Enumeration of Waterborne *Escherichia coli* with Petrifilm Plates: Comparison to Standard Methods

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Abstract

***Escherichia coli* is often monitored in environmental waters as an indicator of the possible presence of human pathogens associated with feces. Petrifilm *E. coli*/coliform count plates (3M, Minneapolis, MN), previously validated for enumerating *E. coli* in food, were tested for monitoring *E. coli* in environmental water. *Escherichia coli* counts in environmental water samples enumerated with Petrifilm were significantly correlated ($R > 0.9$; slope = 0.9–1.0; $p < 0.001$) with counts obtained with three commonly used methods, mTEC (Becton Dickinson, Sparks, MD), m-ColiBlue (Hach, Loveland, CO), and Colilert-18/IDEXX Quanti-Tray 2000 (IDEXX, Westbrook, ME). Blue colonies on Petrifilm plates were most reliably identified as *E. coli* when accompanied by gas formation, as determined by characterization of the colonies on MacConkey agar plates (PML Microbiologicals, Mississauga, ON, Canada) and by polymerase chair reaction (PCR) with *E. coli*–specific primers. The main disadvantage of Petrifilm plates for environmental water testing is the small volume (1 mL per sample) that can be tested; however, the plates appear to be suitable for screening and locating sites that exceed criteria for total body and partial body contact. Simplicity of use and storage, reliability, and relatively low cost make Petrifilm plates suitable for volunteer-based and educational water quality monitoring applications, particularly when used as a preliminary screening method to identify problem sites.**

High levels of bacteria are a concern for many marine, brackish, and freshwater environments. Elevated levels of bacteria in coastal waters are associated with increased risk of gastrointestinal symptoms for recreational swimmers (Cabelli, 1977; Dufour, 1984; Prüss, 1998; USEPA, 1986). Because of known association with fecal matter, levels of *E. coli* bacteria are a key regulatory measure of the healthfulness of recreational waters (USEPA, 1986, 1999a). For fresh waters, the USEPA recommends criteria of 126 *E. coli* colony forming units (cfu)/100 mL for the geometric mean of five samples over a 30-day period and 235 *E. coli* cfu/100 mL for a single sample, but states set their own standards (USEPA, 1986). For example, in Michigan, rivers, lakes, or streams measuring greater than 300 *E. coli* cfu/100 mL on a single day or more than 130 *E. coli* cfu/100 mL for a 30-day geometric mean are considered out of compliance for total body contact (e.g., beaches); 1000 *E. coli* cfu/100 mL is out of compliance for partial body contact (e.g., fishing, boating) (Rule 62; Michigan Department of Environmental Quality, 1999).

Despite the general awareness of the need for monitoring, many places that are suspected to be out of compliance are not monitored due to perceived high cost and complexity of equipment involved in a local monitoring program. Citizen-based volunteer monitoring programs have been developed in several states, such as Iowa (Seigley, 2001), and have been used for purposes of preliminary screening of local waters for identifying problem areas. However, since *E. coli* enumeration methods generally require expensive media or equipment not generally available to volunteers, a convenient, inexpensive method of *E. coli* enumeration is needed for such programs.

This paper describes the testing of Petrifilm *E. coli*/coliform count plates as a new, convenient method for enumerating *E. coli* in environmental waters. Petrifilm plates have previously been described for use in enumerating *E. coli* in food and dairy products (Curiale et al., 1991; Priego et al., 2000; Russell, 2000; AOAC, 2000a,b), and it therefore seemed reasonable to evaluate whether they may also be useful in water testing. Petrifilm plates consist of plastic films with grids that are coated with Violet Red Bile nutrients, a tetrazolium indicator, and gelling agents. The gel contains a β-glucuronidase indicator for confirmed detection of *E. coli*. The present study is a multilaboratory investigation comparing *E. coli* enumeration of environmental water samples with Petrifilm technology with *E. coli* enumeration by methods frequently used by each participating laboratory. The methods to which Petrifilm enumerations were compared were the mTEC, m-ColiBlue, and Colilert-18/IDEXX Quanti-Tray methods.

