

Evaluation of A New Detection and Quantification Method of Total Coliforms and *Escherichia coli* in Water

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Abstract

The discovery of chromogenic and fluorogenic substances for their application in tests has brought about huge advantages over the use of tests based on traditional culture media. In the field of drinking water microbiology, many studies are addressed at reviewing methods of analysis of coliform bacteria and *Escherichia coli*. The main objective of this study was to evaluate a new method of detection and quantification of total coliforms and *E. coli* in water by the Most Probable Number (MPN) technique using as broth culture a chromogenic-fluorogenic medium (CCL) according to the fundamental aspects of the validation guidelines of qualitative and quantitative methods of AOAC International and the standard ISO 16140 by analyzing 99 water samples from different origins. The new method is based on the splitting of specific substrates for glucuronidase and tryptophanase activity by *E. coli*, and for β -galactosidase by *E. coli* and coliform bacteria, and also on the gas production by the coliform group. A method described by ISO 9308-3 was chosen as reference, which is based on the fermentation of lactose with acid production, on the oxidase-negative character of total coliforms (for confirmation) and, additionally, on the production of indole from tryptophan at 44°C (*E. coli* confirmation). By different validation methodologies of alternative methods it was proven that the new chromogenic fluorogenic method using the Most Probable Number, employing methylumbelliferyl- β -D-glucuronide and ortho-nitrophenyl- β -D-galactopyranoside as enzymatic markers for the detection and counting of coliforms and *E. coli*, is equivalent to the procedures described in the ISO reference method for the same purpose in terms of diagnostic accuracy and precision of the count. The composition of the culture medium used allowed for the correct determination of the target microorganisms. The new procedure allowed obtaining results in less time, with less reagents, and more easily.

Keywords: Water; Coliform; *E. coli*; β -D-galactosidase; β -D-glucuronidase; Rapid detection

Introduction

Presently, in the world, the use of alternative reference methods of water and food analysis becomes more important. The discovery of chromogenic and fluorogenic substances for their application in tests has brought about huge advantages over the use of tests based on traditional culture media. In the field of drinking water microbiology, many studies are addressed at reviewing methods of analysis of coliform bacteria and *Escherichia coli* [1-12]. Herrera and Suarez [13] reported that fecal coliforms and enterococci are the most appropriate indicators for the presence of fecal contamination, as

they are ubiquitous and able to stay for long periods of time in water as well as in tropical and subtropical soils (see also, Fujioka et al. [14]). Considering *E. coli* as an indicator of fecal contamination requires the introduction of new methods for the rapid identification of the species of microorganisms, such as that based on 4-methylumbelliferyl- β -D-glucuronide (MUG) [15].

In the National Center of Biopreparations (Cuba), a group of researchers has developed chromogenic and fluorogenic media for detection and enumeration of total coliforms and *E. coli* [16-19]. These culture media and methods proposed were evaluated by various researchers in different applications [20-24].

Council Directive 98/83/EC of the European Union (November 3, 1998) [25] establishes to set concrete official analysis methods for microbiological-type parameters. Reference methods are those that are internationally recognized and widely accepted. Examples of these methods are published in: Standard Methods for the Examination of Dairy Products (official methods in the USA), published by the American Public Health Association (APHA), Environmental Protection Agency of the USA, Food Safety Inspection Service, Department of Agriculture, Compendium of Methods for the Microbiological Examination of Foods [26]; Official Methods of Analysis of the internationally based Association of Official Analytical Chemists AOAC [27]; Bacteriological Analytical Manual of the Food & Drug Administration of the USA (FDA), American Association of Cereal Chemists (AACC) [28]. These methods, in general, correspond to International Organization for Standardization (ISO) standards, and specifically for the case of coliform bacteria and *E. coli* exists the ISO 9308-1:2000 [29]. Some of these methods are cumbersome, sometimes expensive, and may require several steps to achieve reliable counts with verified identifications. Furthermore, achieved time reductions do not meet yet the needs of laboratories and end users. For these reasons, the search continues for alternative methods that accelerate results, reduce costs, facilitate the work, and diminish material burden. These alternative methods can be used provided that a performance study shows that their results are equivalent or superior to the official reference methods. These procedures should facilitate the execution of the tests, save manual labor, decrease waste, facilitate interpretation of results, and preferably increase specificity. Indeed, enzymatic reactions allow for the analysis of more samples. However, among the disadvantages of a method to detect a microorganism by an alternative method, such as the presence/absence (P/A), is that it is still considered only as a screening test, requiring confirmation of positive results by the traditional method. When a new method does not require confirmation, it should be validated under a protocol officially recognized at the national or international level.

