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Development of a multiplex PCR assay for diarrheagenic *Escherichia coli* and *Shigella* spp. and its evaluation on colonies, culture broths, and stool

Mami Taniuchi[1], **Carol C. Walters**[2], **Jean Gratz**[1,3], **Athanasia Maro**[3], **Happiness Kumburu**[3], **Oralak Serichantalergs**[4], **Orntipa Sethabutr**[4], **Ladaporn Bodhidatta**[4], **Gibson Kibiki**[3], **Denise M. Toney**[2], **Lynette Berkeley**[5], **James P. Nataro**[6], and **Eric R. Houpt**[1]

[1]Division of Infectious Diseases and International Health, University of Virginia, PO Box 801340, Charlottesville, Virginia 22908 [2]Division of Consolidated Laboratory Services, Virginia Department of General Services, 600 North 5th Street, Richmond, VA 23219 [3]Biotechnology Laboratory, Kilimanjaro Christian Medical Centre, PO Box 3010, Moshi, Tanzania [4]Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences, 315/6 Rajvithi Road, Phyathai, Bangkok 10400, Thailand [5]Center for Vaccine Development, University of Maryland, 685 West Baltimore Street, Room 480, Baltimore, MD 21201 [6]Department of Pediatrics, University of Virginia, PO Box 800386, Charlottesville, VA 22908

Abstract

Detection of diarrheagenic *E. coli* (DEC) typically depends on identification of virulence genes from stool cultures, not on stool itself. We developed a multiplex PCR assay that detects key DEC virulence genes (*stx1*, *stx2*, *eae*, *bfpA*, *ipaH*, *LT*, *STh*, *aaiC*, *aatA*). The assay involved a multiplex PCR reaction followed by detection of amplicon (s) using Luminex beads. The assay was evaluated on over 100 colony and broth specimens. We then evaluated the assay using DNA extracted from stool, colony pools and gram-negative broths, using stool spiked with known quantities of DEC. Performance of the assay on stool DNA was most quantitative, while stool broth DNA offered the lowest limit of detection. The assay was prospectively evaluated on clinical specimens in Tanzania. Stool DNA yielded higher sensitivity than colony pools compared with broth DNA as the standard. We propose using this assay to screen for DEC directly in stool or stool broths.

Introduction

Diarrhea is a major cause of global childhood mortality, leading to 1.5 million deaths each year or 15% of attributable mortality (Fischer Walker et al. 2010). The list of

Corresponding author address: Mami Taniuchi, Department of Medicine, Division of Infectious Diseases and International Health, University of Virginia, 345 Crispell Dr., MR6 Room 1714, Charlottesville, VA 22908. Telephone: 434-924-5575, Fax: 434-924-0075, mt2f@virginia.edu.

enteropathogens that can cause diarrhea is long and includes bacteria, viruses, protozoa, and helminths. DEC are major bacterial causes, however since *E. coli* are also a major component of normal microflora, identifying those with diarrheagenic potential requires cumbersome phenotypic or genotypic testing for virulence determinants. On the basis of these virulence determinants DEC are often categorized into Shiga-toxin producing (STEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), and an EAEC subset termed Diffusely Adherent *E. coli*. A fatal outbreak of sprout-associated *E. coli* recently occurred in Germany, and this strain had both Shiga-toxin producing (*stx2*) and enteroaggregative features (Rasko et al. 2011).

Given the complexity of phenotypic assays to discriminate the DEC, PCR methods that amplify DEC virulence genes have proliferated, including singleplex PCR, multiplex PCR followed by gel-based analysis, and real-time PCR. The number of virulence targets that one needs to detect is often 8 or more, including chromosomal, plasmid, and phage DNA, thus the multiplex assays often need to be separated into multiple reactions. As examples, the assay of Fujioka et al. for *stx1*, *stx2*, *eae*, *invE, astA*, *aggR*, *ST,* and *LT* genes was separated into two amplifications (Fujioka et al. 2009). Aranda et al. also described two amplifications for *eae*, *bfpA*, CVD432, *LT*, *ST*, *ipaH*, and *stx1* (Aranda et al. 2004). Gel electrophoresis is often used to distinguish amplicons, although band size discrimination can be difficult. Guion et al. described an 8-plex assay for DEC that used a real-time PCR platform and melt curves to discriminate products (Guion et al. 2008). In contrast to these methods, we describe a single multiplex PCR reaction that amplifies 9 virulence targets where amplicons are distinguished using sequence-specific probes on a Luminex platform. Advantages to this technique are the additional layer of specificity due to the probes and the ability to add additional targets given the multiplexing capability of the Luminex platform.