Materials and Methods

Water Sample Sources

Each participating laboratory collected environmental water samples near its location. The source sites were chosen to have a range of bacteria levels ranging from near zero up to relatively high noncompliant levels, based on previous experience at the same sites. Water from Ruddiman Lagoon and tributaries, in the city of Muskegon, MI (43°13′ N, 86°17′ W) was enumerated by Petrifilm and m-ColiBlue methods at the Annis Water Resources Institute. Water from a small tributary of the Grand River in Ottawa County, MI in a rural area near the intersection of 68th Avenue and Leonard Street in Coopersville, MI (43°1′ N, 85°57′ W) was enumerated by Petrifilm and mTEC methods at Grand Valley State University. The tributary is located in a rural area mostly occupied by cattle pastures and was sampled just upstream from its confluence with the Grand River. Water from various sites in the middle Rouge River subwatershed in the Rouge River watershed, in several suburbs west of Detroit, MI (42°22′ N, 83°25′ W) were enumerated by Petrifilm and IDEXX Quanti-Tray/Colilert-18 methods at Wayne State University. In addition,

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Abbreviations: cfu, colony forming units; PCR, polymerase chain reaction.

E. coli colonies on Petrifilm plates from water obtained from various sites in the Clinton River watershed (42°35′ N, 82°55′ W) were used for further characterization of Petrifilm colonies.

Sampling and Enumeration Procedures

All water samples were obtained with sterile bottles or sterile Whirlpak bags (Nasco, Fort Atkinson, WI), transported on ice, and analyzed within 4 h. Samples were tested in various dilutions, as indicated below, to assure that bacteria concentrations were within the appropriate range for each technique (American Public Health Association, 1998).

Petrifilm Procedure. The methodology for the Petrifilm plates was to (i) inoculate and spread 1 mL of water on the gel (see Fig. 1A), (ii) incubate the plate at a temperature of 35 ± 1°C for 24 ± 2 h, and (iii) count the number of blue colonies associated with a small gas bubble. Coliform colonies appear red surrounded by a bubble, due to an indicator dye and the trapping of gas produced by the coliforms by the upper film of the Petrifilm plate. *Escherichia coli* colonies are characterized by a blue precipitate surrounded by a gas bubble; blue colonies with gas are counted as *E. coli*, while blue colonies without gas are not (AOAC Official Methods, as described by the 3M interpretation guide). An example of the results obtained with one such plate is illustrated in Fig. 1B.

m-ColiBlue24 Analysis. The m-ColiBlue24 membrane filtration broth is USEPA approved for analysis of total coliforms and *E. coli* in drinking water (USEPA, 1999b) for enumerating total coliforms and *E. coli* in a proposed rule for ambient waters (USEPA, 2001). *Escherichia coli* colonies are characterized by a blue color due to a reaction between the enzyme β-glucuronidase and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. One-milliliter ambient water samples were diluted with 99 mL of sterile buffered dilution water and 100 mL was filtered through a sterile 47-mm nitrocellulose filter with a pore size of 0.45-μm (Millipore, Bedford, MA). The filter was then placed on an absorbent pad pre-soaked with m-ColiBlue nutrient broth in a Petri plate and incubated at 35 ± 0.5°C for 24 h. Blue colonies were counted as *E. coli*. One-milliliter portions of the undiluted water samples were assayed on Petrifilm plates.

mTEC Technique. The USEPA-approved original *E. coli* method was used (Method 1103.1; USEPA, 2000). Nutrient plates were prepared with dehydrated mTEC agar powder. Environmental water samples were serially diluted 10-fold. One-milliliter samples of each dilution were filtered through a sterile 47-mm nitrocellulose filter with a pore size of 0.45 μm (Millipore) and aseptically placed on mTEC agar plates. Similarly, 1-mL samples of the same dilutions were assayed on Petrifilm plates. The mTEC plates were incubated for two hours at 35 ± 0.5°C and then incubated at 44.5 ± 0.2°C for 22 to 24 h. After incubation, the filter membranes were placed on Whatman (Maidstone, UK) filter paper that had been saturated (1.0 mL) with urea substrate media containing 2.0% urea (w/v) and 0.01% phenol red (w/v). Colonies that remained yellow, yellow-brown, or yellow-green were considered *E. coli*.