Checking, comparing and validating alternative methods is essential for the user, as well as for the company or laboratory that develops them. Although, the recently published ISO 16140:2003 [30] and its 2011 amendments, present a protocol to validate alternative microbiological methods, we still have not reached a global agreement - there are different protocols, depending on whether they are defined by ISO, AOAC International or NordVal. Some studies estimate a growth of 17% of annual sales of kits for alternative methods, compared with 8% of the traditional methods in Latin America. The development of alternative methods will continue and will be useful for surveillance of food by the health authority and by businesses, based on the conference papers presented at the Congress of Science and Food Technology, held in Cordoba, Argentina (2004) (Source: Food Emphasis Latin America, Technical Publications).

The main objective of this study was to evaluate a new method of detection and quantification of total coliforms and *E. coli* in water by the Most Probable Number (MPN) technique using as broth culture a chromogenic-fluorogenic medium (hereinafter CCL) according to the fundamental aspects of the validation guidelines of qualitative and quantitative methods of AOAC International and the standard ISO 16140 [30] by analyzing water from different origins.

Materials and Methods

Samples

The study period ran from February 2009 until November 2012. We obtained a total of 99 water samples from seven geographic locations distant from each other and representative of different origins: 30 samples of water from the main supply (aqueduct), 4 samples from cisterns supplied by water from chlorine-treatment plants, 36 water samples from wells and 4 samples of untreated rivers, 16 samples from chlorinated pools, and 8 samples of de-ionized water. Of these, 9 aqueduct water samples, 4 from pools, and 26 from wells were processed as follows to achieve specific pollution levels for validation purposes. They were treated with 18% sodium hypochlorite solution (from 0.1 to 0.5 mg/L) for subsequent artificial contamination with a pure culture of *E. coli* ATCC 25922 in Tryptone Soy broth incubated at 35°C for 24 h. The cultures were refrigerated (2-8°C) overnight for stabilization. They were standardized to $OD_{550} = 0.25$ corresponding to approximately 3×10^8 CFU/mL in a spectrophotometer (T70 UV/VIS Spectrometer, PG Instruments Ltd., United Kingdom). Decimal dilutions were made in 0.1% (w/v) saline peptone according to the ISO 6887-1 (1999) [31]. Aliquots were taken from the 10^{-6} dilution and inoculated in Tryptone Soy Agar. The dilutions were stored under refrigeration (2-8°C) until the next day, when the recount results were in the plates. Finally, aliquots of 10^{-6} dilution were added to 1 L of water to be analyzed. Water samples were collected in 1000-mL sterile flasks, with 1 mL of 10% (w/v) sodium thiosulfate solution prior to its sterilization in the case of chlorinated water samples. Samples were transported in containers protected (isolated) from heat and temperature. Maximum transportation time did not exceed 6 h at a temperature below 10°C. Samples were processed in the laboratory within 2 h after arrival and were coded with Arabic numerals in consecutive order, both for the collected and the artificially contaminated samples. Subsequently, they were homogenized and used with their natural microbial load, likewise necessary dilutions were prepared: 1 in 10 and 1 in 100, using saline peptone 0.1% (w/v), according to APHA et al. [32]. The stability of the samples was ensured throughout the process without exposure to different temperatures, contamination from other potential sources, or other potentially damaging physical factors.

Levels of Contamination

Five levels were established for the classification of samples depending on the amount of contamination: level 0; level 1, from 1-10 CFU/100 mL; level 2, from 11 to 30 CFU/100 mL; level 3, from 31 to 100 CFU/mL; and level 4, more than 100 CFU / mL.

Procedure

Samples with possible contamination of total coliforms and *E. coli* were tested comparatively by the two methods: alternative (5 assay tubes) using the chromogen-fluorogene CCL [19] and the reference (5 assay tubes) using lactose broth medium (CL), brilliant green bile broth (CBVB), EC medium with MUG (ECMUG) [32]. The culture media were prepared according to Rodríguez et al. [33]. The reference method described in ISO 9308-3 [34] is based on the fermentation of lactose with acid production, in the oxidase-negative character of total coliforms (for confirmation) and, additionally, in the production of indole from tryptophan at 44°C (*E. coli* confirmation). ECMUG was incubated at $44.5 \pm 0.2^\circ\text{C}$ for 24 ± 2 h in a water bath (Gallencamp, United Kingdom). The presence of gas is characteristic for fecal coliforms and blue fluorescence at 366 nm for *E. coli*. Tubes where gas was not produced were considered to be negative. Agar Endo (AE) plates were inoculated from the