Most *E. coli* detection schemes utilize *E. coli* cultures. Specifically, stool is cultured on solid media such as MacConkey agar, a variable number of lactose fermenting colonies are picked to sample a diversity of *E. coli* strains, after which testing for virulence genes occurs. Both the culture and colony picking steps can be problematic. In many settings individuals with diarrhea are treated empirically with antibiotics, which hamper the sensitivity of culture. Picking colonies is tedious and creates an enormous amount of work for the laboratory. Most laboratories, therefore pool colonies for testing (Barletta et al. 2009), however the number of colonies to pool remains an open question. Galbadage et al. found that testing 20 colonies detected ETEC in twice as many individuals as testing 5 (Galbadage et al. 2009). This suggests potentially significant underestimation of DEC based on the colony picking process alone. For these reasons we investigated the performance of our assay on colonies versus broths, where theoretically all growth can be sampled, versus stool itself.

Materials and Methods

Strains, cultured specimens, and spiked materials

Positive control materials used in this study included reference *E. coli* strains EHEC 0157:H7, EPEC 2348/69, EIEC O124, ETEC H10407, and EAEC O42. Other materials included clinical DEC isolates from Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand and stool-containing broth specimens from the Virginia

Division of Consolidated Laboratory Services (DCLS). Negative controls included *E. coli* strain ATCC 25922 (no virulence genes) and water. For sensitivity testing, individual reference strains of known colony forming units (cfu) were spiked into 200 mg aliquots of stool from a healthy DEC-negative donor and stored at −80°C until further processing or DNA isolation. Spiked stool specimens were plated onto MacConkey agar plates and incubated it at 37°C overnight, and inoculated a swab into 2ml of gram-negative (GN) broth followed by incubation for 16 hours in a shaking 37°C incubator.

Clinical stool specimens

Fifty-four fecal samples from inpatients with diarrhea were prospectively tested at Kilimanjaro Christian Medical Center in Moshi, Tanzania. All protocols were approved by the Ethics Committees of KCMC and the institutional review boards of the University of Virginia (UVA). Fresh stool samples were plated onto MacConkey agar using a sterile polyester swab and a GN broth (Becton Dickinson, Franklin Lakes, NJ) was inoculated with a pea-sized piece of stool. After overnight incubation 5 morphologically distinct lactose-fermentingcolonies were picked from the MacConkey agar and subcultured onto Trypticase Soy Agar with 5% Sheep blood (Becton Dickinson, Franklin Lakes, NJ).

DNA extraction

Lactose-fermentingcolonies were picked from the MacConkey agar, subcultured and suspended in 1 ml 0.05% Triton solution in a 2 ml screw top tube. The solution was briefly vortexed, heated at 100°C for 10 min, then centrifuged at 13000 rpm for 3 min, and 5 μl of supernatant was used for PCR. DNA was extracted from the GN broth by boiling or by using the QuickGene-810 and the QuickGene DNA tissue kit S (Fujifilm, Tokyo, Japan; http://www.fujifilm.com/products/life_science_systems/nucleic_acid_isolation/guides/pdf/common/D_Helicobacter_pylori_E.pdf). Stool DNA was isolated from 200 mg of stool using a slightly modified QIAmp Stool Mini Kit protocol (Qiagen Inc., Valencia, CA). Briefly, 1.4 ml of ASL buffer was added to the stool then pretreated by bead beating with 0.15-mm garnet beads (MO-BIO Laboratories, Carlsbad, CA) for 2 min followed by boiling for 7 min before continuing with the manufacturer's extraction protocol. All DNA samples were stored at −80°C prior to use in PCR.

Luminex Multiplex PCR

Accepted virulence genes were targeted for DEC identification. Primer sequences for *stx1* (Hidaka et al. 2009), *stx2* (Paton and Paton 1998), *ipaH* (Vu et al. 2004), *ial* (Frankel et al. 1990), *LT* (Hidaka et al. 2009), *aaiC* (Boisen et al. 2008), and *aatA* (Schmidt et al. 1995) have been described previously (Table 1). Other primers were designed for targets that did not amplify efficiently in multiplex or when the amplicon was not suitable for Luminex detection. Multiple primer and probe combinations were evaluated before the final optimized multiplex PCR reaction. PCR amplification for the 9-plex reaction was performed in 25 μl volume with 12.5 μl of Qiagen Multiplex PCR Master Mix (Qiagen, Valencia, CA, USA) which contains dNTPs and 3 mmol/L $MgCl_2$ final concentration, 0.2 μmol/L of each forward primer, 0.2 μmol/L of each reverse biotinylated (modified with biotin-TEG) primer, 5 μl Q-solution, 0.5–3.5 μl of nuclease free water (NFW) and 2–5 μl of DNA template. PCR was performed in a 9-plex at AFRIMS (*ial* target instead of *ipaH* target) and in a 6-plex at

