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USE OF READYCULT™ - LMX FOR ENUMERATION OF TOTAL COLIFORMS AND *ESCHERICHIA COLI* IN MILK

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ABSTRACT

Readycult™ - LMX is a rapid method to test for the presence of total coliform bacteria and *Escherichia coli* in water, giving results in 24h. It is based on reactions of specific microbial enzymes or indicator nutrients of the medium. The goal of this paper was to study the use of Readycult™ - LMX to detect total coliform and *E. coli* in milk. One hundred twenty five samples of pasteurized and raw milk, collected in Londrina, PR, Brazil, were tested simultaneously by the most probable number (MPN) method using Brilliant Green Bile Lactose Broth (2%) (BRILA) and Readycult™ - LMX. The Readycult™ - LMX test was evaluated for sensitivity, simplicity, and speed of results. There was a significant correlation between the results obtained by the two methods for total coliforms (r: 0.8224) and for *E. coli* (r: 0.8603). The two methods yielded similar results, but Readycult™ - LMX was easier to use. In addition, results were available as early as 24h.

Key words: Readycult™ - LMX, X-GAL, MUG, milk, *Escherichia coli*, total coliforms

INTRODUCTION

The increasing number of outbreaks of food poisoning has highlighted the importance of microbiological control in the food industry. The group of total coliforms is the main indicator of the microbiological quality of water and food (7) and their presence indicates improper treatment or post-disinfecting contamination. *E. coli* is used as a faecal contamination indicator in water and food, as this microorganism is found in the intestine of human beings and warm blooded animals. Its use for water analysis was suggested in 1892 (3). In the milk industry, coliform counts are used to indicate hygiene in milk production and post-pasteurization contamination (9).

Traditional methods to count total coliforms include plating in Violet Red Bile Agar (VRBA) with subsequent biochemical identification of typical colonies and the technique of the Most Probable Number (MPN) using Brilliant Green Lactose Bile Broth (BRILA) for total coliforms and an additional incubation in EC and Tryptone broths for faecal coliforms identification (4). These methods are laborious and the results for total coliforms are obtained only after 48h incubation, while a further period of 48h incubation is needed to check for the presence of faecal coliforms (8). These time periods are incompatible with the perishable nature of milk.

The Readycult™ - LMX system (Merck Co., Darmstadt, Germany) detects coliforms and *E. coli* in water in 24h. This system is based on enzymes that are not part of the microorganisms. The culture medium offers two active combined substrates, 5-Bromo-4Chloro-3-Indolyl b-d-galactopyranoside (X-GAL) and the 4-methylumbelliferyl-b-d-glucuronide (MUG), which simultaneously detect bacteria from the coliform and *E. coli* group, respectively. The total coliforms produce the b-galactosidase enzyme which cleaves the X-GAL releasing the indicator that produces a change of color in the medium from yellow to greenish blue. *E.coli* produces the b-glucuronidase enzyme that hydrolyzes MUG releasing the 4- methylumbelliferyl

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producing fluorescence under UV light (365 nm). Readycult™ - LMX further contains a phosphate and peptone buffer that favors the rapid growth of coliforms, and lauril-sulphate that inhibits Gram positive microorganisms.

In this study, the Readycult™ - LMX system was compared to the standard BRILA culture medium by the MPN technique for enumeration of total coliforms and *E. coli* in milk.

MATERIALS AND METHODS

Sample collection and preparation

One hundred and twenty-five milk samples (seven from raw and 118 from pasteurized milk) collected in Londrina, PR, Brazil, from June 1998 to March 1999 were tested. Samples were kept under refrigeration until analyzed at the Laboratory for Inspection of Products of Animal Origin (LIPOA) at the Department of Preventive Veterinary Medicine, State University of Londrina. For plating, serial decimal dilutions (1:10, 1:100 and 1:1000) of each sample were prepared in 0.85% saline.

Standard methodology for total and faecal coliform enumeration

Total coliforms. The Brilliant Green Lactose Bile Broth (BRILA, Biobrás, Montes Claros, MG, Brazil) was used in the standard method for enumeration of total coliforms. The multiple tube technique, using 3 dilutions in triplicates, was used following Brasil - MARA (1), with readings after 24h and 48h of incubation. The tubes with blurred medium and gas formation in the Durhan tubes were considered positive. The MPN of total coliforms per mL of milk was obtained using the table in the Appendix II of Brasil - MARA (1).

Faecal Coliforms. Faecal coliforms were counted by the standard method, as described in Brasil - MARA (1): 30 µl from each positive tube of BRILA were transferred to tubes containing EC broth (Difco, Detroit, USA) and Triptose broth (Biobrás S/A, Montes Claros, MG, Brazil), and incubated in a water bath at 45.5ºC for 24-48h. Samples that fermented lactose in the EC broth (gas formation in the Durhan tubes) and produced indol in the Tryptone broth were considered positive. The MPN of faecal coliforms in the milk samples was determined using the MPN table of BRASIL - MARA (1).

