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Decoding the multiple tube fermentation method used in coliform testing

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Abstract

The multiple tube fermentation method (MTFM), used worldwide for decades in coliform testing, consists of the detection of the presence of total coliforms as the first step and fecal coliforms as the second step. The results obtained during the MTFM can be used to enumerate the relevant organisms detected in each step in terms of the most probable number (MPN). This research was carried out to find out whether it would be possible to omit the total coliform test and start the MTFM with the fecal coliform test. Minimum concentration of *Escherichia coli* (*E. coli)* in an aqueous suspension was obtained by means of a dilution series and was subjected simultaneously to the two tests. The results indicated that both tests responded identically to *E. coli* by showing no statistically significant differences ($p > 0.05$) between the MPN counts, even to very low numbers of the bacterium, which is often the condition of the samples tested by the MTFM in the real world. Accordingly, it was concluded that there was no scientific requirement to perform the total coliform test to commence the MTFM for the detection and enumeration of *E. coli*, and, instead, the procedure can be initiated with the fecal coliform test without compromising the accuracy of the outcome.

Keywords: Fecal coliform test, Enumeration of *E. coli*, Microbial food and water quality, Multiple Tube Fermentation, Total coliform test

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Introduction

Numerous authors and laboratories throughout the years have employed the laboratory tests collectively known as coliform tests to test the microbiological quality of drinking water and foods. The coliform tests are also used to test bathing waters and, various other samples which are not foods. These may include sources that have no possibility of even inadvertent consumption. However, such sources of samples may also carry the risk of spreading intestinal pathogens, of which the microbiological quality is a matter of concern. Waite (1985) endeavored to evaluate the relative importance of various coliform testing procedures. Among different coliform tests, the multiple tube fermentation method (MTFM) is one of the most widely used. Although the MTFM is one of the oldest coliform tests, and the procedure of the MTFM is widely available in scientific literature, the justification and interpretation of some steps of the test are either vague or absent. This research endeavored to provide explanations of various aspects of the MTFM, and especially to assess whether the initial step of the MTFM, the total coliform test, is essential for the analysis.

The coliform group is a diverse group of bacteria, which is defined as either motile or non-motile Gram-negative non-spore forming bacilli, whose habitats and characteristics may differ but share the ability to ferment lactose, producing acids and gases within 24–48 hrs. at 35–37 °C. The fermentation of lactose needs the beta-galactosidase enzyme, and therefore, coliforms are often described as bacteria that possess beta-galactosidase to produce acids and gases from lactose (Li & Liu, 2019). Some coliforms are members of the human normal intestinal bacterial flora, some are intestinal pathogens, while others live in the inanimate environment. The natural habitat of the coliform bacterium *E. coli* is the intestinal tract of warm-blooded animals, including mammals. As *E. coli* invariably lives in large numbers in the large intestine of all humans, it is always expelled with fecal matter in large numbers (Tenaillon et al., 2010; Singleton, 1999). Under the relatively low nutrient contents in soil or water, the bacterium would not survive for more than a few days, unless it enters a host again. Therefore, as pointed out by Martin et al. (2016) and many others, the presence of live *E. coli* in water or soil is considered an indication of recent fecal pollution. As *E. coli* may survive in foods longer than in water or soil, the presence of *E. coli* in foods is considered an indication of fecal pollution, but not necessarily recent. Similar to *E. coli*, intestinal pathogens expelled with the fecal matter of infected people would also not survive outside the human body for durations close to what *E. coli* survives. The absence of *E. coli* in a sample in numbers exceeding accepted microbiological standards excludes the possibility of fecal pollution, and it, in turn, excludes the possibility of the presence of intestinal pathogens (Microbiology of Food and Animal Feeding Stuffs — Horizontal Method for the Detection and Enumeration of Coliforms — Most Probable Number Technique, 2006). This provides the microbiologist with an opportunity to determine that a test sample poses no threat of intestinal microbial infections by testing only for the presence of *E. coli*, the indicator organism, instead of testing for the presence of a wide range of intestinal pathogens, and this is the specific importance of coliform testing. The presence of one or more *E. coli* in 100 mL is considered worldwide an indication of recent fecal pollution, and therefore, is not microbiologically safe for drinking (Microbiology of Food and Animal Feeding Stuffs — Horizontal Method for the Detection and Enumeration of Coliforms — Most Probable Number Technique, 2006). However, the absence of *E. coli* in 100 mL of drinking water cannot necessarily be regarded as microbiologically safe, because it does not rule out the possibility of the presence of harmful microorganisms that are non-intestinal.