ECMUG- CBVB-positive tubes in the confirming phase of total coliforms, fecal coliforms, and *E. coli*, and were incubated at $35 \pm 2^\circ\text{C}$ for 18-24 h. From AE up to 5 colonies per plate were selected, codifying between typical and atypical: typical (colonies with greenish metallic sheen to reflected light with dark center in the transmitted light or red) and atypical (with other features) and these were inoculated simultaneously on Nutrient Agar (NA) and Cysteine Tryptic Agar (CTA) in order to preserve them for future identification of the colony. If 1 of the 5 colonies was identified as *E. coli* or coliform, was considered as sufficient identification, discarding the rest of the colonies. Otherwise, we proceeded to the identification of the remaining 4 colonies to confirm the presence of *E. coli* or other coliforms. From a total of 1444 colonies from CBVB, 701 were identified (48.5%), and from the 718 from ECMUG, 401 (55.8%) were identified. All colonies were inoculated into culture media for biochemical tests from CTA. NA- plates were incubated for 18 to 24 h at $35 \pm 2^\circ\text{C}$ for Gram staining and cytochrome oxidase testing. From CTA we proceeded to inoculate the culture media with the aim of identifying the microbe: fermentation of dextrose and lactose, and production of hydrogen sulfide (Kligler Iron Agar), lactose fermentation (Lauryl Tryptose Broth), indole production, sulphydric gas, and motility at $35\text{--}37^\circ\text{C}$ (SIM Medium), methyl red and Voges-Proskauer (MR-VP Medium) Simmons Citrate Agar, decarboxylation of ornithine, lysine (amino acid decarboxylation medium), fermentation of cellobiose, inositol, raffinose, sorbitol, xylose (phenol red broth), esculin hydrolysis (Bile Esculin Agar), beta-galactosidase and beta-glucuronidase (CromoCen CC). Where necessary, additional tests of fermentation of melibiose, dulcitol, rhamnose, L(+)-arabinose, D-arabitol (Phenol Red Broth) were performed, as well as of malonate and pyrrolidonyl-beta-naphthylamide (PYR) (Oxoid Ltd., United Kingdom). Where required, biochemical tests were made with the battery of API 20E and API 20NE assays (bioMérieux, France).

CCL is a liquid culture medium constituted by a chromogenic substrate (ortho-nitrophenyl- β -D-galactopyranoside) and another fluorogenic (methyl-umbelliferyl- β -D-glucuronide) to determine, respectively, the activity of enzymes β -D-galactosidase (present in coliforms) and β -D-galactosidase (present in *E. coli*) [19]. The broth also contains a mixture of nutrient bases specially selected, among which are highlighted peptones of different origins and extracts, inhibitors of Gram-positive microorganisms, carbon sources, buffers, and L-tryptophan for *E. coli* confirmation.

First enzyme activity is manifested by the appearance of yellow color in the culture medium and the second by the appearance of blue fluorescence at 366 nm. This method corresponds to new criteria for defining what is a coliform (β -galactosidase positive character) and what is *E. coli* (β -glucuronidase positive character) [35]. That is, the total coliform group includes Gram-negative bacilli, aerobic and facultative anaerobic, fermenting β -galactopyranoside with the production of color change to different shades of yellow within 24 h of incubation at $35 \pm 2^\circ\text{C}$. *Aeromonas* species can develop yellow hues, so that for samples in which their presence is suspected, the CCL broth was supplemented with cefsulodin (Fluka, Switzerland) in solution (5-10 mg/L of prepared medium). *E. coli*: in this method, are considered as such bacteria that ferment β -galactopyranoside with production of the color change to different shades of yellow, produce indole by the Kovac's reagent, and emit blue fluorescence when cultures are exposed to long wave UV light (366 nm) (hydrolysis of β -glucuronide) within 24 h of incubation at $35 \pm 2^\circ\text{C}$. In the medium, Gram-positive microorganisms are

inhibited and the other Gram-negative microorganisms do not develop all the above described characteristics.

Presumptive Phase: For total coliforms detection 5 portions of 10 mL of the test sample were aseptically taken with a pipette, and inoculated into 5 assay tubes with 10 mL of double strength CCL containing inverted Durham tubes. Five portions of 1 mL each of the 1:10 and 1:100 dilutions were taken aseptically, using a pipette, and inoculated into 5 assay tubes containing inverted Durham tubes. The tubes were incubated at $35 \pm 2^\circ\text{C}$ for 24 h and, after this time, growth was observed by the development of turbidity and color of the medium. Response was considered positive if there was growth, yellow color development (positive β -galactosidase reaction). For *E. coli* detection, in cultures from the total coliforms test, we observed the emission of blue fluorescence under UV light at 366 nm. It was considered a positive response to *E. coli* if there was growth, yellow color development (positive β -D-galactosidase reaction), and blue fluorescence at 366 nm (positive β -D-glucuronidase reaction). In parallel, a positive control, *E. coli* ATCC 25922 (MUG and indole positive) was inoculated and, as negative control, *Enterobacter aerogenes* ATCC 13048 (MUG and indole negative) and *Salmonella typhimorium* ATCC 14028 β -galactosidase and β -glucocoronidase negative) were used. For the assessment of the inhibitory capacity of the medium with microorganisms, cefsulodin on β -galactosidase-positive microorganisms not pertaining to coliforms, *Aeromonas hydrophila* ATCC 7966 was used.

