ABSTRACT

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Development of a low-cost absorbent pad test for the detection of *Escherichia coli* in contaminated drinking water
(Under the direction of Dr. Mark D. Sobsey)

Diarrheal disease kills 1.8 million people each year, mostly children under the age of 5 in developing countries. Inadequate sanitation and access to improved drinking water are major correlates of waterborne disease (WHO 2005). There is a compelling need for inexpensive, portable drinking water quality monitoring technology for resource poor and disaster settings. A simple, low-cost absorbent pad test to detect *E. coli* is in development to meet this need. The test is currently capable of enumerating a pure culture *E. coli* at concentrations between 10-1000 CFU per 100 mL in high quality surface water. Test results are not significantly different from USEPA approved methods under these conditions. Mixed bacterial populations such as those found in environmental water samples currently pose a considerable challenge to the ability of the test to accurately enumerate *E. coli* throughout the range of concentrations necessary to adequately assess drinking water safety.

To my friends, whose culinary talents, raucous humor and boundless empathy were indispensable to my sanity and productivity during the long and otherwise ascetic weeks in lab and library.

To my family, whose unconditional love, unfailing support and unholy work ethic comprise the foundation of everything I have ever accomplished.

ACKNOWLEDGEMENTS

Dr. Sobsey

Dr. Christine Stauber

Dr. Lisa Casanova

Dr. Fu-Chi Hsu

Tina Lusk

Douglas Wait

the Sobsey lab at UNC

Thanks for your scientific knowledge, your input and your camaraderie. Your persistent faith in my ability to succeed, your generous sense of humor in light of my propensity to strike out confidently in the wrong direction. I have never been afraid to make mistakes around any of you, and I have learned ever so much more than I would have otherwise.

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LIST OF ABBREVIATIONS

8HQ	8-hydroxyquinoline ß-D-glucuronide
APHA	American Public Health Organization
CFU	colony forming units
CTAB	cetyltrimethylammonium bromide
GAL	ß-galactosidase
GUD	ß-glucuronidase
IBDG	indoxyl ß-D-glucuronide
IPTG	isopropyl ß-D-1-thiogalactopyranoside
ISO	International Organization for Standardization
LTB	Lauryl Tryptose Broth
MF	Membrane Filtration
MPN	Most Probable Number
MTF	Multiple Tube Fermentation
MUG	4-methylumbelliferyl ß-D-glucuronide
MUGal	4-methylumbelliferyl ß-D-galactoside
ONPG	ortho-nitrophenyl ß-D-galactoside
PCR	polymerase chain reaction
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
USD	United States dollars
USEPA	United States Environmental Protection Agency
WHO	World Health Organization
X-Gluc	5-bromo-4-chloro-3-indolyl ß-D-glucuronide

INTRODUCTION

Diarrheal disease kills 1.8 million people every year, mostly children under the age of 5 living in developing countries in Asia and Africa (WHO 2005). Most diarrheal disease can be attributed to inadequate access to safe drinking water and lack of basic sanitation (WHO 2005). In 2002, more than 1 billion people lacked sustainable access to improved water sources, and 2.6 billion lacked access to improved sanitation (WHO 2005). Yet even in places with access to improved water sources, recontamination of drinking water between the source and the point of use may be a significant source of infection (Wright 2004, Mint 2001, Reiff 1996). Disaster-related damage to drinking water and wastewater infrastructure can result in significant and widespread contamination of surface water and drinking water sources (Gupta 2007, Schwab 2007). Thus, identifying safe drinking water sources, drinking water treatment, safe storage, and microbial water quality monitoring at the household level are crucial elements in reducing the burden of waterborne disease in developing countries and in post-disaster areas (Mintz 2001, Clasen 2007, Reiff 1996, Wright 2004).

Microbial water quality monitoring technology is necessary to evaluate the effectiveness and assess the need for point of use interventions to improve microbial water quality. However, resource poor conditions such as those in developing countries or in post-disaster areas often preclude or complicate the implementation of existing microbial water analysis technologies in the field. Standard microbial analysis methods such as membrane filtration and multiple tube fermentation tests require trained labor and costly, cumbersome equipment and reagents. Shipping samples to the nearest water quality laboratory for analysis incurs additional costs, risks significant sample deterioration, delays results, and may not even be feasible in the absence of adequate transport infrastructure and laboratory facilities and personnel. Moreover, the sustainability of point of use interventions to improve drinking water quality may depend on the involvement of the community in monitoring and evaluating the effectiveness and

need for such interventions (Reiff 1996). A simple, low-cost microbial water quality test can make monitoring more widely accessible in times and areas of critical need and may improve the sustainability of interventions to improve drinking water. In addition to having adequate precision and accuracy to assess drinking water quality with respect to international guidelines and standards, this test should have the following qualities:

1. **Low cost.** Current testing technology is available at a cost of a few USD per test, a price that exceeds the daily per capita gross national income in many areas of the world where diarrheal disease is endemic (World Bank 2008). The ability to adequately assess drinking water quality for less than 1 USD per test will significantly expand the accessibility of testing technology.
2. **Simple to perform.** The test should comprise a self-contained, disposable device. The test procedure should require not more than two active steps and should be simple enough that untrained persons (e.g. children) can easily perform the test and achieve accurate, replicable results.
3. **Portable.** The test device should be lightweight and compact. Performance of the test should not require any electricity or apparatus beyond the test device. The test should provide reliable results with room temperature or ambient temperature incubation.
4. **Shelf stable.** The test device may encounter fluctuating and extreme conditions in the course of storage and transport to point of use. It should be reasonably resistant to extreme temperatures, desiccation, humidity and contamination.

The degree to which selected existing low-cost tests meet these criteria is summarized in Table 1. Each of the methods has weaknesses with respect to the above criteria. Some require cold storage, some are technically complicated to perform, and most exceed the 1 USD per test target cost. To overcome the drawbacks of available testing methods and develop a procedurally simple, portable, low cost *E. coli* test that

test format	cost (USD) per test	cost (USD) per 100 mL sample	procedure simplicity	medium storage	electricity required (other than incubation)	portability
Colilert® Quanti-Tray®/2000	$5.25	$5.25	fair	Up to 12 months at 4-30°C	yes	low (tray sealer)
MF on MI agar	$1.94	$19.40	poor	dehydrated agar up to 2 years at 20°C, prepared media until contaminated or dessicated at 6°C	no	low (vacuum and filtration apparatus)
MF on Bio-Rad Rapid *E. coli* II agar	$0.43	$4.30				
Coliscan Easygel	$1.50	$30.00	good	up to 1 year frozen	no	high
3M™ Petrifilm™	$1.44	$144.00	good	18 mo.s sealed refrigerated, ≤ 7 days sealed 38°C, 30 days open refrigerated	no	high

Table 1. Summary of key characteristics of *E. coli* test formats relevant to accessibility and cost, after Lusk 2008. Cost per 100 mL sample for membrane filtration assumes 10 mL sample/plate. Cost per sample for Colilert® Quanti-Tray®/2000 does not incorporate one-time cost cost of tray sealer ($4000). Storage information for Colilert®, MI, Bio-Rad and Coliscan comes from product documentation. Storage information for Petrifilm from 3M Microbiology Technical Bulletin TB-001(http:www.3m.com/intl/kr/microbiology/p_aerobic/tech4.pdf, Accessed 12 Oct 2008).

can be used in resource poor and post-disaster settings, this research was undertaken with the following objectives:

OBJECTIVES

To develop a proof of concept for a microbiological water quality test based on the detection and enumeration of *E. coli* in a water sample applied to an absorbent pad containing a bacteriological culture medium.

To develop a medium for the pad that supports the growth of *E. coli* while inhibiting the growth of competing organisms. The medium should contain a chromogenic ß-D glucuronide that specifically indicates the presence of *E. coli* by producing a colored product upon cleavage by ß-D-glucuronidase.

To optimize the test for use in resource-poor conditions by maximizing its simplicity, portability, and shelf stability and by minimizing its cost.

LITERATURE REVIEW

Indicators of fecal contamination in drinking water

Escherichia coli or *E. coli* is routinely used as an indicator of microbial drinking water quality. An ideal fecal indicator is non-pathogenic, present in large numbers in feces, more or as resistant as pathogens to disinfection and other treatment processes, sufficiently persistent in the environment to be detected when pathogens are present, strongly associated with the presence of pathogens, and easy to detect and quantify (adapted from Hurst et al 2002). No single indicator is ideal for all climates and circumstances, but *E. coli* is the best understood and most commonly used indicator organism. Because *E. coli* generally originate in feces and survive in water comparably to many bacterial pathogens, the presence of *E. coli* in drinking water indicates that other pathogenic organisms of fecal origin may be present.

World Health Organization guidelines for drinking water quality recommend that all water intended for drinking not contain any detectable *E. coli* or thermotolerant coliforms in a 100 mL sample (2006). *E. coli* is not without its weaknesses as an indicator organism. The concentration of *E. coli* in drinking water has not been demonstrated to be a consistent predictor of health outcomes, and the absence of *E. coli* does not rule out the possibility of lingering contamination with pathogenic enteric viruses and protozoa, which are often more persistent in the environment and less susceptible than *E. coli* to many common disinfection methods (Brown et al 2008, Costán-Longares et al 2008, Harwood et al 2005). There is substantial evidence to support the use of alternative indicators such as coliphages, *Clostridium perfringens* and even fecal sterols in lieu of or in combination with *E. coli* (Savichtcheva & Okabe 2006). Despite its weakness as an indicator organism, *E. coli* remains the most useful and widely accepted single organism targeted by tests for monitoring microbial drinking water quality (Edberg et al 2000).

E. coli

Escherichia coli or *E. coli* are enterobacteria, or members of the bacteria family *Enterobacteriaceae*, which includes the familiar genera *Salmonella* and *Shigella* as well as other common members of the intestinal flora. Enterobacteria are rod-shaped, gram-negative, non-sporulating, facultative anaerobes. A subset of enterobacteria known as coliforms are distinguished by their ability to ferment lactose to produce acid and gas. *Escherichia* and the other genera in the fecal or thermotolerant coliform group–*Enterobacter*, *Klebsiella*, and *Citrobacter*–can grow at 44.5˚C. *E. coli* can metabolize a wide variety of substrates and grow in temperatures ranging from 20˚C to as high as 49˚C, though the optimal growth temperature is 37˚C. By virtue of the ease with which these resourceful bacteria can be cultured and genetically manipulated, *E. coli* are perhaps the most studied microorganisms in the laboratory.

While *E. coli* and other enterobacteria may be found in water and soil, *E. coli* are by and large associated with the gastrointestinal tract and with feces. Large numbers of *E. coli* normally colonize the intestinal tracts of humans and other warm-blooded fauna. While the relationships between *E. coli* and its hosts are generally commensal or symbiotic, there exist some pathogenic *E. coli* strains which are responsible for human diseases. Uropathogenic *E. coli* (UPEC) are associated with ascending urinary tract infections, enterotoxigenic *E. coli* (ETEC) are a significant cause of diarrheal disease, and *E. coli* O157:H7 or enterohemmorhagic *E. coli* (EHEC) are responsible for several recent, high-profile outbreaks of infection that resulted in bloody diarrhea and some cases of hemolytic uremic syndrome and kidney failure (Todar 2008). *E. coli* infection and other enteropathogen infections typically occur through the ingestion of food or water contaminated with feces, but interpersonal contact and fecally contaminated objects (fomites) are also possible routes of exposure.

E. coli detection and enumeration techniques

Rapid and culture-free E. coli detection

A number of techniques have been utilized for the rapid detection of *E. coli* in food and water. Fluorescence *in-situ* hybridization and real-time quantitative polymerase chain reaction (PCR) have been used with some success to detect and enumerate *E. coli*. Several immunological and fluorimetric techniques for *E. coli* detection and quantification have also been described in the literature (Rompré et al 2002). These newer techniques produce results in less than four hours and can enumerate non-culturable bacteria and in some cases dead bacteria. The enhanced speed of these techniques stems from the use of direct fluorimetry, luminometry, laser cytometry or fluorescence microscopy for quantification of fluorescence or chemiluminescence at lower levels and higher resolution than is possible with the unaided human eye. These techniques are well-suited to real-time monitoring of recreational waters; however, some of their lower detection limits are not sufficiently low[1] to detect the concentrations of *E. coli* commonly found in drinking water. A separate concentration step such as membrane filtration or enrichment may reduce the lower detection limits of some of these tests. However, this additional step increases test complexity and cost and reduces test portability. Moreover, the inability of some of these tests to distinguish between dead and live bacteria may compromise their ability to predict human health risks. Finally, these rapid tests require highly trained labor, sophisticated laboratory equipment and expensive, perishable reagents, rendering them impractical for field use in resource poor settings.

