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February 22, 2010

Philip Derfler, Assistant Administrator
Office of Policy and Program Development
USDA-Food Safety and Inspection Service
1400 Independence Avenue, SW
Room 350-E Jamie Whitten Building
Washington, DC 20250

RE: Petition Number 09-03: Petition for an Interpretive Rule Declaring all enterohemorrhagic Shiga Toxin-producing Serotypes of *Escherichia coli* (*E. coli*), including Non-O157 Serotypes, to be Adulterants Within the Meaning of 21 U.S.C. § 601(m)(1)

Dear Mr. Derfler:

I am writing to thank you for your letter dated January 27, 2010, responding to my request for an update on the status of the review of the above-referenced petition. I applaud the fact that FSIS has granted the petition's request for expedited review, pursuant to 9 CFR 392.8, and is thus reviewing the petition ahead of other pending petitions that request actions that are not related to food safety.

In your letter you mentioned that the Agency is studying laboratory capacity and available data to determine how it will proceed regarding this issue. Specifically, you state that the Agency "intends to carefully consider all relevant data made available to the Agency on non-O157 STEC to determine how it should address the presence of these microorganisms in or on the products it regulates." In order to aid this process, I would like to submit additional materials regarding non-O157 STEC testing as they become available to me, and will plan on continuing to submit the materials to Mary Porretta. Please let me know if there are additional persons to which I should address these materials.

It is worth noting that your sister agency, the FDA, has already developed and implemented a PCR method to identify isolates that belong to six prevalent EHEC serotypes. FDA scientists detailed their research results in a 2007 paper entitled "Identification of Shiga toxigenic Escherichia coli seropathotypes A and B by multiplex PCR" (see attached). Since the publication of this paper, FDA scientists have additionally developed a method to indetify *E. coli* O45 isolates. With these recent developments, EHEC detection has become more feasible than ever before. As you know, we used these methods in our own study on retail ground beef in 2008 (see attached). As was the case with *E. coli* O157:H7 detection and testing, however, these tests will only be implemented on a widespread basis when FSIS declares that these deadly pathogens are adulterants.

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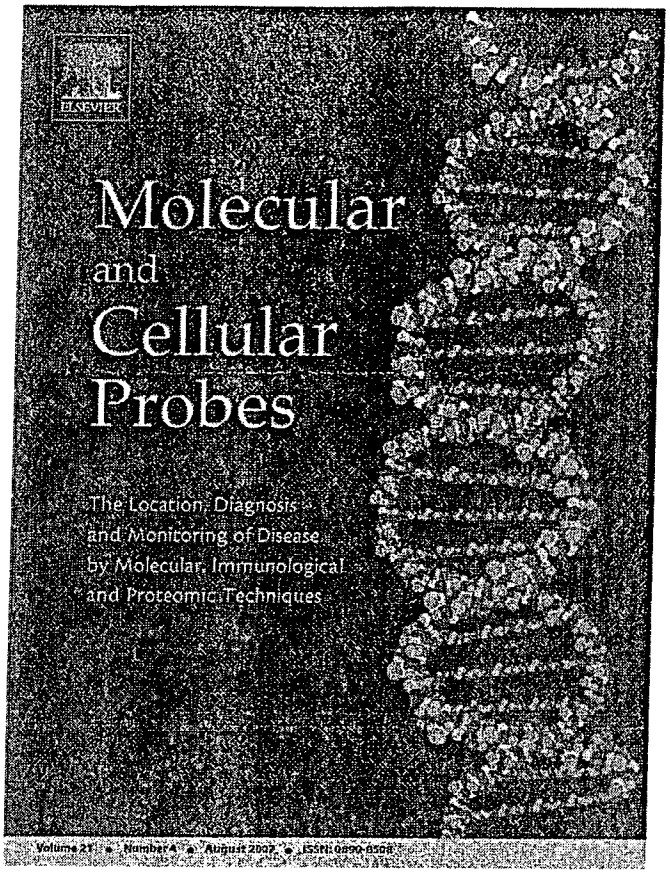
I would like to follow the Agency's progress regarding the development of laboratory capacity to detect and isolate various non-O157 STEC groups, as well as the recommendations of the Agency scientists that have been assembled to study the available data on non-O157 STEC. I would therefore ask that you let me know the best way for me to stay informed about the progress of work on the petition. Ideally, I would like to get written progress reports, but the Agency may not be in a position to routinely generate these. Accordingly, I may write to request updates every three months, and may also make requests under the Freedom of Information Act. Of course, if you have another suggestion as to how I might stay informed about this, please let me know. Thank you.

