at this University. Research in my laboratory focuses on several areas of environmental microbiology, including determination of sources and sinks of *E. coli* and fecal pathogens in soil and aquatic environments, the development and use of microbial source tracking ("MST") molecular technologies to determine sources of fecal bacteria in the environment, the genomics, genetics and physiology of the symbiotic nitrogen fixation process in soybeans and other legume species, and the biodegradation and bioremediation of recalcitrant halogenated herbicides in soil and water. I am the recipient of several awards and honors, including elected Fellow of the American Association for the Advancement of Science (AAAS) (2009), CFANS Distinguished Graduate Teaching Award (2009), CFANS Distinguished

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Diversity and Inclusion Award (2008), Distinguished McKnight University Professorship (2004), elected Fellow of the American Academy of Microbiology (1999), and the Young Investigator Award from the American Society for Microbiology (1990). I have published more than 140 original, peer-reviewed, scholarly publications and several book chapters concerning various aspects of environmental microbiology. Recently I co-organized, co-wrote and, co-edited a book on MST, entitled "Microbial Source Tracking" that was published by the American Society for Microbiology. My work is widely cited by researchers in several scientific disciplines. My laboratory currently has 13 members, including postdoctoral scholars, graduate students, and research technicians. Taking into consideration my accomplishments and publications, I am viewed as one of the leading experts in the areas of MST, genomics, and environmental biology.

2. MST is now an established field of scientific study and is used to determine sources of fecal bacteria and pathogens in the environment. The potential sources of fecal contamination in water, soil, and in sediments include human sewage, and feces from pets, farm animals, wildlife, and waterfowl. Several library- dependent and -independent methods have now been developed for MST studies. While library-dependent MST methods first appeared to be useful tools for analysis of fecal contamination, issues of geographical variation of target organisms, limited library sizes, and genotypic variation amongst the microorganisms tested has limited the use of these methods. In response to these limitations, several laboratories developed library-independent MST methods. These methods rely on the use of DNA fragments or polymerase chain reaction (PCR) primers to identify sources of fecal contaminants in the environment. The majority of

library-independent MST tools rely in the use of PCR primers designed to identify bacteria within the order Bacterioidales and other bacterial families, which comprise the greatest majority of bacteria inhabiting the feces of warm blooded animals. Many Bacteriodales and other intestinal bacteria (e.g. *Brevibacterium;* order *Actinomycetales*) are specific to the animal host they reside in, making them ideal "markers" for MST studies. PCR primers have been developed to specifically detect fecal bacteria originating from swine, cattle, humans, and geese. The majority of researchers and companies involved in MST studies now use library-independent, host-specific PCR-based detection systems (either in a quantitative or qualitative manner) to determine sources of fecal bacteria in the environment. These techniques employ the use of host-specific markers, e.g. PCR primers, to determine sources of fecal pollution. Host-specific markers, targeting 16S rDNA and other genes, have been identified for *E coli*, methanogens, viruses and coliphage, and members of the Bacteriodales. Due to the advantages of library-independent methods, they are now recognized by the vast majority of scientists and practitioners as the "gold standard" for MST analyses of water, soil, and sediment. The host-specific markers used for MST studies have been validated by estimating the proportion of false-positives and false-negatives in the target population, and for sensitivity in detecting these bacteria that are present in low numbers in complex matrices, such as soil and sediment. These well accepted, library-independent, host-specific PCR-based detection methods targeting the 16S rDNA of the Bacteriodales are the same methods employed by Dr. Harwood in development of the poultry biomarker.

3. In April 2008, I was contacted by Dr. Valerie (Jody) Harwood (University of South Florida) and Camp Dresser & McKee to examine and validate the use of a

Brevibacterium–derived biomarker gene (in the form of DNA primers), that when used with the polymerase chain reaction (PCR) technique, was purported to specifically identify fecal contamination originating from poultry litter. In exchange for our validation work, my laboratory was to be reimbursed for lab expenses and out of pocket costs associated with the validation. I personally received no salary remuneration for my involvement in these studies.

4. The study we did was done with minimal collaboration with North Wind Inc., who helped design the DNA primers we utilized. They provided us with three sets of blindly-labeled (i.e., we did not know the source of the samples) DNA samples for evaluation. The blind samples were numbered and separated into three sets and labeled as "A", "B" & "C". The blinded samples were evaluated by using conventional PCR and quantitative PCR (qPCR) for their ability to serve as templates for the synthesis of DNA using the designated primers. This is indicative of the presence of DNA corresponding to the presence of fecal contamination.

5. The protocol we used was initially provided by North Wind and was followed as written, except that we used an Applied Biosystems ABI Prism 7000 Real-Time PCR machine instead of the BioRad Chromo4 Real-time Module as our sequence detection system. Initial results obtained using the provided quantitative PCR (qPCR) protocol indicated that our instrument was not optimal for this analysis as the method was written due to limited band intensity and the formation of primer dimers. Thus, the protocol was optimized to better perform with the reagents and equipment we were using (i.e., Applied

Biosystems ABI Prism 7000 Real-Time PCR machine). The primers, as designed by Harwood and North Wind were always used in our studied. Modifications to the North Wind's protocol included: a decrease in the volume of sample used, increase in the temperature and time of the elongation phase of PCR amplification, and reduction in the concentration of primers used. These modifications served to reduce: the impact of inhibitors in samples, primer dimers, and production of secondary products. The final protocol used in our analyses is shown in the accompanying report (attachment 1).

