A HealthTech Report

Efficacy of Standard Methods for Detection of Fecal Contamination at a Range of Nonstandard Temperatures

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HealthTech Report to USAID

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Abstract

Standard, reliable testing for fecal contamination remains inaccessible to the majority of the world's population. Evaluation of water sources in developing countries (and following emergencies or natural disasters) cannot rely on electricity, skilled personnel, or a controlled laboratory environment. There is an urgent need for a simple, specific, and reliable field test that can be used in a variety of conditions. In an effort to test the viability of currently available *Escherichia coli* detection assays at an assortment of temperatures, we undertook this study at the request of USAID in collaboration with the University of North Carolina. Fifteen strains of *E. coli* were each inoculated onto five standard and nonstandard tests and incubated at a range of five temperatures. Of the tests studied, membrane filtration with MI Agar gave the best performance for ß-glucuronidase detection, ß-galactosidase detection, and *E. coli* specificity across all temperatures. Petrifilm™ produced the best growth of *E. coli* at all temperatures, but lacked specificity. Colilert® did not perform well outside its optimized temperature range, so would be of limited usefulness in low-resource settings. The nonstandard tests, MacConkey II (MAC II) agar and EC Medium multiple-well fermentation, gave inconsistent results and would not be useful for a reliable test in uncontrolled conditions. The results of this study have been submitted for publication in *Applied and Environmental Microbiology.*

Introduction

The World Health Organization (WHO) estimates that more than 1 billion people are still using potentially harmful sources of water.[1] In recent emergencies, including the tsunami in December 2004, and the earthquake in Pakistan in October 2005, there has been little capacity to test water for fecal contamination and make informed management decisions about drinking water sources, treatment, and protection.[2,3] These situations highlight the need to assess the available tools for measuring fecal contamination in drinking water in the absence of conventional laboratory facilities and skilled personnel.

Standardized protocols of testing for the presence of coliform bacteria in drinking water in the United States require a detection limit of 1 CFU/100 mL. All current methods approved by the US Environmental Protection Agency (EPA), including broth enzyme substrate methods such as Colilert® (IDEXX, Westbrook, ME); Colisure® (IDEXX, Westbrook, ME); Colitag® (CPI International, Santa Rosa, CA); E*Colite® (Charm Sciences, Malden, MA); Readycult® (EMD Chemicals, Gibbstown, NJ); or Standard Method 9221 (LTB, P/A broth, M-Endo-EC-MUG); as well as membrane filtration methods such as Standard Method 9222B (M-Endo/LES-Endo-NA-MUG), EPA method 1604 (MI Medium); m-ColiBlue24 (Hach, Ames, IA); or Chromocult (EMD Chemicals, Gibbstown, NJ)[4] involve testing under standard conditions and equipment common in the developed world. Incubators for maintaining temperatures standard for growth of

E. coli (35ºC and/or 44.5ºC), sterilizing equipment such as an autoclave, membrane filtration equipment, long-wave UV lamps for detection of fluorescence, an assortment of sterile plastic or glass containers, and a sterile laboratory environment are common requirements of these standard methods.[5] However the vast majority of humanity (approximately 80%) lives within the developing world—defined by the US Bureau of the Census as India, Asia, sub-Saharan Africa, Latin America, the Near East, and North Africa—and thus is not served by such water testing facilities and protocols.[6] Without access to the supplies, facilities, and energy necessary for these standard methods of testing, water quality assessment is not readily available to the most populated regions of the world. As a result, pathogenic waterborne diarrhea causes approximately 2 million deaths each year, most often in children under five years of age.[7] There is a critical and urgent need for simple, effective, and affordable field tests to detect fecal contamination of water in the developing world in both everyday and emergency situations.

The most commonly used sentinel microbes for detection of fecal contamination are coliforms, specifically fecal coliforms, and *E. coli* in particular. The term "coliforms" refers to several genera of bacteria belonging to the family Enterobacteriaceae that are able to ferment lactose after 24 hours at 35°C.[5] Conventionally, these included Escherichia, Enterobacter, Klebsiella, and Citrobacter. However, this is a controversial grouping as it is not in accordance with current taxonomical criteria.[8] The American Public Health Association therefore defines a coliform as "all aerobic and facultatively anaerobic, Gram-negative, non-spore forming rod-shaped bacteria that ferment lactose with acid and gas production."[5] Coliforms have long been recognized to be a poor indicator of fecal contamination for untreated water, as the definition includes species often found in unpolluted water and soil.[8] However, because water treated for human consumption should contain no microorganisms, coliform detection is usually sufficient for testing drinking water.