DCLS (targets included were *stx1*, *stx2*, *LT*, *STh*, *eaeA*, *bfpA*). PCR cycling condition consisted of an initial 15-min 95°C step followed by 40 cycles of 30 sec at 94°C, 30 sec at 60°C, 60 sec at 72°C, and a final 10-min extension 72°C step. Positive controls and negative controls (NFW and ATCC 25922 DNA template) were included in every run.

Luminex detection

Luminex detection was performed using amplicon specific internal probes as previously described (Taniuchi et al. 2011a). Probes were amine modified at the 5′ terminus with 12 carbon spacers and covalently hybridized to the carboxylated Luminex beads using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride. Previously published real-time probe sequences were utilized for detection of *ipaH* (Vu et al. 2004), *ial* (Frankel et al. 1990), and *LT* (Hidaka et al. 2009) amplicon. All other probes were designed for this study (Table 1). Hybridization of the amplicon to the beads was performed at 50°C for 35min using the Oligonucleotide Hybridization Protocol from Luminex (Luminex 2006). After addition of streptavidin PE to the bead-amplicon complex, detection was performed on either a Bioplex 200 (Bio-Rad, Hercules, CA, USA) or a Luminex 100 (Luminex Corporation, Austin, TX, USA) instrument.

Comparison Methods

For initial assay evaluation (Table 2), isolates or broths were identified by existing methods. The AFRIMS specimens were tested by DNA probe hybridization assay using digoxigenin labeled probes for *LT* (Sommerfelt et al. 1990), *STh* and *STp* (Sommerfelt et al. 1990), *ial* (Echeverria et al. 1990), *stx1* and *stx2* (Macario and Conway de Macario 1990), *bfpA* (Donnenberg et al. 1992; Giron et al. 1993), and *eae* (Jerse and Kaper 1991; Donnenberg et al. 1993). The DCLS broths were tested using in-house methods (PCR, Enzyme Immunoassay, and culture). Other strains (ATCC isolates 35401, 43893, and 11175, EDL 933, and O42 strains) and the clinical specimens in Tanzania were tested using an in-house multiplex PCR followed by gel electrophoresis (for primers and protocol see supplemental Table 1), which target the same genes as the PCR-Luminex assay (Luscher and Altwegg 1994; Cebula et al. 1995; Schmidt et al. 1995; Nguyen et al. 2005; Boisen et al. 2008). Briefly, the 9-plex PCR amplification was performed in 25 μl total volume consisting of 12.5 μl of Qiagen Multiplex PCR Master Mix (Qiagen, Valencia, CA, USA) which contains dNTPs and 3 mmol/L $MgCl_2$ final concentration, 0.2 μmol/L of each forward primer, 0.2 μmol/L of each reverse primer, 5 μl Q-solution, 3.5 μl of nuclease free water (NFW) and 2 μl of DNA template. The cycling conditions were the same as the 9-plex amplification for the PCR-Luminex assay described earlier. Amplicons (range 147bp to 881bp) were detected by electrophoresis on the E-gel system (Life Technologies, Grand Island, NY, USA) using 2% agarose precast E-gels and E-gel Low Range Quantitative DNA Ladder (Life Technologies, Grand Island, NY, USA). Positive and negative controls (NFW and ATCC 25933 DNA template) were included with each run.

Statistical analysis

Luminex data was reported as median fluorescence intensity (MFI). We used a corrected MFI (cMFI = [MFI (sample) – MFI (background)]/MFI (background)] to accommodate for testing on different Luminex platforms under both high and low photomultiplier tube

voltages and different softwares (Luminex 100 IS Software v. 2.3 in Thailand and Bioplex v. 5.0 at UVA and Tanzania). A cMFI cutoff of 3.0 was used at all sites except at DCLS which used 2.0. Where replicates or multiple cMFI are shown, data is reported as mean ± SEM. The cMFI values in clinical specimens were compared using Mann Whitney test (SPSS, Chicago, IL, USA). The correlation between cMFI and log cfu/200 mg of stool was calculated by Pearson correlation.