Colilert-18/IDEXX Quanti-Tray Method. Use of Colilert-18 with Quanti-Tray 2000 trays to enumerate *E. coli* is described in USEPA (2001). Fifty-milliliter ambient water samples from the sampling sites were diluted fivefold. Then, out of 103 mL of the diluted sample, three 1-mL samples were assayed on Petrifilm (1 mL on each plate out of the 103 mL diluted sample), and the remaining 100 mL was added to Colilert-18 and assayed in IDEXX Quanti-Tray 2000 trays, according to the manufacturer's instructions. Quanti-Tray samples were incubated 20 h at 35.5°C, after which fluorescent (β-glucuronidase positive) wells on the Quanti-Tray were counted, to calculate the most probable number (MPN) of *E. coli* cfu/100 mL in the diluted sample, according to a chart supplied by the manufacturer.

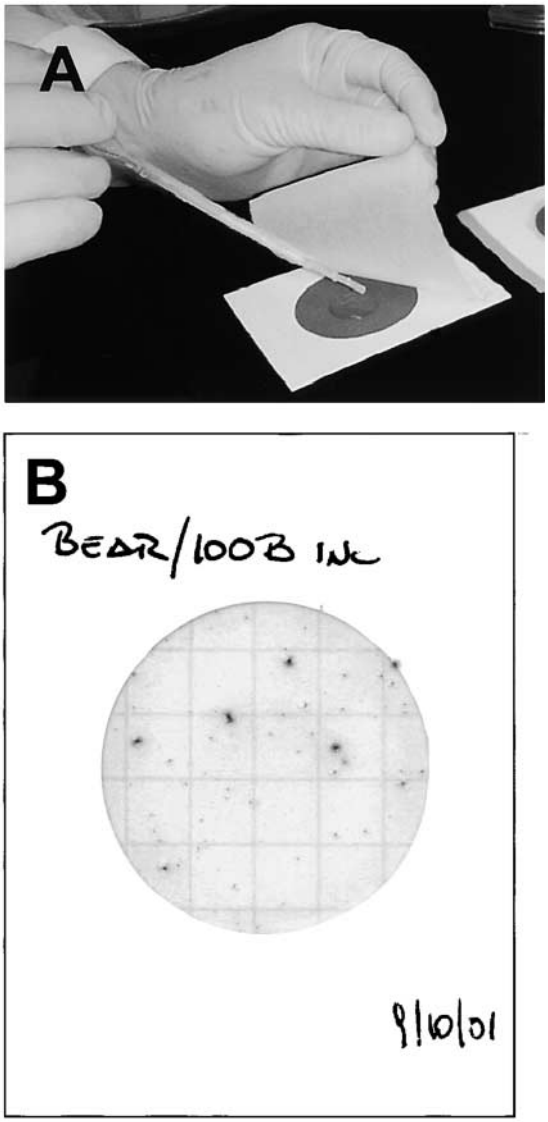

Fig. 1. Application of water to a Petrifilm plate and resultant bacterial growth. (*A*) The top film is lifted while a 1-mL water sample is applied with a sterile transfer pipet. (*B*) Petrifilm plate after a 24-h incubation with a 1-mL environmental water sample. Dark spots in this grayscale image were blue in the original.

Further Characterization of Petrifilm Colonies

Blue colonies were picked from Petrifilm plates with sterile inoculating loops and streaked onto MacConkey agar plates. MacConkey plates were incubated for 24 h at 35.5°C, the presence of pink (i.e., lactose-fermenting, as expected for *E. coli*) colonies was noted, and then well-isolated pink colonies were inoculated into Colilert-18 medium. After culturing for 20 h at 35.5°C, the presence of yellow color and fluorescence was noted, sterile glycerol was added to a final concentration of 14%, and then the culture was frozen until further analysis. For the polymerase chain reaction (PCR), 1 μL of the thawed culture was subjected to thermocycling (anneal, 60°C; synthe-