Readycult™ - LMX

Total coliforms. *The Readycult*™ - LMX system was adapted to the MPN multiple tube method to quantify total coliforms in milk, using 3 tubes of media for each diluiton (1:10; 1:100; 1:1000). The dehydrated culture medium was dissolved in 100mL of bi-distilled sterilized water and distributed in sterile test tubes (9.0mL/tube), which were inoculated as described for BRILA. After incubation at 35ºC for 24h, the tubes that presented color alteration from the normal yellow to greenish blue were considered positive. The MPN result for total coliforms was determined using the MPN table of Brasil - MARA (1).

Escherichia coli

The tubes that were positive for total coliforms were observed for fluorescence at UV light (365 nm) and for production of indol after addition of Kovacs reagent (Laborclin, Curitiba, PR, Brazil). Positivity for both tests indicated a positive result for *E. coli*. The MPN of *E. coli* was calculated using the MPN table of Brazil - MARA (1).

Statistical analysis

The MPN results were converted to $\log_{10}$ and submitted to statistical evaluation using Excell 97 and Statistica. The Kappa test (6) and a probabilistic model (2,5) were used.

Checking for false-positive and false negative results in Readycult™ LMX and BRILA

Sixty four positive and negative tubes of Readycult™ - LMX and 46 of BRILA were streaked on MacConkey agar (Bekton & Dickinson, Cockeysville USA) and Standard Plate Count Agar (PCA) (Biobrás S/A, Montes Claros, MG, Brazil) plates. When needed, colonies were submitted to Gram staining, oxydase tests and to the BAC-TRAY I identification system (Difco, Detroit, USA).

Readycult™ - LMX sensitivity assessment

The Readycult™ - LMX sensitivity was determined through testing of milk inoculated with different quantities of *E. coli* (3 up to $3x10^8$ cells/mL). The exact concentration of added *E. coli* was determined by plating in Plate Count Agar and incubation at 35ºC for 48h.

RESULTS AND DISCUSSION

Taking the tests for total coliforms, 59 (47.2%) of the 125 milk samples did not present growth in either Readycult™ - LMX or BRILA, 47 (37.6%) were positive in both media, 15 (12.0%) were positive only in Readycult™ - LMX and 04 (3.2%) were positive only in BRILA. Taking BRILA as the standard procedure, the relative sensitivity of the Readycult™ - LMX method was 91.15%, and the relative specificity 79.72% (Table 1).

Table 1. Distribution of 125 milk samples according to results for total coliforms as detected by Brilliant Green Bile Lactose Broth (BRILA) and by Readycult™ - LMX.

Culture Media		BRILA		Total
		Positive	*Negative*	
Readycult™ - LMX	*Positive*	47	15	62
	Negative	04	59	63
Total		51	74	125

relative sensitivity = 47/51 = 91.15%; relative specificity = 59/74 = 79.72%; concordance of tests = 47+59/125 = 84.80%.

Among 66 samples with counts for total coliforms, in 37 (56.06%), 14 (21.21%) and 15 (22.72%) the MPN in Readycult™ - LMX was higher, lower and coincident with that of BRILA, respectively. The correlation (r) between the methods was 0.8224 with a mean variance of 0.1405 (Fig. 1).

The differences among the results presented by the two methods and the large number of samples in which the counts in Readycult™ - LMX were greater than in BRILA indicated the need to check these results.

Seven (5.6%) out of the 125 samples analyzed for faecal coliform and *E. coli* were positive in Readycult™ - LMX, EC and Tryptone broths simultaneously. Other seven (5.6%) were positive only in Readycult™ - LMX, four (3.2%) were positive only in EC and Tryptone broths and 107 (85.6%) were negative in these media (Table 2). The relative sensitivity of Readycult™ - LMX was 63.64% (07/11), and the relative specificity 92.10% (107/114). The coincidence of these results was 93.44%.

Table 2. Distribution of 125 milk samples according to results for faecal coliforms and *E. coli* as detected by EC, Tryptone Broths and Readycult™ - LMX.

Culture Media		EC/Tryptone		Total
		Positive	*Negative*	
Readycult™ - LMX	*Positive*	07	07	14
	Negative	04	107	111
Total		11	114	125

relative sensitivity = 07/11 = 63.64%; relative specificity = 107/114 = 92.10%; concordance of tests = 07+107/125 = 93.44%.

Other methods, using the same substrates, were evaluated by Venkateswaran *et al.* (10) with similar results for sensitivity and specificity, probably because the standard methods were considered 100% correct, being the differences imprecisions of the alternative methods.