Culture-based *E. coli* test methods

The American Public Health Association (APHA) and International Organization for Standardization (ISO) standard methods for enumerating *E. coli* are traditional culture-

[1] The lower detection limits of some of these tests can be sufficiently reduced for drinking water by use of a separate concentration step such as membrane filtration or by a separate enrichment step. However, this additional step increases test complexity and cost and reduces test portability.

based membrane filtration (MF) and multiple tube fermentation (MTF) methods. Culture-based methods are relatively inexpensive, have reliably low detection limits, and use readily available reagents and equipment. They generally require at least 18 hours of incubation at 35˚C to produce results that can be enumerated with the naked eye, though there is some evidence that longer incubation periods at nonstandard temperature can produce comparable results (Lusk 2008). Culture-based methods enumerate *E. coli* by either direct colony count or Most Probable Number (MPN) estimation.

Direct colony count methods

Standard membrane filtration methods enumerate *E. coli* using direct colony counts. Solids from water samples are deposited on a 0.45 µm porous membrane by the application of a vacuum. The filtrate is discarded and the membrane is incubated atop a solid culture medium (e.g. MI agar or Bio-Rad Rapid *E. coli* II agar) or atop an absorbent pad containing a liquid culture medium (e.g. MI broth). The absorbent pad alternative circumvents the time-consuming process of pouring agar plates and obviates the storage of prepared agar media, which quickly degrades in quality under inappropriate sterility, humidity and temperature conditions. The culture medium contains substrates that are both selective and differential for the growth of *E. coli*, which multiply within 24 hours to form colonies of sufficient size for enumeration with the naked eye.

Coliscan Easygel and 3M™ Petrifilm™ methods are convenient and popular for field use by virtue of their portability and simplicity. Up to 5 mL of sample water can be mixed directly with Coliscan Easygel liquid medium, which contains pectin and solidifies after contact with a disposable petri dish pre-treated with a calcium-based crosslinking agent (US Patent 4282317). 3M™ Petrifilm™ was designed for use with food samples but has been evaluated as a test for water samples (Schraft & Watterworth 2005). A 1 mL water sample hydrates a monolayer of dehydrated gel medium adhered in a flat card and covered with a flap of transparent plastic film. Both tests, like agar plates, must be incubated for at least 18 hours at 35˚C before results can be enumerated.

MPN methods

The Most Probable Number (MPN) enumeration method uses a maximum likelihood estimation statistical model to estimate bacterial concentrations based on the presence or absence of bacteria in replicate serial dilutions of a water sample. Multiple tube fermentation, which has become synonymous with MPN, is carried out by incubation of the sample with a liquid culture medium in test tubes or other sterile containers. The volumes and replicate numbers of the samples are selected to give desired or adequate statistical power, and the medium contains a specific indicator for the presence of target bacteria. MPN is at best a semiquantitative method, requiring a large number of a replicates to ensure adequate test precision. Multiple tube fermentation can be labor and materials intensive because of the need to make many volumetric measures and dilutions. IDEXX Colilert® Quanti-Tray®/2000 simplifies the MPN procedure considerably with the use of a single 100 mL sample subdivided by heat sealing into a multi-well tray. MPN in the Colilert® Quanti-Tray®/2000 format can detect as few as 1 CFU per 100 mL. However, use of the Quanti-Tray®/2000 also necessitates the purchase of a tray sealer, which is expensive and difficult to transport and requires electricity to operate.

E. coli and total coliform culture media

The accuracy and sensitivity of culture-based methods rely heavily on the ability of the culture medium used to accurately indicate the presence of target organisms while suppressing the growth of contaminating organisms. The selectivity and specificity of a medium may be increased by the following means:

5. Adding substances, e.g. detergents and antibiotics, that inhibit the growth of competing organisms
6. Restricting available substrates to reduce the variety of organisms that can grow on the medium,
7. Providing substrates that produce a recognizable signal, e.g. color change, to

signify the presence of the target organism.

Figure 1. Absorbent pad test with X-Gluc and Colilert® exposed to long-wave UV. Dark spots are X-Gluc hydrolysis product, halos are diffused MUG hydrolysis product.

Defined substrate methodology uses specific enzyme substrates to reduce the need for harsh inhibitory agents such as bile salts, which can hamper recovery of injured and stressed bacteria. Colilert®, a defined substrate medium, incorporates antibiotics to inhibit gram positives. It also contains two specific indicator molecules that change color when metabolized by the target organism and serve as its sole carbon sources, making Colilert® medium both selective and differential. Indicator molecules used in the specific detection and enumeration of *E. coli* detection exploit several *E. coli*- and coliform-specific phenotypes, in particular, the expression of the enzyme ß-D-glucuronidase (GUD). Between 94-96% of tested *E. coli* strains express GUD (Manafi 2000). GUD catalyzes hydrolysis of ß-D-glucuronides to yield an aglycone and free glucuronic acid, which *E. coli* can utilize as a carbon source. GUD activity can be visibly demonstrated with fluorogenic or chromogenic ß-D-glucuronides. 4-methylumbelliferyl ß-D-glucuronide (MUG) is the most widely used fluorogenic glucuronide. The liberated aglycone fluoresces blue when irradiated with UV light at 366 nm (Figure 1). Indoxyl-ß-D glucuronide (IBDG), 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-Gluc), and other substituted indolyl glucuronides are hydrolyzed to produce indole or substituted indoles that dimerize in the presence of oxygen to yield insoluble indigoid pigments in colors ranging from deep blue to pink (Kiernan 2007). The aglycone liberated from 8-hydroxyquinoline ß-D-glucuronide chelates Fe(II) and Fe(III) ions to produce a black precipitate (James 1988).

Members of the coliform group, which includes *E. coli*, exhibit inducible expression of ß-galactosidase (GAL). Chromogenic and fluorogenic ß-D-galactosides such as ortho-nitrophenyl ß-D-galactoside (ONPG) and 4-methylumbelliferyl-ß-D-galactoside (MUGal) can be used to detect GAL activity. The latest generation of commercial *E. coli* culture

medium	GAL substrate	GUD substrate	other coliform indicators
Colilert® (IDEXX)	ONPG† (yellow)	MUG† (blue UV fluorescent)	--
MI agar (USEPA Method 1604)	MUGal† (blue UV fluorescent)	IBDG† (indigo)	--
Rapid *E. coli* II agar (Bio-Rad)	X-Gal† (blue)	RoseGluc† (pink)	--
Coliscan Easygel (Micrology Labs)	SalmonGal† (pink)	X-Gluc† (blue)	--
3M™ Petrifilm™	--	X-Gluc§ (blue)	triphenyltetrazolium dye (red) lactose (bubbles)§

Table 2. Use of indicator substances to elicit characteristic phenotypes in *E. coli* and coliforms in selected commercial culture media. †Manafi 2000. §Curiale et al 1991

media include both a chromogenic or fluorogenic ß-D-galactoside and a chromogenic or fluorogenic ß-D-glucuronide for the simultaneous detection and enumeration of total coliforms and *E. coli*, respectively (Table 2). Presumptive *E. coli* are identified by a combination of both GAL and GUD expression phenotypes on a simultaneous detection medium. For example, on Coliscan Easygel, *E. coli* form purple colonies, a combination of the red color from SalmonGal and the blue color from X-Gluc. No additional biochemical tests are required to confirm the identity of presumptive *E. coli* detected on these media.

The use of enzymatic activity to detect *E. coli* has significant limitations. GUD is expressed by organisms other than *E. coli*, and selective agents must be incorporated into culture media to suppress growth of these organisms in order to make GUD substrates adequately specific indicators of the presence of *E. coli*. The physical condition of the bacteria may influence GUD expression, so GUD substrate tests may underestimate bacterial concentrations if there are many damaged or stressed bacteria in the sample (Martins et al 1993). In addition, chromogenic and fluorogenic GUD and GAL substrates are by far the most costly components of culture media (Appendix B). Tests using chromogenic enzyme substrates for *E. coli* detection are convenient for field use because the results can be enumerated without specialized equipment or complex

statistical tables and do not require additional confirmation tests. When assessed in a selective medium that supports *E. coli* growth, GUD activity is a convenient, sensitive, and specific means of detecting and enumerating *E. coli* (Rice 1990).

Strengths and limitations of existing E. coli tests

When material costs are normalized for 100 mL samples, the gold standard membrane filtration and Colilert® Quanti-Tray®/2000 methods are the most cost effective *E coli* test methods for drinking water (Table 1). However, both test formats are still several dollars too expensive to be accessible to much of the developing world. Moreover, procedural complexity and requirements for expensive, cumbersome equipment preclude the use of these test formats in resource poor conditions.

The 3M™ Petrifilm™ and Coliscan Easygel tests are both procedurally simple and portable. What Petrifilm™ and Easygel gain in simplicity and portability, they lose in their power to detect low numbers of bacteria. Direct colony count methods are limited by the number of colonies that can be individually enumerated on a given surface area. In practice, the number of distinct and enumerable colonies is limited to about 100 colonies per 6 cm agar plate or Petrifilm™ card, and about 300 colonies on a 10 cm agar plate. The wide range of sample volumes that can be examined on a single agar plate using membrane filtration, corresponds to a wide range of detectable concentrations of bacteria, with a lower limit equal to 1 CFU divided by the volume of sample that can reasonably be filtered through a single membrane. Most drinking water is not too turbid to permit membrane filtration of several hundred or thousand mL. Thus, membrane filtration methods often give lower detection limits of less than 1 CFU per 100 mL. In contrast, the lower detection limits for Petrifilm™ and Easygel are 100 and 20 CFU per 100 mL, respectively. These test formats quickly become cost prohibitive when many Petrifilm™ cards or Easygel kits must be purchased to assess an adequate volume of water to reasonably ensure its safety for drinking (Table 1).

Among currently available *E. coli* tests, Petrifilm™ most nearly meets the criteria outlined above for a drinking water quality monitoring technology optimized for resource poor conditions. This research was conducted in pursuit of a test format with the shelf stability, portability and simplicity of Petrifilm™ but also with the additional capacity to accommodate a significantly larger sample volume at the same or lower cost. One potential means of increasing the sample volume of a culture based test method without reducing its portability or increasing its cost, is to incubate the water sample with a selective and differential culture medium in an absorbent pad. The use of readily available materials such as cellulose pads helps minimize the material and manufacturing costs of the test format. Furthermore, the absorbent pad format lends itself to the use of dehydrated liquid culture media, which in addition to being commercially available are significantly more shelf stable than prepared solid media. An absorbent pad test format was assembled from dehydrated media, cellulose pads and other readily available components and was evaluated using the materials and methods outlined below.

MATERIALS

Test microorganisms

E. coli test strains

E. coli ATCC strain 11303 (*E. coli* B) was originally obtained from ATCC. Other *E. coli* isolates were provided by Tom Whittam at Michigan State University and propagated by Tina Lusk at the University of North Carolina at Chapel Hill. University Lake water spiked with pure cultures of the strains noted in Table 3, was obtained from Lusk and used in the pilot experiment and in

Accession #	Strain #	Host
TW11566	MT2a	Unknown
TWO3283	ECOR 20	Steer
TWO4576	RO-9	Chicken
TWO7334	931107	Seagull

Table 3. *E. coli* strains obtained from Tina Lusk as pure culture spikes in University Lake water for use in pilot testing and background media

background media evaluation experiments. *E. coli* B was used for all other experiments when pure *E. coli* culture was required.

Propagation of E. coli B stocks

Frozen stock of *E. coli* B was obtained from Tina Lusk in the laboratory of Mark Sobsey at the University of North Carolina-Chapel Hill. It was streaked for isolation onto MacConkey agar (Difco). After 24 hour incubation at 35˚C, a single red colony was inoculated into a sterile flask of TSB. This culture was incubated with shaking at 100 rpm for 24 hours at 35˚C, centrifuged for 5 minutes at 14000 rpm and 10˚C, and resuspended in TSB with 20% glycerol. Bacterial stocks were stored at -20˚C and used for all experiments requiring *E. coli* B spiking into test water.