6. Blind samples were received from North Wind by our lab, on dry ice, in three shipments on 4/10/2008, 4/11/2008 and 4/23/2008. There were a total of 63 samples, comprising three sets: Set "A" contained 32 samples, set "B" had 12 samples, and set "C" contained 18 samples. The sample vials were blindly labeled, and we were not aware which represented DNAs from contaminated sites, controls, or from other sources. We also received a control sample of marker-specific DNA (the *Brevibacterium* poultry litter biomarker) that contained 108 copies per µl of PCR product in a pCRII-TOPO plasmid. This was used as a positive control for all studies and served to "calibrate" the method on our instrument. Prior to PCR analyses, the concentration of DNA in each sample, which were measured, DNA concentrations from the samples obtained were 0.7 to 358.7 nanograms per microliter (ng/µl). A stock of each sample, when possible, was made and stored in a freezer at -20°C. The original stocks were stored at -80°C. All PCR reactions were done in triplicate, and positive (control sample of cloned, marker-specific DNA) and negative (water sample without DNA) controls were used in all tests.

7. Based on the initial studies, a final PCR protocol was adopted and used to determine concentrations of marker gene in the environmental samples. The total reaction mixture volume was 25 µl, and diluted sample, containing 5 ng of DNA was added in a 5 µl volume. The remaining volume of each reaction consisted of master mix containing 1x FastStart SYBR Green Master Mix with ROX (Roche), 75 nM each of the forward and reverse primers, 5% DMSO and nuclease free water to reach 20 µl The thermal cycler protocol 50°C for 5 minutes, and 95°C for 15 minutes; followed by 40 cycles of 30 seconds of dissociation at 95°C, 30 seconds of annealing at 60°C, and 60 seconds of elongation at 75.6°C. After each elongation step the fluorescence of each well was measured. Following amplification there was an incubation at 50°C for 5 minutes, followed by a melting curve step measuring the dissociation of double-stranded DNA between 65°C and 95°C. The qPCR studies were performed on an ABI-Prism 7000 sequence analyzer in optically clear 96 well microplates.

8. Triplicate reactions were run for all samples along with one matrix spike experiment. In the matrix spike experiment 10^4 copies of control plasmid was added to the reaction. The matrix spike was used to gauge inhibition in samples which were below the threshold for detecting *Brevibacterium*. No sample experienced complete inhibition or an order of magnitude drop in the expected concentration during any of the experiments. Therefore inhibition of the qPCR reaction was not a concern with these samples.

9. Four sets of plates were run to accommodate all of the samples. Each plate had its own separate set of controls. The limit of detection (L.O.D.) was taken to be the point with the highest R^2 value in the Ct versus log copy number curve. The L.O.D. was taken to be

50 copies per reaction. The suggested measure of repeatability was for samples to have a relative standard deviation below 20. Samples where the concentration of each triplicate was below the limit of detection were taken to be negative, independent of the relative standard deviation.

10. All samples were analyzed by qPCR and to be considered positive for the poultry biomarker the following criteria needed to be met: (1) the melting curve for the PCR product in that region had to be greater than that seen in the non-template control and greater than the melting curve generated by the 50 copy positive control (the LOD); (2) if a sample's fluorescence was below that of the zero copy (negative control) samples, it was considered negative for the presence of the poultry marker gene; (3) samples producing maximum fluorescence below that produced in the 50 copy reaction were considered below our limit of detection and reported as negative for the marker gene; and (4) all samples producing melting curves with fluorescence at or above the 50 copy per reaction LOD were considered positive.

11. Based on these criteria, the following results were obtained:

a. The entire set of "B" samples were positive for the presence of the marker gene, and this gene was present in high concentration, in the order of tens of thousands of copies of the marker gene per 5 ng of sample DNA. Thus, these samples contained the marker gene and were consistent with contamination with poultry litter or poultry feces.

b. The following samples were below the limit of detection for the *Brevibacterium* marker gene that is stated to be indicative of poultry litter contamination: C01, C06, C07, C08, C09, C10, C11, C13, C14, C15, C16, C17, C18, C23, C24 and C25 (proxies for sample C04). Two of three replicate analyses of samples C02, C03, C04, C05, and C12 were positive for the presence of the marker gene, and the average of all three replicates were quantitatively above the limit of detection. Consequently, these samples were viewed as containing the marker gene.

c. The entirety of set "A" was below the limit of detection and was considered negative for the presence of the marker gene. Thus, these samples likely were likely not contaminated with poultry litter or poultry feces.

12. On May 12, 2009, I received from Dr. Harwood the decoded list (unblinding) of samples we analyzed with specific reference to their origin and comparison to results obtained from North Wind Inc. and our laboratory at the University of Minnesota. Results of this analysis indicated the samples labeled as "Set B" originated from poultry litter samples and we correctly identified 100% of these samples as reacting, in a quantitative manner, with the LA35F and LA35R PCR primers provided by North Wind, Inc. Sample set C consisted of DNAs from soil fields receiving poultry litter (C1, C2, C11, and C25), run-off from field receiving poultry litter (C3-C7), reference DNA samples with no known poultry impact (C16-C18) with and ground water (C8). Sample C9, C10, C12-C15, C23 and C24 were from surface water. Using our modifications of the North Wind's protocol, we correctly identified 3 of 5 samples from run-off from spread fields, 1 of 4 DNA samples from soil originating from fields that received poultry litter, 3 of 3 reference samples with no known poultry impact (negative controls), and 6 of 11 samples in the remainder of the C samples. The LA35F and LA35R primers gave no reaction with a DNA sample from cow feces (our negative control). The results obtained during our validation studies were very similar to those obtained by North Winds Inc., with the exception of two surface water samples. Based on these analyses, I conclude that that LA35F and LA35R PCR primers are a reliable tool to identify contaminated poultry litter and its presence in soil and water samples.

13. The validation studies done in collaboration with Dr. Harwood and North Winds Inc. are being used for the Master of Science thesis dissertation of Daniel Norat, a student in the Microbial Engineering Graduate Program at the University of Minnesota. Mr. Norat has presented results of these studies, in the form of a poster presentation, at the North Central Branch Meeting of the American Society for Microbiology that was held on Oct 17-18, 2008 at St. Cloud, MN. Our laboratory's contribution to the studies outlined here and in the attached report to Camp Dresser & McKee, Inc. will be included in a manuscript to be submitted for peer review by Dr. Harwood. My student Daniel Norat and I will be co-authors on this submitted manuscript.