Although coliforms other than *E. coli* are not indicative of fecal pollution, the presence of an unusually high number of total coliforms in one place may also be considered indicative of the possibility of fecal pollution, as it may be possible for *E. coli* also to be present as a member among a large population of coliforms. Therefore, the total number of coliforms in a sample, the Total Coliform count (TC), is also a parameter that is sometimes assessed.

As an intestinal organism of mammals, *E. coli* can ferment lactose, the milk sugar. It can live in temperatures above the average environmental temperature in the intestinal tract, and under the alkalinity and osmolarity provided there by bile salts. Therefore, the growth of *E. coli* is selectively promoted, while selectively suppressing the growth of many other bacteria, in a liquid growth medium that contains lactose as the sole organic substrate, bile salts, is incubated at a temperature slightly above the average temperature of the environment. This principle is followed for the selective detection and enumeration of *E. coli* by the MTFM.

A test to detect and enumerate *E. coli* must be responsive to the bacterium even when its numbers are very low, which is often the situation in water. Therefore, the conventional MTFM begins with the total coliform test, a step using liquid growth media that enriches the growth of *E. coli* to produce observable growth in the media. The basis of the MPN principle for enumeration in the MTFM is the capability of a single cell to produce an observable growth. The enrichment provides the sizable populations needed to start the steps of the MTFM for the detection of *E. coli*. The first step of the MTFM, which is to test the presence of coliforms, is known in literature (Bacteriological Analytical Manual Chapter 4: Enumeration of *Escherichia coli* and the Coliform Bacteria October 2020 Edition, 2020) as the 'Presumptive Test', because it presumes that *E. coli* could be present. However, as what the test precisely shows is the presence of members of the coliform group of bacteria in total, the term 'total coliform test' was used in the study to limit ambiguity. The results of this step are always presented in the literature as the Total Coliform (TC) count.

Although *E. coli* grows at 37 °C, the body temperature of the human host, 37 °C is not the optimum growth temperature of the bacterium. Medvedova et al. (2021) reported that the optimum growth temperature of the *E. coli* strain they tested was 41.1 ± 0.8 °C, and the maximum growth was observed at 48.3 ± 0.9 °C. *E. coli* grows well at 44 °C, a temperature under which many other coliforms are eliminated. Therefore, the incubation temperature used during the second step of the MTFM, the fecal coliform test, is 44 °C. The second step of the MTFM is known as the 'Confirmed Test' in the literature (Bacteriological Analytical Manual Chapter 4: Enumeration of *Escherichia coli* and the Coliform Bacteria, October 2020 Edition, 2020). What this second step shows is the presence of fecal coliforms, and, therefore, the term 'fecal coliform test' was used in the study to limit ambiguity.

In the fecal coliform test, fecal coliforms grow at 44 °C, fermenting lactose in the presence of bile salts, producing acids and gases within 24-48 hrs. However, as some non-fecal bacteria, including the soil bacterium *Enterobacter*, display the same characteristics, any bacterium that gives a positive result for the fecal coliform test is not necessarily of fecal origin. Therefore, specific tests such as testing for the colony appearance on Eosin methylene blue agar, Indole test, Methyl red test, Voges-Proskauer test, and citrate utilization test are needed to determine whether the coliform present is *E. coli*. All those tests of the MTFM carried out after the fecal coliform test are carried out for the specific purpose of identifying the bacterium *E. coli*. However, those steps together are conventionally known as the 'Completed Test' in the literature (Bacteriological

Analytical Manual Chapter 4: Enumeration of *Escherichia coli* and the Coliform Bacteria, October 2020 Edition, 2020), considering the fact that those are the tests that complete the MTFM.

Both the total coliform test and fecal coliform test of the MTFM are carried out for the same twin purposes. Both tests, if negative, save time and resources needed to test specifically for *E. coli*. In addition, both tests enrich the bacterium, a precondition for specific testing for the bacterium. The objective of this study was to assess whether the total coliform test can be eliminated by starting the MTFM with the fecal coliform test.

The chemical characteristics of the two media used in the two steps are similar. The major difference between the two tests is the incubation temperature, which is 37 °C for the total coliform test and 44 °C for the fecal coliform test. Therefore, this study determined whether even the usual low levels of *E. coli* found, if present, in the samples tested in the real world, would produce the same results in the test for total coliforms and the test for fecal coliforms, irrespective of the difference in incubation temperature and other minor differences between the two media. If the same number of *E. coli* cells, including very low numbers, produces the same results for both tests, without being negatively affected by the higher temperature of the fecal coliform test, it would be possible to streamline the procedure by starting the MTFM with the fecal coliform test, by eliminating the total coliform test, thereby reducing the burden on resources and accelerating the detection and enumeration of *E. coli*.