Very truly yours,

William Marler, Esq., on behalf of:

Marler Clark LLP, PS
Outbreak, Inc.
The Family of June Dunning
Megan Richards
Shiloh Johnson

cc: Mary Porretta



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Short communication

Identification of Shiga toxigenic *Escherichia coli* seropathotypes A and B by multiplex PCR

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Abstract

A multiplex PCR assay was developed to identify the six clinically important enterohemorrhagic *Escherichia coli* (EHEC) serotypes classified in seropathotypes A and B and to differentiate these from Shiga toxigenic *E. coli*. The assay simultaneously detects genes for Shiga toxin (*stx*) and intimin (*eae*), including allelic variants of both genes, 16S internal amplification control, as well as unique sequences in the *wzx* genes that are specific for serotypes O157, O26, O111, O103, O121 and O145. PCR analysis of 40 representative strains showed that the assay correctly identified the virulence genes, if present, and the respective O antigen type of all the strains, including some atypical EHEC, as well as enteropathogenic *E. coli* and *E. coli* strains examined.
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Keywords: Multiplex PCR; Enterohemorrhagic *E. coli*; Seropathotypes

There are over 200 Shiga toxin (Stx)-producing *Escherichia coli* (STEC) serotypes, but many have not been implicated in illness [1]. Enterohemorrhagic *E. coli* (EHEC), however, is a subset of STEC and is comprised of recognized human pathogens. EHEC infections result in hemorrhagic colitis, which may progress into the life-threatening, hemolytic uremic syndrome (HUS). Serotype O157:H7 is the prototypic EHEC that causes most of the food-borne outbreaks, but other EHEC serotypes are increasingly causing illness worldwide [1]. For example, O111 and O26 are the second most common causes of HUS in the US [2] and Europe [3], respectively, and atypical sorbitol-fermenting O157 (SFO157) are also causing HUS in Europe [4].

EHEC are phenotypically diverse, so diagnostic assays have to test samples first for Stx and, if positive, it is plated and several colonies are pooled and retested until an Stx-producing isolate is obtained and serotyped. This approach is tedious but also cannot distinguish EHEC from the STEC serotypes that have not been implicated in illness. Similarly, O type is not correlated with pathogenicity, so serotyping may also be of limited use, as some *E. coli* and EHEC have the same O type. To better delineate EHEC, Karmali et al. [5] proposed to classify STEC into seropathotypes based on incidence, outbreak frequency and severity of illness. Seropathotype A consisted of O157:H7 and its non-motile (NM) variants; B had other EHEC serotypes that have often caused severe illness; C had strains that have caused outbreaks but are rare; and D and E had strains that did not cause severe illness or are non-pathogenic. Phylogenetic studies of STEC showed good correlations between seropathotypes and incidence of disease [6].

The major virulence factors carried by seropathotype A and B strains are Stx and intimin and these two factors can be reliable predictors for occurrence of HUS [7,8]. Stx_1 and Stx_2 are commonly implicated in infections but there are many alleles, especially of Stx_2, [1] and some may also cause human illness. Intimin enables bacterial attachment to epithelial cells and is also a virulence factor of enteropathogenic *E. coli* (EPEC). There are many intimin alleles that are carried by various EHEC and EPEC strains [9]. We developed a multiplex PCR to simultaneously detect major EHEC virulence genes and the O-type genes of STEC in seropathotypes A (O157:H7 and O157:NM)

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and B (O26:H11, O111:NM, O121:H19, O145:NM and O103:H2) that comprises the majority of EHEC serotypes of public health concern.

The genetic sequences of stx_1, stx_2 and stx_2 alleles were compared to design a single primer pair (Table 1) that was confirmed to detect both stx_1 and stx_2, and based on sequence analysis, also detects stx_{2c}, stx_{2d} and stx_{2e} genes. Similarly, a single primer pair specific for the homologous regions within 15 different intimin (*eae*) alleles (Table 1) was designed and confirmed by PCR to detect 13 different *eae* alleles. The Wzx protein (*wzx*) is required for O lipopolysaccharide export across the plasma membrane [10] and there is poor Wzx and *wzx* homology among serotypes, hence, *wzx* sequences unique to each serotype were used to design O type-specific primers (Table 1). A primer pair to 16S rRNA was also included as internal PCR amplification control.