14. It should be noted, that while the MST PCR-based method we evaluated and validated was not published at the time of our analyses, it does not detract from its usefulness nor render it scientifically unsound. A large number of methods, especially those developed by companies, and now considered standard methods, have never been published in scientific journals. These methods are recognized by the scientific community as being valid. In contrast, the mere fact that a scientific method is published in a revered scientific journal does not establish its validity or usefulness, the literature is replete with methodology and data that are in fact not useful, incorrect, and have been judged not reproducible by others. In my experience as an editor and associate editor of several journals, if a method or scientific principle has been rejected for publication by scientific referees for that journal, it does not mean that the material seeking to be published is unsound or incorrect, only that in the opinion of the referees and editor that the material either did not meet certain standards for that journal or was not of interest to

the readers of that journal. In many cases, manuscripts submitted and subsequently rejected for publication in a peer-reviewed journal are published elsewhere after modification by writing or by the addition of additional data or interpretation suggested by reviewers of the journal initially not granting publication.

I declare under penalty of perjury, under the laws of the United States of America, that the foregoing is true and correct.

Executed on the 26th day of May, 2009.

Michael J. Sadowsky, Ph.D

ATTACHMENTS

Draft Final Project Report to Camp Dresser & McKee, Inc.

Quantification of Poultry Litter-Specific Marker Genes in Environmental DNAs and Optimization of a QPCR Method to Detect *Brevibacterium* Marker Genes in Environmental Samples

May 26, 2009

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Summary

Our lab was contacted to validate the use of a *Brevibacterium*–derived biomarker gene specific for poultry litter. The study was done in collaboration with North Wind Inc., who designed the primers, and provided us with three sets of blindly labeled DNA samples. The blind samples were were numbered and separated into three sets labeled "A", "B" & "C". The qPCR protocol provided by North Winds was followed as written, except that we used an Applied Biosystems ABI Prism 7000 Real-Time PCR machine instead of the BioRad Chromo4 Real-time Module as our sequence detection system. Initial results obtained using the provided quantitative PCR (qPCR) protocol and our instrument were not usable, due to excessive variance in readouts. Thus, the protocol needed optimization to better perform with the reagents and equipment we were using.

Four changes were made to the original protocol. First, the quantity of DNA from environmental samples was decreased in order to reduce the effects of inhibitors present in the samples. Secondly, the temperature of the elongation phase of amplification was increased to remove or prevent nonspecific secondary products which formed during the PCR reaction. Thirdly, the amount of time for product elongation was increased to ensure that PCR generated more actual product than secondary products. Lastly, the concentration of primers was decreased to reduce the production of primer dimers, which was a major cause of the production of secondary products.

Based on these studies, the final protocol adapted by us to determine concentrations of a *Brevibacterium*-based poultry marker gene in the environmental samples was as follows: The reaction mixture (25 µl) contained master mix containing 1x FastStart SYBR Green Master Mix with ROX (Roche), 75 nM each of the forward and reverse primers, 5% DMSO, a diluted sample containing 5 ng of DNA in a 5 µl volume, and nuclease free water to reach 20 µl final volume. The thermal cycler protocol used was as follows: 50°C for 5 min, and 95°C for 15 min, followed by 40 cycles of 30 sec of dissociation at 95°C, 30 sec of annealing at 60°C, and 60 sec of elongation at 75.6°C. For qPCR, fluorescence of each well is measured after each elongation step. Following amplification there was an incubation at 50°C for 5 minutes, followed by a melting curve experiment measuring the dissociation of samples between 65°C and 95°C. The qPCR studies were performed on an ABI-Prism 7000 sequence analyzer in optically clear 96 well microplates. Based on qPCR, the protocol adopted had a limit of detection of 50 copies per 25 µl reaction volume.

With the protocol optimized we were able to find that in the blind samples from set "B" were contaminated with poultry feces while isolates from sets "A" & "C" were not contaminated.

RESULTS AND DISCUSSION

I. Receipt and Initial Analysis of Samples

Receipt and storage of environmental extracts. Samples containing DNA from environmental extracts were received from North Wind lab on dry ice in three shipments on 4/10/2008, 4/11/2008 and 4/23/2008. There were a total of 63 samples, comprising three sets. Set "A" contained 32 samples, set "B" had 12 samples, and set "C" contained 18 samples. The sample

vials were blindly labeled, and we were not aware which represented DNAs from contaminated sites. We also received a control sample of marker-specific DNA (the *Brevibacterium* poultry litter biomarker) that contained 10^8 copies per µl of PCR product in a pCRII-TOPO plasmid.

Prior to PCR analyses, initial studies were done to determine the concentration of DNA in each sample, which were measured using a Thermo Scientific NanoDrop1000 Spectrophotometer (Wilmington, DE). Results in Table 1 show that DNA concentrations from the environmental samples ranged from 0.7 to 358.7 ng/µl.

Table 1: Concentration of DNAs from Environmental Samples.

Sample ID	ng/ul	260/280	260/230
A01	41.45	1.47	0.61
A02	43.64	1.72	0.96
A03	17.89	1.4	0.51
A04	66.63	1.65	1.05
A05	12.54	1.58	0.52
A06	48.39	1.52	0.59
A07	38.71	1.63	0.89
A08	110.3	1.67	0.93
A09	23.44	1.8	1.15
A10	27.88	1.5	0.82
A11	28.25	1.55	0.83
A12	50.21	1.81	1.6
A13	56.24	1.47	0.79
A14	48.51	1.75	1.35
A15	65.6	1.86	1.76
A16	3.47	1.57	0.59
A17	8.52	1.69	0.96
A18	83.66	1.14	0.67
A19	5.93	1.5	0.61
A20	3.29	1.32	0.47
A21	2.6	1.06	0.3
A22	2.26	1.24	0.29
A23	3.69	1.23	0.27
A24	1.72	0.96	0.31
A25	22.04	1.74	1.38
A26	55.72	1.83	1.7
A27	1.2	1.42	0.29
A28	0.95	1.03	0.23
A29	6.58	1.67	0.72
A30	9.89	1.54	0.73
A31	0.7	0.76	0.18
A32	2.58	1.33	0.44

