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Research Article

The Need for Confirmation in Coliform and *E. coli* Enumeration in Foods

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Abstract: For food and environmental analysis, coliform bacteria and especially *E. coli* should be determined and enumerated rapidly, correctly and economically. Even if the results are obtained either by traditional or improved techniques, the results require confirmation, meaning that these techniques are not reliable. Confirmation tests need additional time and/or cost. In this study, 500 food samples of 10 various types were analyzed for their natural coliform contamination by the standard MPN method and *E. coli* by two MUG based MPN techniques. Enumeration results were statistically analyzed to determine whether confirmation tests for coliform and *E. coli* analysis are necessary or not according to the results of three statistical reliability analyses: Pearson's correlation coefficient (r), Cronbach's alpha (α) and determination coefficient (r^2). The results clearly showed that BGB broth confirmation for LST broth in coliform analysis and indole test confirmation for MUG test in *E. coli* analysis are not necessary ($p < 0.0001$).

Key Words: Coliforms, *E. coli*, MUG, LST broth, indole test, BGB broth

Gıdalarda Koliform ve *E. coli* Sayımında Doğrulama Gereği

Özet: Gıda ve çevre örneklerinin analizinde koliform bakterilerin ve özellikle *E. coli*'nin analizinde sayım sonuçlarının hızlı, doğru ve ekonomik olarak belirlenmesi önemlidir. Standart ya da hızlı ve gelişmiş yöntemlerle yapılabilen bu sayım sonuçlarının doğrulanma gereği bu yöntemlerin yeterince güvenilir olmadığını göstermektedir. Ayrıca, doğrulama testleri ilave zaman ve analiz giderine yol açmaktadır. Bu çalışmada 5 adedi doğrudan süt ürünü olmak üzere 10 farklı gruba dahil 500 gıda doğal koliform grup kontaminasyonun belirlenmesi açısından standart EMS yöntemiyle, *E. coli* kontaminasyonunun belirlenmesi için ise MUG esaslı EMS yöntemiyle analiz edilmiştir. Sayım sonuçları *E. coli* sayımında doğrulama testlerine gerek olmadığı açısından istatistik olarak incelenmiştir. Pearson's korelasyon katsayısı (r), Cronbach's alfa (α) ve belirleme katsayısı olmak üzere yapılan 3 farklı istatistik analiz sonuçlarına göre koliform grup bakteri sayımında Brilliant Green Bile Broth ile doğrulama yapmaya, *E. coli* sayımında ise MUG testi sonucunun indol testi ile doğrulanmasına gerek olmadığı açık bir şekilde görülmüştür.

Anahtar Sözcükler: Koliform, *E. coli*, MUG, LST Broth, indol test, BGB Broth

Introduction

Coliforms were historically used as indicator microorganisms to serve as a measure of fecal contamination, and thus potentially of the presence of enteric pathogens in foods. Although coliform bacteria themselves are not pathogenic, their presence indicates possible fecal contamination and the corresponding presence of intestinal pathogens responsible for a variety of diseases. Within the coliforms *Escherichia coli* is of interest since when present it indicates that recent fecal contamination has occurred with the possibility of accompanying enteric pathogens (1-3).

Rapid and sensitive methods for the detection and enumeration of target microorganisms in clinical, applied

and environmental microbiology have become more effective over the last decade (2,4).

Currently, coliform analysis takes 3 d according to both the International Standards Organization (ISO) and the Bacteriological Analytical Manual/Association of Official Analytical Chemists (BAM/AOAC) methods by the most probable number (MPN) technique. The analysis requires a minimum of 24 h and for gas negative tubes an additional 24 h incubation of inoculated lauryl sulphate tryptose (LST) broth at 37 ºC. All gas positive tubes should be transferred into brilliant green bile (BGB) broth and incubated at the same temperature for 24 h, in order to confirm the presumptive coliform results obtained from LST broth (5,6).

