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Variability in the Characterization of Total Coliforms, Fecal Coliforms and *Escherichia coli* in Recreational Water Supplies of North Mississippi, USA

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Abstract The fecal coliform, *Escherichia coli*, is a historical organism for the detection of fecal pollution in water supplies. The presence of *E. coli* indicates a potential contamination of the water supply by other more hazardous human pathogens. In order to accurately determine the presence and degree of fecal contamination, it is important that standard methods approved by the US Environmental Protection Agency are designed to determine the presence of *E. coli* in a water supply, and distinguish *E. coli* from other coliform bacteria (e.g. *Citrobacter, Klebsiella* and *Enterobacter*). These genera of bacteria are present not only in fecal matter, but also in soil and runoff water and are not good indicators of fecal contamination. There is also ambiguity in determining a positive result for fecal coliforms on M-FC filters by a blue colony. When all variations of blue, including light blue or glossy blue, were examined, confirmation methods agreed with the positive M-FC result less often than when colonies that the technician would merely call "blue", with no descriptors, were examined. Approximately 48 % of M-FC positive colonies were found to be *E. coli* with 4 methylumbelliferyl-β-D-glucuronide (MUG), and only 23 % of samples producing a positive result on M-FC media were found to be *E. coli* using API-20E test strips and current API-20E profiles. The majority of other M-FC blue colonies were found to be *Klebsiella* or were unidentifiable with current API-20E profiles. Two positive M-FC colonies were found to be *Kluyvera* with API-20E, both of which cleaved MUG and produced fluorescence under UV light, a characteristic used to differentiate *E. coli* from other fecal coliforms.

Keywords Membrane filter · API-20E · Standard methods · Indicator

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Coliform and fecal coliform bacteria have been used as indicators of fecal contamination of potable water supplies since the early 1900s (Edberg et al. 2000). While the mere presence of fecal coliforms in water bodies does not necessarily pose immediate health dangers, the premise is that their presence may also indicate the possibility of more threatening pathogens found in feces, including viruses (Leboffe and Pierce 2002; Noble et al. 2003). *Escherichia coli* is an indicator of potential fecal contamination because it inhabits the intestine of warm-blooded animals. Species of *Klebsiella*, such as *K. pneumoniae*, are fecal coliforms because they are present in the gastrointestinal tract of warm blooded mammals, but they are not good indicators of fecal contamination because they may also be present in non-fecal sources such as soil (Holt et al. 1994; McLellan et al. 2001; Struve and Krogfell 2004). While the pathogenic nature of environmental isolates is uncertain, recent studies indicate that such isolates are just as pathogenic as *Klebsiella* of a gastrointestinal source (Struve and Krogfell 2004). Species of the coliform *Kluyvera* are essentially *E. coli* phenotypically, demonstrating β-glucuronidase activity and positive indole production; however they are citrate positive (Koneman et al. 1992; McLellan et al. 2001).

In 1973, Standard Method 9222 D Fecal Coliform Membrane Filter Procedure (M-FC) was approved by the US Environmental Protection Agency (US EPA) to assist in determination of water quality impairment using enumeration of fecal coliforms. Since 1998, the state of Mississippi has developed 207 fecal coliform total maximum daily loads (TMDLs) for the state's impaired water bodies (US EPA 2013). Data used to determine initial fecal coliform impairment was generated from the application of Standard Method 9222 D, M-FC. All current US EPA approved methods for enumeration of fecal coliforms rely on bacterial growth, either noted by colony formation or estimated by the most probable number (MPN) index, in selective and differential media that include indicators of biochemical activity. A determined percentage of "positive" colonies or broth cultures are then confirmed to be fecal coliforms by additional biochemical tests. Selective factors can include addition of surfactants, such as bile, and dyes to inhibit growth of non-fecal microorganisms at 44.5°C incubation. Differentiation factors rely on the ability of fecal coliforms to utilize lactose as a fermentation carbon source while producing a gas. This lactose utilization can be made visible by a color change with a pH indicator dye or by gas collected in an inverted Durham tube. An additional method designed to enumerate *E. coli*, 9221 F. *Escherichia coli* Procedure (Proposed), relies on the enzyme, β-glucuronidase, which enables *E. coli* to cleave a fluorogenic substrate, 4-methylumbelliferyl-β-D-glucuronide (MUG) (APHA 2005).