Sample ID	ng/ul	260/280	260/230
B01	185.87	1.84	1.85
B02	190.66	1.77	1.47
B03	83.66	1.69	1.25
B04	226.07	1.85	1.79
B05	249.05	1.85	1.83
B06	292.3	1.86	1.9
B07	340.61	1.86	1.95
B08	281.7	1.85	1.84
B09	357.5	1.85	1.9
B10	263.7	1.87	1.97
B11	216.77	1.86	1.88
B12	358.71	1.86	1.94
B13	354.37	1.86	1.97
C01	6.34	1.66	1.29
C02	27.72	1.92	1.82
C03	182.3	1.77	1.63
C04	249.69	1.82	1.88
C05	195.7	1.81	1.77
C06	48.16	1.78	1.58
C07	49.59	1.74	1.53
C08	7.46	1.52	0.83
C09	28.49	1.58	1.22
C10	11.7	1.73	1.55
C11	4.15	1.83	0.92
C12	2.58	1.65	0.49
C13	139.58	1.78	1.59
C14	3.14	1.4	0.31
C15	1.11	1.03	0.27
C16	2.39	1.51	0.35
C17	5.89	1.85	0.64
C18	3.53	1.6	0.23

In preparation for PCR, a 3 ng/µl stock of each sample, when possible, was made and stored in a freezer at -20°C. The original stocks were stored at -80°C.

Lyophilized, oligonucleotide, forward and reverse PCR primers (sequence provided by North Wind) LA35F and LA35R, respectively, for the target genes were ordered from Integrated DNA

Technologies, Inc (Coralville, IA). The primers were reconstituted to a stock concentration of 100μM in nuclease free water. Sub-stocks of 10μM for use in reactions were made from the master stock. The oligonucleotide primers were stored in a freezer at -20°C.

Generation of standard curve for the LA35 *Brevibacterium*-specific primer set. Before examining environmental DNAs for the concentration of target sequences, an initial experiment was done to evaluate the quantitative PCR (qPCR) protocol provided by North Wind. These studies were done on 5/22/08 using varying concentrations of the control plasmid DNA as PCR template. The protocol called for 25 μl reactions, where 5 μl is a diluted DNA sample containing 15 ng of sample. The remaining volume of each reaction is a master mix containing 1x FastStart SYBR Green Master Mix with ROX (Roche Applied Science, Indianapolis, IN), 0.5 μM each of the forward and reverse primers, 5% DMSO, and nuclease free water to reach 20 μl. Five μl samples containing 0, 10, 100, 10^3, 10^4, 10^5, 10^6 & 10^7 copies per reaction of the control cloned *Brevibacterium* DNA in plasmid pCRII-TOPO were used as template. All experiments were run in triplicate. The PCR reaction conditions used were as follow below.

The thermal cycler protocol started with an incubation step at 50°C for 5 minute and 95°C for 15 minutes. That was followed by 40 cycles of 30 seconds of dissociation at 95°C, 30 seconds of annealing at 60°C, and 30 seconds of elongation at 72°C. After each elongation step the fluorescence of each well was measured. Following amplification there was an incubation at 50°C for 5 minutes followed by a melting curve experiment measuring the dissociation of samples between 65°C and 95°C. The qPCR reactions were performed on an Applied Biosystems ABI PRISM 7000 Sequence Detection System (Foster City, CA) using optically clear 96 well microplates.

Results in Figures 1 and 2 showed that most of the standard samples did not appear to have amplified and the curve of when the cycle threshold (Ct) was crossed for a given concentration of plasmid DNA showed a high limit of detection, between 10^5-10^6 copies per 25 μl reaction.

Figure 1: Fluorescence curves for *Brevibacterium* clone standard for 5/22/08

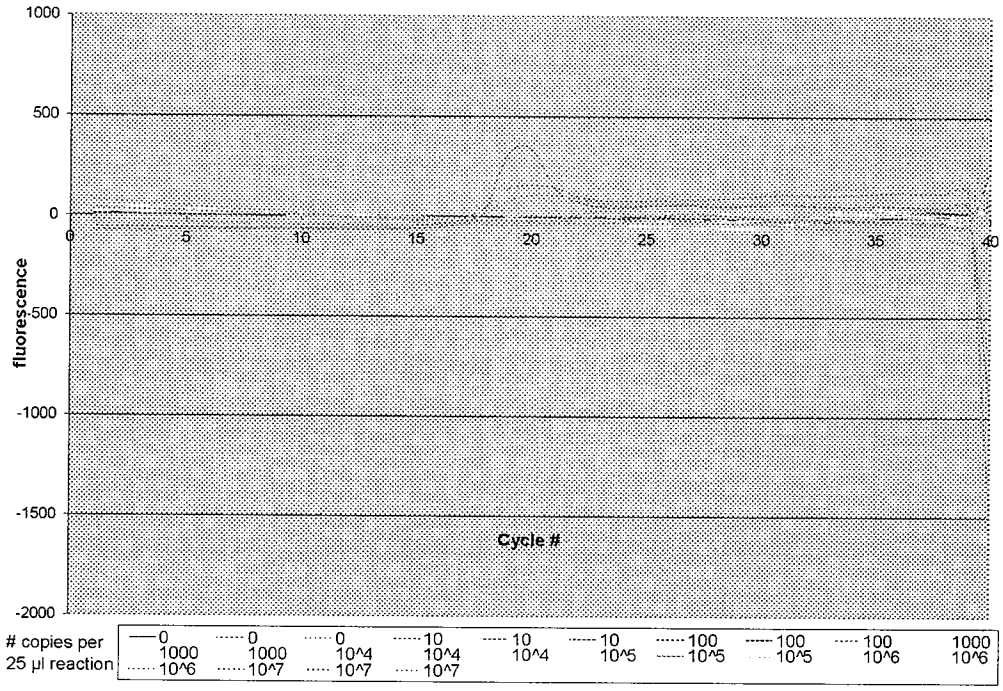
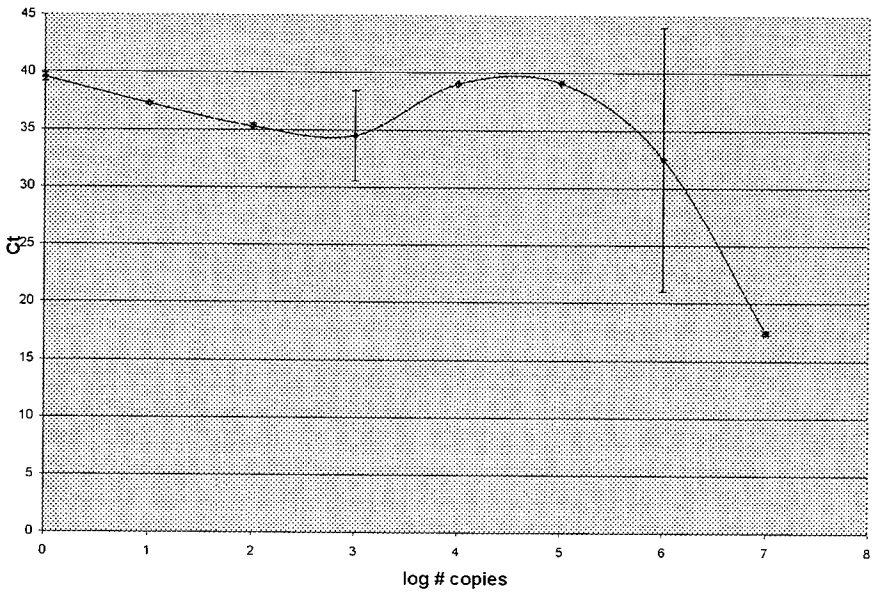