E. coli analysis takes 6 d and 10 d for ISO and BAM/AOAC, respectively, when the MPN method is used since the *E. coli* analysis can be done 4 d after fecal coliform determination. The ISO method determines *E. coli* by indole formation in tryptone water while BAM/AOAC requires IMViC tests (5,6).

Although both coliform and *E. coli* enumeration results are undoubtedly correct, analyzing perishable foods for coliforms and *E. coli* by standard methods is totally impossible because of the long analysis time. Hence, not only for coliforms and *E. coli*, but also for most bacteria many rapid and sensitive methods have been improved. Among them, the 4-methylumbelliferyl β-D-glucuronic acid (MUG) technique was evaluated as a rapid, specific, inexpensive and sensitive method for *E. coli* analysis. The MUG substrate is cleaved by β-glucuronidase (β-GUR) enzyme to a fluorescent end product (7,8). This reaction is easily determined by a 366 nm long wave UV lamp. MUG can be used with both liquid and solid as well as for membrane-filter media (9-11). While some solid media, such as eosin methylene blue (EMB) agar, did not provide satisfactory results, violet red bile (VRB) agar, EC broth, BGB broth and particularly LST broth incorporated by MUG were evaluated as equal to or better than the current methods in detecting *E. coli* (9,10,12,13).

The MUG method has been criticized for some false negative and false positive results. In addition to *E. coli*, some strains of *Salmonella*, *Shigella*, *Enterobacter* and *Klebsiella* are determined to be β-GUR positive and thus these strains are responsible for false positive results in *E. coli* analysis. In contrast, some strains of *E. coli* such as *E. coli* O157:H7 serotype do not possess this enzyme and thus give false negative results. False positive reactions may be eliminated easily by an indole test. While *E. coli* is β-GUR and indole positive, others, *Salmonella*, *Shigella*, *Enterobacter* and *Klebsiella*, are indole negative. The indole test is easy to apply and can be used directly in LST + MUG broth cultures (14). The ratio of false MUG negative strains of *E. coli* is not clear. Although recent research reported by Chang et al. (15) showed that 34% of *E. coli* strains of human fecal origin were β-GUR negative, the majority of reports have shown that 94-97% of human and environmental *E. coli* strains produce β-GUR enzyme (8). In spite of the problems caused by false positive and false negative reactions, the fluorogenic assay remains a much more sensitive and rapid method. Nowadays many standard analysis organizations including BAM/AOAC have accepted the MUG method as the standard analysis method (6).

This study is a part of our project entitled "Research on Fecal Coliforms in Foods". During the studies 10 different foods were analyzed for the natural contamination of coliforms and *E. coli* by standard and modified methods. The purpose of this study was to discover the necessity of confirmation tests in coliform and *E. coli* analysis so analysis time and cost may be saved. Both presumptive and confirmed enumeration results of coliforms and *E. coli* were analyzed by using three different statistical reliability tests.

Materials and Methods

Materials

Fifty samples each of pasteurized milk, yogurt, butter, cheese, ice cream, salad, delicatessen product, cookies, spices and fresh fruit and vegetables were enumerated for their natural coliform bacteria contamination. All samples were collected from local markets and open-air bazaars.

Methods

Samples were analyzed for total coliforms according to ISO directives (5) with a single change using LST broth + MUG media instead of standard LST broth. As there were numerous studies on MUG incorporated media, there were no objections to the direct use of LST broth + MUG media (7,9,13). All samples (10 gml^{-1}) were homogenized and then serially diluted 10-fold by good laboratory practice. Pasteurized milk, ice cream, yogurt and butter samples were inoculated directly. Since

negative coliform results were obtained from many samples, some of the pasteurized milk, ice cream, yogurt and butter samples were incubated at 37 ºC for 1-3 h to increase the number of existing coliforms up to countable levels. Samples that included uncountable levels of coliforms either very low (actually 0 MPN/g-ml) or very high and samples resulted in suspicion, were deleted from the analysis for statistical reasons. These samples were reanalyzed. When these repeated samples are included, a total of 1083 food samples were analyzed. From all five consecutive dilutions (from 10^{0} to 10^{-4} or 10^{-1} to 10^{-5}), 1 ml was inoculated to each 3 LST broth + MUG media and incubated at 37 ºC for 24-48 h. Gas positive tubes were marked and positive tubes of three consecutive dilutions were transferred into BGB broth (Merck) and incubated at 37 ºC for 24 h for the confirmation of these presumptive coliform counts.