Verification of enzyme expression and activity based on substrate hydrolysis

GAL and GUD activity were verified by inoculation of E. coli B into 100 mL of hydrated Colilert® (IDEXX) medium with incubation for 24 hours at 35˚C. Strains were observed for the yellow product of hydrolysis of ONPG by GAL and for the fluorescent product of MUG hydrolysis by GUD as evidence of the expression of active forms of these bacterial enzymes.

Creation of working monocultures

Pure cultures of *E. coli* B for use in experiments were made by inoculating from frozen stocks into 25 mL TSB and incubating overnight at 35˚C with rotary shaking at 105 rpm. A 100 µL volume of this culture was added to 25 mL fresh TSB and incubated with rotary shaking at 105 rpm at 35˚C for 1.5-2.5 hours. Once the culture's OD_{580} reached 0.1-0.5, indicating that bacterial growth was in log phase, the log phase culture was chilled in an ice water bath, maintained at 4˚C, and used for within one week.

Sewage as a source of *E. coli* and mixed coliforms

Raw municipal sewage was obtained from the influent sampler at the OWASA

reclamation plant off Mason Farm Road in Chapel Hill, NC. and used to spike University Lake water samples with a mixed population of fecal coliforms of predominantly human origin. The sewage was stored at 4˚C and used within one week of collection.

Test waters

University Lake water

Test water was a surface water obtained from University Lake in Carrboro. University Lake is a 200 acre impoundment that serves as a drinking water source for Chapel Hill and Carrboro, North Carolina. University Lake water was collected in January 2007 and July 2008 and stored at 10˚C for up to 18 months until use..

Experimentally contaminated surface water

Surface water samples from Northeast Creek and Booker Creek were used as naturally occurring contaminated water samples to assess the pad test. Northeast Creek is a major Durham county waterway that receives more than 1 million gallons per day if discharge from the Durham County Wastewater Treatment Plant. Northeast Creek drains heavily developed areas in the city of Durham and Research Triangle Park before terminating in Jordan Lake. Booker Creek is a shallow, naturally occurring creek 5.7 miles in length. It flows through a mixture of undeveloped and residential areas in northwest Chapel Hill, into Lake Ellen and Eastwood Lake, and under highly developed portions of east Chapel Hill before merging with Bolin Creek. Surface water samples from these creeks were obtained from Leigh-Anne Krometis in June 2008 and stored overnight at 4˚C before use.

E. coli spike

A pure culture of *E. coli* B (ECB) was diluted in University Lake water to give a target concentration of 2-150 CFU/mL depending on the experiment. The pure culture was titered by spread plating on TSA with 18-24 hours incubation at 35˚C. This initial titer

was used to calculate the volume of *E. coli* culture to be spiked into water samples to achieve the target concentration of *E. coli* for each experimental run.

Sewage spike

Fecally contaminated surface water was simulated by adding sewage to University Lake water to achieve a target concentration of presumptive *E coli* in the spiked water ranging from 2-150 CFU/mL depending on the experiment. To determine the *E. coli* titer in the sewage, samples were assayed on Bio-Rad Rapid *E. coli* II agar using membrane filtration and incubated for 18-24 hours at 35˚C. This titer was used to calculate the volume of sewage needed to spike a volume of University Lake water to achieve a target concentration of presumptive *E. coli*.

As with the *E coli* B monocultures, a sewage spike was freshly made for each experiment, and an entire series of replicate experiments was conducted with a single batch of sewage whenever possible. The measured bacterial concentration was calculated based on experimental results and this revised figure was used to calculate volumes for sewage spikes in subsequent experiments using the same batch of stored sewage. Regardless of initial bacterial concentration, the concentration of bacteria in sewage samples typically ranged between 10^3-10^4 CFU/mL after the first day of storage and remained within ±0.2 log for the next six days. Sewage samples were discarded after a week of storage.

In the following material, "sample" refers to volumes of natural or contaminated water, with or without bacterial spike, which are combined with test or benchmark medium and incubated with the goal of assaying enumerable *E. coli* bacteria.

Liquid bacteriological media

Whenever possible, liquid bacteriological media were obtained in dehydrated form from Difco and prepared according to package directions. The media that were not available in prepackaged form were composed from individual shelf reagents according

to formulations in the Difco/BBL manual (see Appendix A for media composition). Indicator dyes such as basic fuchsin and bromthymol blue were omitted from media formulations when they interfered with the visualization of the blue X-Gluc hydrolysis product.

METHODS

Benchmarks and controls

Absorbent pad tests results were compared to two USEPA approved methods for the enumeration of *E. coli*: a MPN method using Colilert® Quanti-Tray®/2000, and a direct colony count method using membrane filtration on Bio-Rad Rapid *E. coli* II agar plates. Results from these methods were used to quantify the accuracy and precision of bacterial numbers spiked into test waters.

Samples were assayed using the Quanti-Tray®/2000 according to the manufacturer's instructions. After 18-24 hours incubation at 35˚C, the numbers of yellow (total coliforms) and fluorescent (*E. coli*) chambers were tallied and the Most Probable Number of *E. coli* per 100 mL sample was calculated using the MPN calculator provided by the manufacturer.

On Bio-Rad agar, purple (presumptive *E. coli*) and light blue (presumptive total coliforms) colonies were enumerated after incubation for 18-24 hours at 35˚C.

University Lake water or sample diluents were used as negative controls in all test formats (pads, Colilert® Quanti-Tray®/2000 and Bio-Rad membrane filtration).

Wet and dry protocols for media application to pads

Wet application

Samples were applied to absorbent pads (Topco Top Care® pantiliners). All pads were handled with flame-sterilized forceps and placed in 1 quart Ziploc® bags or in sterile 3.75" x 7" 7 oz Nasco Whirl-Pak® bags in a decontamination hood. Forceps were

used to remove the wax paper from the adhesive sides of the pads. The pads were placed at the lowest possible point in the bags, which were then pressed flat from the outside to help the pad adhere to the bag. Bagged pads were stored for up to 24 hours in a clean 1 gallon Ziploc® bag before use.

Wet protocol A:

Sterile 2X concentrated liquid medium was combined with an equal volume of spiked water to give a final *E. coli* concentration between 20 and 50 CFU/pad. A 10 mL volume of this mixture was pipetted onto each bagged pad, giving a 5 mL sample volume per pad. The bags were sealed and incubated flat at 35˚C for 16-24 hours.

Wet protocol B:

The wet application protocol was adjusted to allow for the use of 1X media. For each set of three test pads, 1 mL of spiked University Lake water was mixed thoroughly with 30 mL of 1X medium. This procedure diluted the medium by only about 3% but also reduced the sample volume to 0.32 mL/pad. A 10 mL volume of the resulting mixture was pipetted onto each of 3 bagged pads. The bags were sealed and incubated flat at 35˚C for 16-24 hours.

Dry application

A 10 mL volume of medium was pipetted onto individual absorbent pads. The pads were placed absorbent side up on aluminum foil in a desiccator oven at 60˚C and allowed to dry for 8-14 hours. The dried pads were placed in a decontamination hood and exposed to disinfecting UV light (280 nm) for 5-10 seconds The wax paper was removed with sterile forceps to reveal the adhesive backing before the pads were placed inside sterile 3.75" x 7" (7 oz.) Nasco Whirl-pak® bags and lightly adhered to one side of the bag interior. The bagged pads were stored in a large, clean, airtight bag away from light and heat for no more than 24 hours before use. These pads with pre-dried media are hereafter referred to as "dried X pads" or "dried X" where X is the name of the

medium applied to the pad before desiccation.

To use, 10 mL of sample were pipetted directly onto each dried pad to re-hydrate the medium. The bags were sealed and incubated flat at 35˚C for 16-24 hours.

Chromogen choice and concentration

Chromogenic glucuronides were dissolved at various concentrations in hydrated Colilert® medium, combined with sample and applied to pads using wet protocol A. The chromogens and concentrations tested were: 8HQ at 0.1 and 0.2 g/L, X-Gluc at 0.15 and 0.3 g/L and IBDG at 0.16 and 0.32 g/L. The results were photographed after 16 hours and 24 hours incubation and qualitatively assessed with respect to colony resolution. Colony resolution was assessed according to the following criteria:

- color intensity
- spot size
- spot spacing
- color diffusion (smearing)

Each pad was assigned a score on a scale from 1 to 4 based on the above criteria. The scores correspond to the following rubric:

1: no contrast between background and colored spots; no enumeration possible

2: some individual spot or the suggestion of individual spots of intense coloration visible, but background smearing precludes accurate enumeration

3: distinct spots with some diffusion of color and little background smearing,, enumeration is possible with some uncertainty

4: distinct spots with minimal diffusion and no background smearing; facile enumeration

Colony resolution scores of less than 2.5 were associated with colonies that could not be enumerated with sufficient accuracy to make numerical comparisons between the pad test and the Bio-Rad and Colilert® benchmarks.

Chromogen as sole carbon source

X-Gluc was dissolved in M9 minimal salts medium at concentrations of 0.15 g/L, 0.3 g/L and 0.6 g/L. A filter sterilized, concentrated lactose solution was added to some M9 medium with 0.3 g/L X-Gluc to give final lactose concentrations of 2%, 1%, and 5%. The six media were combined with sample, applied to pads using wet protocol A, and incubated overnight. The results were photographed after 24 hours and 36 hours incubation and qualitatively assessed with respect to colony resolution based on the previously specified criteria. When possible, colony counts were compared to results for *E. coli* concentrations from the same samples using Colilert® Quanti-Tray®/2000 analysis and Bio-Rad membrane filter plates.

Liquid background media comparison

Concentrated bacteriological media were mixed with spiked samples (target spike of 10 CFU/mL E. coli) and applied to pads using wet protocol A. After overnight incubation, the pads were qualitatively assessed with respect to colony resolution based on specified criteria. When possible, colored colonies were enumerated and the results compared to *E. coli* results from the same samples using Colilert® Quanti-Tray, Colilert® pad and Bio-Rad plates. The media compared were:

- **Colilert®** (IDEXX)
- **mEndo** (Difco/BBL manual formulation, omitting basic fuchsin)
- **mTEC** (Difco/BBL manual formulation, omitting bromthymol blue and bromcresol purple)
- **Tryptic soy broth (TSB)** (Difco, also modified to include yeast extract and lactose in lieu of dextrose)
- **Lauryl Tryptose broth (LTB)** (Difco)
- **mT7** (Quelabs) with and without penicillin G

LTB alteration, wet vs. dry application

Based on preliminary experiments by Fu-Chi Hsu, LTB was tested in standard formulation with 0.3 g/L X-Gluc and the following alterations:

- (none)
- 0.001% CTAB
- 0.02% IPTG
- 0.001% CTAB and 0.02% IPTG (Personal communication, Fu-Chih Hsu).

The four media formulations were combined with samples spiked with either *E. coli* B or sewage (target *E. coli* spike 50 CFU/pad) and applied to pads using wet protocol B or the dry application protocol. After 18 hours incubation the pads were photographed and qualitatively assessed with respect to colony resolution based on specified criteria. When possible, colored colonies were enumerated and the results compared to results from *E. coli* analysis of the same samples using Colilert® Quanti-Tray®/2000, Colilert® pad and Bio-Rad plates.

Solid media comparison

Dried pads were made with Easygel, MI agar without cefsulodin, Bio-Rad Rapid E. coli II agar, or LTB containing 0.3 g/L X-Gluc and 0.25%, 0.5% or 1.0% guar gum. These dried pads were hydrated with samples spiked with E. coli B or sewage (target E. coli spike 5 CFU/mL). After 18 hours of incubation at 35˚C, the pads were photographed and qualitatively assessed with respect to colony resolution based on specified criteria. When possible, colored colonies were enumerated and the results compared to results from *E. coli* analysis of the same samples using Colilert® Quanti-Tray®/2000 and Bio-Rad plates.

Sample water pasteurization and filtration

A single sewage spike was subdivided into four equal volumes and each volume

subjected to one of the four treatment regimes intended to reduce concentrations of culturable bacteria:

1. no treatment
2. membrane filtration through 0.45 µm Millipore membrane filter
3. 25 minutes pasteurization in a 65 deg C water bath
4. 25 minutes pasteurization in a 65 deg C water bath followed by membrane filtration through 0.45 µm Millipore membrane filter

The resulting waters were then spiked with *E. coli* B (target spike 10 CFU/mL) and served as test samples for *E. coli* analysis by LTB pad, Bio-Rad plates, and Colilert® Quanti-Tray®/2000. After 18 hours of incubation at 35˚C, the pads were photographed and qualitatively assessed with respect to colony resolution based on specified criteria.