A mix of all nine primer pairs were made using the concentrations shown in Table 1. DNA templates were made by suspending a colony in 100 µl of water and heated for 5 min in a boiling water bath or by DNA extraction using the Masterpure DNA Purification kit (EpiCentre, Madison, WI). The 50 µl PCR mix contained 1 × *Taq* Polymerase buffer (Qiagen, Valencia, CA), 3 mM $MgCl_2$, 300 µM of dNTP, 2 µl of crude template or 100 ng of DNA, 1 × primer mix and 2.5 U of HotStar*Taq* (Qiagen). The "touchdown" PCR [11] conditions were 95 °C for 15 min; 10 cycles of 95 °C for 30 s, 68–59 °C (–1 °C/cycle) for 20 s, 72 °C for 52 s, followed by 35 cycles of 95 °C for 30 s, 59 °C for 20 s, 72 °C for 52 s and a 72 °C for a final 1 min extension. Amplicons were examined by agarose gel electrophoresis in 1 × Tris–borate–EDTA buffer, pH 8.2 or with the Agilent DNA 1000 kit and the 2100 BioAnalyzer (Agilent, Palo Alto, CA).

Multiplex EHEC PCR was tested using a panel of strains (Table 2), and the data for selected strains are shown in Fig. 1. Both crude and purified DNA gave satisfactory results, but the latter gave cleaner bands. Also, it was essential to run a 1.75% agarose gel for at least 2 h at 125 V to resolve all the bands (Fig. 1, bottom), but a faster and more practical alternative was to use the Agilent BioAnalyzer, which used only 1 µl of the post-PCR mix and effectively resolved all the products within 45 min (Fig. 1, top).

The multiplex EHEC PCR assay reliably detected the presence of *stx*, *eae* and the O type for each of the strains tested, by yielding amplicons consistent with the serotypes and virulence genes carried by each strain (Table 2). The panel included Stx non-producing O157:H7 (43888 and TT12B) and O111 (01277 and 43887) strains and atypical SFO157 strains (493-89 and 9160) (Fig. 1 and Table 2). Analyses of eight *E. coli* that did not carry any of the target genes showed no bands except for the 16S amplicon (Table 2). Both EPEC and EHEC include O26 and O111 serotypes, hence an O26 EPEC (01200) and two other EPEC strains (not shown) were tested. EPEC were distinguished from EHEC by the presence of *eae* but the absence of *stx* bands (Fig. 1, ln. 2). To study if there were primer conflicts, a pool of DNA from all six serotypes was tested. As expected, the intensity of *stx* and *eae* bands was enhanced by the presence of multiple copies, but all the expected bands, except for the O111 product, were still

Table 1
Primers and concentrations used in the multiplex EHEC PCR assay

Gene[a]	Primer	nM	Sequence	Amplicon (bp)
stx1/2	SRM128	240	CTGATTGTTGAGCGAAATAATTTATATGTG	528
	SRM129	240	TGATGATGACAATTCAGTATAACTGCCAC	
eae	SRM103	120	GTTCACTGGACTTCTTATTACCG	482
	SRM104	120	ATCGTCACCAGAGGAATCG	
16S	VMP5	120	AGAAGCACCGGCTAACTC	204
	VMP6	120	CGCATTTCACCGCTACAC	
wzx	5′O26	400	ACTCTTGCTTCGCCTGTT	268
	3′O26	400	CAGCGATACTTTGAACCTTAT	
wzx	5′O103	200	TATCCTTCATAGCCTGTTGTT	320
	3′O103	200	AATAGTAATAAGCCAGACACCTG	
wzx	5′O111.3	200	GTTGCGAGGAATAATTCTTCA	829
	3′O111.2	200	CCATAGATATTGCATAAAGGC	
wzx	5′O121	100	GTAGCGAAAGGTTAGACTGG	651
	3′O121	100	ATGGGAAAGCTGATACTGC	
wzx	5′O145.6	200	TTGAGCACTTATCACAAGAGATT	418
	3′O145.B	200	GATTGAATAGCTGAAGTCATACTAAC	
wzx	5′O157	300	GCTGCTTATGCAGATGCTC	133
	3′O157	300	CGACTTCACTACCGAACACTA	

GenBank Accession numbers for each of the gene targets are shown below the table.