Fecal coliforms are defined as coliforms that can ferment lactose at elevated temperatures (44.5°C). *E. coli* is considered to be the only veritable fecal coliform, however, as all other thermo-tolerant coliforms have been isolated from waters free of fecal contamination.[5,8] Because of this, *E. coli* is currently held to be the truest representative of the bacterial indicators for fecal contamination. The ability to identify *E. coli* from a variety of sources is an essential requirement for any drinking water test procedure.

Defined substrate technology has long been the norm for detection of a microbe. Composed of indicator nutrients that detect the action of a target enzyme, defined substrates can reliably indicate the presence of a microorganism by its specific metabolic activity.[9] ß-glucuronidase is considered to be the most promising indicator for the presence of *E. coli*, as most strains (>90%) carry the enzyme. [9,10,11] Moreover, ß-glucuronidase is specific only to *E. coli* and some Shigella and Salmonella strains.[5,9] The majority of standard *E. coli* tests therefore contain indicators for the presence of both ß-glucuronidase and ß-galactosidase (lactose fermentation).[5,9]

In this series of experiments, we investigated whether *E. coli* could be cultured at temperatures that might be ambient in developing world countries and whether tests that indicate the presence of *E. coli* can be performed at these temperatures. This represents the first step towards development of a reliable test for widespread use in nonstandard conditions.

Materials and Methods

***E. coli* test strains.** All isolates (with the exception of ATCC#25922, which was obtained from ATCC, Manassas, VA) were provided by Dr. Mark Sobsey of the University of North Carolina. Of the 34 *E. coli* strains available, 15 were chosen based on source variability. Table 1 summarizes the host species and/or geographical origin of each isolate.

TABLE 1. Serotyping and source information for all tested *E. coli* strains.

Accession #	Strain Name	O	H	K	Class	Host	Locale	Date	Clinical
TW02282	MT-512	2				chicken	France	1972	trachea
TW02283	MT-513	2				chicken	France	1972	salphinx
TW02284	MT-515	78				chicken	France	1972	lung
TW06930	92-1232	141	12			goose	USA (N.Y.)		feces
TW03284	ECOR-21	121				steer	Bali		healthy
TW03289	ECOR-29	150	21			k-rat	USA (Nev.)		healthy
TW02053	ECOR-33	7	21		ECORr5	sheep	USA (Calif.)		healthy
TW03292	ECOR-34	88	NM		ECORr5	dog	USA (Mass.)		healthy
TW02060	ECOR-45	ON	M			pig	Indonesia		healthy
TW03298	ECOR-47	OM	18			sheep	New Guinea		healthy
TW02066	ECOR-67	4	43			goat	Indonesia		healthy
TW11566	MT2a					environment (soil)	Puerto Rico		
TW11569	MT4a					environment (soil)	Puerto Rico		
TW11573	MT7c					environment (soil)	Puerto Rico		
ATCC 25922	DSM 1103; NCIB 12210					Clinical Isolate	Seattle	1946	

Water testing assays. The study was composed of two standard ß-glucuronidase assays—Colilert® (IDEXX, Westbrook, ME) and membrane filtration with MI Agar (BD-BBL, Sparks, MD)—as well as two nonstandard (and less expensive) assays using 4-methylumbelliferyl-p-glucuronide (MUG) as the indicator nutrient. EC Medium with MUG (Difco, Sparks, MD) was chosen for comparison with Colilert®, and MAC II Agar with MUG (BD, Sparks, MD) was used in contrast with MI Agar. A Petrifilm™ (3M™ Microbiology, St. Paul, MN) assay was also included.

All test protocols were adapted from the standard methods recommended by either the manufacturer or the EPA.[5,12,13,14,15] The Colilert® assay consisted of three snap packs (pre-measured for 100 mL samples) combined within 300 mL of *E. coli*-inoculated water, and divided by 1 mL samples into five 48-well culture plates. Similarly, 11.1 g of dry EC Medium was added to a 300 mL sample and transferred to 48-well plates. Prior to experimentation, the EC Medium was tested repeatedly for sterility by incubation of uninoculated medium at 37°C for 24 hours. Petrifilm™ consisted of 1 mL water samples transferred onto five Coliform Count Plates. For the agar assays, a total of ten 100 mL samples were run through a 0.45 µm filter and transferred to agar medium. MI Agar and MAC II agar were purchased pre-plated, and alcohol-sterilized forceps were used in handling of the filters.