Methodology

Preparation of a dilution series of *E. coli*

A dilution series of *E. coli* was prepared using the following procedure. 100 mL of Nutrient Broth (HiMedia Laboratories Pvt. Ltd., India) was inoculated using a loopful of *E. coli* grown at 37 °C for 18 hrs. on Nutrient Agar (HiMedia Laboratories Pvt. Ltd., India), and was incubated at 37 °C for 18 hrs. An aliquot of 100 µL of the broth culture was transferred aseptically into 1000 mL of sterile distilled water and dispersed evenly by shaking for 5 min. From this original suspension, 20 mL was transferred into 180 mL of sterile distilled water and dispersed evenly, achieving a 10-fold dilution of the original suspension (dilution of 10^1). 20 mL from the 10-fold dilution was then transferred into another 180 mL of sterile distilled water and dispersed evenly, achieving a 10^2-fold dilution of the original suspension (dilution of 10^2). The same was repeated until 10^8-fold dilution was achieved (dilution of 10^8).

Total coliform test

The total coliform test and the fecal coliform tests were conducted in parallel to determine whether even very low numbers of *E. coli,* a condition achieved by preparing the dilution series, could grow into large observable populations without a difference during both tests within the same duration of incubation, irrespective of the temperature difference between the two tests and the differences between the two media. The *E. coli* suspensions of the dilutions of 10^5, 10^6, 10^7, and 10^8 were subjected to the two tests. For each test, taking inocula from each suspension, 15-tube fermentation cultures were prepared using the standard procedure. The number of *E. coli* in each suspension was determined by following the MPN principle. For the total coliform test, a 10 mL inoculum from each suspension was inoculated separately into 5 test tubes, each

containing 10 mL of 2X MacConkey Broth (HiMedia Laboratories Pvt. Ltd., India) to prevent excessive dilution of the medium. Then, 1 mL inoculum from each suspension was inoculated separately into another 5 test tubes, each containing 10 mL of MacConkey Broth. Next, a 0.1 mL inoculum from each suspension was inoculated separately into another 5 test tubes, each containing 10 mL of MacConkey Broth. The 15-tube fermentation cultures prepared in this manner from each suspension were incubated at 37 °C for 24 hrs. *Bacillus subtilis* (ATCC 6633), a non-coliform, and an uninoculated medium were used as the controls of the test.

Fecal coliform test

The same procedure as detailed above for the total coliform test was conducted in parallel for the fecal coliform test at 44 °C using the Brilliant Green Bile Broth (Oxoid, Thermo Fisher Scientific Inc., USA) as the medium. *Klebsiella pneumoniae* (ATCC 4352), a coliform of non-fecal origin, and an uninoculated medium were used as the controls of the test.

Comparing the growth of *E. coli* in the total coliform test with that in the fecal coliform test

Both the total coliform test and the fecal coliform test were conducted in triplicate, and the results of the two tests were compared to determine whether *E. coli* could grow identically under the different physical and chemical conditions of the two tests. In each test, the 15-tube fermentation cultures prepared using inocula taken from each suspension were observed for the number of positive tubes in each row of 5 tubes. A standard MPN table according to Lipps et al. (2023) was used to determine the number of *E. coli* in each suspension, which was expressed in terms of the MPN of *E. coli* in 100 mL of the suspension.

Statistical Analysis

All experiments were conducted in triplicate. A two-sample t-test was carried out to determine significant differences ($p \leq 0:05$) between the means. Data were analyzed using Minitab statistical software (Version 17 for Windows).

Results

Growth of *E. coli* in the total coliform test and the fecal coliform test

The growth of *E. coli* under the conditions for the total coliform test and the fecal coliform test are given in Table 1. The dilution of 10^5 showed that the suspension had 93.3 ± 15.3 (n=3) MPN of *E. coli* per 100 mL compared to the fecal coliform test with 110.0 ± 20.0 (n=3) MPN of *E. coli* per 100 mL. The growth of *E. coli* showed no significant difference ($P > 0.05$), indicating that the culture conditions did not affect the growth (Table 1).