For tubes positive for the production of yellow color (for the case of total coliforms) or yellow color and blue fluorescence (in the case of *E. coli*) the confirmatory element was observed.

Confirmatory Phase: For total coliforms, in some countries, the definition of total coliforms by the applicable standard involves producing gas from lactose. The MPN method with the use of CCL provides no gas formation from lactose contained in the medium; however, we decided to include this indicator during the study in order to compare its inclusion in those cases in which regulation do require it. Thus, in this way the total coliforms can be confirmed by the increase in turbidity of the medium and its yellow color as well as by the production of gas. The response is considered positive in one variable if there is production of gas. For *E. coli*, the cultures determined to be positive and with blue fluorescence we proceeded with the indole reaction (positive for this microorganism). For this assay, 3 mL of culture were taken with a sterilized pipette and transferred to a sterilized assay tube, adding 0.2-0.3 mL of Kovac's reagent. A response was considered positive for *E. coli* if growth was detected, accompanied by the appearance of a yellow color (positive β -D-galactosidase reaction), blue fluorescence at 366 nm (positive β -D-glucuronidase reaction), and a red color ring in the indole test.

Confirmation of the samples as part of the comparison of methods (Phase of complementary comparison):

As known, even standardized methods can generate false positives or false negatives. As part of the comparison protocol (not as part of the methods per se), an additional stage was designed only for *E. coli* within the framework of this protocol. Plates of AE were inoculated from the CCL positive tubes for *E. coli* to be incubated at $35 \pm 2^\circ\text{C}$ for 18-24 h. Inoculation of AE plates with the isolated colonies took place in the following manner: in aseptic conditions, an inoculation loop of

3 mm in diameter was introduced in the CCL-positive, tilting it and sinking the loop to 0.5 cm, the first quadrant of the AE was seeded, the loop was sterilized (flamed) again and the application continued. Up to 5 colonies were selected per plate, coding them as typical or atypical: typical (bright metallic green upon reflection of light with a dark center in the transmitted light) and atypical: having different characteristics. They were simultaneously inoculated in NA and in Iron Kliger agar medium (AHK) to preserve for future identification. When necessary, the colonies were kept in the CTA. If 1 of the 5 colonies was identified as *E. coli* or coliform, this was considered as sufficient identification, discarding the rest of the colonies. Of a total of 1610 colonies from the CCL test, 773 (48%) were identified.

Phase identification: All colonies were inoculated into culture media for biochemical tests from CTA. NA-plates were incubated for 18 to 24 h at $35 \pm 2^\circ\text{C}$ for Gram staining and cytochrome oxidase test. From the CTA-supplemented medium, we proceeded to inoculate the following media with the aim of identifying the germ: all Gram-negative cultures underwent IMViC tests in the culture media, Tryptone Water (TW), Medium MR-VP (MR-VP), Simmons Citrate Agar (CS) and checking gas production in CLT with Durham tubes. Where necessary, identification was strengthened in an additional step, especially to identify non-target microorganism colonies, with additional biochemical tests, similar to those used for the reference method.

Calculations and Statistical Analysis: For comparative analysis, MPN values were taken before and after confirmation with the indicated biochemical tests of the isolated colonies in both methods and the tubes were analyzed according to the Standard Methods [32]. For most comparative statistical analyses, logarithmic transformation was used according to Excel (Microsoft). The statistical analysis was performed with the help of SmartVal Program [36], based on the definitions described in EN ISO 16140 [30].

Determined Parameters: The index of the most probable number (MPN) per 100 mL was calculated for total coliforms, fecal coliforms, and *E. coli* by both methods according to the table described in Standard Methods [32]. In cases considered negative, the result was expressed as <1.8 and for statistical purposed it was considered equal to 1. Different recommended parameters were determined for the validation of alternative microbiological methods for water and food, contained mainly in ISO 16140 [30] and ISO/TR 13843 [37], in "Protocol for the validation of alternative microbiological methods" [38]: true positives (TP), true negative (TN), positive deviation (PD), negative deviation (ND), analysis of discordant results, relative sensitivity (RS), relative specificity (RSP), relative accuracy (RA), Kappa index (k), inclusivity, and exclusivity.

Relative Accuracy, Relative Specificity, and Relative Sensitivity: Paired data of the alternative method and the reference (Table 1) were tabulated and performance parameters were calculated from the tabulation (Table 2 x 2).