Titration

University Lake water was spiked with sewage to give total coliform concentrations of 0.5 CFU/mL, 5 CFU/mL and 50 CFU/mL. Each sewage spike was then spiked with cultured *E. coli* B to a concentration of 5 CFU *E. coli*/mL, giving *E. coli* B:total coliform ratios of 10:1, 1:1, and 1:10. The samples were applied to dried pads made with LTB containing 0.5% guar gum, and were enumerated for total coliform and *E. coli* concentrations in Colilert® Quanti-Tray®/2000 and on Bio-Rad plates.

Statistical analysis

All statistical analyses were conducted using InStat Prism 5 for Mac OS.

Liquid media comparison, wet protocol A.

Colony resolution scores were averaged across triplicate pads with the same medium using the same batch of spiked test water. These averages were then grouped by medium to give three average scores per medium, and compared using one-way analysis of variance and Tukey's post-test.

Liquid media comparison, wet protocol B, *E. coli* spike.

E. coli colony counts were averaged among triplicate pads with the same medium and test water batch. *E. coli* counts on triplicate Bio-Rad plates using the same test water batch were also averaged. These averaged counts and the Colilert® Quanti-Tray®/2000 MPN results were normalized to CFU/mL and log transformed. Averaged counts from different test formats using the same batch of test water, formed matched pairs for repeated measures analysis of variance.

Liquid media comparison, wet protocol B, sewage spike

E. coli colony counts were averaged among triplicate pads with the same medium and test water batch. *E. coli* counts on triplicate Bio-Rad plates using the same test water batch were also averaged. These averaged counts and the Colilert® Quanti-Tray®/2000 MPN results were normalized to CFU/mL, log transformed, and compared using one-way analysis of variance and on a pairwise basis using paired t-test.

Solid media comparison

E. coli colony counts were averaged among 10 pads with the same medium formulation using the same batch of test water. *E. coli* counts on triplicate Bio-Rad plates using the same test water batch were also averaged. These averaged counts and the Colilert® Quanti-Tray®/2000 MPN results were normalized to CFU/mL and compared using one-way analysis of variance.

Titration

The effect of the concentration of non-*E. coli* total coliforms on *E. coli* titers was assessed by repeated measures analysis of variance on log transformed mean *E. coli* titers from membrane filtration on Bio-Rad plates.

RESULTS

Absorbent pad method development and initial screening

Initial screening

A pilot experiment demonstrated that *E. coli* would grow and hydrolyze a chromogenic ß-D-glucuronide substrate in a liquid medium applied to an absorbent pad. Colilert® medium was selected as the background medium for the pilot experiment because it does not require sterilization and can be hydrated with only the sample water. Colilert® contains ONPG and MUG to specifically indicate the presence of total coliforms and *E. coli*, respectively. The hydrolysis products of both indicators are highly water soluble and diffuse readily in the absorbent pad, prohibiting enumeration of distinct colonies. Moreover, visualization of the MUG hydrolysis product requires a long-wave (366 nm) UV lamp and a darkroom, which would impair test portability and raise its cost. Thus IBDG, a chromogenic ß-D-glucuronide, was added to Colilert® at a concentration of 3.2 g/L, based on its inclusion at the same concentration in MI agar, the USEPA standard medium for simultaneous detection and enumeration of *E. coli* and total coliforms (USEPA Standard Method 1604). This modified medium was hydrated with University Lake water spiked with *E. coli* TW11566. A 10 mL volume of this mixture was applied to an absorbent cellulose pad and sealed inside a 1 quart plastic Ziploc® bag. Wood fiber cellulose pads were chosen because they are inexpensive, biodegradable, and readily available in a variety of shapes, sizes and textures. Topco Top Care® pantiliners are of a convenient size and thickness and can accommodate up to a 10 mL sample volume. Ziploc® and Nasco Whirl-Pak® bags are inexpensive, sturdy, disposable, portable, inert, reasonably leakproof, transparent, and readily available in sizes that accommodate Top Care® pantiliners. After 18 hours of incubation at 35˚C, blue-green spots approximately 4 mm in diameter were visible against a yellow background color (Figure 2, top right). These spots were presumably colonies of GUD-

expressing *E. coli*. Accurate colony enumeration was difficult because of the high concentration of colonies on the pad (more than 100) and some diffusion of the colored hydrolysis product away from the colonies.

Subsequent screening experiments

Attempts were made to improve the consistency and readability of absorbent pad test results by varying the following test characteristics:

1) Chromogenic substrate choice and concentration

2) Background medium formulation

3) Mode of medium application to pad

E. coli B was chosen as the representative *E. coli* strain for these experiments because it is GUD and GAL positive and grows readily on many commercial bacteriological media. However, naturally contaminated water samples contain a mixture of microorganisms in addition to *E. coli*. In order to more realistically simulate naturally contaminated surface water samples, small amounts of untreated human sewage were added to University Lake water. Simulation of contamination was necessary because it was difficult to find a consistent supply of surface water naturally contaminated with measurable and predictable amounts of *E. coli*.

Several chromogenic ß-D-glucuronides producing insoluble hydrolysis products were compared to IBDG with respect to colony resolution in the absorbent pad test format. Because most available *E. coli* media are yellow to brown in color, only blue and black chromogens were tested to maximize contrast between colored colonies and background medium coloration. X-Gluc and IBDG both have insoluble blue hydrolysis products and the 8-hydroxyquinoline liberated from 8HQ forms a black precipitate in the presence of Fe(III) ions (James 1988). 8HQ was tested at a starting concentration of 0.2 g/L based on its inclusion at that concentration in sorbitol MacConkey agar by Reinders et al for the detection of *E. coli* O157 (2000). X-Gluc was tested at 0.3 g/L based on its

inclusion in various commercially available agars at concentrations ranging from 0.15 to 0.5 g/L (Difco™/BBL™ manual 2007).

Colilert® considerations

Colilert® performed reasonably well as a background medium in the pilot experiment and was initially designated as a benchmark for experiments comparing the performance of different background media. However, Colilert® will not be used on a permanent basis for the absorbent pad tests for the following reasons:

1) Cost: The Colilert® medium is a proprietary formulation controlled by IDEXX Inc. It is considerably more expensive than several other *E. coli* media and too expensive to be included in what is intended to be a low-cost technology.
2) Stability: Dehydrated Colilert® medium contains at least one heat labile antibiotic. The preparation of absorbent test pads with pre-dried medium involves hydration, heating and desiccation of the hydrated medium. It is doubtful that Colilert® would be as effective after being subjected to such conditions, whereas several other *E. coli* media can be autoclaved or boiled without adversely impacting their performance.

Alternative background media

Other candidate background media were selected from among existing liquid *E. coli* media on the basis of their cost, availability, appearance and composition. Media containing bile salts were excluded because it was thought that amphipathic bile salts would solubilize the insoluble colored products of chromogen hydrolysis and reduce the readability of the pad test results. Darkly colored media were initially ruled out on the basis of the need for adequate visual contrast between background and colored colonies. Some dark media were included in the experiments if they could be adequately lightened by excluding colored indicator dyes. It is unknown whether the exclusion of these dyes affected other properties of these media besides their visible color.

Media concentration

The addition of nonsterile dehydrated culture media directly to spiked test water, such as in the pilot test with Colilert®, maximized the amount of sample that could be applied to each pad. However, with the exception of Colilert®, none of the media tested were sufficiently selective (defined substrate or containing selective antibiotics) to justify their use without sterilization. Hence, pad test method development proceeded with sterilized liquid media. Alternative background media were initially evaluated by combining 2X concentrated liquid media with spiked test water. The use of more highly concentrated media would increase the volume of sample that could be applied to each pad; however, coagulation, darkening and precipitation of media components was observed in some 10X liquid media after autoclaving. Dilution to standard concentration did not restore autoclaved 10X media to the original color or uniformity seen in media that were autoclaved at standard concentration, whereas 2X concentrated media did not precipitate or coagulate upon autoclaving, but still gave a sample volume of 5 mL per pad. 5 mL sample volumes compared favorably to 1 mL per test for 3M™ Petrifilm™ and to 5 mL per test for Coliscan Easygel, two of the most comparable existing *E. coli* enumeration technologies.

After the first phase of background media evaluation demonstrated poor colony resolution for all media, measures to improve colony resolution were prioritized over maximization of sample volume. Many 2X concentrated media were still significantly darker in color than media that were autoclaved at standard concentration, even after dilution of the 2X concentrated media to standard concentration. Hence, the pad test protocols were modified to include the use of 1X media, reducing the sample size to 0.32 mL per pad but increasing the visible contrast between colored colonies and background media enough to consistently allow enumeration of colonies.

Glucuronides as a sole carbon source

Because all candidate media were designed or were known to support the growth of organisms other than *E. coli*, selectivity was a key concern in the choice and alteration of background media. Defined substrate formulations such as Colilert® limit the range of available nutrients to support *E. coli* growth while suppressing the growth of interfering organisms. Because few fecal microorganisms can grow with only glucuronic acid as a carbon source, this property can be exploited to increase the selectivity of a test medium. Hence, a set of experiments assessed the ability of X-Gluc to function as a sole carbon source for *E. coli*. M9 minimal salts medium was chosen as the background medium for these experiments because it does not contain a carbon source in its basic formulation. It was supplemented with X-Gluc only or with both X-Gluc and lactose to simulate the many defined substrate media that include both a galactoside and a glucuronide to indicate the presence of both total coliforms and *E. coli*, respectively.

Medium modification to reduce smearing

Although several test media yielded satisfactory results with University Lake water spiked with *E. coli* B, significant diffusion of the blue X-Gluc hydrolysis product ("smearing") precluded accurate enumeration of colored colonies in nearly all experiments with sewage spiked water or naturally contaminated surface water samples. The absorbent pad test format was further modified in an effort to reduce smearing by:

1) adding CTAB, a detergent with some selective antimicrobial activity that ostensibly improves water absorption by the pad;
2) switching from the simultaneous application of liquid media and sample to the pad, to the application of water samples to pads that were pre-treated with dehydrated medium;
3) adding hydrocolloids such as agar or guar gum to stabilize and increase the viscosity of the medium, thereby reducing the diffusion of the colored hydrolysis

product. The medium on 3M™ Petrifilm™ is a hydrocolloid gel (Petrifilm™ patent). Guar gum was chosen for its high cold water solubility, ready availability and low cost. Bio-Rad and MI agars were tested because they were readily available as prepackaged media and because the latter is a USEPA standard medium and the former approximates the latter in performance as a culture medium for membrane filtration and enumeration by direct colony count (USEPA Method 1604, Lusk 2008).

In addition, experiments were conducted with water samples spiked with pasteurized and filtered sewage in efforts to characterize the cause or causes of colony smearing. Titration experiments were conducted to determine the threshold concentration of competing microorganisms that led to significant colony smearing.