Protocol. Each *E. coli* strain was tested according to the following protocol. A frozen isolate was first thawed and subcultured once for optimum recovery of microorganisms. The sample was then used to create 10 mL, ten-fold serial dilutions of 1:1,000 (10^{-3}) through 1:10,000,000 (10^{-7})

in sterile deionized (DI) water. A 30-mL 10^{-8} dilution was created from the 10^{-7} sample, and this was used to generate two 10^{-10} samples: 600 mL and 1,000 mL.

The 1,000-mL sample was divided into 100-mL rations, and dispensed into 0.45-µm membrane filters mounted on vacuum filtration units. All filters were then carefully transferred to plated medium—five to MI Agar plates, and five to MAC II agar with MUG.

The 600 mL sample was poured into two glass bottles, 300 mL in each. One container received three Colilert® snap packs, and 11.1 g EC Medium with MUG was added to the other. Each was then used to fill five 48-well plates (one for each temperature), 1 mL in each well.

The Petrifilm™ medium was inoculated using the 10^{-8} dilution—due to the small sample volume required for the test; no growth was observed at a lower microbial concentration (10^{-10} dilution) during preliminary testing. Samples of 1 mL were taken from the remaining 10^{-8} dilution and carefully inoculated onto five Petrifilm™ plates.

Incubation temperatures. All tests were then incubated for 24 hours at a range of five temperatures: room temperature (~23°C), 30°C, 37°C, 40°C, and 45°C. This array was determined primarily by the observed patterns of *E. coli* growth during preliminary experimentation, the lower and upper limits of which were established to be approximately room temperature and 45°C, respectively. The optimal *E. coli* incubation temperature (37°C) was included as a comparator, and the remaining intermediate temperatures were then chosen to represent a spectrum between these values.

To control for humidity, all tests were incubated in the presence of a source of water. Room temperature incubation was therefore achieved by placing the tests inside sealed plastic containers with a moist paper towel, and then placed in a drawer to control for light exposure. The 30°C, 37°C, and 40°C tests were placed in water-jacketed incubators set at their respective temperatures. Finally, the 45°C tests were placed inside a dry incubator, once again using wet paper towel-lined containers to adjust for humidity.

Results

All tests were observed in terms of the presence or absence of a particular trait, and all traits were counted in terms of the number of strains that were conclusively positive.

FIGURE 1. Number of strains exhibiting growth at five temperatures in each test system.

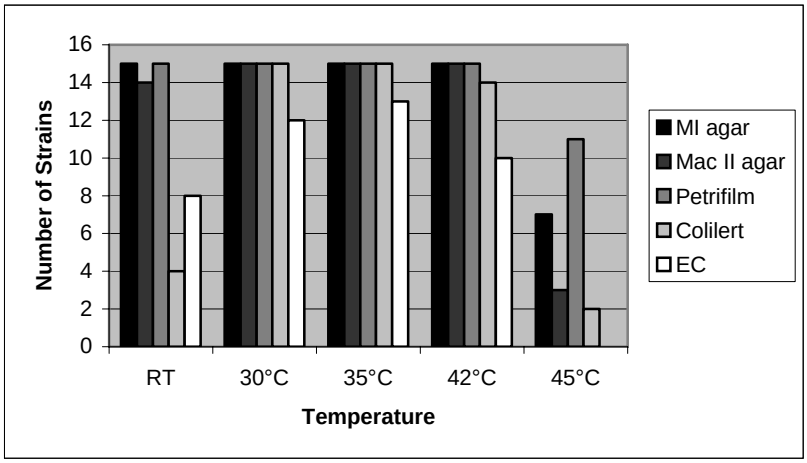
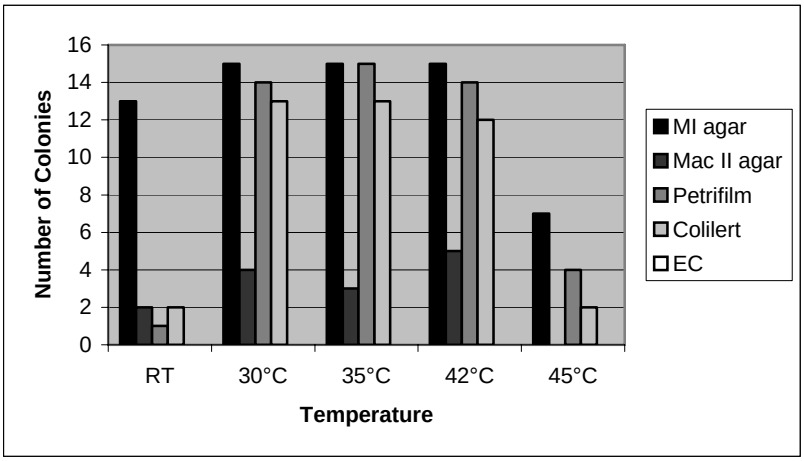


FIGURE 2. Number of strains that exhibited *E. coli*-positive results in each test system at various temperatures.