The calculation was made as follows:

$$\text{Relative Accuracy (RA)} = \frac{(\text{PC} + \text{NC})}{N} \times 100\%$$

$$\text{Relative Specificity (RS)} = \frac{\text{NC}}{N-} \times 100\%$$

$$\text{Relative Sensitivity (RS)} = \frac{\text{PC}}{N+} \times 100\%$$

Where:

N: total number of samples ($\text{NC} + \text{PC} + \text{PD} + \text{ND}$)

N-: total number of negative results by the reference method ($\text{NC} + \text{PD}$)

N+: total number of positive results by the reference method ($\text{PC} + \text{ND}$)

Table 1. Paired results of the reference method and the alternative method

Responses	Positive by the reference method (R+)	Negative by the reference method (R-)
Positive by alternative method (A+)	++ Concordance of positive (PC)	Positive deviation (PD) (R-/A+)
Negative by alternative method (A-)	+/- Negative deviation (ND) (A-/R+)	Negative concordance (NC)

Confidence intervals: Calculating confidence intervals for the number of samples tested was calculated as set forth below:

For each percentage (p) of the RA, RS and RSP and if $10\% < p < 90\%$, the approximate confidence interval (CI) is taken for two tails at 95%, being CI (at 95%) = with $n = N, N+, N-$, respectively, for p (in %) = RA, RS, RSP, where p: percentage of the index study (p of RA, or RS, or RSP), n: number of samples studied for the index, for example or RA, take N, for RS, take N+ and RS, it takes N. For the case where $p > 90\%$, we calculated the lower confidence limit of 95% (one-tailed), with $n \approx N, N+, N-$, respectively, for p (in%) = RA, RS, RSP. For the latter case is considered preferable to use the binomial table for $n = 10, 20, 30, 40, 50, 60$.

Discordant Results: Discordant results were analyzed by the McNemar test, using the number of positive deviations (PD) and negative deviations (ND). First we calculated the number of discordant results (Y) as follows: $Y = \text{PD} + \text{ND}$ and determined if both methods are different based on the balance Sensitivity vs. Specificity: for $Y < 6$, (less than 6 disagreements): there is no test available and we did not proceed the analysis; and for $6 \leq Y \leq 22$ (6 to 22 disagreements), m was determined as the lowest value of PD and ND. If $m \leq M$ for a given Y, both methods are considered different at an $\alpha < 0.05$ (2-tailed).

Interpretation of Results: Given the number of positive and negative deviations, we assessed the ability of the alternative method to provide greater or lesser amount of true positive results compared to the reference method and performed a comparative assessment between the results obtained for naturally contaminated samples and those artificially contaminated.

Results and Discussion

Total Coliforms: Qualitative comparison of total coliforms/Relative Accuracy, Sensitivity and Specificity/Global Analysis of the Samples

A total of 97 samples were analyzed with different levels of contamination according to the ISO 16140 [30] methodology. Overall analysis results of the samples are presented in Table 2.

Resulting indicators with their respective confidence intervals were:

Relative Accuracy = $94.85 \pm 0.90\%$
 Relative Sensitivity = $94.74 \pm 0.90\%$
 Relative Specificity = $95.24 \pm 0.80\%$

As shown, relative sensitivity is high compared with the reference method and the relative accuracy is high and greater than 90%.

Table 2. Results of the global analysis of water samples for total coliforms

Responses	Positive by the reference method (R+)	Negative by the reference method (R-)
Positive by alternative method (A+)	72	1
Negative by alternative method (A-)	4	20

The value for relative specificity was because with the alternative method the presence of coliforms was detected in 1 sample, whereas with the reference method this was negative. The confirmation with biochemical tests of colonies isolated according to CCL in the discrepant samples are shown below: in one of the samples analyzed with the reference method no total coliforms were detected, and with the new alternative method, colonies of *Aeromonas* were detected, resulting false positive. These findings are consistent with results obtained by other authors [39,40], who found interference with coliform counts in agar medium with chromogenic substrate with other β -D-galactosidase-producing bacteria [41,42] such as species of *Aeromonas* and *Vibrio*. Moreover, for the 4 samples negative by the alternative method, the reference method yielded: the group *Serratia liquefaciens* and *Enterobacter aerogenes* in one of the samples; in another, *Enterobacter sakazakii* was identified, the other two samples revealed *E. coli* colonies.

Likewise, we used a similar statistical analysis to determine the Kappa (k) index from chance (frequency) of occurrence that takes into account the discrepancies as and the concordances as a whole and demonstrates the strength of the alternative method with respect to the standard. As the resulting Kappa index (0.86) was greater than 0.8, it was concluded that the agreement between the two methods is almost perfect [38].

Discordant results analysis was not implemented for lack of any statistical test for such a small number discrepancies. We can say that in the indicators of the MPN method, using the CCL medium, is equivalent in terms of sensitivity, specificity, and accuracy in the analyzed samples.