Chromogenic substrate choice and concentration

Colony resolution scores obtained with X-Gluc and 8HQ were comparable (average colony resolution score 2.5/4, n=3) , and the scores were slightly higher than scores obtained using IBDG (average score 2.17, n=3). X-Gluc was selected for use in all subsequent experiments based on its higher resolution score, ready availability in

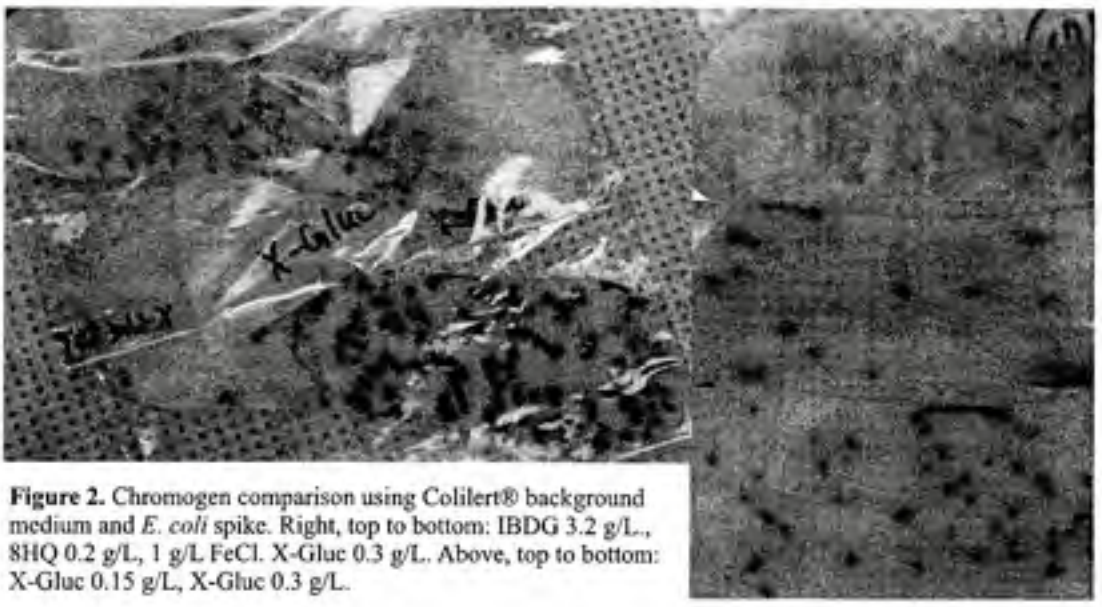

Figure 2. Chromogen comparison using Colilert® background medium and *E. coli* spike. Right, top to bottom: IBDG 3.2 g/L., 8HQ 0.2 g/L, 1 g/L FeCl. X-Gluc 0.3 g/L. Above, top to bottom: X-Gluc 0.15 g/L, X-Gluc 0.3 g/L.

large quantities (>1 g), and relatively low cost compared to IBDG and 8HQ. Comparison of colony resolution using varying concentrations of X-Gluc demonstrated that 0.3 g/L gave intensely colored results, while 0.15 g/L and 0.6 g/L gave significantly paler and only slightly more intensely colored results, respectively (Figure 2, left). A concentration of 0.3 g/L balanced colony color intensity with cost minimization. Based on these results, X-Gluc was used in all subsequent pad experiments at 0.3 g/L unless otherwise noted.

Chromogen as sole carbon source

X-Gluc was evaluated as the sole carbon source for the growth of E. coli on absorbent pads. For comparison, some test pads were supplemented with lactose as an additional carbon source. After 18 hours of incubation at 35˚C, no colored colonies were visible on pads containing only X-Gluc. On pads containing both lactose and X-Gluc, pale blue colonies were visible, but colony resolution was not sufficient for enumeration of bacteria (Figure 3, leftmost triplet). After 40 hours of incubation, colonies on the X-Gluc only pads were enumerable (Figure 3, rightmost triplet), but by gross visual examination were present in lower numbers than on the pads containing X-Gluc and lactose (Figure 3, middle triplet). Insufficient quantitative data were collected to make statistical

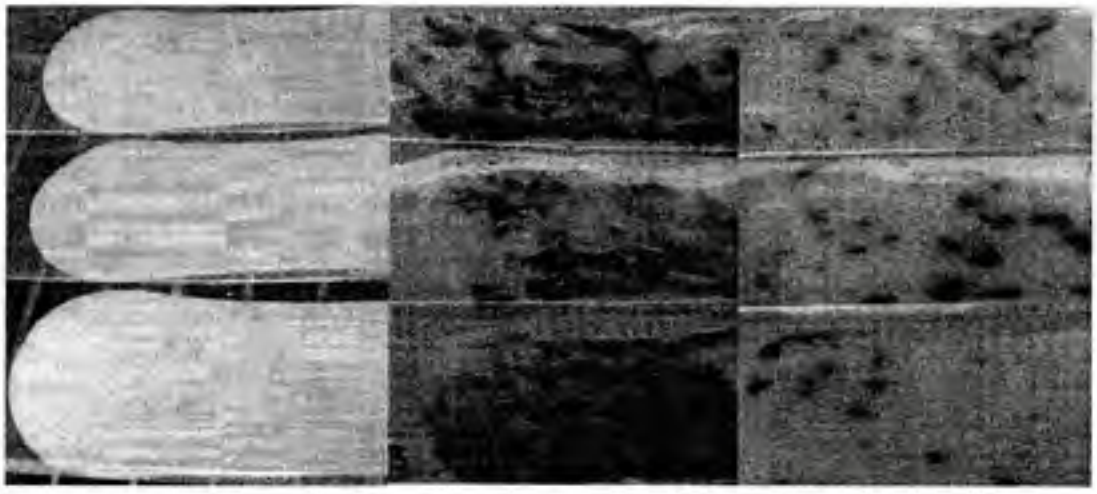

Figure 3. M9 medium with X-Gluc as sole carbon source or with both lactose and X-Gluc as carbon sources. Leftmost triplet, top to bottom: M9 + 0.3 g/L X-Gluc with 2%, 1%, or 0.5% lactose, after 18 hours incubation. Middle triplet, top to bottom: same pads after 40 hours incubation. Rightmost triplet, top to bottom: M9 + 0.15 g/L, 0.3 g/L, and 0.6 g/L X-Gluc, after 40 hours incubation.

comparisons between E. coli counts on test pads and benchmark tests. More than 24 hours of incubation at 35°C exceeds the incubation time of currently available tests and lengthens the amount of time before water quality results are available. This is an important drawback in resource poor conditions. Furthermore, incubation at ambient temperatures in the absence of temperature controlled incubators and a stable power supply may need to be longer than the times that provide reliable results with temperature controlled conditions. Longer incubation times would make the test even less desirable.

Liquid background media comparison

The test pad method development proceeded with the evaluation of several candidate background media: mEndo, mTEC, TSB, modified TSB, mT7, and mT7 with penicillin G. These media were potentially less selective than a defined substrate formulation, but could also support robust *E. coli* growth with less than 24 hours of incubation. They were assessed using wet protocol A with University Lake water spiked with *E. coli* B to 4 CFU/mL or 20 CFU per pad, to allow for adequate spacing between colored colonies for enumeration. There were no significant differences (p=0.75) between the mean colony resolution scores of liquid media tested using wet protocol A with University Lake water spiked with pure cultures of various strains of *E. coli* to 4 CFU/mL or 20 CFU/pad (Figure 4). Furthermore, none of the tested pad

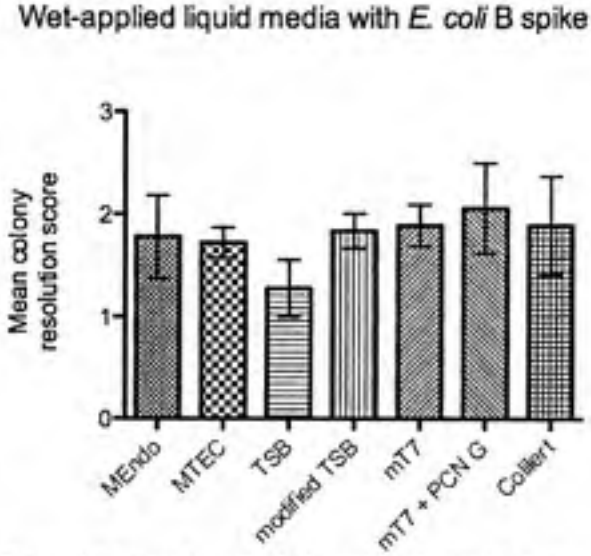

Figure 4. Mean colony resolution scores for liquid media applied to pads via wet protocol A with *E. coli* B spike. Columns represent averages of three averaged batches of triplicate pads using the same batch of test water. Bars are standard error of the mean.

media had a mean colony resolution score greater than 2 in these experiments (Figure 4) Colony resolution scores of less than 2 were associated with colonies that could not be enumerated accurately, so numerical comparisons could not be made between *E. coli* titers on the pad test and on Bio-Rad and Colilert® benchmarks. The colonies on these pads could be enumerated in some replicates, but there were an insufficient number of enumerable replicates to make statistical comparisons between colony counts on test pads and Bio-Rad and Colilert® counts.

The liquid media were significantly darker in color when prepared and autoclaved at 2X or higher concentrations, even after dilution to standard concentration. These darker media reduced contrast between colored colonies and the background coloration. Hence, the wet application protocol was modified to allow for the use of standard concentration (1X) media.

In a personal communication, Dr. Fu-Chih Hsu noted good results with LTB and X-Gluc in the wet application pad test format (March 17, 2008). LTB had not yet been tested in this laboratory in the pad test format, so it was evaluated against the Colilert® pad benchmark in a set of experiments using the modifeed wet protocol, wet protocol B. Test waters were spiked with either *E. coli* B or sewage to a target bacterial concentration of 62.5 CFU/mL, or 20 CFU per pad. Both Colilert® and LTB produced colony resolution scores above 2.5 in experiments with test water spiked with *E. coli* B (Figure 5, top). Because they gave similar colony resolution scores, bacterial numbers from Colilert® and

Figure 5. Qualitative comparison of Colilert and LTB wet protocol B pads with E. coli B and sewage spikes. Top: Colilert pad (left) and LTB pad (right) with *E. coli* B spike at ~20 CFU/pad. Bottom: Colilert

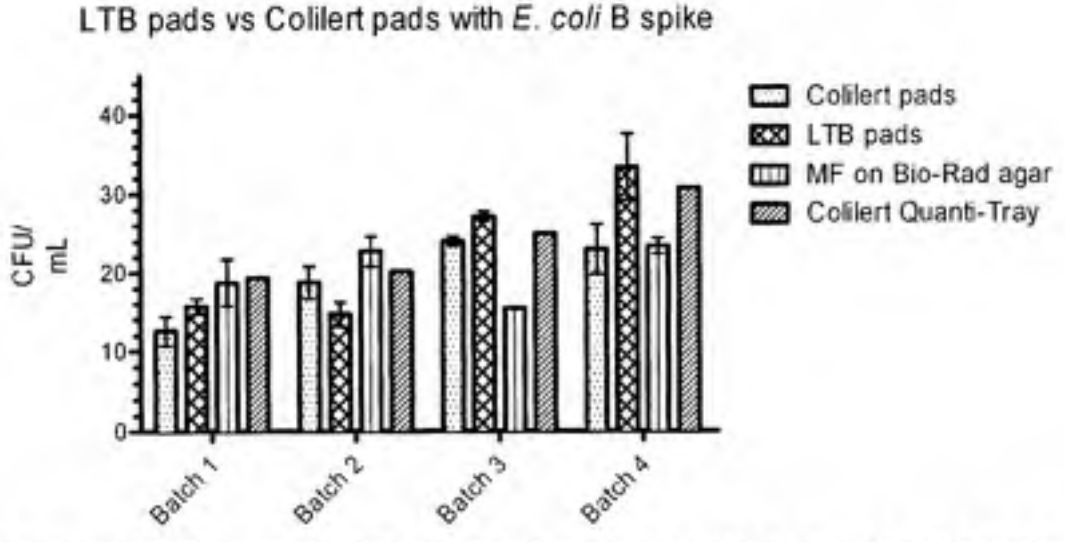

Figure 6. Mean colony counts for LTB and Colilert® applied to pads via wet protocol B with *E. coli* B spike. n=3 for pads and Bio-Rad plates. Columns represent averages of triplicate pads or plates using the same batch of spiked water. Error bars are standard error of the mean.

	mean log diff.	p < 0.05?	95% CI of diff
Colilert® pads vs LTB pads	-0.051	No	-0.2596 to 0.1578
Colilert® pads vs Colilert® Quanti-Tray®/2000	-0.091	No	-0.2999 to 0.1174
Colilert® pads vs MF on Bio-Rad agar plates	-0.018	No	-0.2263 to 0.1910
LTB pads vs Colilert® Quanti-Tray®/2000	-0.040	No	-0.2490 to 0.1683
LTB pads vs MF on Bio-Rad agar plates	0.033	No	-0.1754 to 0.2419
Colilert® Quanti-Tray®/2000 vs MF on Bio-Rad agar plates	0.074	No	-0.1350 to 0.2823

Table 4. Tukey pairwise comparisons between *E. coli* titers obtained from different test formats for the same test water spiked with *E. coli* B. .