Growth of isolates. Figure 1 displays the performance of each medium across all temperatures in terms of bacterial recovery. Each of the 15 strains was inoculated onto 5 sets of tests, and each set was incubated at one of 5 temperatures. Growth was determined either by colony formation (for the agar and Petrifilm™ assays) or by the presence of visible turbidity or color change (for the two broth tests).

E-coli positive tests. Figure 2 shows the number of strains that produced a positive result for *E. coli* when inoculated onto each medium. Only those strains that exhibited clear ß-glucuronidase and ß-galactosidase activity were considered positive. For instance, positive wells for Colilert® were yellow (ß-galactosidase) and fluorescent (ß-glucuronidase)[14], and EC Medium positives exhibited fluorescence (ß-glucuronidase) and gas production (ß-galactosidase). Positive colonies for MI Agar were blue (ß-glucuronidase) and fluorescent (ß-galactosidase)[13], while MAC II agar positives were pink (ß-galactosidase) and fluorescent (ß-glucuronidase). *E. coli* colonies on Petrifilm™ appear as blue spots (ß-glucuronidase) surrounded by gas bubbles (ß-galactosidase).[15]

Membrane filtration with MAC II agar. As can be seen in Figure 1, membrane filtration supplemented with MAC II agar produced significant growth across all temperatures for a high number of strains. However, this assay also produced unclear results for ß-glucuronidase activity. Upon exposure to a long-wave (366 nm) UV lamp, some strains presented with luminous orange and white colonies. Of the many strains tested, however, none produced a colony exhibiting distinct blue-green fluorescence. This accounts for the low incidence of *E. coli*-positive results shown in Figure 2, as only the strains producing white fluorescence were regarded as positive for ß-glucuronidase—most likely they were exhibiting fluorescence with interference.

EC Medium with MUG. EC Medium produced the least growth across all temperatures, with the exception of room temperature. The turbidity of the culture interfered with accurate readings of fluorescence. Furthermore, the gas produced by lactose fermentation was not observable in multiple-well fermentation, and therefore the EC broth exhibited no *E. coli*-positive results at any temperature. EC Medium would therefore not be of use in a field test, nor would any medium that indicates enzyme activity through gas.

Colilert®. Colilert® performed moderately well across all temperatures and was the only broth with any observable sensitivity to *E. coli*. Positive results were very easily distinguished in all tested strains. However, both efficacy and bacterial recovery dropped sharply at room temperature and 45°C.

Membrane filtration with MI Agar. Only MI Agar gave consistent and accurate detection of *E. coli* throughout all trials. Using this test, all *E. coli* samples at temperatures between 30°C and 42°C clearly exhibited both β-glucuronidase and β-galactosidase activity. Furthermore, more strains showed enzyme function at room temperature on MI Agar than with any other assay.

Petrifilm™. Although most of the tested *E. coli* strains showed good recovery on Petrifilm™, the test was only moderately sensitive to *E. coli* (as defined by the color change and production of gas) at temperatures other than 37°C. Petrifilm™ gave the best results for growth across all temperatures, however, and thus may be of use in the field under suboptimal conditions.

FIGURE 3. ß-glucuronidase enzyme activity on each medium in terms of the number of exhibiting strains.