Total Coliforms: Quantitative Indicators An equivalence analysis based on the evaluation of the maximum allowable deviation ($-D = 20\%$) of the alternative method (for one tail), relative to the reference method according to ISO 17994 [44] (Anonymous, 2004) was also explored. It was based on comparison of the MPN obtained with the alternative method with the MPN

obtained with the reference method. Results with MPN values of <1.8 were excluded. As a first step the expanded uncertainty was calculated at 22.33.

$$U = kS_x = \frac{2s}{\sqrt{n}} = 22,33$$

Lower limit

$$x_L = \bar{x} - U = -13,04$$

Upper limit

$$x_H = \bar{x} + U = 31,62$$

Relative Mean Difference = 9.29

Test of hypothesis: Methods did not differ because X_L is greater than -20 and less than 0; and X_H is greater than 0, according to ISO 17994 [44] (Anonymous, 2004).

Total Coliforms: Regression Analysis: Regression analysis was performed based on the results of individual data of the CCL-MPN/100 mL at 24 h of the confirmed values. Logarithmic transformation was also performed. The absence of growth (MPN <1.8 , was transformed, taking the log of value 1 and a unit value was added to all the obtained values). Regression results are given in the following: regression coefficient was 1.0855, which implied to apply the calculation method for an orthogonal regression (ISO 16140) [30] (Anonymous, 2002). The regression equation resulted to be: $y = 0.04 + 1.09x$.

It was observed that the intercept is -0.04 , close to zero, and the slope was 1.09 close to 1. This indicated that the MPNs from the alternative method were slightly higher than those of the reference, suggesting a high degree of linearity. Variance analysis yielded an F value of 2.99, lower than the critical (3.05), so goodness of fit (linearity) was proven.

Escherichia coli

E. coli: Qualitative comparison/Relative Accuracy, Sensitivity and Relative Specificity/Global analysis: A total of 99 samples from all contamination levels were analyzed according to the methodology of ISO 16140 [30] (Anonymous, 2003). Overall analysis results of the samples are presented in Table 3.

Resulting indicators with their respective confidence intervals were:

Relative Accuracy = $96.00 \pm 0.94\%$
 Relative Sensitivity = $92.18 \pm 0.88\%$
 Relative Specificity = $100.00 - 1.00\%$

Table 3. Results of the global analysis of water samples for *E. coli*

Responses	Positive by the reference method (R+)	Negative by the reference method (R-)
Positive by alternative method (A+)	48	0
Negative by alternative method (A-)	3	48

As can be seen, the relative sensitivity is very high compared to the reference method, relative accuracy is higher than 90%. The relative specificity value was due to the absence of positive deviations. Confirmation with biochemical tests on colonies isolated through CCL in the discrepant samples are as follows: for the 3 negative samples through the alternative method, *E. coli* was identified through the reference method, but not by the alternative method. An analysis of discordant results was not performed because there is no minimum number (m) of discordant results. We can state that in terms of specificity that the MPN method, using the CCL medium exceeds the reference MPN, and is equivalent in terms of sensitivity and accuracy. Likewise, a similar statistical analysis was used to determine the Kappa (κ) index from the expected chance (frequency) of occurrence and which took into account the discrepancies and concordances as a whole, and demonstrated the strength of the alternative method as compared to the standard. Kappa = 0.92 was greater than 0.8, implying that the agreement between both methods was almost perfect [38], which is the best criterion for the acceptance of an alternative method. It was concluded from this set of indicators that both methods are equivalent in determining the absence or presence of *E. coli* in different water samples with different contamination levels.

***E. coli*: Quantitative Indicators:** Equivalence analysis was based on a single tail of the maximum acceptable deviation (-D = 20%) of the alternative method, with respect to the reference according to ISO 17994 [43] (Anonymous, 2004).

The analysis was based on comparing the MPN obtained by the alternative method with the MPN obtained by the reference method. Included were the results with MPN values <1.8, as MPN = 1.0, when both methods gave the same result. As a first step we calculated the expanded uncertainty $U = 12.10$, its Lower Limit $x_L = \bar{x} - U = -11.80$ and Upper Limit $x_H = \bar{x} + U = 12.41$ for a Relative Mean Difference = 0.31. The hypothesis test showed that the methods do not differ since X_L was greater than -20% and less than 0; and X_H was greater than 0.

***E. coli*: Regression analysis:** Regression analysis was performed based on the analysis of individual data of the CCL MPN/100 mL at 24 h of the confirmed values. Logarithmic transformation was performed. The absence of growth (MPN <1.8, was transformed by taking the log of the value 1 and a unit was added to all other values).

The regression results are listed as follows:

The regression coefficient was = 1.53, the regression equation resulted to be $y = 0.02 + 0.99x$.

It was observed that the intercept is 0.02 and the slope 0.99. This indicated that the values of the alternative MPN were slightly higher than the reference, suggesting a high degree of linearity. The analysis of variance showed a value of F (1.81) much smaller than the critical (3.05), so goodness of fit (linearity) was proven.

Summary of the obtained main indicator values: The following tables show the values obtained for the different parameters studied, the acceptance criteria, and the final evaluation of each.