Samples were analyzed for *E. coli* by two methods.

a) Fluorescence reactions were checked by using a 366 nm long wave UV hand lamp (Merck) in gas positive LST broth + MUG media. All of the fluorescence positive tubes were marked as presumptive *E. coli*. An indole test was used to confirm these positive ones. Positive tubes of three consecutive dilutions were used for standard MPN calculations (5,14).

b) Gas positive LST + MUG broth tubes were transferred into EC broth (Merck) and incubated at 44.5 ºC for 24-48 h. EC positive tubes were inoculated into 2.5 ml LST + MUG broth (Merck) and those tubes incubated at 37 ºC for 24 h and checked for fluorescence reactions in the same manner. All fluorescence positive tubes marked as presumptive *E. coli* and an indole test was used for confirmation. Positive tubes of three consecutive dilutions were used for standard MPN calculations (5,14).

All presumptive and confirmed enumeration results of both coliform and *E. coli* (as $\log_{10}$ values) were statistically compared by Pearson's correlation coefficient (r), Cronbach's alpha (α) and determination coefficient (r^2) analysis. For this purpose SPSS 9.0 for Windows was used.

Results

Confirmation of coliform counts

Reliability analyses for $\log_{10}$ values of presumptive and confirmed results of total coliforms are given in Table 1. According to the three different analyses there is no difference ($p < 0.001$) between LST broth + MUG results and BGB broth results for the 10 different food groups each consisting of 50 food samples (16).

During this study 1083 samples were analyzed. Mainly, five consequent dilutions of each sample and three tubes of each dilution, a total of 16,425 LST + MUG broth tubes, were inoculated for coliform enumeration. From all gas positive tubes, 4261 of them were evaluated according to the standard MPN procedure and transferred to BGB broth; 4173 of these tubes (97.93%) also gave positive gas reaction in this medium.

Confirmation of *E. coli* counts

Table 2 shows the reliability analysis results of *E. coli* counts by method (a). Similar to coliform reliability analysis, there is no difference ($p < 0.0001$) between presumptive (MUG reaction) and confirmed (indole reaction) results. Additionally, a total of 2520 tubes were evaluated as MUG positive and 2493 of them (98.93%) confirmed by the indole test. Since there is a high level of reliability ($p < 0.0001$) between presumptive and confirmed results for coliform counts in all 10 groups of food, there is no need to discuss the figures that varied according to food groups.

Table 1. Reliability analysis for the Presumptive vs. confirmed results for coliforms.

Food Type	R	r^2	a
Pasteurized milk	0.9964	0.9928	0.9982
Yogurt	0.9962	0.9924	0.9981
Cheese	0.9966	0.9932	0.9983
Butter	0.9931	0.9862	0.9966
Ice cream	0.9937	0.9874	0.9969
Salads	0.9934	0.9868	0.9967
Delicatessen products	0.9976	0.9952	0.9988
Cookies	0.9932	0.9864	0.9966
Spices	0.9932	0.9864	0.9966
Fruit and vegetables	0.9926	0.9853	0.9963
TOTAL	0.9955	0.9910	0.9978

* For all the figures $p < 0.0001$

Table 2. Reliability analysis for the presumptive vs. confirmed results for *E. coli* (Method a).