Results

We have previously developed PCR-based Luminex assays for enteric viruses, protozoa, helminths, and bacteria (Liu et al. 2011a; Liu et al. 2011b; Taniuchi et al. 2011a; Taniuchi et al. 2011b). In this work we adopted the same general principles of targeting conserved regions of virulence genes, designing short fragments to maximize PCR efficiency, and placing probes near one of the primer regions in order to shorten the distance from bead-probe to fluorophore-primer. We chose to target commonly accepted genes for EHEC (*stx1*, *stx2*, *eae*), EPEC (*eae*, *bfpA*), EIEC (*ipaH* or *ial*), ETEC (*LT*, *STh*), and EAEC (*aaiC*, *aatA*). We sourced primers from the literature where possible, however ultimately the assay required new primers for certain targets (*eae*, *STh*). Likewise, probes were designed new for many targets (*stx1*, *stx2*, *aaiC*, *aatA*) and most required significant refinement including 3 iterations for *aaiC*, *stx1*, and *bfpA*, and 2 for *aatA*, *stx2*, and *STh*.

We first tested the assay on DEC specimens of known pathotype confirmed with existing assays. This entailed use of laboratory strains, clinical isolates, and broths tested by DNA probes or in-house PCR assays targeting different regions of the genes (see Materials and Methods). The comparison of the Luminex protocol with the comparator methods revealed a sensitivity of 100% and specificity of 94–100% (Table 2). We further examined specificity of the assays by testing pure cultures or clinical specimens containing rotavirus, sapovirus, astrovirus, norovirus, *Campylobacter, Vibrio, Salmonella, Shigella, Aeromonas, Yersinia, Entamoeba histolytica*, *E. dispar, Cryptosporidium*, *Cystoisospora, Cyclospora, Ascaris, Ancylostoma, Necator, Strongyloides, Trichuris*, and found no detection on these materials with any of the assays (data not shown).

Since the assay was able to detect the virulence genes in cultures, we then evaluated how well the assay could detect the genes across specimen type. Many laboratories test for DEC from MacConkey colony pools, so we compared this material with DNA isolated from stool directly and from GN broths. To do so we spiked $10^1 – 10^8$ cfu of EHEC O157:H7, EPEC 2348/69, EIEC O124, ETEC H10407, and EAEC O42 strain into aliquots of a single DEC PCR-negative healthy volunteer stool sample. These spiked stool samples were then cultured on MacConkey and 5 colonies picked or grown in GN broth overnight, as described in the Materials and Methods. DNA was extracted from these colony pools, broths, and the parental stool sample, and all extracts were tested using the multiplex PCR-Luminex assay in triplicate. This yielded several results (Figure 1). First, across all targets, there was no false positive detection of EHEC, EPEC, EIEC, ETEC, or EAEC with the other targets in the assay. Second, the stool itself yielded a quantitative correlation for most targets. Namely, a log-linear correlation between cfu/g of *E. coli* and Luminex cMFI was statistically significant for EHEC across *stx1*, *stx2*, *eae*, for EPEC across *eae* and *bfpA*, for EAEC for

aatA, for ETEC across *LT* and *STh*, and EIEC. By contrast, the DNA extracted from broths and from colonies exhibited no statistically significant correlation between cfu/g of input stool and cMFI ($P > 0.05$). Third, using the standard cMFI cutoff for positivity of 3.0, broths yielded detection of 10^1 for most targets, however detection of ETEC was lost at 10^2 cfu/200 mg stool (i.e., cMFI was < 3.0) and was low for *STh*. In contrast stool and colonies yielded detection at 10^3 cfu/200 mg stool (i.e., cMFI was > 3.0) for most targets. Fourth, there were clearly different levels of detection for the different targets; for instance, the *STh* assay offered lower cMFI than the *LT* assay for this *STh*/*LT* positive ETEC isolate in stools, broths, and isolates ($P < 0.05$ comparing mean cMFIs across all dilutions).

We then prospectively evaluated the assay across the three specimen types in a field setting in Tanzania that experiences a diverse range of DEC. Given the previous finding that broths should be most sensitive, this specimen type was our standard. When stool was compared to broths we observed a sensitivity of 67–100% and specificity of 89%-100% across the assays (Table 3). Among discrepant samples, we frequently observed higher target fluorescence in specimens that were positive in both GN and stool versus specimens that were positive in only one specimen type, however differences were not statistically significant. The performance of the assay in colony DNA was also examined and this specimen type exhibited more discrepancies than stool DNA, with sensitivities of 33–50% and specificities of 89–100% (data not shown).

Discussion

This work describes an assay to detect diarrheagenic *E. coli* in stool or broths. We combined an accurate multiplex PCR method to detect characteristic DEC virulence genes, and evaluated the performance of the assay across specimen types – from stool to colonies to broths. We view this as important step towards the goal of directly identifying DEC in stool samples without the current limitations of colony picks or requiring phenotypic methods.