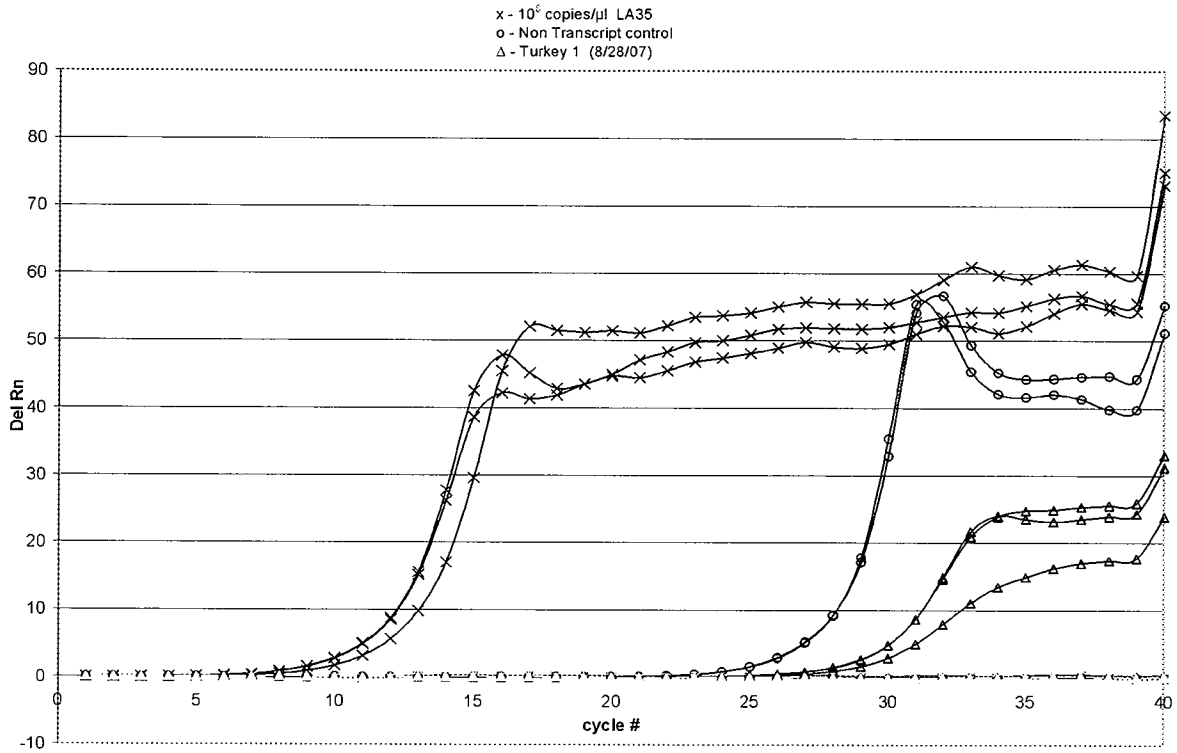
Figure 2: Ct vs. gene copy number relationship for *Brevibacterium* clone standards for 5/22/08



Based on these results, a qPCR experiment was performed on 5/23/08 to determine how the primer set was performing using the above conditions for three samples, a non-template control (i.e. 0 copies of DNA), 5 µl of 10^8 copies per µl of control DNA, and 5 µl of a turkey fecal DNA extract isolated on 8/27/07 from a single turkey at the Minnesota State Fair (Figure 3). Results of this study (Figures 3 and 4) indicated two problems. Firstly, the turkey feces DNA sample gave a lower signal (greater Ct value) than the water sample, and secondly, the cycle threshold

for the water sample at 22 of 40 cycles, was too low for a non-template control sample (which should not yield a signal before about 35 cycles). These results indicated that qPCR protocol provided us was not working for this qPCR instrument, and consequently, that qPCR procedure would need to be optimized for the ABI PRISM 7000 Sequence Detection System instrument that we were using.

Figure 3: Fluorescence curve of reaction run on 5/23/08



Because the North Wind qPCR protocol was not working for this sequence analyzer, it was realized that the procedure would have to be optimized for the ABI Prism instrument.