LTB in pad format were compared to bacterial numbers when samples were analyzed using the benchmark methods of Bio-Rad membrane filtration and Colilert® MPN. Data in Figure 6 and Figure 7 are grouped to show *E. coli* titers from the same spiked water assayed using different test formats. The average *E. coli* titers across test formats differed significantly between batches of water spiked with *E. coli* B (p=0.04), indicating that repeated measures analysis of variance would be a more powerful statistical test of this data than one-way analysis of variance. There were no significant differences between mean *E. coli* titers from Colilert® pads, LTB pads, Bio-Rad plates and Colilert® Quanti-Trays in the experiments using test waters spiked with *E. coli* B (repeated

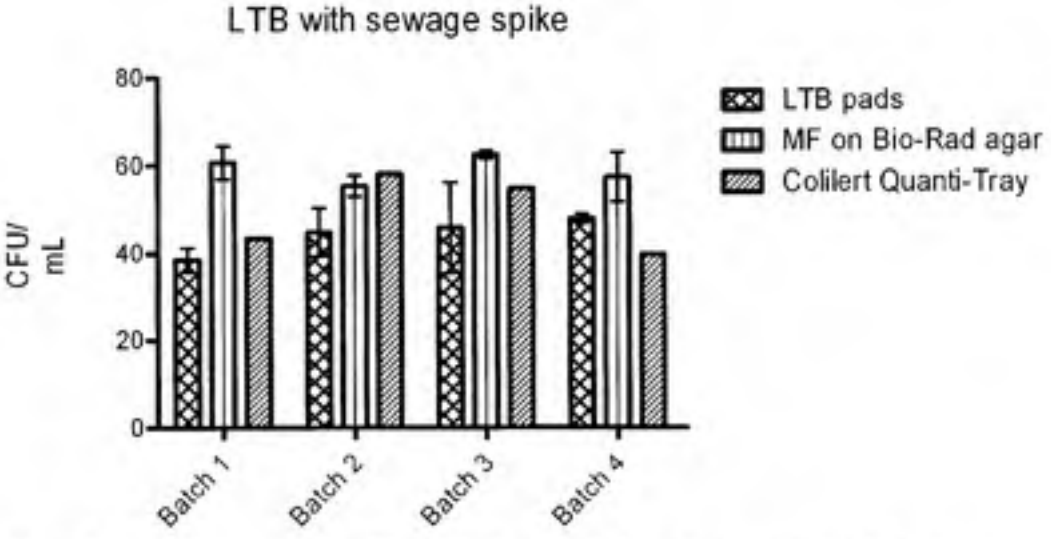

Figure 7. Mean colony counts for LTB and Colilert® applied to pads via wet protocol B with sewage spike. n=3 for pads and Bio-Rad plates. Columns represent averages of triplicate pads or plates using the same batch of spiked water. Error bars are standard error of the mean.

	mean log diff.	Means significantly different?	95% CI of diff	paired t-test p value
LTB pads vs Colilert® Quanti-Tray®/2000	-0.040	No	-0.1445 to 0.06460	0.38
LTB pads vs MF on Bio-Rad agar plates	-0.125	Yes	-0.2296 to -0.02050	0.02
Colilert® Quanti-Tray®/2000 vs MF on Bio-Rad agar plates	-0.085	No	-0.1897 to 0.01946	0.13

Table 5. Tukey pairwise comparisons and paired t-tests between *E. coli* titers obtained from different test formats for the same test water spiked with sewage.

measures analysis of variance p=0.56) (Figure 6, Table 4).

When test waters were spiked with sewage, the test pad format using Colilert® medium did not give sufficient colony resolution for enumeration (Figure 5, bottom). Using test waters spiked with sewage, colony counts from LTB in pad format were not significantly different from results from Colilert® MPN (paired t-test p=0.38), but were significantly lower than counts on Bio-Rad plates (one-way analysis of variance p=0.02, paired t-test p=0.02, r^2=0.89). *E. coli* titers from the same sewage spiked test water in Colilert® MPN and Bio-Rad plates did not differ significantly between Colilert® MPN and Bio-Rad plates (paired t-test p=.13) (Figure 7, Table 5).

Time scale of color development

In order to determine the optimum incubation period for LTB pads, LTB pads were observed for colony resolution after 12, 14, 16, 18, 20, 22, and 24 hours of incubation at 35˚C with test water spiked with *E. coli* B. All LTB pads achieved maximum colony resolution scores after 16-18 hours of incubation, but became too smeared to accurately enumerate after 20-24 hours (Figure 8). In all subsequent pad tests, samples were incubated for 18 hours.

Figure 8. Time scale of color development on wet protocol A LTB pads with *E. coli* B spike. (Left to right) After 18 hours, 20 hours, 22 hours, and 24 hours incubation.

LTB alteration, wet vs dry application

In the next set of experiments, LTB was supplemented with either CTAB, IPTG, or both and tested with a sewage spike by both wet protocol B and dry protocol. The target concentration of the sewage spike was raised to 100 *E. coli* CFU/pad to increase the power to detect statistically significant differences in colony counts. No medium formulation tested gave a mean colony resolution score higher than 2, and no significant difference in mean colony resolution was observed between different formulations or between the wet and dry formats (Figure 9). However, the dry format test was observed to absorb and distribute the sample more evenly across each pad, and could accommodate a larger sample volume than the pads prepared using wet protocol B (10 mL vs. 0.32 mL).

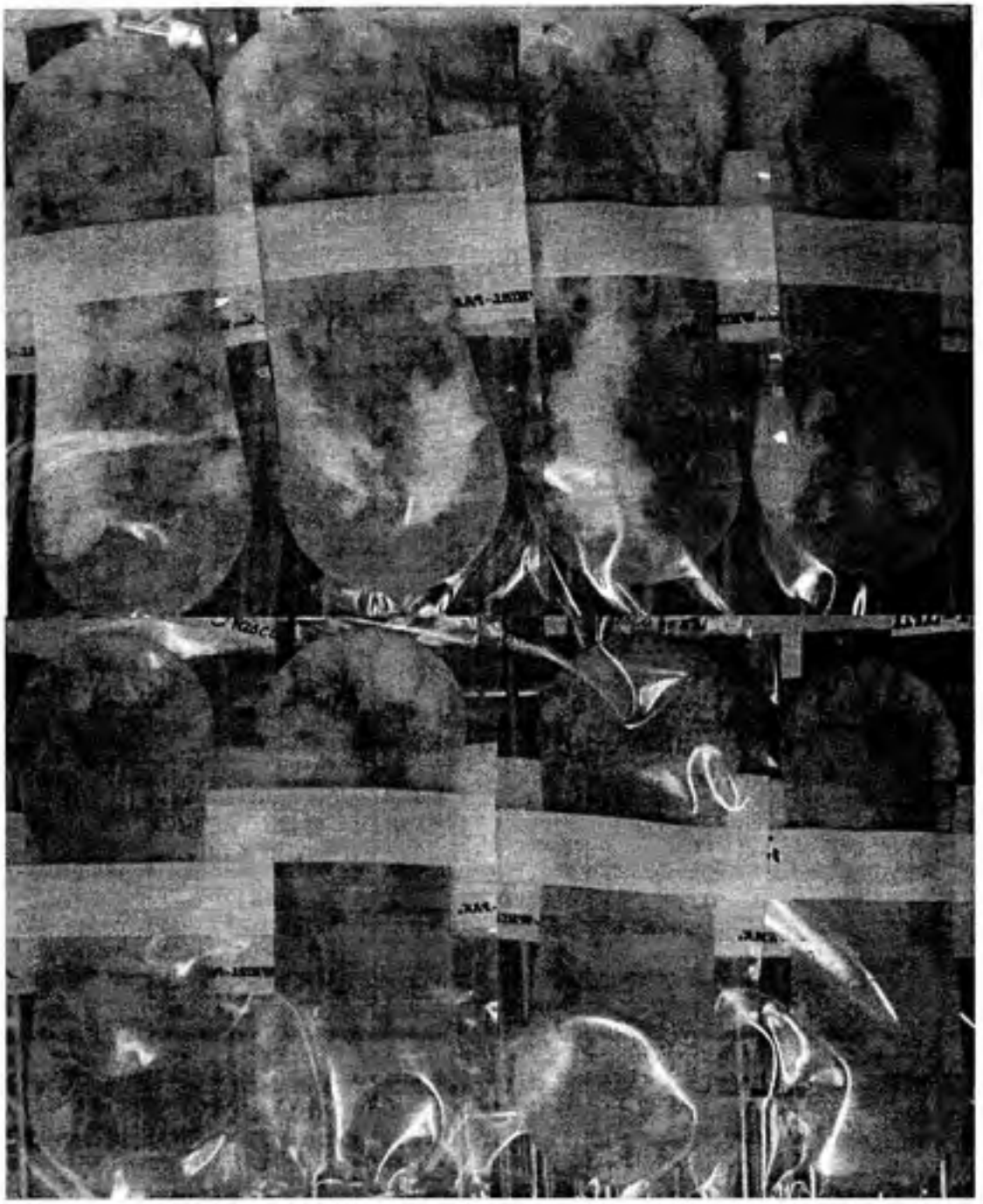

Figure 9. Qualitative comparison of wet and dry applications of LTB formulations. Top row: pads using wet protocol B (left to right) LTB only, LTB + CTAB, LTB+IPTG, LTB + IPTG + CTAB. Bottom row: pads using dry protocol, same media as pads directly above. Note dry patches on wet protocol pads.

Solid media comparison

The next set of experiments demonstrated that colony resolution on the dry format pad test could be increased and stabilized with the addition of hydrocolloids to the test medium. Easygel, MI agar without cefsulodin, Bio-Rad Rapid *E. coli* II agar, and LTB supplemented with guar gum at 0.1%, 0.25%, and 0.5% were tested using the dry application protocol with University Lake water spiked with *E. coli* B or sewage to 5 *E. coli* CFU/mL or 50 *E. coli* CFU per pad. The agar and Easygel pads did not absorb as much sample as the guar gum pads. Easygel did not produce enumerable colonies with either *E. coli* spike (Figure 10, far left). None of the solid media gave enumerable results with sewage spiked test water (Figure 10, bottom row). Dried MI agar pads, dried Bio-Rad agar pads and dried LTB pads with 0.5% or 1.0% guar gum all achieved the maximum colony resolution score of 4 with test water spiked with *E. coli* B after 18 hours of incubation. Dried MI agar pads produced significantly lower *E. coli* titers than the dried LTB guar gum pads even when the sample volume for the MI agar pads was decreased to a volume that the agar pads could absorb completely (Figure 11). LTB pads with 0.5%

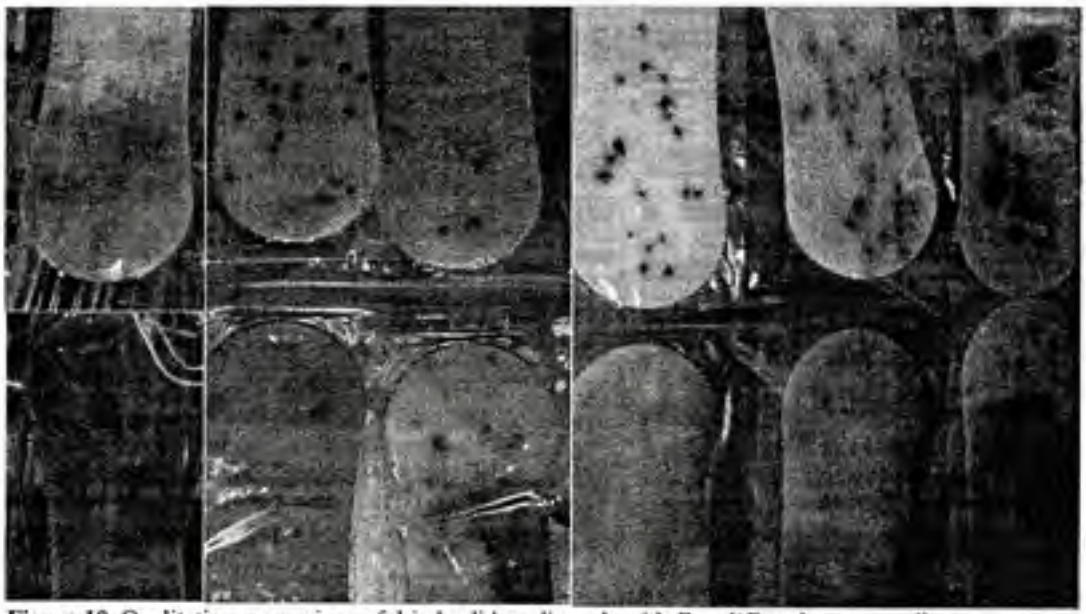

Figure 10. Qualitative comparison of dried solid media pads with *E. coli* B and sewage spikes. Top row: *E. coli* B spike, (left to right) Easygel, Bio-Rad agar, MI agar, LTB + 0.5% guar gum, LTB + 0.25% guar gum, LTB + 0.1% guar gum. Bottom row: sewage spike, same media as top row.

guar gum and 1.0% guar gum with an *E. coli* B spike produced *E. coli* titers comparable to the Colilert® Quanti-Tray®/2000 and Bio-Rad plate benchmarks (one-way analysis of variance p=0.49) (Figure 11, Table 6). The colonies on 1.0% guar gum pads were smaller in diameter and less intense in color than the colonies on the 0.5% guar pads produced with University Lake water spiked with *E. coli* B (Figure 12, left and center). In addition, dried LTB + 0.5% guar gum and LTB + 1.0% guar gum pads with *E. coli* B spiked University Lake water were still enumerable (colony resolution score >2) after 42 hours of incubation (Figure 13). Colony counts on these pads at 18 hours and at 42 hours were identical.