The genes targeted in this assay deserve discussion. Shiga-toxin producing *E coli* (STEC) are food-borne pathogens that have the potential to cause hemorrhagic colitis and hemolytic uremic syndrome, and are important to detect because antibiotics are not beneficial and may increase the risk of complications (Wong et al. 2000). STEC contain lysogenic bacteriophages that produce one or two shiga toxins, *stx1* and *stx2*, the latter of which has several variants (De Baets et al. 2004). Our assay utilized published primers for both of the toxins that had been tested on 28 and 52 STEC strains, respectively, including known variants (Paton and Paton 1998; Hidaka et al. 2009). STEC are of important public health concern in developed countries, witness the recent Germany outbreak, and our assay exhibited 94–100% sensitivity/specificity on 73 *stx1* and 37 *stx2*-positive stool broths submitted to the Virginia state laboratory and confirmed by in-house methods (Table 2). Currently the assay will be used for screening selected broth specimens for STEC at this laboratory.

A subset of STEC has the capacity to attach to and efface intestinal epithelial cells, a pathology mediated by the adhesin intimin encoded by *eae*. The same pathology and gene is also found within EPEC. Intimin sequence can be variable (Blanco et al. 2006) and indeed

this target was relatively problematic in our hands, requiring multiple redesigns. Typical EPEC also contain a plasmid-borne *E. coli* adherence factor that contains *bfpA*, while "atypical" EPEC strains do not. Such atypical EPEC are more prevalent in many settings (Ochoa and Contreras 2011). Our spiking studies suggested that *eae* was more readily detected in the EHEC strain (detected 10^3) than EPEC (detected 10^5). This was surprising because it is a chromosomal gene, and was not due to sequence variations because we sequenced both *eae* amplicons and they were identical (data not shown).

Enteroinvasive *E. coli* were readily detected by targeting *ipaH* or *ial*, and in our hands the assays were interchangeable. These two genes are widely used for detection and are found on the invasion plasmid (Echeverria et al. 1992). The *ipaH* gene is also present on the chromosome thus we favored it for most of our work. The gene is exclusively found in *Shigella* and EIEC, and although there are sequence differences between these species, we targeted the conserved core region (bp 1065–1128). The assay yielded robust amplification and detected 10^2 cfu of EIEC bacteria per 200mg stool sample. We have also used this assay for detection of *Shigella* (Liu et al. 2011a), with similar sensitivity.

ETEC are common causes of diarrhea in children of the developing world. After adherence to the intestinal mucosa, ETEC produce one or both of two enterotoxins, heat-labile enterotoxin (*LT*) and heat-stable enterotoxin (*ST*). Globally it is estimated that 26% of strains contain *LT*, 45% contain *ST*, and the rest contain both (Isidean et al. 2011). The *LT* sequence is relatively conserved across human isolates. Two main subtypes of *ST* have been found in humans, *STh* (or *STIb*) and *STp* (or *STIa*). *STh* appears more common and has been reported to be the only diarrhea-associated subtype in some studies, while others have found both *STh* and *STp* to be associated with diarrhea (Steinsland et al. 2002; Bolin et al. 2006). *ST* was a difficult target given substantial sequence variation within GenBank and there appears to be some debate on published assays for *ST*. We first tried an assay based on the primers of Nguyen et al. (Nguyen et al. 2005), the comparator method of supplemental Table 1, which amplifies both *STh* and *STp* through wobble primer. This worked however Luminex signal was low and probe choices were limited. We thus redesigned the assay to enhance the signal, although this required preferential redesign for *STh*. For those interested in the rarer subtype *STp* we would recommend a singleplex test for this explicitly.

Although the HEp-2 adherence assay remains the defining feature of EAEC, we focused on the subset of EAEC strains that harbor the AggR regulon of virulence factors (the "typical" EAEC), as members of this group have been proven to be virulent in volunteer and epidemiologic studies. Typical EAEC carry key factors on a virulence plasmid and a chromosomal pathogenicity island, so we chose to detect one factor from each of these genetic elements (Czeczulin et al. 1999). *aatA* is a constituent of the EAEC virulence plasmid pAA2, corresponds to a widely used EAEC probe (Baudry et al. 1990), and has been found to be highly specific for EAEC versus other *E. coli* (Monteiro et al. 2009). *aaiC* encodes a secreted protein of the EAEC pathogenicity island AAI, which is coordinately regulated by the AggR activator, along with pAA plasmid-encoded factors (Dudley et al. 2006).