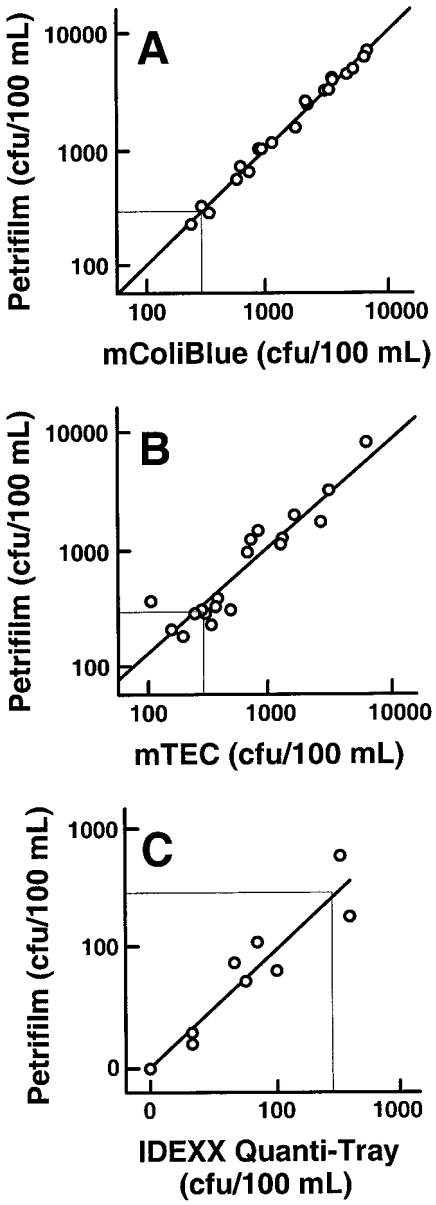

Fig. 2. Comparisons of Petrifilm to three standard methods for *E. coli* enumeration of environmental water samples. Data were normalized to 100 mL and transformed with log[cfu/100 + 10] prior to linear regression. Vertical and horizontal lines in each graph indicate 300 cfu/100 mL, the Michigan maximum whole body contact limit. (*A*) Comparison with the m-ColiBlue method, with 20 water samples from Ruddiman Lagoon and tributaries. The regression line has slope = 1.01, $R = 0.995$, and $p < 0.001$. (*B*) Comparison with the mTEC method, with 19 water samples from a tributary to the Grand River. The regression line has slope = 0.91, $R = 0.93$, and $p < 0.001$. (*C*) Comparison with the Colilert-18/IDEXX Quanti-Tray method, with nine water samples from the Middle Rouge River. The regression line has slope = 0.908, $R = 0.935$, and $p < 0.001$.

sis, 72°C; melt, 94°C; 30 cycles) with the following *E. coli*–specific primers: 298F, 5′-AATAATCAGGAAGTGATGG AGCA-3′; and 884R, 5′-CGACCAAAGCCAGTAAAGTA GAA-3′, which amplify a segment of the β glucuronidase gene. Identity of the PCR products was confirmed by sequencing.

Statistics

Regression analysis was done on log-transformed data after multiplication by appropriate factors to take into account the amount of dilution, so that counts in 100-mL volumes were being compared, and adjusted upward by a small constant to prevent taking the logarithm of zero. The log transformation used was $\log_{10}$(cfu/100 mL + 10 cfu/100 mL). For Petrifilm, mTEC, and m-ColiBlue assays, in which 1 mL was the volume assayed, the transform was therefore log(counts × 100 + 10). For IDEXX Quanti-Tray assays, in which the assayed volume was 100 mL, the transform was log(most probable number + 10). The means of log-transformed values of duplicate or triplicate assays or the individual log-transformed values when measured without replicates were used in subsequent correlations, and in calculations of method means, standard deviations, and statistical significance with paired *t* tests. Statistical tests were performed with Sigmastat 2.0 (Jandel Scientific, 1995) software. For linear regression, Petrifilm data was used as the dependent variable and the "standard" measurement method as the independent variable. A repeat of the analysis with a simple log transform and adjusting only the zero count samples to 10 cfu/100 mL to avoid log(0) yielded essentially identical conclusions.