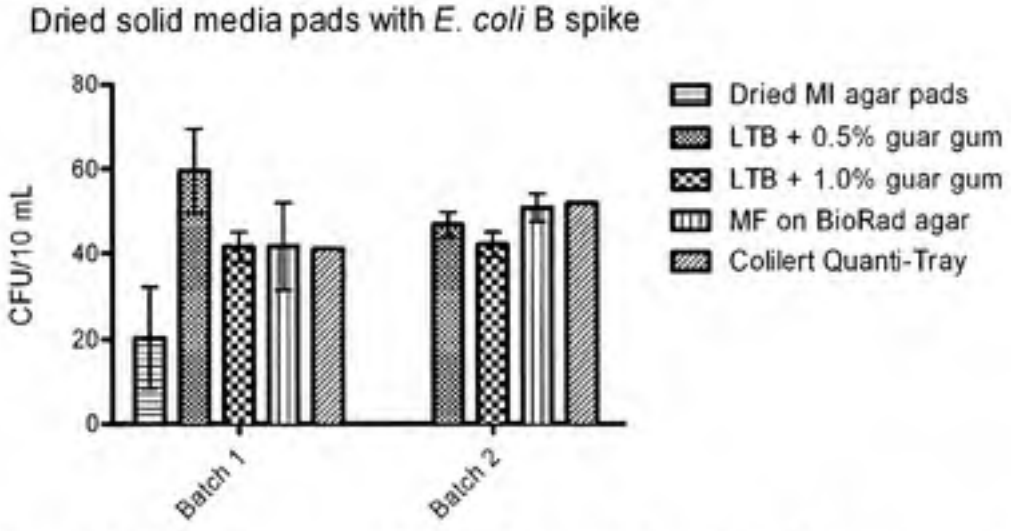

Figure 11. Mean colony counts on dried solid media pads with *E. coli* B spike. Columns represent average of 10 duplicate pads or 3 Bio-Rad plates using same batch of test water. Bars are standard error of the mean.

	log mean diff.	p<0.05?	95% CI of diff
LTB + 0.5% guar gum vs LTB + 1.0% guar gum	0.101	No	-0.1407 to 0.3435
LTB + 0.5% guar gum vs MF on Bio-Rad agar plates	0.060	No	-0.1823 to 0.3019
LTB + 0.5% guar gum vs Colilert® Quanti-Tray®/2000	0.059	No	-0.1836 to 0.3006
LTB + 1.0% guar gum vs MF on Bio-Rad agar plates	-0.042	No	-0.2837 to 0.2005
LTB + 1.0% guar gum vs Colilert® Quanti-Tray®/2000	-0.043	No	-0.2850 to 0.1992
MF on Bio-Rad agar plates vs Colilert® Quanti-Tray®/2000	-0.001	No	-0.2434 to 0.2409

Table 6. Tukey pairwise comparisons between mean *E. coli* titers on dried solid media pads and Colilert Quanti-Tray and Bio-Rad membrane filtration benchmarks.

Figure 12. Qualitative comparison of dried LTB and solid media pads with *E. coli* B spike after 18 hours incubation. (Left to right) Dried LTB + 1.0% guar gum, dried LTB + 0.5% guar gum, dried LTB.

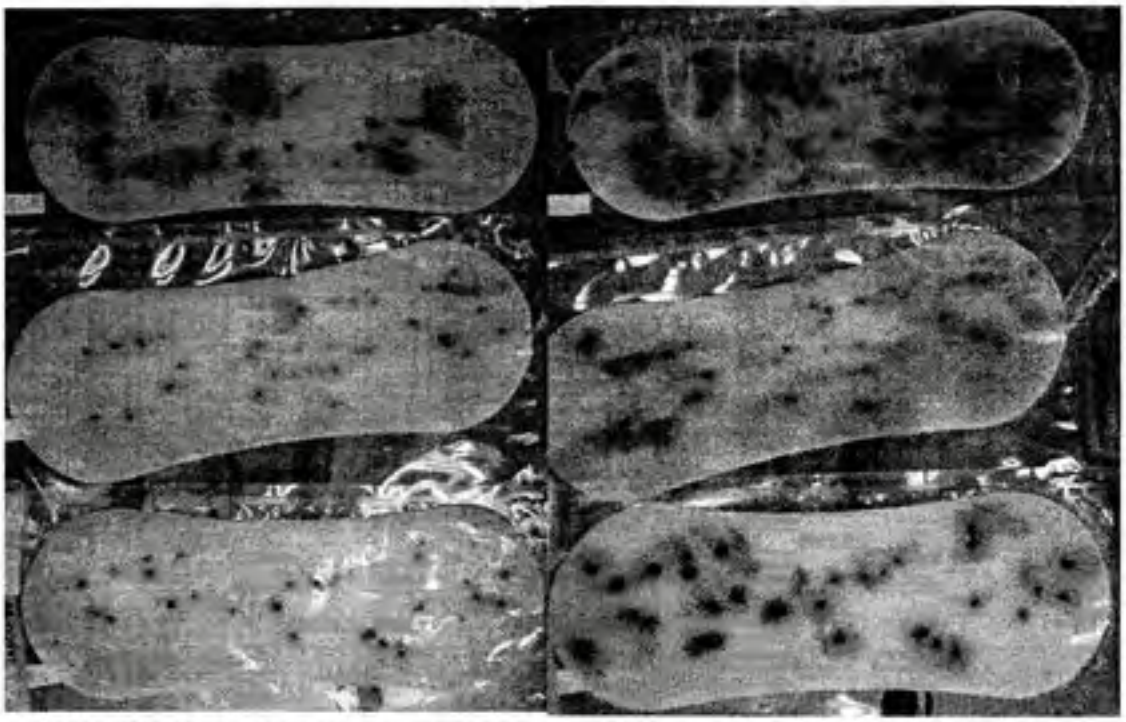

Figure 13. Stability over time of colony resolution on dried LTB + guar gum media. (Left) After 18 hours incubation. Top to bottom: 0.1% guar gum, 0.25% guar gum, 0.5% guar gum. (Right) Same pads after 42 hours incubation.

Pasteurization and titration

Pasteurization, filtration, or both pasteurization and filtration before sample inoculation produced colony resolution from test waters spiked with sewage comparable to colony resolution from test waters spiked with *E. coli* B (Figure 14). This was observed

on dried LTB pads, dried LTB with 0.5% guar gum pads, and dried MI agar pads. Experiments designed to titrate the numbers of background organisms in test waters using dried LTB pads with 0.5% guar gum showed that background smearing increased and colony resolution decreased with increasing ratios of non-*E. coli* total coliforms to *E. coli* (Figures 15, 16). The ratio of *E. coli* to total coliforms had no significant effect on the number of *E. coli* detected by membrane filtration on Bio-Rad agar (repeated measures analysis of variance p=0.37) (Figure 17).

Figure 14. Pasteurization and filtration experiment. Qualitative comparison of dried LTB pads with various waters spiked with *E. coli* B (target 5 CFU/mL). (Left to right) University Lake water, pasteurized and filtered sewage spike, filtered sewage spike, pasteurized sewage spike, and untreated sewage spike (10 CFU/mL total *E. coli*).

Figure 15. Titration experiment. Dried LTB + 0.5% guar gum pads with varying ratios of sewage total coliforms to total *E. coli*. (Left to right): 10:1 total coliforms:*E. coli*, 1:1, 1:10, control (*E. coli* B in University Lake water).

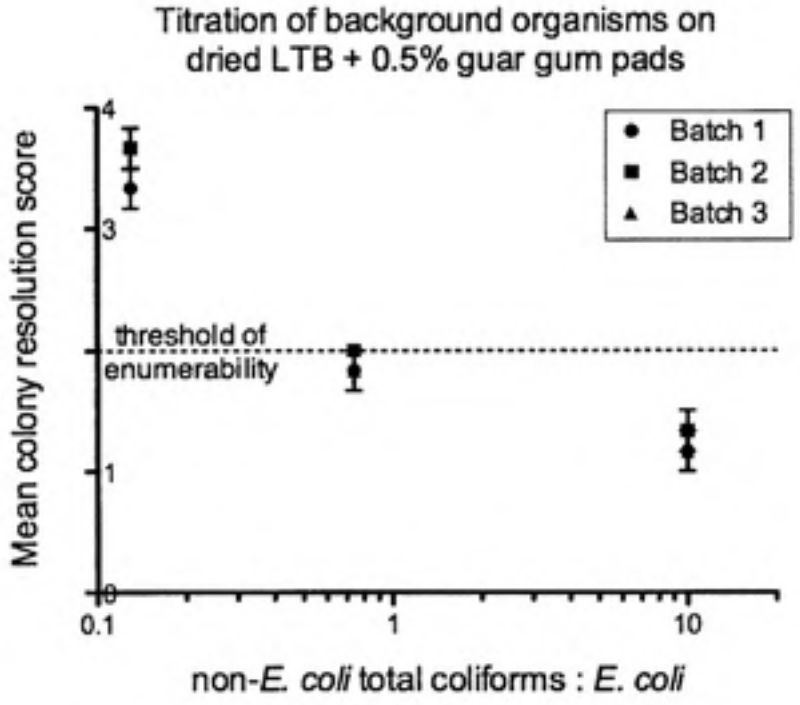

Figure 16. Titration of background organisms in test waters on dried LTB + 0.5% guar gum pads. Mean colony resolution scores for triplicate pads are plotted with standard error bars against the ratio of non-*E. coli* total

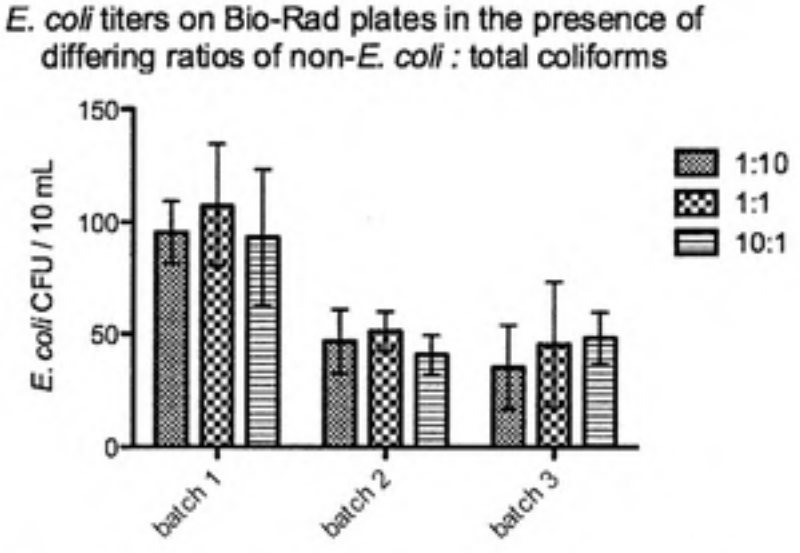

Figure 17. *E.* coli titers by membrane filtration on Bio-Rad plates in the presence of differing ratios of non-*E. coli* total coliforms. Columns represent mean *E. coli* titers from membrane filtration in triplicate on Bio-Rad plates.