II. Optimization of qPCR Protocol for Detection of Poultry-Specific Marker Gene using an ABI PRISM 7000 Sequence Detection System.

The first optimization experiment done was to extend the elongation time of the qPCR reaction from 30 seconds to 1 minute. This was intended to ensure that there was sufficient time for amplification of the PCR product. This experiment was performed on 5/26/08. All other parameters were held constant. Results in Figure 4 show that extending the annealing temperature produced sigmoid curves whose fluorescence increased with increasing DNA concentration.

Figure 4: Fluorescence curves for *Brevibacterium* clone standards for 5/26/08

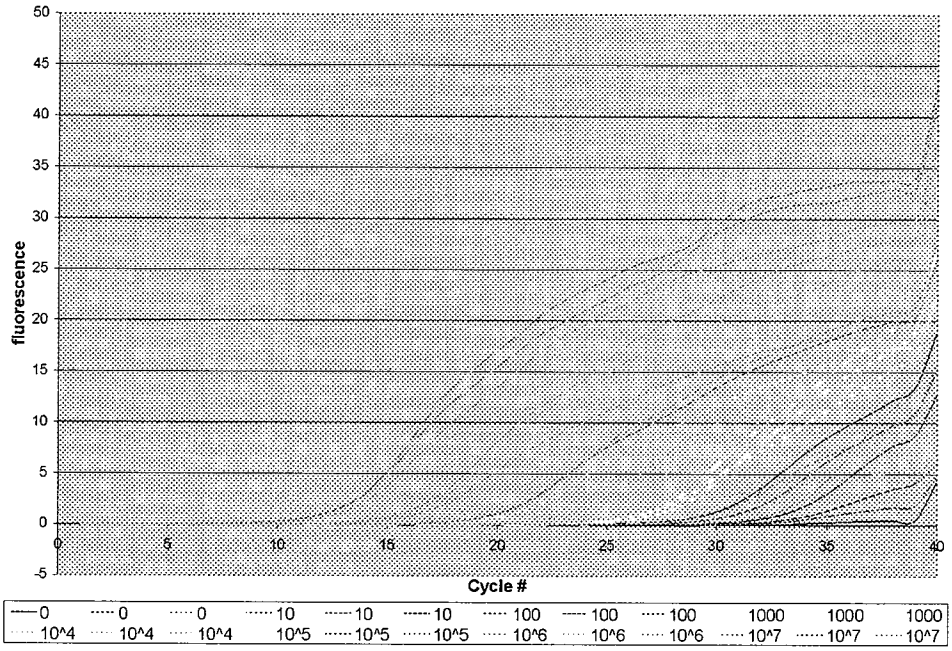
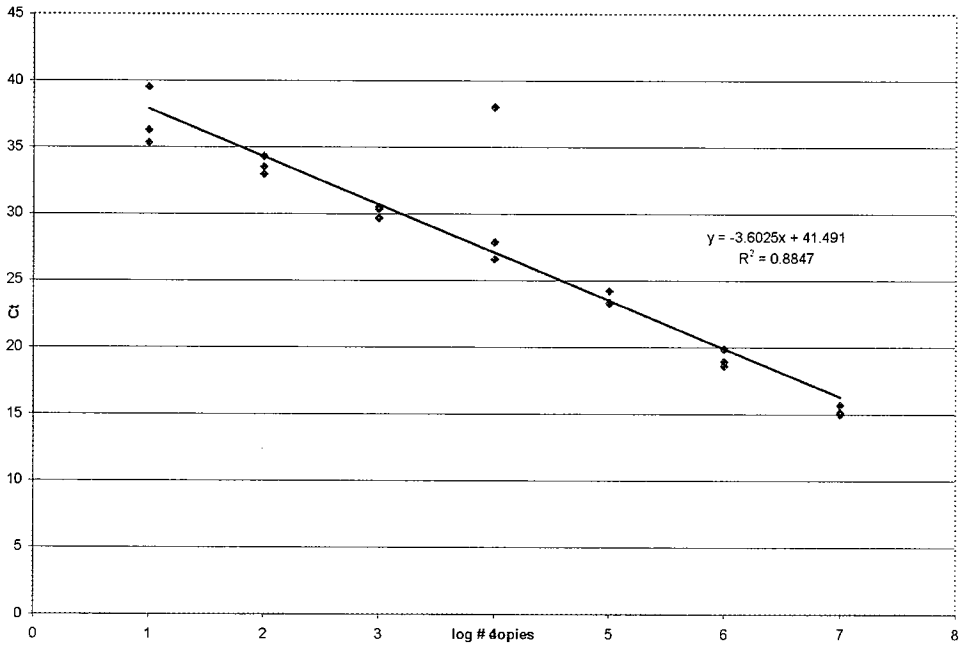


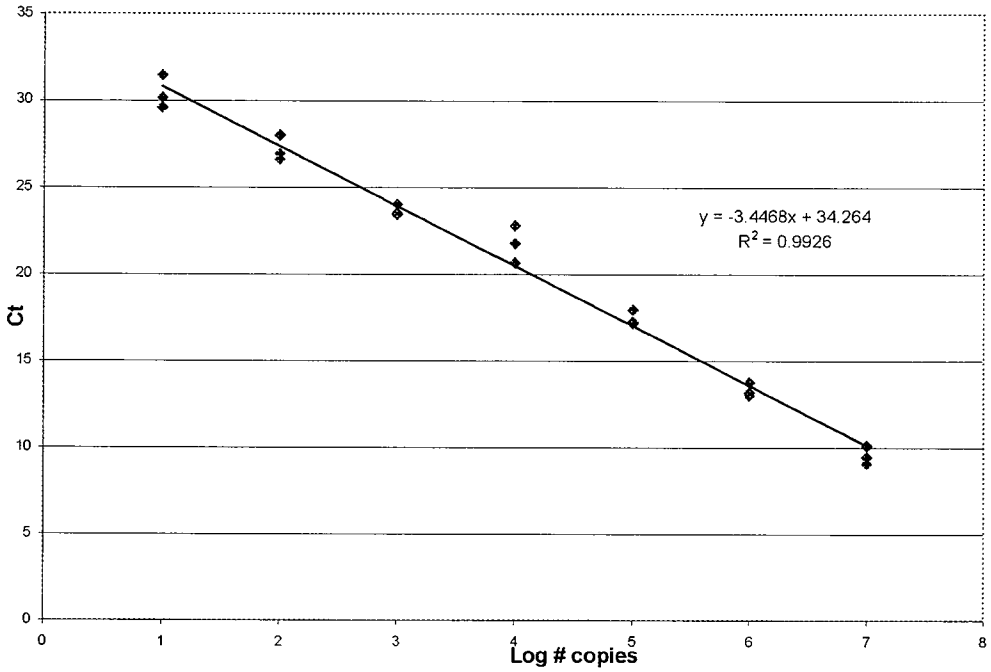
Figure 5: Ct curve for 5/26/08 at a instrument-determined threshold of 5.149285