When Readycult™ - LMX was used for *E. coli* enumeration, the MPN was coincident with that obtained by the standard method in 110 samples (88.0%). In 11 samples (8.8%), the MPN in Readycult™ - LMX was greater than that in the standard method, while in 4 (3.2%), the MPN in the Readycult™ - LMX was lower than that in the standard method. The correlation between the two methods was 0.8603 and the mean variance was 0.1947 (Fig. 2).

The checking tests indicated that there was no false positive or false negative results in Readycult™ - LMX, but false negative results were detected in the standard media. Due to the low number of samples that were positive for faecal coliforms and *E. coli*, statistical analysis of results was not possible, indicating the need of a larger sampling.

The sensitivity tests with inoculated milk indicated that Readycult™ - LMX was able to detect *E. coli* in all concentrations that were tested. In addition, the color of positive Readycult™ - LMX tubes was easily visualized. Any alteration to greenish blue or similar tones indicated a positive result. The opacity of the milk did not interfere in color production or fluorescence.

Results in this study indicate that Readycult™ - LMX, used in the MPN format, is a good alternative for total coliform and *E. coli* detection and enumeration in milk, with the advantage of being easy to use. Results are easily visualized, and the time required for MPN results for both groups of microorganisms is only 24h. No false positive or false negative results were observed.

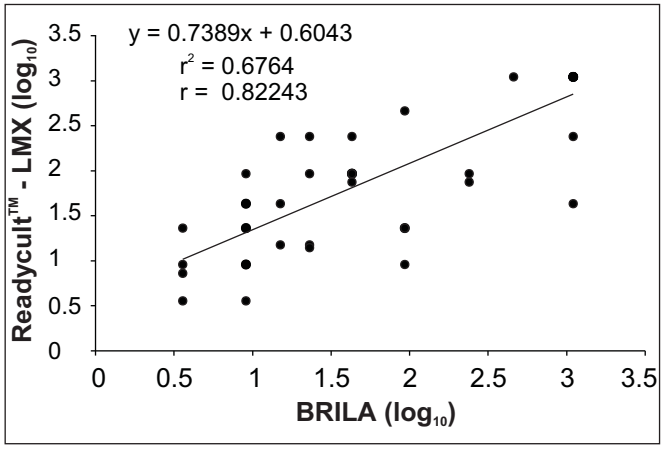

Figure 1. Dispersion of the MPN results of coliforms in milk obtained by the Readycult™ - LMX and by the standard method (BRILA).

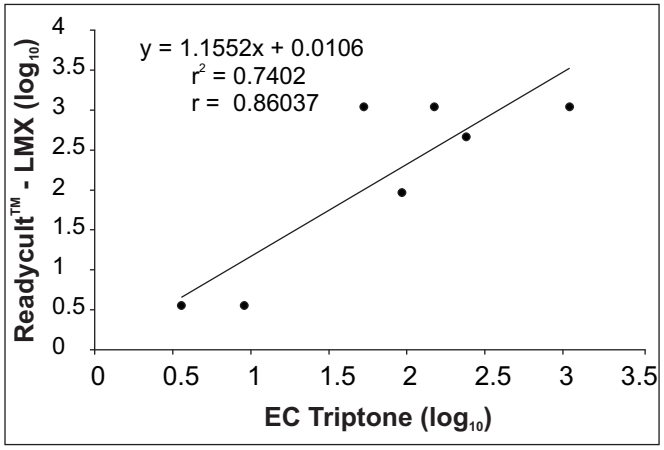

Figure 2. Dispersion of the MPN results of *Escherichia coli* in milk obtained by the Readycult™ - LMX and by the standard method (EC and Tryptone).

RESUMO

Uso do Readycult™ para enumeração de coliformes totais e *Escherichia coli* em leite

O Readycult™ - LMX é um método rápido, que permite resultados em 24h para detecção de coliformes totais e *Escherichia coli*. Indicado para detecção destes microrganismos em água, baseia-se em reações de enzimas específicas dos coliformes com nutrientes-indicadores do meio. O objetivo deste trabalho foi estudar sua utilização para enumeração de coliformes e *E. coli* em leite. 125 amostras de leite cru e pasteurizado coletadas na cidade de Londrina, PR, Brasil, foram submetidas à determinação do Número Mais Provável (NMP) de coliformes totais e *E. coli* pela técnica de tubos múltiplos, utilizando-se simultaneamente o Caldo Lactosado Bile Verde Brilhante (CLBVB) e o Readycult™ - LMX. A análise estatística indicou uma correlação entre os resultados obtidos pelos dois métodos de 0,8224 para coliformes totais e de 0,8603 para *E. coli*. Observou-se que o Readycult™ - LMX e o CLBVB deram resultados semelhantes, mas o Readycult™ - LMX foi mais fácil de ser utilizado. Além disso, os resultados foram mais rapidamente obtidos (24h).

Palavras-chave: Readycult™ - LMX, X-GAL, MUG, leite, *Escherichia coli*, coliformes totais

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