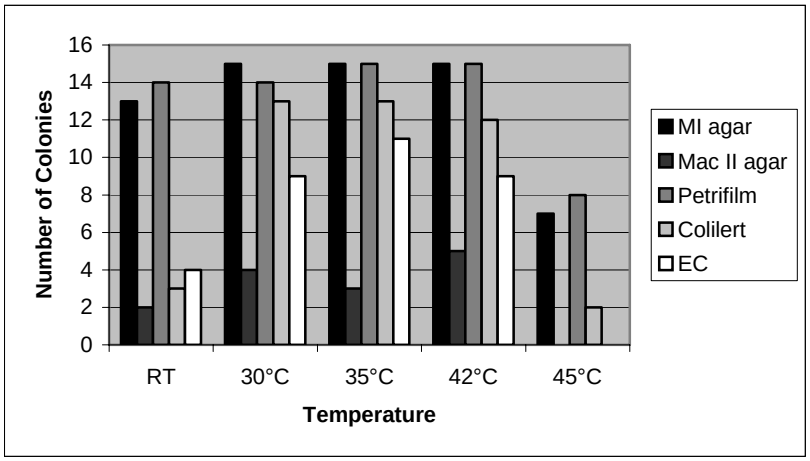
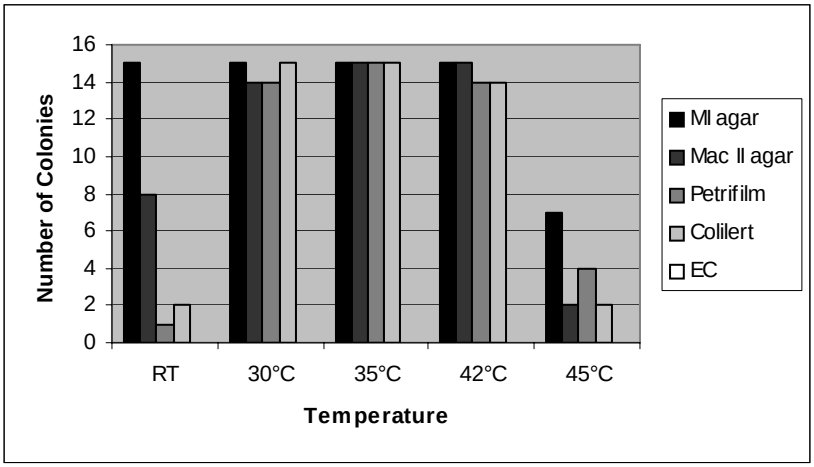


FIGURE 4. ß-galactosidase enzyme activity on each medium in terms of the number of exhibiting strains.



Enzyme activity. As seen in Figure 3, β-glucuronidase gives the strongest performance on MI Agar and Petrifilm™ followed by Colilert® and EC Medium. The best results for ß-galactosidase activity are seen in Figure 4—MI Agar, Petrifilm™, and Colilert®. In appraising the most effective *E. coli* indicator, ß-galactosidase seems to be the limiting factor for Petrifilm™ (as well as EC Medium, due to lack of observable gassing), whereas all other tests are curbed by ß-glucuronidase activity.

Discussion

E. coli can be cultured at temperatures ranging from approximately 22°C to 40°C. Some strains could be cultured at 45°C, but not all. Preliminary testing at 4°C demonstrated no visible growth at 24 hours. The commercial tests and media used have been optimized for use at 35°C ± 2°C. However, growth could be observed at temperatures from 22°C to 42°C using these tests.
Of the standard tests currently available, Petrifilm™ is the most portable and cost-effective option (less than one dollar per test). More testing is required to investigate the strains unable to produce gas at 30°C, However, the Petrifilm™ test reliably detects *E. coli* at a range of 20 to 200 CFU/100 mL across a broad range of temperatures. Furthermore, it does not require UV light for data collection and allows for direct counting of *E. coli* concentration as an indicator of fecal contamination.

A simple broth presence-absence test would not be sufficient to determine the extent of fecal contamination, which could lead to needless sterilization of water with bacterial populations that are within tolerable limits. Most probable number (MPN) methods have a built-in positive bias that often accounts for over-estimation of bacterial concentration and would be too expensive and problematic for field testing. The standard method using MI plated agar would also be inappropriate for use in the field, as it requires a vacuum for membrane filtration, is extremely expensive, and is too large for transport.

According to the data collected in this study, however, direct inoculation of MI Agar with a water sample may yield the most reliable results. The next phase of research would therefore consist of a more direct comparison of the sensitivity, selectivity, and specificity of MI Agar and Petrifilm™. Future studies could also investigate the possibility of creating a type of Petrifilm

plate assay using MI Agar for greater cost-effectiveness, as well as ease in transportation and distribution.

Other nonstandard and standard media could also be explored. Those that do not require gassing or fluorescence as indicators would be especially useful for testing in low-resource settings. Temperatures between 4°C and 22°C are also possible in low-resource settings, for example in Kashmir in the winter, and thus it would be useful to investigate a variety of tests at a wider range of temperatures. The possibility of testing for other fecal bacteria could also be examined, especially in light of recent evidence regarding the high prevalence of non-fecal *E. coli* in tropical waters.[16,17] Fecal streptococcus, in particular, has shown promise as an alternative fecal indicator and could also be studied at a range of temperatures.[8]

Acknowledgements

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