A comparison of results in Figures 5 and 6 show that lowering the threshold for detection from ~5.15 (Figure 5) to 0.2 (Figure 6) increases the R^2 value from 0.88 to 0.99 in the fit of the standard curve of control DNA concentrations. In the example with the higher threshold, the limit of detection was 10^4 copies per reaction, due to that concentration having a relative standard deviation above 20. When the data were analyzed with a lower threshold, no data point had a relative standard deviation above the threshold of 20, and in this analysis the limit of

detection is approximately 10 copies per reaction. For these reasons, a cycle threshold of 0.2 was used for all further experiments unless otherwise noted.

Figure 6: Ct curve for 5/26/08 at a threshold of 0.2

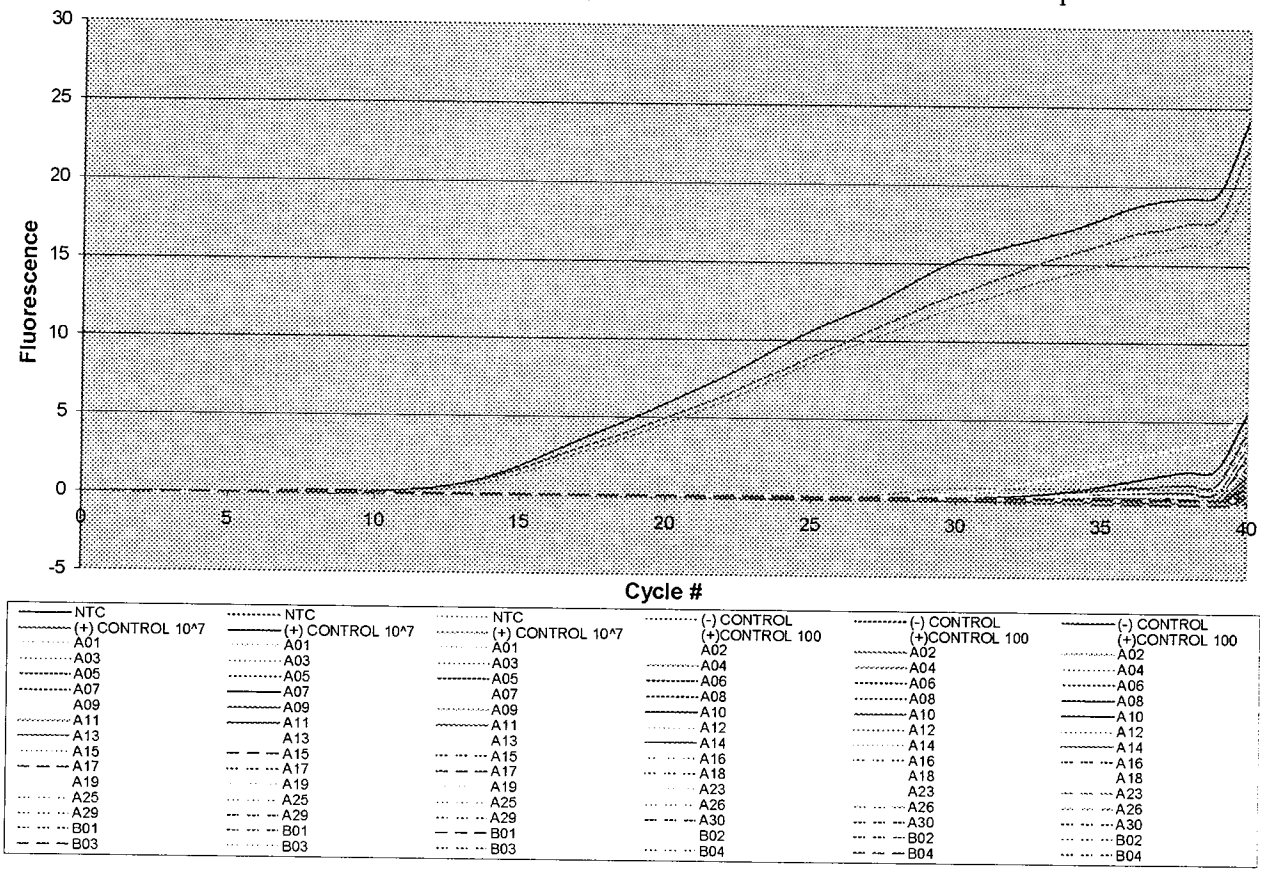


The above described set of qPCR standard curve experiments was repeated using DNA extracted from the *Brevibacterium* clone as a template, both with and without extending the elongation time. The results of these experiments matched previous results, in which a short (30 sec) elongation time gives a sporadic fluorescence curve, while the reaction with the longer elongation time had the characteristic, expected sigmoid functions for fluorescence versus time. Thus, the extended elongation time (1.0 min) for the qPCR reaction was used in all subsequent studies.