These methods are not without error. The M-FC procedure relies on the subjective interpretation of the development of a blue colony, rather than a color change, as an indication of a positive result (APHA 2005). Determination of colony color on a membrane filter can be difficult and is often subjective. While the elevated temperature of 44.5°C is designed to select only *E. coli*, other thermotolerant fecal coliforms, such as *Klebsiella* and *Kluyvera*, can also grow at this temperature. Many of the thermotolerant fecal coliforms, not exclusively *E. coli*, have the enzyme β-glucuronidase, thus giving them the ability to cleave MUG (Alonso et al. 1999; McLellan et al. 2001). To determine the efficacy of these procedures as applied to surface water bodies in north Mississippi, these methods were utilized for the initial isolation of fecal coliforms. A biochemical test kit developed for clinical applications (API-20E) was utilized as the de facto standard for determination of taxa within the *Enterobacteriaceae*. Since 1986, *E. coli* and enterococci have been the two bacterial water quality indicators used to monitor and assess recreational waters (US EPA 1986).

The purpose of this study was to demonstrate that US EPA approved standard methods for isolating *E. coli* and determining the level of fecal contamination may lead to erroneous conclusions. Only DNA sequencing of the bacterial 16S rRNA gene could truly confirm identity of isolates. Therefore, this study was designed to analyze the use of current US EPA approved standard methods in north Mississippi watersheds and the conclusions made with the use of such methods.

Materials and Methods

Routine water samples were obtained from nine north Mississippi streams (Coles Creek, Persimmon Creek, Skuna River, Turkey Creek, Otoucalofa Creek, Bearman Creek, Bridge Creek, Little Hatchie River, and Upper Hatchie River) on a monthly basis for 1 year, and 58 pure cultures from "positive" M-FC colonies were then confirmed by US EPA approved standard methods 9260F, 9221 B, 9221 E. 1, 9221 F. 1 and their subsequent verification methods discussed in standard method 9020B.9 (APHA 2005).

"Positive" M-FC colonies required the technician to subjectively interpret the development of a blue colony which varies in intensity (APHA 2005). These 58 samples included what the technician characterized as 25 glossy light blue colonies, 5 blue matte colonies, 22 blue colonies, and 6 glossy blue colonies. Additionally, 2 *E. coli* standards were examined, for a total of 60 samples tested. Isolates were grown on trypticase soy agar (TSA). After isolate purity was determined by streaking to the third generation on TSA, Gram-stains (Difco, Detroit, MI) were performed on the 60 samples. All were Gram-negative rods, warranting further testing.

Sixty isolates were inoculated onto Levine-Eosin Methylene blue (L-EMB) (Difco, Detroit, MI) agar plates. Inoculated plates were incubated at 35°C for 24 h. Plates were then examined for growth and production of a metallic sheen, indicating *E. coli* (9260 F. Pathogenic *E. coli* and 9221 B.3 Standard Total Coliform Fermentation Technique Completed Phase).

Isolates were inoculated into lauryl tryptose broth (LTB) (Difco, Detroit, MI) containing inverted Durham tubes and incubated at 35°C. Inverted Durham tubes were inspected for gas bubbles after 24 and 48 h of incubation. All tubes with any gas bubbles in the inverted Durham tubes were counted as positive according to 9221 B. Standard Total Coliform Fermentation Technique and 9020B. 9 Intralaboratory Quality Control Guidelines Verification.

E. coli broth (EC) with MUG (Difco, Detroit, MI) tubes with inverted Durham tubes were inoculated from the 60 samples and incubated at 44.5°C for 24 and 48 h. An additional 24 h of incubation was included to reduce the number of false negatives in MUG. All inverted Durham tubes with any bubbles were counted as positive for the

ability to ferment lactose in EC broth. Tubes were also held under ultraviolet (UV) light to determine ability of bacteria to cleave MUG when incubated at 44.5°C for 24 h. All tubes that fluoresced under UV light were counted as positive for the ability to cleave MUG [9221 E. 1 Fecal Coliform Procedure Fecal Coliform Test (EC Medium), 9221 F. 1 *E. coli* Test (EC–MUG medium), 9020B. 9 Intra-laboratory Quality Control Guidelines Verification.]

Each of the 60 isolates from TSA was suspended in API solution (biomérieux Vitek, Inc., Hazlewood, MO) and vortexed. A humid environment was created in the API-20E test by adding water to the bottom cover, containing honeycombed wells. The test strip was placed in the bottom cover. The suspension was added to 20 cupules on the test strip containing different substrates. In some of the cupules, an anaerobic environment was created using mineral oil. Top covers were placed on the strips, which were incubated at 37°C for 24 h. An oxidase test was also performed by dropping 1–2 drops of oxidase reagent (Difco, Detroit, MI) onto isolates placed on sterile cotton swabs. A purple color change on the cotton swab was considered a positive result for the oxidase test, indicating that the sample bacterium contained cytochrome C. If the media required reagents, they were added after incubation. From color changes on the API-20E test strips, the sample was considered positive or negative for its ability to utilize each substrate. These series of positives and negatives, along with the oxidase results, were used to assign a seven digit number to each isolate. The seven digit numbers were compared to the Analytical Profile (version 4) for the identification of the isolate (Leboffe and Pierce 2002; Robinson et al. 1995).