[a] *stx* (stx_1, AB035142; stx_2, X07865; stx_{2c}, AJ567994; stx_{2d}, AJ567997; stx_{2e}, AJ567999; stx_{2f}, AJ270998); *eae* (α, M58145; β, AF200363; δ, AJ875027; ε, AF116899, AF530554; η, AJ308550, AJ876652; γ, AF071034; κ, AJ308552; τ, AJ308551; λ, AJ715409; μ, AF530553, AJ705049; ν, AJ705050; ο, AJ876648; θ, AF449418; ξ, AF530556, AF715407, AJ705051; and ζ, AJ271407); O26*wzx* (AF529080); O103*wzx* (AY532664); O111*wzx* (AF078736); O121*wzx* (AY208937); O145*wzx* (AY647260); O157*wzx* (AF061251).

Table 2
EHEC multiplex PCR results of EHEC strains examined

Strain	Source[a]	Serotype[b]	*stx*	*eae*	O157	O26	O111	O121	O145	O103	16S
35150	ATCC	O157:H7	+	+	+	–	–	–	–	–	+
43888	ATCC	O157:H7	–	+	+	–	–	–	–	–	+
43895	ATCC	O157:H7	+	+	+	–	–	–	–	–	+
6321	FDA	O157:H7	+	+	+	–	–	–	–	–	+
6391	FDA	O157:H7	+	+	+	–	–	–	–	–	+
MA6	FDA	O157:H7	+	+	+	–	–	–	–	–	+
G5101	CDC	O157:H7	+	+	+	–	–	–	–	–	+
TT12B	Takeda	O157:H7	–	+	+	–	–	–	–	–	+
493–89	TW	SFO157	+	+	+	–	–	–	–	–	+
9160	TW	SFO157	+	+	+	–	–	–	–	–	+
400	FDA	O26:H11	+	+	–	+	–	–	–	–	+
13C07	FDA	O26:H11	+	+	–	+	–	–	–	–	+
13C57	FDA	O26:H2	+	+	–	+	–	–	–	–	+
13C60	FDA	O26:H11	+	+	–	+	–	–	–	–	+
6438	FDA	O26:H11	+	+	–	+	–	–	–	–	+
01200	TW	O26:NM	–	+	–	+	–	–	–	–	+
05554	TW	O26:NT	+	+	–	+	–	–	–	–	+
00971	TW	O26:H11	+	+	–	+	–	–	–	–	+
403	FDA	O111:NM	+	+	–	–	+	–	–	–	+
405	FDA	O111:NM	+	+	–	–	+	–	–	–	+
00186	TW	O111:H8	+	+	–	–	+	–	–	–	+
01387	TW	O111:H8	+	+	–	–	+	–	–	–	+
01277	TW	O111:H2	–	+	–	–	+	–	–	–	+
07809	TW	O111:NM	+	+	–	–	+	–	–	–	+
13C56	FDA	O111:NT	+	+	–	–	+	–	–	–	+
43887	ATCC	O111:NT	–	+	–	–	+	–	–	–	+
13B96	FDA	O121:H19	–	+	–	–	–	+	–	–	+
08023	TW	O121:H19	+	+	–	–	–	+	–	–	+
08868	TW	O121:H19	+	+	–	–	–	+	–	–	+
01671	TW	O121:H7	–	–	–	–	–	+	–	–	+
07927	TW	O121:NM	+	+	–	–	–	+	–	–	+
07596	TW	O145:NM	+	+	–	–	–	–	+	–	+
07865	TW	O145:H18	+	–	–	–	–	–	+	–	+
08087	TW	O145:NM	+	+	–	–	–	–	+	–	+
13C62	FDA	O103:H2	+	+	–	–	–	–	–	+	+
04112	TW	O103:H6	+	+	–	–	–	–	–	+	+
04162	TW	O103:H6	+	+	–	–	–	–	–	+	+
07990	TW	O103:H6	+	+	–	–	–	–	–	+	+
07920	FDA	O103:H2	+	+	–	–	–	–	–	+	+
07597	FDA	O103:H2	+	+	–	–	–	–	–	+	+
8 *E. coli* strains	NA	–	–	–	–	–	–	–	–	–	+

[a]ATCC, American Type Culture collection; TW, STEC Center; NA, not applicable.
[b]NM, non-motile; NT, H type undetermined.

observed (data not shown). The absence of the O111 band remains unknown, but as the assay is intended for isolate identification and characterization, interference by mixed populations is remote.