With a working qPCR protocol now in hand, a qPCR analysis was performed on 6/3/2008 using 15 ng DNA as template per reaction for environmental samples A01-B04 provided by North Wind.

Results in Figure 7 showed that only a small number of DNAs from the environmental samples crossed a threshold of 0.2. For all samples, the average Ct was lower then the average Ct of a negative control of DNA extract from cow feces. This result suggests that there may be inhibitors present in the environmental DNAs that interfered with the qPCR reactions at the 15 ng concentrations used. This was the DNA concentration suggested in the North Wind protocol provided us.

Figure 7: Fluorescence curve for 6/03/2008 experiments on blind environmental samples.



III. Varying concentration of DNA to avoid the effect of PCR inhibitors. To determine if qPCR inhibitors were present in DNA samples, conventional PCR was performed with the LA35 primer set on 6/3/2008. In our studies, we used DNA from a cow fecal extract as a positive control for fecal bacteria and for a negative control for reaction with the poultry-specific PCR primers. The cow fecal sample used to extract DNA for the positive control came from a farm in south-eastern Minnesota in March of 2008. The PCR reaction conditions used was based on the PCR protocol for 16S rDNA universal bacterial primers given to us by North Wind. Each 25 µl reaction contained the following: 1 µl of unadjusted (as provided by North Winds) template DNA, 1 x Promega clear GoTaq PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM each of the forward and reverse LA35 primers, 0.02 U per µl of Taq, and sterile water to 24 µl. The thermal cycler protocol started with a 5 minute incubation at 94°C, followed by 40 cycles of 1 minute of dissociation at 94°C, 1 minute of annealing at 53.5°C, and 50 seconds of elongation at 72°C. Following incubation was a 7 minute final elongation step at 72°C, and samples were held at 4°C.

Figure 8(a): Conventional PCR of 1 µl environmental samples with LA35primers

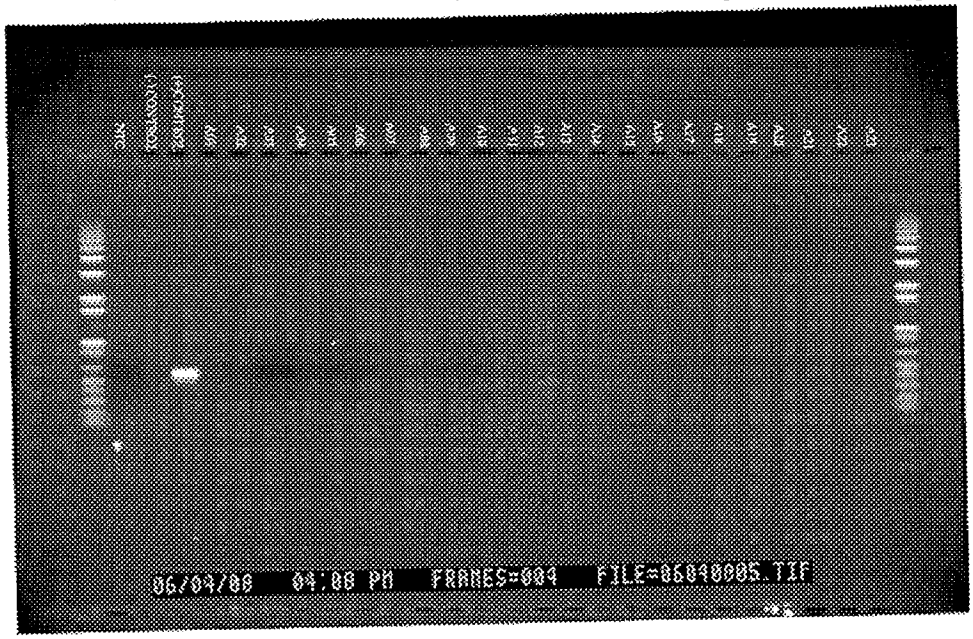
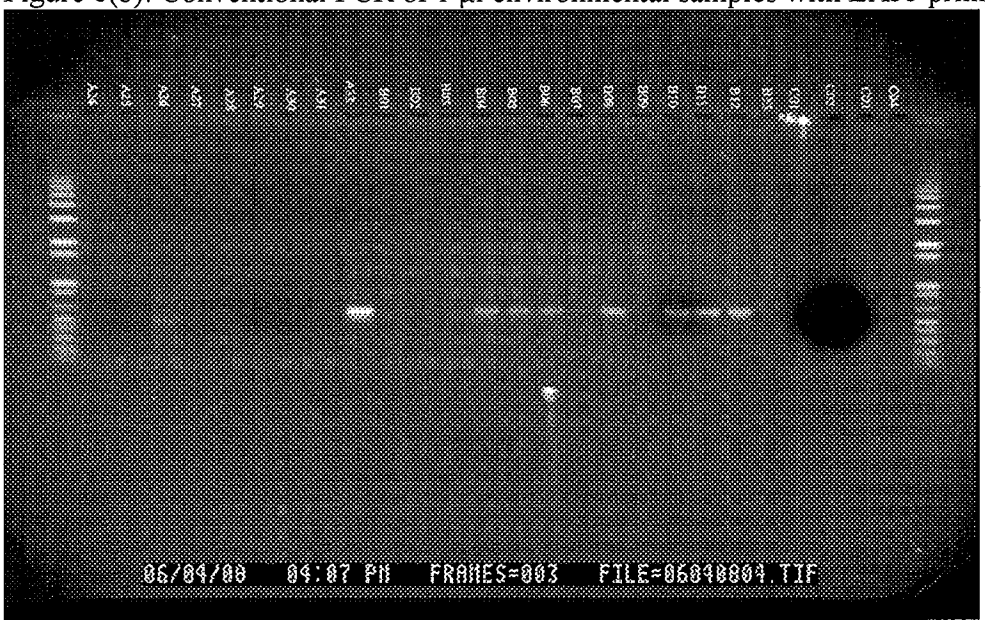



Figure 8(b): Conventional PCR of 1 µl environmental samples with LA35 primers