Abstract

Objective: to compare an alternative method to the most probable number (MPN) test for the detection of total coliform present in manually expressed human milk.

Methods: 343 samples of manually expressed human milk from flasks donated to the Human Milk Bank of Instituto Fernandes Figueira - IFF were sent to the Laboratory of Food Control of IFF. The samples were used for comparing both methods, i.e., the most probable number (MPN) method, as described in the “Standard methods for the examination of dairy products”, and the alternative method.

Results: coliforms were detected in 31.2% of the samples analyzed, with populations ranging from $3.0 \times 10^0$ to $1.1 \times 10^4$ total coliform MPN/mL. The comparison between classical and alternative methods showed similar results regarding the presence of coliform microorganisms in expressed human milk samples. The alternative method detected the presence of total coliform in all contaminated and in four noncontaminated samples according to the MNP method.

Conclusions: the alternative test allows the detection of the presence or absence of coliforms and it is useful for the quality control of pasteurized flasks containing manually expressed human milk manipulated at human milk banks. Therefore, we conclude that the alternative test can be used in the routine of human milk banks as a substitute for the MNP method, since its cost is equivalent to 1/7 of the cost of the traditional method.


Introduction

The cause-effect relationship in the transmission of diseases specifically related to foods contaminated with fecal material was initially described by Von Fristsch in 1880, when Klebsiella sp was identified in human feces. Later, the relationship between fecal microorganisms and gastrointestinal diseases was established by Escherich, who described Bacillus coli, currently known as Escherichia coli, suggesting that such microorganism could be used as an indicator of fecal contamination.1

Later on, researchers found out that different pathogens occurred discontinuously and at variable concentrations in feces. These researchers believed, however, there could be an indicator that would reveal the presence of these agents in fecal material.2

In 1892, Shardinger suggested that coliforms could be used as indicators of fecal contamination, since they can be more easily obtained than other enteric species such as Salmonella.3

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By definition, the group of total coliforms includes unsporulated, gram-negative, lactose-fermenting bacteria, with production of acid and gas at temperatures between 32 and 37°C.4-6 Fecal coliforms, in their turn, are a subgroup of total coliforms, whose natural habitat is the intestinal tract of homeothermic animals, which are able to ferment lactose and produce acid and gas at 44.5°C.6,7 Fecal coliforms can indicate more precisely whether foods have been contaminated by fecal material.

The group of coliforms consists of bacteria of a limited number of genera, basically including Escherichia, Klebsiella, Citrobacter, Serratia, Erwinia and Enterobacter.8

Since fecal coliforms are a subgroup whose detection is sequential and depends on positive results for total coliforms, they indicate more precisely the possible presence of enteric pathogens. Among the microorganisms isolated through fecal coliform tests, E. coli is the most frequently found,5-7,9 and is the classic indicator of the presence of enteric pathogens in foods,10 especially in expressed human milk.11

When defining microbiological indicators for food quality control, one should use a simple culture method and an indicator that is economic and that does not present false results. On top of that, the detection technique should be easily performed and reproducible. According to these requirements, the most probable number (MPN) is the most frequently recommended method in literature.11-13

In summary, the presence of total coliforms clearly indicates the lack of proper food handling practices and serves as an alert to the possible presence of more pathogenic and hardly detected microorganisms. Fecal coliforms, in their turn, indicate more accurately the presence of other microorganisms that are found together with E. coli in fecal material.14

The present study aims at comparing classic methods for the detection of total coliforms15 with an alternative method, in addition to assessing the cost and operational safety of this method.

Methods

Sample collection of expressed human milk

The donors of expressed human milk (EHM) at the Human Milk Bank of Instituto Fernandes Figueira (BLH-IFF) were asked to collect the milk at home after washing their hands with soap and water, brushing their fingernails, and washing their breasts with drinkable water. After that, they either expressed the milk manually or used a previously sterilized breast pump.

Afterwards, the donors transferred the milk directly into sterile flasks supplied by BLH/IFF and stored them in the refrigerator or freezer until before transportation to the Human Milk Bank, where the flasks were thawed in a bain-marie at 40°C before pasteurization.

In the present study, 343 samples of expressed human milk were obtained. The samples were collected according to the previously described procedures and sent out to the Laboratory of Food Control of the Instituto Fernandes Figueira. The samples were used to compare the methods described next.

1 – Detection of total coliforms by the classic method:

The most probable number technique was used as described in the Standard Methods for the Examination of Dairy Product.16 The samples were shaken for at least 20 seconds on a vortex shaker before the tests.

2 – Detection of coliforms by the alternative method:

Four samples of EHM were inoculated (one milliliter each), and after having been shaken for 20 seconds, they were independently pipetted into a test tube containing 10ml of brilliant green lactose bile broth 2% (Merck), prepared at the concentration of 50g of culture medium for each liter of newly-distilled water and distributed into test tubes containing a Durham test tube. Afterwards, the samples were autoclaved at 121°C for 15 minutes.

After inoculation, the tubes were incubated at 36 ± 1 °C for 24-48 hours and the presence or absence of coliforms was assessed by the formation of gas in Durham tubes, presumptive test.

Using the positive tubes, preparations (40g/liter of newly-distilled water) were seeded in new tubes of brilliant green bile broth with a bacteriological loop calibrated at 1/100, according to the manufacturer’s recommendations, and then incubated at 36 ± 1 °C for 24-48 hours. The presence or absence of coliforms was assessed by the formation of gas in Durham tubes, confirmatory test.