A major focus of this work was examining the performance of the assay in stool vs. broths vs. colonies, because ultimately we desired an assay that was workable on stool. Using spiked specimens we found detection of all targets in stool to be less sensitive than detection in broth, which is logical because broth allows for an additional round of amplification due to bacterial replication and the dilution of the inhibitory substances present in stool. However we found a substantial number of Tanzanian samples that were positive in stool only, not broth (for example, 9 of 54 specimens tested by *ipaH*). The significance of such stool+/culture- materials will need a significant amount of future study. Plausible explanations include a false positive result, low levels of DNA in stool of no significance, or prior antibiotic use rendering culture negative (essentially all individuals in Tanzania were on antimicrobials), the latter of which would be highly clinically relevant. The sensitivity of our assay in stool, with detection down to 10^3 cfu/200 mg for most targets, is similar to or better than previous reports that have tested stool DNA directly (Persson et al. 2007; de Boer et al. 2010; Barletta et al. 2011).

We also found DNA extracted directly from stool to be more quantitative than DNA extracted broth, again logical since the overnight broth amplification plateaus. The significance of pathogen quantitation in stool in predicting diarrhea is an area of active investigation, but there are emerging reports of its relevance in norovirus and EPEC infections (Phillips et al. 2009; Barletta et al. 2011). Evaluation of the role of quantitation in direct stool samples is warranted.

Our results reveal little benefit in picking colonies for *E. coli*, in that this technique was less sensitive than DEC detection from broths, no more sensitive than directly testing stool, and much more cumbersome. Thus we would propose using the assay to test on stool or broths for DEC as a screen, using broths if sensitivity is desired or stool itself if quantitation is desired, and if the screen is positive then one would have the option of continuing to culture the specimen in order to isolate the exact lactose fermenting bacterial strain for subsequent testing as necessary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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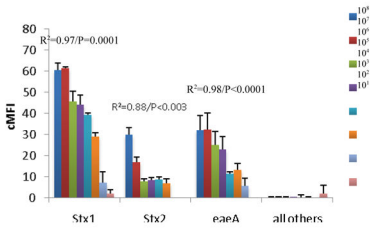
Fig 1A



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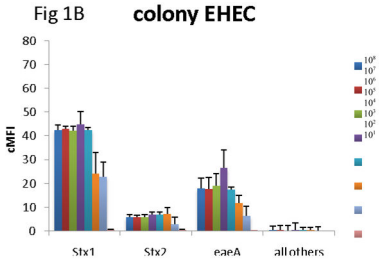

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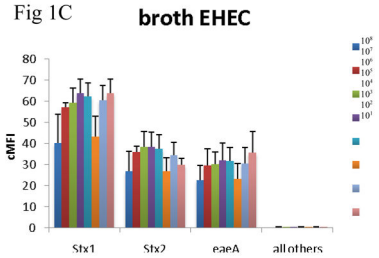


Fig 1D **stool EPEC**

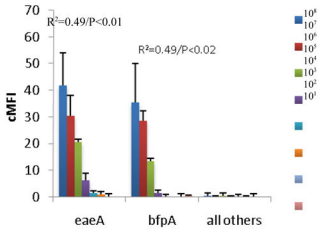


Fig 1E **colony EPEC**

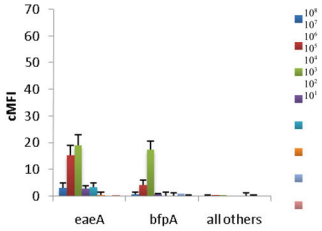
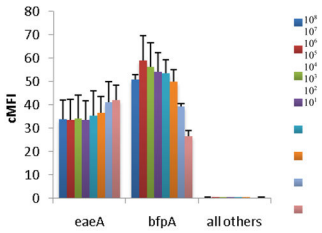


Fig 1F **broth EPEC**



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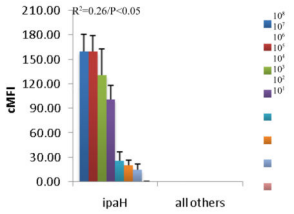

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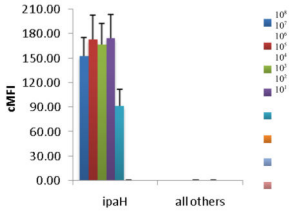
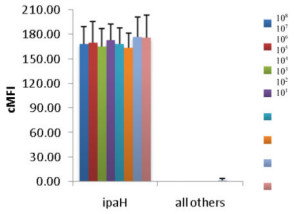