Results

Comparison of Petrifilm Results with Other Enumeration Methods

Comparison with m-ColiBlue. Samples from Ruddiman Lagoon and tributaries were enumerated in triplicate with Petrifilm and in duplicate with m-ColiBlue. Water samples were collected from four sites on five occasions, for a total of 20 water samples. Correlation of the counts obtained with the two methods is illustrated in Fig. 2A. Counts ranged from as low as 200 cfu/100 mL to as high as 7000 cfu/100 mL. Linear regression of counts determined from Petrifilm assays versus counts determined with m-ColiBlue, after log transform, gave a slope of 1.01, $R = 0.995$, and $p < 0.001$.

The variability of data obtained with the Petrifilm method was assessed by determining the 95% confidence intervals of each triplicate measurement. The 95% confidence intervals averaged 17% of their corresponding mean values.

To test for bias one way or the other for the values determined by Petrifilm versus the m-ColiBlue method, the mean values obtained by each method for each water sample were compared with a paired *t* test. The overall method means and standard deviations of the log-transformed counts were 3.207 ± 0.451 for Petrifilm and 3.202 ± 0.458 for m-ColiBlue. The mean difference of paired measurements was −0.0054 ± 0.0455, indicating no significant difference between the results obtained with the two tests ($p = 0.60$, paired *t* test).

Comparison with mTEC. *Escherichia coli* in water samples from a tributary to the Grand River were enumerated in triplicate with both Petrifilm and mTEC methods. Two water samples were collected on each of nine occasions, and one water sample was collected on a tenth occasion, for a total of 19 samples. Linear regression of the Petrifilm data against the mTEC data, illustrated in Fig. 2B, gave a slope of 0.911, $R = 0.933$, and $p < 0.001$.

The 95% confidence intervals of Petrifilm triplicates averaged 39% of their corresponding mean values. Similarly, the 95% confidence intervals for the mTEC triplicates averaged 53% of their corresponding means. A

paired *t* test indicated no consistent differences between paired measurements made with the Petrifilm and mTEC methods on the same water samples ($p = 0.38$; method means: Petrifilm, 2.84 ± 0.46; mTEC, 2.81 ± 0.47; difference between paired samples, 0.035 ± 0.169).

Comparison with Colilert-18/IDEXX Quanti-Tray. Samples collected from nine sites on the Middle Rouge River were diluted fivefold and then enumerated in triplicate by Petrifilm and in one 100-mL sample, by Colilert-18/IDEXX Quanti-Tray. After transformation for comparison of equivalent 100-mL volumes, the regression of the Petrifilm results versus IDEXX results gave a slope of 0.908, $R = 0.935$, and $p < 0.001$ (Fig. 2C). Paired tests by the two methods were not significantly different from one another ($p = 0.80$; method means: Petrifilm, 1.81 ± 0.55; IDEXX, 1.79 ± 0.56; difference between paired samples, 0.018 ± 0.200); however, the average 95% confidence interval for the Petrifilm triplicates was quite large, equal to 101% of the corresponding means. The large 95% confidence intervals, compared with their corresponding means, reflect the fact that several of the water samples had low counts (0–2 colonies per plate) and therefore a variation of one or two colonies per Petrifilm plate produced a large percentage change of this measure.

Gas Formation as a Criterion for Identifying *E. coli* Petrifilm Colonies

As noted in the Materials and Methods, the AOAC Prescribed Method, recommended by 3M, requires that only blue colonies with gas bubble formation be counted as *E. coli*. To determine the importance of this criterion in accurately determining the correct number of *E. coli* colonies, the proportions of gas forming and non-gas-forming blue colonies were counted in several experiments. In addition, gas-forming and non-gas-forming colonies were picked from Petrifilm plates and characterized further.