Analysis of total coliforms: Tables 4 and 5 show respectively, the qualitative and quantitative performance indicators for total coliform bacteria in water samples.

In Tables 5 and 6 we show the qualitative and quantitative performance indicators for *E. coli* in water samples.

Detection of coliforms by conventional methods is based on the acid and gas production from lactose. But occasionally, fecal coliforms, particularly *E. coli*, lack the enzyme hydrogenase and do not produce gas. For these reasons, the current trend is to define coliforms by the existence of the enzyme β -D-galactosidase and β -D-glucuronidase in 94-96% of *E. coli* [44].

Relative specificity for total coliforms and *E. coli* was $95.24 \pm 0.80\%$ and 99-100%, respectively. Other authors refer in their studies 93.1% and 95.7% of specificity for total coliforms and *E. coli*, respectively, in the culture medium with the chromophore indoxyl- β -D-glucuronide and fluorophore 4-methylumbelliferil- β -D-galactopyranoside, reporting 4.3% false-positive and false-negative [2].

Table 4. Summary of the main performance indicators of the new method of Presence/Absence for the determination of total coliforms in water samples.

Qualitative parameters (Presence/Absence)

Evaluated Parameter	Result obtained	Acceptance Criteria
Relative Accuracy	$94.85 \pm 0.90\%$	> 90%
Relative Sensitivity	$94.74 \pm 0.90\%$	> 90%
Relative Specificity	$95.24 \pm 0.80\%$	-
Kappa index	0.86	> 0.80
Discrepancies	5 in 97	There is no statistical criterion

Table 5. Summary of the main performance indicators of the new method of counting total coliforms in water samples.

Quantitative Parameters (count)

Parameter evaluated	Result obtained	Acceptance criteria
Hypothesis of equivalence based on limits and dispersion	$X_L = -13.04$; $X_H = 31.62$	$-20 \leq X_L \leq 0$, $X_H > 0$
Regression	High correlation and linearity $a \approx 0$, $b \approx 1$ $F_{ca} < F_{table}$	$a = 0$, $b = 1$, $F_{ca} < F_{table}$

F_{ca} : Stadigraph calculated F Fisher; F_{table} : Stadigraph F of theoretical Fisher

Analysis of *E. coli*

Table 6. Summary of the main performance indicators of the new method of Presence/Absence for the determination of *E. coli* in water samples.

Qualitative parameters (Presence/Absence)

Evaluated Parameter	Result obtained	Acceptance Criteria
Relative Accuracy	96.00 ± 0.94%	> 90%
Relative Sensitivity	92.18 ± 0.88%	> 90%
Relative Specificity	100,00 to 1.00%	-
Kappa Index	0.92	> 0.8
Discrepancies	3 en 99	No statistical criterion

Table 7. Summary of the main performance indicators of the new method of counting *E. coli* in water samples

Quantitative Parameters (Count)

Parameter evaluated	Result obtained	Acceptance criteria
Hypothesis of equivalence based on limits and dispersion	XL = -11.80, XH = 12.41	-20 ≤ XL ≤ 0; XH > 0
Regression	High correlation and linearity A ≈ 0, b ≈ 1 Fca; <Ftable	a = 0, b = 1, Fca; <Ftable

Fca: Statigraph calculated Fisher F; Ftable: Stadigraph F of theoretical Fisher

Results According to NordVal

The scatter plot for total coliforms according to the NordVal protocol is presented in Figure 1.

As can be seen and was expected, dispersion of data was higher at lower concentrations of total coliforms in the sample. Likewise, an aberrant result was observed between values of 1.5 and 2 log MPN, which, when analyzed its possible elimination did not change significantly the regression equation that describes the dependence between both methods. Despite the discrepancies in values, the resulting regression equation showed equivalence between the two counting methods, as the value of the intercept (0.052) did not differ significantly from 0 and the slope (0.973) was not significantly different from 1. The statistical analysis for testing the hypothesis of linearity showed that the calculated X was less than the critical and, therefore, linearity in the regression existed. Comparison of the average values by both methods is shown in Figure 2.

The mean by the alternative method was part of the range of the median for the reference method ± 2SD and the standard deviation of the alternative method was framed within the expanded uncertainty interval of the reference method, so it was considered that the two methods are equivalent for the quantification of total coliforms, by the multiple tubes technique, in water samples.

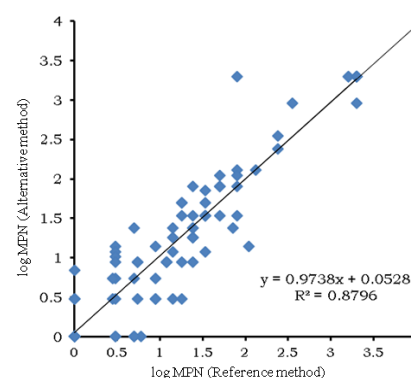


Figure 1 Scatter graph around the regression equation for total coliforms.