Results were converted to numbers by using a 1 as a positive result and a 0 as a negative result. For each of the above methods, a ratio of M-FC blue colonies to an actual positive result (as confirmed by L-EMB, LTB, EC with MUG, and API-20E) was calculated. Ratios were then used with Students *t* test to determine whether any statistical significance existed ($\alpha = 0.05$) between M-FC and LTB, M-FC and EC, LTB and EC, EMB and MUG, and MUG and API-20E. .

Results and Discussion

Positive results with LTB only indicate the isolate is a total coliform. LTB was the least selective method of all those utilized, even less selective than the original M-FC method. Of the positive M-FC isolates, 96 % yielded positive results for total coliforms using LTB. EC, like M-FC, tests for fecal coliforms. Slightly more selective than LTB, a surprising 45 % of M-FC positive isolates tested negative for fecal coliforms using EC. Moving toward more selectivity, both MUG and L-EMB can identify positive colonies as *E. coli*. Of the 58 positive M-FC colonies, 54 % were negative for *E. coli* in both MUG and L-EMB.

Table 1 Percentages of positive results for each method based on colony description with original 58 M-FC isolate numbers in parentheses

Isolate description	LTB	EC	L-EMB	MUG	API-20E
Glossy light blue (25)	88	32	8	8	4
Glossy blue (6)	100	33	17	17	0
Blue with no descriptors (22)	95	86	86	86	45
Blue matte (5)	100	60	100	100	20

Using the API-20E method (most selective) on the 58 positive M-FC colonies resulted in 23 isolates (40 %) that were unidentifiable; 20 isolates (34 %) identified as *Klebsiella*; 12 isolates (21 %) identified as *E. coli*; 2 isolates (3 %) identified as *Kluyvera*; and 1 isolate (2 %) identified as *Serratia*.

When blue colonies (positive results for the M-FC method) were described in different ways, not all descriptions yielded similar results with the different confirmation methods (Table 1). For example, blue colonies described as glossy were identified as *E. coli* with API-20E profiles only 4 % of the time. Only 32 % of glossy, light blue colonies were confirmed to be fecal coliforms by the EC method. Of these same glossy, light blue colonies, only 8 % were identified as *E. coli* on L-EMB agar plates, and only 8 % were MUG positive (Table 1). If the glossy colonies were not light blue, they were confirmed to be fecal coliforms 33 % of the time by the EC method and identified as *E. coli* 17 % of the time on both L-EMB agar plates and in MUG. Those colonies described as blue matte were more likely to be confirmed as fecal coliforms. Both L-EMB agar plates and MUG broth yielded positive results on 100 % of the isolate colonies, but only 60 % were found to be fecal coliforms in EC broth (Table 1). When blue matte colonies were tested using API-20E test strips, only 20 % of their profiles indicated they were *E. coli*.

There is a disparity between colonies testing positive for MUG and L-EMB and colonies testing positive for *E. coli* with the API-20E system. Only 47 % of colonies testing positive for *E. coli* in MUG were confirmed using API-20E. Using current API-20E profiles, 37 % of colonies were not capable of being identified. Ten percent of MUG positive colonies were identified as *Klebsiella* and 7 % were identified as *Kluyvera* with API-20E profiles. False negatives on MUG were much less of a problem. Only one isolate of MUG negative samples was found to be *E. coli* with API-20E. Many of the *Klebsiella* species, but none of the *Kluyvera* species were found to be MUG negative.

Table 2 Selective and differential properties of standard methods used for detection of fecal coliforms and *E. coli*

Media	Selective chemical[a, b]	Carbon source differential[a, b]	Detecting[a]	Indicator[a]	Positive result[a]
M-FC	1 % rosolic acid in 0.2 N NaOH (10 mL/L H_2O); Bile salts (1.5 g/L H_2O)	Lactose (12.5 g/L H_2O)	Fecal coliforms	Aniline blue media; pH 7.4 ± 0.2	Blue colony at acidic pH
EC	Bile salts (1.5 g/L H_2O)	Lactose (5 g/L H_2O)	Fecal coliforms	Inverted Durham tube	Gas in Durham tube
EC with MUG	Bile salts (1.5 g/L H_2O)	MUG (0.05 g/L H_2O)	*E. coli*	UV light	Fluorescence under UV light
L-EMB	Eosin (0.4 g/L H_2O); Methylene Blue (0.065 g/L H_2O)	Lactose (10 g/L H_2O)	*E. coli*	Eosin and Methylene Blue media; pH 6.9-7.3	Metallic sheen at acidic pH
LTB	Sodium Lauryl Sulfate (0.1 g/L H_2O)	Lactose (5 g/L H_2O)	Total coliforms	Inverted Durham tube	Gas in Durham tube