Proportions of gas-forming and non-gas-forming colonies were determined in four experiments. In one experiment, five water samples collected from Ruddiman Lagoon and tributaries were assayed in triplicate on Petrifilm plates. On the resultant 15 Petrifilm plates a total of 149 blue colonies were present, of which 107 were blue with gas (72%) and 42 blue colonies exhibited no gas formation (28%). For samples from the Middle Rouge River, blue colonies with gas accounted for 64% of a total of 64 blue colonies counted on 27 plates. For water samples from the Clinton River watershed, blue colonies with gas accounted for 86% of 190 blue colonies observed on 27 Petrifilm plates in one study and for 89% of 355 blue colonies observed on 30 plates in another study. On individual plates the proportion of non-gas-forming blue colonies ranged between 0 and 50%.

Blue colonies from Petrifilm plates of two of the above experiments were streaked onto MacConkey plates and the proportion of Petrifilm colonies producing pink colonies on the MacConkey plates was determined (Fig. 3A). For both experiments 100% of the blue colonies with gas produced pink colonies on MacConkey plates. In fact, in most cases, only pink colonies were present on the MacConkey plates. In contrast, for blue colonies without gas, in one experiment (water from Ruddiman Lagoon and tributaries), only 2 of 7 blue colonies without gas produced pink colonies on the MacConkey plates, and in the other experiment (Clinton River samples), only 6 of 13 no-gas blue colonies produced pink colonies of normal morphology. In both experiments, blue colonies without gas yielded a significantly lower proportion of MacConkey plates with pink colonies than observed for blue colonies with gas (Fisher exact test, $p < 0.002$).

Finally, from the Clinton River samples, bacterial clones from Petrifilm blue colonies with gas that were subsequently isolated on MacConkey plates were subjected to PCR with *E. coli*–specific primers. All 16 isolates produced the expected amplified product for *E. coli*, of which 12 are illustrated in Fig. 3B, and subsequently confirmed as coding for the *E. coli* β glucuronidase gene in comparison with a reference sequence (AE000257, Bases 6765 to 7351) in Genbank (data not shown).

Discussion

Although Petrifilm plates have previously been validated for use in detecting *E. coli* contamination of food (Curiale et al., 1991; Priego et al., 2000; Russell, 2000), they have not been tested extensively for use in detecting *E. coli* in environmental waters. The present study provides a comparison of *E. coli* enumeration in environmental water obtained with Petrifilm plates with three commonly used commercially available tests. Petrifilm results were highly correlated ($R > 0.9$) and equivalent (slope approximately = 1.0; no differences on paired *t* test) to mColiBlue, mTEC, and Colilert/IDEXX Quanti-Tray tests. Analysis of differences between blue colonies with and without gas on the Petrifilm plates suggest that due care in evaluating the presence of gas bubbles is necessary in counting colonies. More extensive testing of the Petrifilm method to determine rates of false positives, false negatives, efficacy in additional types of water samples, etc. could provide further validation of the use of Petrifilm plates. Nevertheless, the simplicity of using Petrifilm plates indicates that it may be a suitable method for citizen-based testing and environmental education programs.

Several characteristics of Petrifilm that make it suitable for volunteer-based monitoring of *E. coli* include ease of use, reasonable accuracy, sensitivity in an appropriate range, safety, low cost, ease of storage, and long shelf life. With three simple steps, as outlined in Materials and Methods, the Petrifilm method is easy to perform in both the laboratory and the field. Although the Petrifilm plates in this study were all inoculated in the laboratory, comparable results inoculating Petrifilm plates in the field have been found in our other studies and by volunteers (Ram, 2001). The dry gel on the plates sets up quickly with the addition of water, enabling the plate to be handled without spillage within a minute or two of inoculation. In other experiments with a range of incubation times (24– 48 h) and temperatures, we have also found a good correlation with professional tests,