Because of assay kinetics and multiple targets, most PCR or real-time PCR (rt-PCR) assays for EHEC only target O157:H7 and a few other notable serotypes or have to use a series of assays for broader detection. For example, one assay used a multiplex PCR to detect stx_1, stx_2, *eae* and the enterohemolysin gene (*ehxA*) and another multiplex PCR to identify serotype-specific genes [12]. A 5′-nuclease rt-PCR for eight clinically important STEC had to use one assay to detect stx_1 and stx_2, followed by separate and individual assays to identify O26, O55, O91, O111, O103, O113, O145, O157 and H7-specific genes [13]. The EHEC multiplex PCR described here offers the advantage that all six clinically significant EHEC in seropathotypes A and B, two major EHEC virulence genes, including allelic forms, plus an internal amplification control can be detected simultaneously in one reaction. The assay is based on the premise that strains with only an O-type amplicon are generic *E. coli*. Strains with O-type and *eae* bands but no *stx* are presumably EPEC, while those that have *stx* but no *eae* are STEC of that serotype. But, a strain carrying *stx*, *eae* and an O-type amplicon is very likely an EHEC of that serotype.

PREVALENCE OF NON-O157 ENTEROHAEMMORRHAGIC ESCHERICHIA COLI IN RETAIL GROUND BEEF IN THE UNITED STATES

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Purpose Enterohaemorrhagic *Escherichia coli* (EHEC) are associated with human diseases ranging from diarrhea to hemolytic-uremic syndrome (HUS). E. coli O157:H7 is the EHEC most often isolated in patients in the US causing an estimated 73,000 illnesses annually. Non-O157 EHEC also causes illness and outbreaks. More than 37,000 illnesses are attributed to non-O157 EHEC serotypes. In the US the most common non-O157 EHEC serogroups in humans are O26, O45, O10, O111, O121, and O145. The incidence of non-0157 EHEC is increasing despite limited laboratory testing. The purpose of this study was to determine the prevalence of non-O157 EHEC in retail ground beef in the US.

Materials and Methods Meat samples were selectively enriched for *E. coli* O157, EHEC, and Salmonella spp. then tested by IEH Polymerase Chain Reaction (PCR) test kits. Sample enrichments screened positive by PCR were culture confirmed by isolating the target organisms. The organisms were confirmed and samples were scored as positive.

Each sample (200g) was weighed into a sterile Whirlpak, 69oz. filter bag followed by the addition of 200-250ml IEH Enrichment Broth. The sample was macerated by hand homogenizing. An additional portion of IEH Enrichment Broth was added to bring broth volume to 500ml. The bag was mixed until uniformly homogenized. Enrichment bags were incubated at 42°C for 24 hours. To minimize contamination, after enrichment incubation time was completed, the broth was well mixed and a 10ml aliquot was removed to sterile test tube as working stock.

Aliquots of enrichment were tested for typical gene signals using IEH-*E. coli* EHEC Test System. Presumptive positive cultures were subjected to cultural confirmation. Presumptive colonies (lactose positive, *E. coli* type colonies) were picked to a gridded Washed Sheep Blood Vancomycin-Cefsulodin-Cefixime (BVCC) plate to test for hemolysin activity. All presumptive target positive colonies were tested by PCR test kit for gene markers indicative of EHEC. Colonies testing positive for EHEC by PCR were checked for purity on MacConkey agar, confirmed as latex agglutination negative for *E. coli* O157, and confirmed by PCR for E. coli serotype specific markers. Colonies confirmed as EHEC by both PCR and negative latex agglutination were scored as positive.

Results and Discussions A total of 1216 retail ground beef samples were tested for the presence of EHEC. Twenty-three samples (1.9%) were positive for non-O157 EHEC strains. Serotypes included O26 (n=6), O103 (n=7), O113 (n=1), O121 (n=6) and O145 (n=3). All but the EHEC isolate serotype O113 were *Stx* and *eae* positive. The O113 strain was $Stx2d_{act}$ and Subtilase positive.

Prevalence of 1.9% non-9157 EHEC in the retail ground beef supply shows the need for public health agencies in the US to increase awareness regarding these pathogens. The data clearly show that clinical and public health laboratories should routinely screen human and environmental specimens for the presence non-O157 EHEC.