[a] APHA 2005

[b] Leboffe and Pierce 2002

A significant difference ($p \leq 0.05$) existed between the M-FC blue colonies and glossy light blue colonies. Likewise, a significant difference was present between the M-FC blue colonies and glossy blue colonies ($p \leq 0.05$). No significant differences existed between the blue colonies and the blue matte colonies. Significant differences also existed between the M-FC method and both the LTB ($p \leq 0.05$) and EC ($p \leq 0.001$) methods. The significance of this difference is critical as these results further illustrate the unreliability of such methods for identifying fecal coliforms. The least selective LTB method (total coliforms) should have encompassed all the fecal coliforms identified through the M-FC and EC methods. The EC method was significantly different from the LTB method ($p \leq 0.001$), while the MUG method was significantly different from the API-20E method ($p \leq 0.05$).

To confirm that positive M-FC blue colonies are fecal coliforms, US EPA requires ten blue colonies to be confirmed each month from one positive water sample using LTB and EC broth (9221B. 3 and 9221 E); however, LTB can only confirm the blue colony is a coliform. The EC broth method looks for the ability of a bacterium to ferment lactose, producing gas, when incubated at 44.5°C for 24 ± 2 h (APHA 2005; Gaudet et al. 1996). This method is unable to differentiate *E. coli* from other genera of coliforms from non-fecal sources such as *Klebsiella, Citrobacter, Serratia, Enterobacter, and Serratia* (Holt et al. 1994).

To yield a positive result only indicative of *E. coli*, MUG is added to EC broth (Gaudet et al. 1996; Stender et al. 2001). EC with MUG is the US EPA approved standard method (9221 F) for the detection of *E. coli* in recreational water supplies (APHA 2005). US EPA approved standard methods require that 5 % of MUG positives and negatives be confirmed with indole and citrate media (9221 B. 3). However, Alonso et al. (1999) demonstrated that using β-glucuronidase activity (which cleaves MUG) as the method for enumerating solely *E. coli* also showed positive activity from species of *Citrobacter*, *Enterobacter* and *Klebsiella*. These MUG positive non-*E coli* species may justify the expense of a multiple test system (Nataro and Kaper 1998), such as API-20E, or time spent on an antibody-based method for determining the identity of an unknown coliform (Pyle et al. 1995). When API-20E strips are inoculated with unknown bacteria a signature pattern is developed for the unknown based on substrates utilized which may or may not produce visible color changes. This can be compared to a database of previously tested enteric bacteria, although not all environmental isolates are represented, leaving many bacteria unidentifiable (Gaudet et al. 1996; Leboffe and Pierce 2002).

Table 2 shows the selective and differential properties of US EPA approved standard methods for the detection of total coliforms, fecal coliforms, and *E. coli*. Since all coliforms can ferment lactose, methods that examine bacterial ability to ferment lactose are not sufficient to confirm the bacteria as a *E. coli*. Similarly, since all fecal coliforms, more descriptively referred to as thermotolerant fecal coliforms, are able to grow at the elevated temperature of 44.5°C, using this elevated temperature to enumerate only *E. coli* is not 100 % effective (Alonso et al. 1999; McLellan et al. 2001).

API-20E yielded many profiles that could not be identified by their current index, but they also identified species of *Klebsiella* and, to a lesser extent, *Kluyvera*, that MUG identified as *E. coli*. Other diagnostic tests, such as biolog that utilize results of 96 biochemical tests, may be more beneficial; however, as with API20, not all environmental isolates are represented in the profile database. Antibody-based methods may be even more accurate at identifying only *E. coli* in recreational water supplies (Pyle et al. 1995).

Confirmation methods presently used are not sufficiently definitive to narrow down the fecal coliforms to just *E. coli*. From these results, it is clear that an additional confirmation method, such as API-20E (Robinson et al. 1995) or an antibody-based method (Pyle et al. 1995), should be used before a technician can be reasonably certain that the bacteria present in the water is from a fecal source. Additionally, molecular typing methods such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), or repetitive-element PCR provide even more accuracy in the determination of *E. coli* (Foley et al. 2004).

In 1986, due to several studies that suggested a greater correlation between enterococci and *E. coli* presence in a water and bather illness than total coliform presence and bather illness, the US EPA recommended using enterococci as the sole indicator of fecal contamination (Noble et al. 2003). This was an attempt to eliminate the problems with using fecal coliform procedures for determining the level of water quality. However, this was not universally implemented, largely because enterococci will not survive for extended periods of time in water supplies (Parveen et al. 1999). While the expense of current molecular methods may hinder their immediate implementation in regulatory practice, results of this study demonstrate current methods are not always sufficient to determine actual contamination of recreational water supplies by *E. coli*.

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