Although the proposed test is used to detect coliforms in pasteurized EHM, no samples of pasteurized EHM were used in the present study, since there is an extremely small incidence of coliforms in pasteurized milk. This would hinder the comparison between the two tests and would compromise the efficiency of the alternative test.

Data analysis: The data were analyzed by the Epi-Info 5.117 and SPSS for Windows18 software programs.

Results

Coliforms were detected in 30% of the samples, with populations ranging between 3.0 x 100 and 1.1 x 104 total coliforms MPN/ml (Table 1). The alternative test detected the presence of total coliforms in all of the contaminated samples and in one uncontaminated sample, according to the MPN technique (Table 2).
Discussion

Practice has shown us that we cannot apply any screening plan for the microbiological analysis of pasteurized EHM, since each flask submitted to the milk bank was obtained from a donor whose hygiene and health conditions may vary from one sample to another. Therefore, the secondary contaminating flora contained in the flasks donated by the same donor is well diversified.

Since the EHM is not mixed, it is not possible to use the concept of food lots, as in the food industry. This way, the analysis of all flasks of EHM is mandatory after pasteurization, involving a vast number of samples and, consequently, a great number of culture media and staff requirements.

In addition, it is impossible to analyze all the samples of pasteurized EHM in search of a wide variety of pathogenic microorganisms due to the costs of such operations, especially because milk banks are regarded as units that do not buy or sell human milk and, therefore, need to suit their activities to low costs and lower response time, based upon the speed of recycling of their EHM stock. This shows that tests for the detection of coliforms in pasteurized products to be distributed are extremely important.

Table 1 - Distribution of coliforms in 343 samples of manually expressed human milk analyzed through the most probable number technique

<table>
<thead>
<tr>
<th>Counting range (MPN/ml)</th>
<th>n. of samples</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1 (absence)</td>
<td>240</td>
<td>70.0</td>
</tr>
<tr>
<td>1</td>
<td>—10</td>
<td>16</td>
</tr>
<tr>
<td>10</td>
<td>—100</td>
<td>99</td>
</tr>
<tr>
<td>100</td>
<td>—1,000</td>
<td>11</td>
</tr>
<tr>
<td>&gt; 1,000</td>
<td>67</td>
<td>19.5</td>
</tr>
<tr>
<td>Total</td>
<td>343</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2 - Results of the comparison between two tests performed in 343 samples of expressed human milk for the presence of coliforms

<table>
<thead>
<tr>
<th>Alternative test result</th>
<th>MPN test result</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive*</td>
<td>Positive</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>04</td>
</tr>
<tr>
<td>Negative</td>
<td>00</td>
<td>236</td>
</tr>
<tr>
<td>Total</td>
<td>103</td>
<td>240</td>
</tr>
</tbody>
</table>

* The difference observed in the positive results could be explained by the loss of 0.67 ml during the successive dilution process in the classical test.

New methods that allow carrying out a great number of analyses have to be sought. These new methods should offer increased safety and lower costs as well, in comparison with conventional techniques.

After inoculation, the final concentrations of the culture medium in both methods were basically the same. The main differences lay in the amount and forms of inoculation of the analyzed samples. The largest detection of coliforms by the alternative method can be attributed to the higher amount of inoculated samples. In this case, when using 4 ml of sample, we add 0.67 ml more than in the classic test, with three series and three dilutions, employing 3.33 ml of EHM samples. The difference in favor of the alternative test could be explained by the loss of 0.67 (4-3.33) ml during successive dilutions.

All positive results were submitted to a confirmatory test, and the observed growths were cultivated on McConkey agar and were classified by traditional microbiological techniques, which confirmed the presence of coliforms in the four samples that yielded positive results on the alternative test and negative results on the MNP test. The different results obtained through both tests can be explained by the irregular distribution of microorganisms on the liquid media and/or by the greater amount of product analyzed by the alternative test.

The proposed test does not quantify the population of coliforms; it only indicates results expressed in terms of presence or absence which, respectively, correspond to positive and negative results. This way, when this method is used to determine coliforms in the analyzed samples of pasteurized EHM, we miss the chance of determining the most probable number of these microorganisms in this product. However, for the basic purpose of this analysis, the mere presence of coliforms in expressed human milk, regardless of their type or amount, would be absolutely undesirable, since it would make the product improper for consumption by preterm infants.

The detection of coliform microorganisms in pasteurized products indicates the existence of inappropriate practices in the processing of expressed human milk. Therefore, the information supplied by the laboratory can only be used to show the causes of such abnormality, when the history of the product under analysis is known.16

The recontamination of products by handling or pasteurization failures can be found and corrected at any units that handle pasteurized EHM, based on the results of coliform count.19

In addition, the most relevant aspect of this study is concerned with the savings the alternative method offers. The classic method requires at least three series and three dilutions, totaling a consumption of 3.6g of culture medium. The alternative method uses only one test tube, with 0.5g of the culture medium per analyzed sample, thus cutting down costs on staff and on materials, such as pipettes, test tubes, and others.
The comparison of the classic test with the one proposed here showed similar results in terms of detection of coliform microorganisms in samples of expressed human milk. These results are important because they prove the efficiency and safety of the alternative test.

Since the alternative test allows confirming the presence or absence of coliforms with increased reliability, it can be used in the quality control of pasteurized EHM flasks handled at human milk banks, especially because the cost of this test corresponds to 1/7 of the value of the conventional test.

References


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