Fig 1I **broth EIEC**



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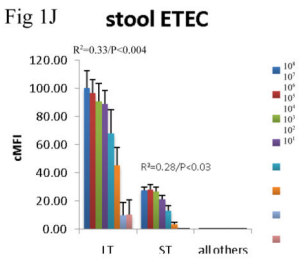

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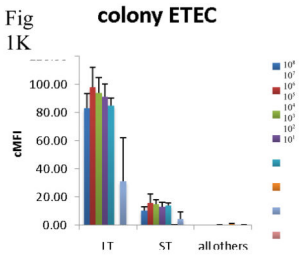

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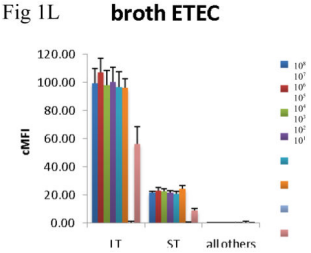

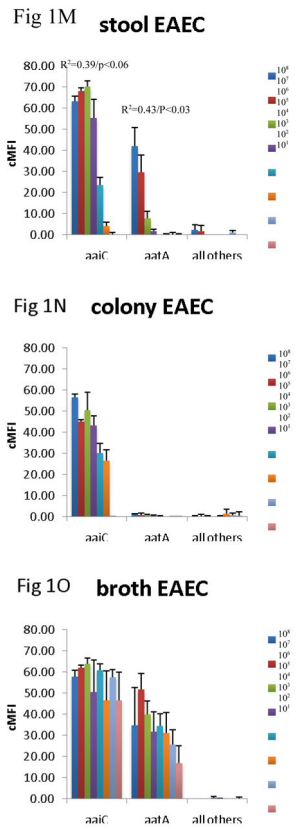

Figure 1.

Comparison of stool DNA, broth DNA, and colony DNA in detection of DEC. Aliquots of a single stool sample were spiked with serial dilutions of EHEC 0157:H7 (AC), EPEC 2348/69 (D–F), EIEC O124 (G–I), ETEC H10407 (J–L), and EAEC O42 (M–O) as indicated. Spiked amounts are indicated in the legends and units are cfu per 200 mg of stool. Spiked samples were then processed by culture in GN broth and on MacConkey agar followed by picking 5 colonies. DNA was extracted from the direct stool versus colony pools versus GN broths and subjected to multiplex PCR in triplicate followed by Luminex detection. Data show cMFI values + SEM on the y axes for the targets indicated on the x axes. cMFI > 3.0 was considered positive. The y axes shown are different for each *E. coli* pathotype. Linear regression revealed R^2 and P values as indicated.

Table 1

Primer and probe sequences

Organism	Target	Reference	Forward, Reverse- biotinylated, Probe	Sequence (5′→3′)
EHEC	*stx1*	(Hidaka et al. 2009)	EH132F	ACTTCTCGACTGCAAAGACGTATG
		(Hidaka et al. 2009)	EH132Rbt	ACAAATTATCCCCTGAGCCACTATC
		this work	EH132P	CTCTGCAATAGGTACTCCA
EHEC	*stx2*	(Paton and Paton 1998)	EH255F	GGCACTGTCTGAAACTGCTCC
		(Paton and Paton 1998)	EH255Rbt	TCGCCAGTTATCTGACATTCTG
		this work	EH255P	GGGGAGAATATCCTTTAATA
EPEC/EHEC	*eae*	this work	EHEC179F	GTAAAGTCCGTTACCCCAACCTG
		this work	EHEC179Rbt	CAAAGCGCACAAGACTACCA
		this work	EHEC179P	GCACATAAGCAGGCAAAATAGC
EPEC	*bfpA*	this work	EP300F	GGAAGTCAAATTCATGGGGG
		this work	EP300Rbt	GGAATCAGACGCAGACTGGT
		this work	EPEC300P	GCTGCAACCGTTACCGCAGG
EIEC	*ipaH*	(Vu et al. 2004)	EI64F	CCTTTTCCGCGTTCCTTGA
		(Vu et al. 2004)	EI64Rbt	CGGAATCCGGAGGTATTGC
		(Vu et al. 2004)	EI64P	CGCCTTTCCGATACCGTCTCTGCA
EIEC	*ial*	(Frankel et al. 1990)	EI320F	CTGGTAGGTATGGTGAGG
		(Frankel et al. 1990)	EI320Rbt	GGAGGCCAACAATTATTTCC
		(Frankel et al. 1990)	EI320P	CCATCTATTAGAATACCTGT
ETEC	*LT*	(Hidaka et al. 2009)	ET62F	TTCCCACCGGATCACCAA
		(Hidaka et al. 2009)	ET62Rbt	CAACCTTGTGGTGCATGATGA
		(Hidaka et al. 2009)	ET62P	CTTGGAGAGAAGAACCCT
ETEC	*STh*	this work	ET172F	TTCACCTTTCGCTCAGGATG
		this work	ET172Rbt	AGCACCCGGTACAAGCAG
		this work	ET172P	ATTACTGCTGTGAATTGTG
EAEC	*aaiC*	(Boisen et al. 2008)	EA215F	ATTGTCCTCAGGCATTTCAC
		(Boisen et al. 2008)	EA215Rbt	ACGACACCCCTGATAAACAA
		this work	EA215P	GTAGTGCATACTCATCATTTAAG
EAEC	*aatA*	(Schmidt et al. 1995)	EA237F	CTGGCGAAAGACTGTATCAT
		this work	EA237Rbt	TTTTGCTTCATAAGCCGATAGA
		this work	EA237P	TGGTTCTCATCTATTACAGACAGC