Similarly, the dilution of 10^6 showed 12.3 ± 1.5 (n=3) MPN of *E. coli* per 100 mL for the total coliform test and 16.7 ± 5.5 (n=3) MPN of *E. coli* per 100 mL for the fecal coliform test. The MPN indices given by the suspension dilution of 10^6 for the two tests also had no significant ($P>0.05$) difference in growth under the two different culture conditions (Table 1).

The dilution of 10^7 showed 2.0 ± 0.0 (n=3) MPN of *E. coli* per 100 mL each for both the total coliform test and the fecal coliform test, with no significant (P >0.05) difference between the growth of *E. coli* under the two growth conditions (Table 1). No growth was observed in all 15 tubes (n=3) under both test conditions for the suspension dilution of 10^8, indicating that the dilution contained no *E. coli*. Accordingly, the number of *E. coli* present in the dilution of 10^7 was estimated to be 1 to 9, the lowest achievable concentration of the bacterium. The fact that even the lowest achievable number of *E. coli* produced identical results in the two tests showed that there was no difference between the two tests in supporting the growth of *E. coli*.

Table 1: MPN of *E. coli* in 100 mL of the suspensions

***E. coli* suspensions**		**Number of positive tubes from the 15-tube series**					
Dilution of	**Coliform test**	**5 tubes inoculated with 10 mL of the suspension**	**5 tubes inoculated with 1 mL of the suspension**	**5 tubes inoculated with 0.1 mL of the suspension**	**MPN of *E. coli* in 100 mL of the suspension (Mean ± SD)**		**The difference between the two MPNs**
10^5	Total coliform test (n=3)	5	3	0	80	93.33 ± 15.28	*p* = 0.32, >0.05 95% CI= (-23.6754 to 57.0154)
		5	3	1	110		
		5	2	2	90		
	Fecal coliform test (n=3)	5	4	0	130	110 ± 20.00	
		5	3	1	110		
		5	2	2	90		
10^6	Total coliform test (n=3)	3	1	0	11	12.33 ± 1.53	*p* = 0.26, >0.05 95% CI= (-4.8366 to 13.4966)
		2	3	0	12		
		3	1	1	14		
	Fecal coliform test (n=3)	4	2	0	22	16.66 ± 5.51	
		3	2	1	17		
		3	1	0	11		
10^7	Total coliform test (n=3)	1	0	0	2	2.00 ± 0.00	*p* = 1.00, >0.05 95% CI= (1 to 1)
		0	1	0	2		
		1	0	0	2		
	Fecal coliform test (n=3)	0	1	0	2	2.00 ± 0.00	
		1	0	0	2		
		1	0	0	2		

The MPNs of the three successive ten-fold dilutions of the bacterial suspension showed approximately a successive ten-fold reduction of growth (Table 1), indicating that there were no errors in the preparation of the dilution series. No control tests produced growth, confirming the accuracy of the tests.

The results showed that both tests supported the growth of *E. coli* identically, proving that the differences in the physical and chemical conditions of the two tests, including the difference in the incubation temperatures, do not exert any different influence on the growth of *E. coli.* Accordingly, it is possible to start MTFM with the fecal coliform test, without any deviation of the outcome if it were to start with the total coliform test.

Discussion

Although the MTFM is employed worldwide for the detection and enumeration of *E. coli*, there is limited literature validating several aspects of the procedure.

This research concluded that the total coliform test can be omitted, and that the MTFM can be initiated with the fecal coliform test, since either test, if negative, would permit, in the same manner, to conclude that what was tested has no possibility of containing *E. coli*. In addition, the growth conditions of either test equally enriched the growth of *E. coli* without any difference. However, the high cost needed to run an incubator routinely at 44 °C to perform the fecal coliform test, instead of at 37 °C needed to perform a total coliform test, justifies performing the total coliform test first in the MTFM. Performing the total coliform test first in routine laboratory testing may cost less than performing the fecal coliform test instead.

Conclusions

Both the total coliform test and the fecal coliform test responded identically, giving the same growth results even for very low numbers of *E. coli*, the numbers that are within the range of numbers of *E. coli* typically expected to be detected and enumerated in real-world conditions. The physical and chemical conditions of the fecal coliform test, including the higher temperature, did not harm the *E. coli* cells inoculated into it and, instead, promoted the growth of the bacterium in the same manner as those conditions of the total coliform test. Accordingly, the study concludes that the initial total coliform step could be omitted without compromising the degree of sensitivity of the MTFM, thus streamlining the procedure, reducing resource use, and accelerating detection.

Conflict of interest statement

The authors declare that there was no conflict of interest in conducting this research work.

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