DISCUSSION

At this stage in method development, the absorbent pad test in the dried solid media formulation with LTB and guar gum gives sufficient colony resolution to allow enumeration of pure culture *E. coli* spiked at concentrations between 10 and 1000 CFU per 100 mL into high quality surface water containing few or no interfering microbes. *E. coli* titers obtained from test waters using the pad test were not significantly different from titers obtained using two gold standard USEPA approved methods for *E. coli* enumeration. Pads with wet-applied LTB gave sufficient colony resolution to allow enumeration of *E. coli* from sewage diluted with high quality surface water to concentrations between 10 and 500 CFU per 100 mL. *E. coli* titers in these test conditions were not significantly different from Colilert® Quanti-Tray®/2000 but were significantly lower than on membrane filters incubated on Bio-Rad plates. However, the absorbent pad test lacks adequate colony resolution to accurately and precisely enumerate *E. coli* in environmental water samples (Figure 18). Under controlled testing conditions using pure culture *E. coli* the absorbent pad test gives sufficient colony resolution to allow enumeration of *E. coli,* and the resulting numbers are not significantly different from USEPA approved methods. These result suggest that further improvements to the absorbent pad format could produce a pad test that can enumerate *E. coli* in naturally contaminated waters.

Figure 18. Dried LTB pads after 18 hours incubation with naturally contaminated water samples from Northeast Creek (top) and Booker Creek (bottom).

The largest single hurdle to achieving a field ready test is the smearing of the colored X-Gluc hydrolysis product, which interferes with colony enumeration. Results from the pasteurization and titration experiments demonstrate that background

organisms are likely a major cause of the smearing. These results suggest three sets of options for reducing smearing. First, alterations to the physical properties of the test may reduce smearing. The absorbent pad allows significantly more diffusion and movement of bacteria and media components than is possible on the surface of solidified agar or on membrane filters. There may be other pad materials or types of cellulose pads that allow less diffusion of medium and sample, although these materials may absorb less liquid and thus force a tradeoff between sample volume and colony resolution. The pad material itself could be treated with substances that enhance its ability to bind the colored product, although this approach may also reduce the absorbency of the pad or inhibit *E. coli* growth. A transparent top layer to which the chromogen adheres might serve the same purpose, but the material for this layer must not to impact *E. coli* growth or GUD expression.

Results demonstrated that one of the tested hydrocolloids, guar gum, may reduce the mobility of the colored product without significantly lowering colony counts. An advantage of using guar gum is that unlike agar, it does not reduce the volume of sample that can be examined using the test pad. However, the use of serological pipettes to aliquot media onto pads placed a practical limit on the concentration of guar gum in the medium. It was not possible to make pads with guar gum concentrations higher than 1.0% using the desiccation technique described in the dry application protocol. Higher concentrations of guar gum or other hydrocolloids may be more successful at containing the spread of the colored product without significantly lowering colony counts. Authors of the patent for the Petrifilm™ test format recommend guar gum concentrations between 0.8 and 5% (US Patent 4565783). Xanthan gum, another cold water soluble hydrocolloid, is another candidate hydrocolloid as it is known to interact synergistically with guar gum in a manner that may enhance the ability of both substances to contain the spread of the colored products of chromogenic glucuronide hydrolysis. The extremely low cost of hydrocolloids (less than 1/10 of 1 cent per test) also makes them excellent candidates for

incorporation in the low-cost absorbent pad format.

A second set of options to reduce smearing consists of alterations to the test medium to reduce the mobility of the bacteria themselves. Some species of *E. coli* and many species of enterobacteria are known to be flagellated and motile. *Proteus mirabilis*, for example, is well known for its swarming behavior. D'Mello and Yotis found that sodium deoxycholate (SDO) at 0.5 or 1.0 g/L completely inhibited *P. mirabilis* swarming (1987). They also noted a slowing effect on *E. coli* growth at concentrations as low as 0.001 g/L, and Fujisawa and Mori found that SDO at 3 g/L inhibited GUD activity in lysed *E. coli* (1996). Fujisawa and Mori also found that GUD activity was enhanced in intact *E. coli* by a tenfold lower concentration of SDO (0.3 g/L), probably by increasing permeability of the bacterial plasma membrane to GUD substrates (1996). SDO is also a bile salt with detergent properties, implying that at some concentrations it may solubilize the colored chromogen hydrolysis product enough to lower colony resolution. These findings suggests that SDO may contribute beneficial properties to the absorbent pad test, but it must be studied to establish a useful range of concentrations that inhibit bacterial motility without reducing *E. coli* growth, GUD activity or colony resolution in the pad test format.

The third and most extensive set of options to reduce smearing are measures to reduce or eliminate competing organisms. Many culture media already employ a variety of traditional measures to achieve this selectivity. LTB already contains sodium lauryl sulfate, a detergent which at low concentrations inhibits the growth of gram positives with little impact on *E. coli*. However, detergents, like bile salts, may also solubilize the colored chromogen hydrolysis product and reduce GUD activity in injured bacteria, resulting in lowered colony resolution and underestimation of *E. coli* numbers in samples containing viable but injured *E. coli*. Antibiotics can increase medium selectivity without interfering with the use of chromogenic enzyme substrates. However, antibiotics are both heat labile and expensive, so their inclusion in the test medium runs counter to the stated

goals of increasing shelf stability and reducing cost.

Restricting available nutrients in the manner of a defined substrate medium can increase medium selectivity without the addition of antibiotics or detergents. M9 minimal salts medium containing a chromogenic glucuronide as a sole carbon source required impractically long incubation periods (40 hours, versus 18-24 hours for USEPA standard culture-based methods) to achieve enumerable results. However, M9 is not designed specifically to support the robust growth of *E. coli*. A medium formulation that supports enhanced *E. coli* growth and suppresses the growth of contaminating bacteria might be achieved by formulating an entirely new medium, or by making stepwise alterations to existing media formulations. Either approach precludes the use of existing commercially available media and adds both time-consuming elements and uncertainty to the development of the pad test. These approaches should not be undertaken unless other routes of pad test improvement prove unsuccessful in producing a test that meets the criteria outline above.

One crucial property of the absorbent pad test that has not yet been addressed is its statistical power, which is largely determined by the sample volume that can be examined in a single test. The current maximum allowable test volume of 10 mL is dictated by the size and absorbency of the pad and, to a lesser degree, the absorbency of some of the medium components. The pad must be thin to avoid bacterial growth inside the pad where it cannot be visualized. A pad of the same material thickness but greater surface area would allow for a larger sample size. Portability could be retained with a pad and bag that can be folded numerous times for transport, then unfurled at the testing site to maximize its usable surface area. Increasing the size of the test would, of course, increase its material cost.

Another determinant of the statistical power of the absorbent pad test is the consistency and precision with which it enumerates *E. coli*. Though presence-absence information on a 100 mL sample is enough to determine whether water meets WHO

drinking water guidelines, the ability to accurately enumerate between 1 and 100 *E. coli* in a 100 mL water sample is useful to determine the need for or effectiveness of water treatment interventions and for evaluating any predictive relationship between E. coli concentrations and health outcomes. The consistency and precision with which the absorbent pad test enumerates this range of bacterial concentrations is impacted considerably by the presence and concentration of other microbial and chemical contaminants. The 10 mL volume absorbent pad test enumerated *E. coli* at concentrations between 10 and 1000 CFU per 100 mL when the water samples were high quality surface water artificially contaminated with pure culture *E. coli*. However, it was impossible to enumerate *E. coli* in surface water naturally contaminated with *E. coli* at 5 CFU/mL (50 CFU/100 mL) and in high quality surface water artificially contaminated with sewage at the same *E. coli* concentration.

Background contamination varies widely between sampling sites. The absorbent pad test in its current format might permit *E. coli* enumeration with drinking water samples from some locations, and yield only presence-absence information in others. Test performance standards were originally set with the goal of achieving maximum applicability by making it possible to enumerate *E. coli* in any drinking water sample. It was thought that the use of dilute raw sewage to mimic naturally occurring fecal contamination at a relatively high concentration would provide a rigorous performance standard for the pad test. However, separate versions of the pad test could be tailored to meet the needs of different sampling locations and users at reasonable cost. More information about the nature of naturally occurring background contamination is needed to set appropriate performance standards for the test and for different end users. Different sources of artificial contamination such as actual human feces, and a wider variety of waters with naturally occurring *E. coli* contamination should be assessed.

The test is quite portable and simple to use in its current configuration. With the exception of the chromogenic enzyme substrates, all of the test components are known

to be shelf stable for at least one year in sealed containers in typical laboratory countertop conditions (room temperature, low humidity). Dried LTB + 0.5% guar gum pads gave enumerable results with *E. coli* B spiked water after one week to four months of storage with minimal disturbance in a lab drawer at room temperature. Colony counts on stored pads have yet to be compared to enumeration results obtained using gold standard methods. When the test is optimized to reduce smearing and produce sufficient colony resolution for enumeration of *E. coli* in naturally contaminated waters, test performance at nonstandard incubation temperatures should be assessed.

A version of the absorbent pad test with its current physical properties and the ability to enumerate *E. coli* with sufficient precision and accuracy in naturally contaminated water samples would be a significant improvement over existing low-cost *E. coli* enumeration technologies. The current cost per test based on standard U.S. sources for all materials is 66 cents per 10 mL test, which makes it comparable to Colilert® or Bio-Rad membrane filtration for 100 mL volumes (Table 1). In comparison to these gold standard tests, the absorbent pad test format yields much less consistent results but is considerably more portable and simpler to use. Lower cost sources for test components have yet to be explored, but it should be noted that the chromogenic glucuronide substrate accounts for 70% of the cost of the test, so the search for a less expensive source of chromogenic glucuronides should be prioritized over cost savings investigations into any other material (Appendix B).

CONCLUSIONS

These results demonstrate that a simple, portable, low-cost absorbent pad method for enumerating *E. coli* can be performed using currently available materials. Further development and optimization are required to address technical problems with the method that were identified in these experiments. An optimized test can be evaluated in the field with respect to its ability to assess interventions for improving water quality at

the point of use. In addition, the test may be used to explore relationships between *E. coli* levels in water and human health risks. This work demonstrates that the absorbent pad *E. coli* test has the potential to become a robust, widely available tool for assessing drinking water quality at the point of use in resource poor and disaster settings.

Key findings

- Absorbent pad with predried LTB and X-Gluc at 0.3 g/L allows detection and enumeration of *E. coli* in surface water with little background contamination
- Colony resolution is significantly and adversely impacted by the presence and concentration of contaminating organisms
- Inclusion of guar gum at 0.5 or 1.0% improves colored colony resolution and lengthens time window during which *E. coli* enumeration is possible

Suggested avenues for investigation

- Improve colony resolution with LTB-based test medium by including:
  - Higher concentrations of guar gum (2.0 - 3.0%)
  - Alternative hydrocolloids e.g. xanthan gum
  - Inhibitors of bacterial motility
  - Alternative pad materials
  - Treatments for pad material that may sequester colored chromogen hydrolysis product without affecting *E. coli* growth
- Improve selectivity of medium by developing defined substrate medium formulation
- Assess consistency of performance of pad test with more representative water samples including:
  - Surface water artificially contaminated with alternative sources of simulated fecal contamination, e.g. human feces
  - Surface water samples with naturally occurring *E. coli* contamination from

international and local field trials

- Investigate effect of storage conditions and length of storage on test performance

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APPENDIX A: Liquid media composition

	nitrogen sources							sugars	
(in grams)	tryptose	proteose peptone #3	casitone	thio-peptone	NH_4Cl	soytone	yeast extract	lactose	dextrose
M9					1				
mEndo*	10		5	5			1.5	12.5	
mTEC*		5					3	10	
TSB			17			3			2.5
TSB (mod)			17			3	3	5	

					salts				
(in grams)	*bile salts*	*Na-DOC*	*SDS*	*Tergitol-7*	*NaCl*	*Na_2HPO_4*	*KH_2PO_4*	*K_2HPO_4*	*$NaSO_3$*
M9					0.50	6.78	3.00		
mEndo*		0.1	0.05		5.00		1.38	4.38	2.10
mTEC*		0.1	0.2		7.50		1.00	3.30	
TSB					5.00			2.50	
TSB (mod)			0.1		5.00			2.50	

APPENDIX B: Per test cost determination

Material	Source	Price (USD)	Quantity	Quantity per test	Per test cost (USD)
LTB	Weber Scientific online, www.weberscientific.com	69.40	500 g	.356 g	0.0494
X-Gluc	Gold BioTechnology®, www.goldbio.com	619.00	5 g CHA salt	.0037 g CHA salt	0.4581
Guar gum	Source Naturals via Amazon.com	6.11	453.6 g	.05g	0.0007
Top Care® Pantiliners	Harris Teeter supermarket	2.99	64	1	0.0467
Nasco 7 oz Whirl-Pak® bags	Nasco online store, www.enasco.com	51.25	500	1	0.1025
				TOTAL	0.6574