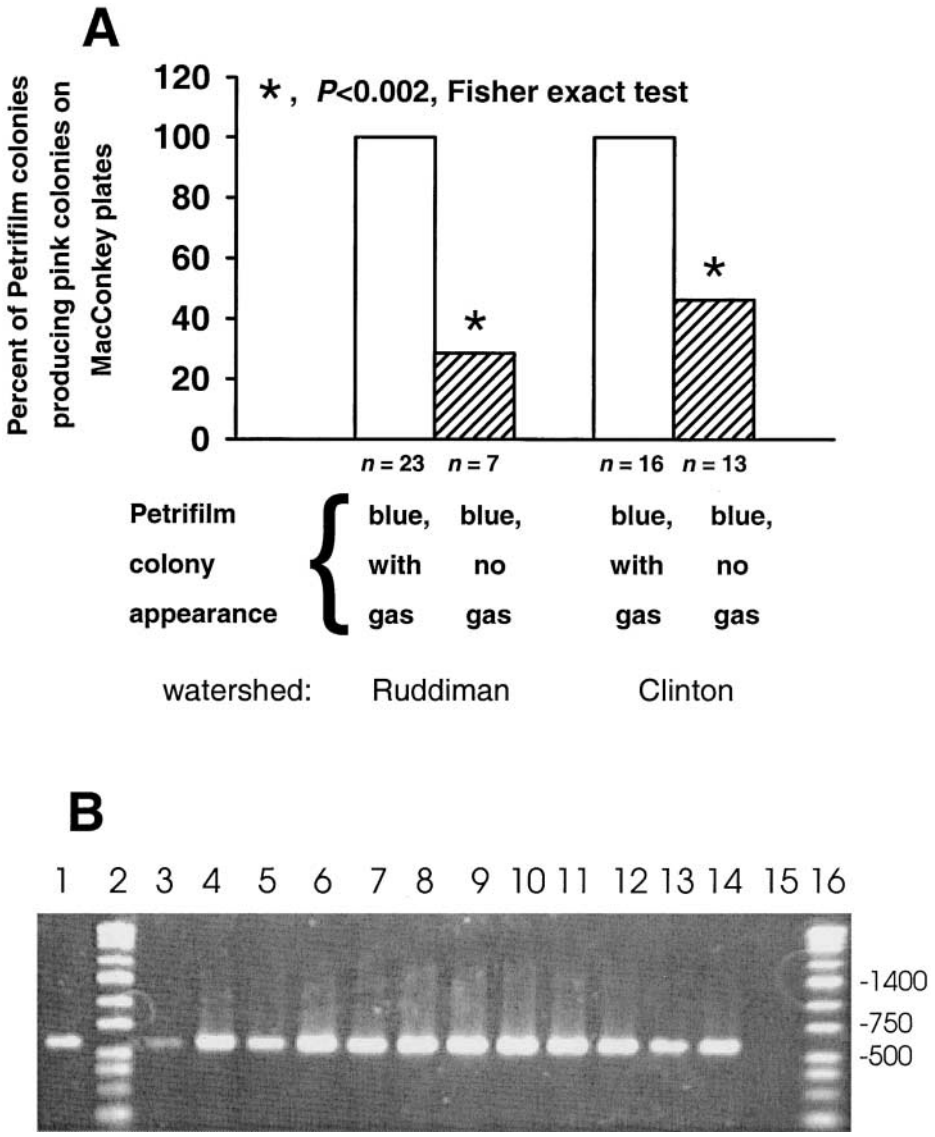

Fig. 3. Characterization of Petrifilm colonies. (*A*) Proportions of blue Petrifilm colonies that produced pink colonies when grown on MacConkey plates, as a function of whether the blue colonies also produced gas. The number of colonies (*n*) streaked onto MacConkey plates from Petrifilm plates used to enumerate *E. coli* from two experiments (Ruddiman and Clinton watersheds) is shown below each bar. *, $p < 0.002$, Fisher exact test. (*B*) Polymerase chain reaction (PCR) products obtained from pink colonies grown on MacConkey plates from the "blue, with gas" Clinton watershed samples in (*A*), amplified with Primers 298F and 884R. Lane 1, *E. coli* positive control; Lanes 3 through 14, amplification of 12 of the isolates; Lane 15, negative control (water instead of bacterial isolate). Sizes of selected bands on the DNA calibration ladders (Lanes 2 and 16) are indicated.

giving needed versatility for volunteer use (C.R. Merino and J.L. Ram, unpublished data, 2001). The overall film packet is compact (7.7 × 10.1 cm), thin (about 1 mm), and stackable, so many plates fit easily into an incubator. The plastic cover sheet readily protects the user from the growing bacterial colonies. The cost, at approximately $1.10 per plate in lots of 500, is less than Colilert but not as inexpensive as mTEC media; however, since Petrifilm does not require filtration apparatus, vacuum source, and space for pouring plates, its convenience may make it preferred in volunteer-based or educational testing situations.