Food Type	R	r^2	a
Pasteurized milk	0.9942	0.9884	0.9971
Yoghurt	0.9985	0.9970	0.9993
Cheese	0.9987	0.9974	0.9994
Butter	0.9982	0.9964	0.9991
Ice cream	0.9990	0.9980	0.9995
Salads	0.9980	0.9960	0.9990
Delicatessen products	0.9989	0.9978	0.9995
Cookies	1.0000	1.0000	1.0000
Spices	0.9994	0.9988	0.9997
Fruit-vegetables	0.9987	0.9974	0.9994
TOTAL	0.9985	0.9970	0.9993

* For all the figures $p < 0.0001$

All the results of the three reliability analyses of 2.5 ml LST + MUG broth tubes were 1.000 for all 10 food groups and also it was 1.000 for total foods. It was not necessary to give those all 1.000 figures in a table. In other words, all the 2418 fluorescence positive tubes were confirmed by the indole test.

Discussion

High level correlations have also been obtained previously (16-18). During studies on fecal coliforms for different kinds of foods, the correlation between coliform counts in LST + MUG broth and BGB broth for confirmation was minimal $r = 0.947$ ($p < 0.05$) and $r = 0.943$ ($p<0.05$), respectively.

Although both BAM/AOAC (6) and ISO (5) coliform MPN enumeration methods indicate that gas production in LST broth is a presumptive result, it must be confirmed by using BGB broth. The results (Table 1) clearly showed that there is no need for this confirmation. According to the reliability tests (16):

- Pearson's correlation coefficient: Measures the degree of linearity between two variables such as x and y (actually presumptive and confirmed results).

- Cronbach's alpha: Defines the repeatability of the measure between two groups or variables. If all items are perfectly reliable and measure the same thing, then the coefficient of Cronbach's alpha will be 1.000.

- Determination coefficient: Determines the variation in one of the variables by the variation in the other variable. This is the square of Pearson's correlation coefficient.

In other words, from the statistical point of view for coliform analysis, these methods can be used instead of each other; actually LST broth results are adequate and do not require confirmation by BGB broth. Accordingly, the 24 h confirmation time and the cost of media and labor will be saved by the clear adequacy of LST broth alone.

In spite of using the LST + MUG broth instead of standard LST broth in this research it is clear that standard LST broth will also not be required for the confirmation by BGB broth.

In this research it is clearly shown that an indole test is not necessary for the MUG reaction's confirmation. The statistical discussion above is also valid for this section. In short the fluorescence reaction in LST broth + MUG is adequate and does not require an additional indole test. Similar to these results, Schindler (12) indicated that the indole test for the confirmation of presumptive fecal coliforms might be omitted and the examination with LST + MUG broth can be recommended.

Even if someone is anxious about false positive reactions due to some *Salmonella* and *Shigella* strains, this should not be important at the point of food analysis because these bacteria are even less desirable. Additionally, not as a rule but in general, if a sample contains *Salmonella* and/or *Shigella* it also contains *E. coli* because of fecal contamination. Although 2493 of 2520 MUG positive tubes were confirmed by the indole test, the 27 unconfirmed results should not be evaluated as *Salmonella* and/or *Shigella*, because specific isolation and identification tests were not applied for these 27 results.

It is interesting that in the fluorescence results of 2.5 ml LST broth + MUG tubes inoculated by EC broth cultures (method b) all of the tubes were confirmed by the indole test. Because the results of reliability tests showed a high level of similarity between presumptive and confirmed enumeration results of method (a), it is not possible to discuss the difference between the confirmed tubes between two methods.

Since the indole test takes only a few minutes, excluding this test for the confirmation of the

fluorescence reaction will not save analysis time. However, the cost of this test is very important. While confirmation of standard LST broth by BGB broth increases the analysis cost approximately twofold, with confirmation of fluorescence reaction in LST + MUG broth by indole test the analysis cost increases approximately fivefold. Routine analysis of foods requires economical approaches. Accordingly, confirmation by indole test is unnecessary.

In conclusion, high correlation values were obtained between presumptive and confirmed results for coliform enumeration. This reliability showed that BGB confirmation of LST broth is unnecessary. Similarly, confirmation of fluorescence results by indole test due to probable false positive MUG reaction is also unnecessary. LST + MUG broth can be used confidently for coliform and *E. coli* analysis without any confirmation being required. By this method, coliforms and *E. coli* can be enumerated rapidly, correctly and cheaply if fecal coliform analysis is not required.

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