Table 2

Performance of assay versus comparator methods on isolates and broths

Target Gene	Isolates in-house+	Isolates in-house−	Broth in-house+	Broth in-house−	Total in-house+	Total in-house−	Sensitivity	Specificity
Stx1 Luminex+	5	0	68	2	73	2	1.00	0.96
Stx1 Luminex−	0	25	0	29	0	54		
Stx2 Luminex+	4	0	33	0	37	0	1.00	1.00
Stx2 Luminex−	0	21	0	66	0	87		
eae Luminex+	10	0	76	3	86	3	1.00	0.94
eae Luminex−	0	25	0	20	0	45		
bfpA Luminex+	3	0	0	2	3	2	1.00	0.98
bfpA Luminex−	0	26	0	97	0	123		
ipaH Luminex+	1	0			1	0	1.00	1.00
ipaH Luminex−	0	6			0	6		
ial Luminex+	4	0			4	0	1.00	1.00
ial Luminex−	0	24			0	24		
LT Luminex+	6	0	0	1	6	1	1.00	0.99
LT Luminex−	0	27	0	98	0	125		
ST Luminex+	3	0	0	0	3	0	1.00	1.00
ST Luminex−	0	27	0	99	0	126		
aaiC Luminex+	3	0			3	0	1.00	1.00

Target Gene	Isolates in-house+	Isolates in-house−	Broth in-house+	Broth in-house−	Total in-house+	Total in-house−	Sensitivity	Specificity
aaiC Luminex−	0	25			0	25		
aatA Luminex+	5	0			5	0	1.00	1.00
aatA Luminex−	0	25			0	25		

Table 3

Evaluation of assay on clinical specimens

Target Gene	GN+	GN−	Sensitivity	Specificity
Stx1 Stool+	7 ($cMFI_{GN}$=9.7±2.4, $cMFI_{Stool}$=12.1±1.6)	3 (cMFI=16.3±4.5)	0.88	0.93
Stx1 Stool−	1 (cMFI=3.3)	43		
Stx2 Stool+	0	0	N/A	1.00
Stx2 Stool−	0	54		
eae Stool+	11 ($cMFI_{GN}$=11.6±3.2, $cMFI_{Stool}$=18.8±5.1)	2 (cMFI=7.6±3.4)	0.73	0.95
eae Stool−	4 (cMFI=6.7±1.1)	37		
bfpA Stool+	0	4	N/A	0.93
bfpA Stool−	0	50		
ipaH Stool+	5 ($cMFI_{GN}$=7.6±1.9, $cMFI_{Stool}$=10.5±1.9)	5 (cMFI=9.5±2.8)	0.71	0.89
ipaH Stool−	2 (cMFI=3.3±0.0)	42		
LT Stool+	4 ($cMFI_{GN}$=6.7±1.0, $cMFI_{Stool}$=14.4±6.2)	0	0.67	1.00
LT Stool−	2 (cMFI=3.2±0.0)	48		
ST Stool+	1	0	1.00	1.00
ST Stool−	0	53		
aaiC Stool+	7 ($cMFI_{GN}$=19.4±5.6, $cMFI_{Stool}$=21.5±2.3)	2 (cMFI=18.7±14.6)	0.88	0.96
aaiC Stool−	1 (cMFI=14.6)	44		
aatA Stool+	15 ($cMFI_{GN}$=11.5±1.5, $cMFI_{Stool}$=10.7±1.3)	4 (cMFI=9.6±2.7)	0.83	0.89
aatA Stool−	3 (cMFI=6.7±2.5)	32		