The correlations illustrated in Fig. 2 indicate that Petrifilm plates give a reasonably accurate *E. coli* count. The variability observed with Petrifilm can be considered in relation to compliant levels of *E. coli*. For example, in the high count range (>300 cfu/100 mL), such as those samples analyzed in Fig. 2A and 2B, the 95% confidence interval averaged <40% of the mean, indicating that triplicate measurements having a mean > 500 cfu/100 mL are significantly greater than the Michigan total body contact allowable limit of 300 cfu/100 mL (e.g., a mean of 501 cfu/100 mL would have 95% confidence limits approximately 200 cfu/100 mL greater than and smaller than the mean of 501 cfu/100 mL, placing 300 cfu/100 mL outside the 95% confidence limit, i.e., significantly different). Conversely, in the low count range (<300 cfu/100 mL), such as the samples analyzed in Fig. 2C, the 95% confidence intervals averaged 101% of the mean, suggesting that triplicate measurements averaging <145

cfu/100 mL are significantly less than 300 cfu/100 mL. For preliminary screening of water samples, consistent observations of Petrifilm plates having zero or one colony (corresponding to 0 or 100 cfu/100 mL) would be good indicators that the actual *E. coli* level (as measured by standard enumeration tests) is <300 cfu/100 mL.

These 95% confidence intervals, while substantial, can be compared with the variability inherent in other methods. For example, a membrane filtration measurement of water having 300 cfu/100 mL would typically use a 10-fold dilution to yield 30 cfu on the filter (to be in the count range of the method). As noted in American Public Health Association (1998; Method 9222) "membrane counts really are not absolute" and are assumed to follow a Poisson distribution. For a count on the filter of 30 colonies, the 95% confidence interval would be ±10.9, or 36% of the number counted (American Public Health Association, 1998; Method 9222). For the IDEXX Quanti-Tray, the manufacturer provides a table of 95% confidence intervals. These vary over a broad range of counts; however, a representative comparison for this paper would be the average 95% confidence interval for the samples measured with Quanti-Trays in Fig. 2C, which averaged approximately 55% of their corresponding means, as calculated from the manufacturers' table. Thus, the 95% confidence intervals for the counts obtained with triplicate Petrifilm enumerations were comparable with that obtained with membrane filtration methods and the IDEXX Quanti-Tray in the high count range but were more variable than other methods when *E. coli* densities were <300 cfu/100 mL.

Petrifilm plates appear to be useful as a first step in obtaining environmental *E. coli* isolates. In the present study, blue colonies with gas were easily removed from Petrifilm plates and streaked on other nutrient media to isolate individual clones. The isolates obtained from 16 different Petrifilm colonies in this manner all produced PCR products consistent with their being *E. coli*. In this and other studies (J.L. Ram, unpublished data, 2001), Petrifilm plates have been a convenient first step in obtaining environmental *E. coli* isolates for sequencing.

The main disadvantage of the Petrifilm system is that only 1 mL of water can be used directly, giving less precise measurements in samples containing low numbers of *E. coli*. It may be possible to combine a preliminary concentration step on a filter with the Petrifilm technique; however, this would somewhat negate the simplicity desired for a citizen-based testing method. For improved precision in enumerating water samples with low numbers of *E. coli*, replicates can be used as in the present study. Overall, the simplicity, reliability, and relatively low cost of the Petrifilm plates make them suitable for citizen-based and educational monitoring of *E. coli*, particularly when used as a preliminary screening method to identify problem sites at which more extensive testing can be done by professional water-testing laboratories.

Acknowledgments

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