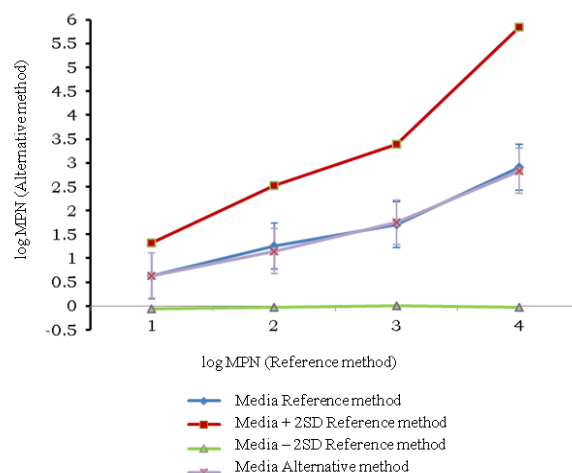


Figure 2 Distribution graph of means and standard deviations of the new method and the reference method for total coliforms.

E. coli

The scatter graph for *E. coli* according to the NordVal protocol is presented in Figure 3.

Just as for total coliforms, data scatter was larger at lower concentrations of *E. coli* in the sample. The resulting regression equation showed equivalence between the two counting methods, since the value of the intercept (0.019) was not significantly different from 0 and the slope (0.960) was not significantly different from 1. The statistical analysis for testing the hypothesis of linearity showed that the calculated X was less than the critical and, therefore, there was linearity in the regression. Comparison of the average values by both methods is shown in Figure 4. The mean by the alternative method was set within the range of the average for the reference method ±2SD and the standard deviation of the alternative method was also set within the expanded uncertainty interval of the reference method, so it was concluded that the two methods are equivalent for the quantification of *E. coli* by the multiple tubes technique in water samples.

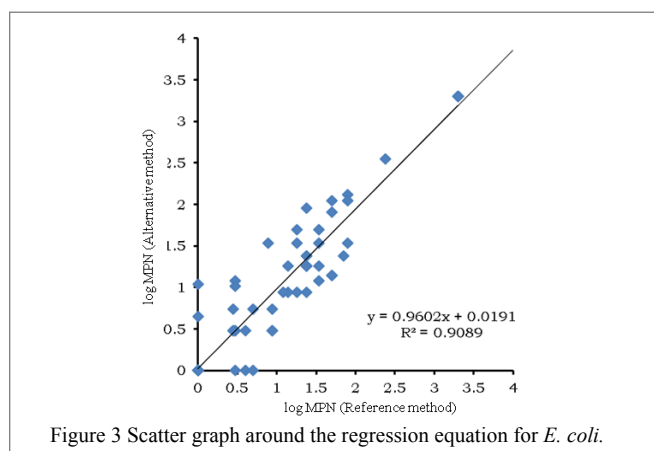


Figure 3 Scatter graph around the regression equation for *E. coli*.

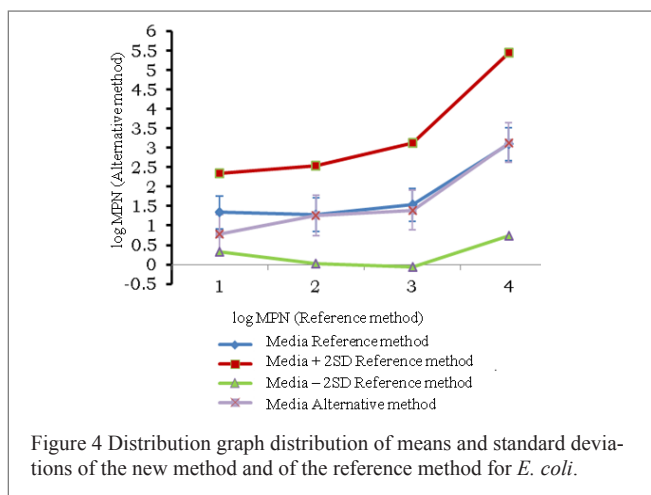


Figure 4 Distribution graph distribution of means and standard deviations of the new method and of the reference method for *E. coli*.

Competing Interests

The authors have declared that no competing interests exist.

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Conclusions

By different validation methodologies of alternative methods it was proven that the new chromogenic fluorogenic method using the Most Probable Number, employing methylumbelliferyl- β -D-glucuronide and ortho-nitrophenyl- β -D-galactopyranoside as enzymatic markers for the detection and counting of coliforms and *E. coli*, is equivalent to the procedures described in Standard Methods for the same purpose in terms of diagnostic accuracy and precision of the count. The composition of the culture medium used allowed for the correct determination of the target microorganisms, in a slightly superior way than the reference method. The new procedure allowed obtaining results in less time, with less reagents, and more easily.

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