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Detection of Bacteria Contaminating Milk and Milk Products in Tanzania by Conventional and Rapid Methods

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Abstract: The aim of the present study was to evaluate the performance of rapid methods for identifying contaminating bacteria in milk and milk products in Tanzania. A total of 60 milk and milk product samples were collected along the dairy value chain in Morogoro Municipality. The samples included raw and boiled milk, local fermented milk, and industrial processed milk products. Laboratory analyses were conducted using the conventional tests (culture and confirmatory tests) as well as rapid methods (3M petrifilm plate and polymerase chain reaction (PCR). Total bacteria count and identification of *Escherichia coli* as well as *Staphylococcus aureus* were performed. Results had indicated that there is positive correlation (R=1) between the 3M petrifilm and conventional methods. On the other hand, the correlation between PCR and the conventional method was poor (R=-0.078 and -0.15 for *E. coli* and *S. aureus* identification respectively). Furthermore, the PCR identified more positive samples (8 for *E. coli* and 12 for *S. aureus*) compared to the conventional method (2 for *E. coli* and 5 for *S. aureus*). No significant difference ($p<0.05$) was observed between 3M petrifilm and the conventional methods in tested boiled milk, local fermented milk, and industrial processed milk products; however, significant differences ($p>0.05$) were observed in raw milk by both methods. Based on these results and the time to obtain them, it is confirmed that rapid methods performed better than the conventional methods for determining contamination and identifying bacteria in milk and milk products and are hence recommended for routine laboratory analysis in Tanzania.

Keywords: Dairy Value Chain, 3M Petrifilms, Polymerase Chain Reaction (PCR), *Escherichia coli*, *Staphylococcus aureus*

1. Introduction

The dairy value chain supports the livelihood of many households worldwide [1]. Both the economic and nutritional values of dairy products are enormous [2, 3]. Globally and in Africa, the demand for milk and milk products has been growing rapidly due to increasing urbanization, population rise, and economic growth, among other factors [4, 5]. However, milk being a nutritious food, serves as an excellent medium for the growth of various microorganisms when suitable conditions exists [6]. Microorganisms cause milk spoilage, resulting in poor quality and limited shelf life, which may cause milk-borne diseases among consumers [7].

In Tanzania, several pathogenic bacteria of public health importance have been isolated from milk and milk products, including *Brucella abortus*, *Clostridium spp*, *Escherichia coli*, *Mycobacterium spp*, *Salmonella spp*, and *Staphylococcus aureus* [8, 9]. These bacteria have been associated with the infection of various diseases, such as typhoid fever, brucellosis, diarrhea, tuberculosis (TB), and allergies among consumers of milk in the country [9, 10]. Furthermore, most of the milk produced in Tanzania has a higher total bacterial count than the limits set by the East Africa Community [11]. This situation has denied lucrative markets for dairy products

for stakeholders and has created fear among consumers in the country [8]. This is one of the major reasons for the high importation of dairy products in the country, costing millions of dollars each year and possibly the main reason for the poor export of dairy products (Tanzania Dairy Board reports, unpublished). It is therefore important that surveillance be done with the major goal of improving the quality of products along the production and supply chains.

In Tanzania, milk testing is mainly conducted for raw milk that is channeled through the formal supply system, which is approximately 2.7 percent of the total country production [12]. In this system, the main platform tests are sensory (organoleptic), alcohol, and Clot on Boiling at the collection and receiving points of raw milk, with no further test along the supply chain. A few institutions and major processing plants in the country use conventional culture methods to determine the levels and types of contaminating microorganisms in milk [8]. The current conventional methods are both time consuming and complex [13] and hence alternative methods should be suggested. In this study, the performance of rapid milk tests, which can be used in institutions and at local collection centres, was assessed against conventional methods. The rapid methods include the Micro Biological Survey (MBS) method, Flow cytometry (FCM) method, Polymerase chain reaction (PCR), and the Petrifilm™ system [13-17]. These methods have been used in developed countries to improve surveillance along the dairy supply chain since they are more time-efficient, labor-saving, and able to reduce human errors [14]. The aim of this study was to evaluate the applicability and performance of the rapid methods in determining the contamination levels and identification of microorganisms in the Tanzanian dairy value chain.

2. Materials and Methods

2.1. Collection of Samples

A total of 60 milk samples were collected along the dairy value chain in Morogoro Municipality (Table 1). Approximately 50 ml of each sample was collected and placed in sterilized falcon tubes. They were then placed in a cool box packed with ice blocks (4°C) for temporary storage during sampling and for transport to the College of Veterinary Medicine and Biomedical Sciences (CVMBS) of Sokoine University of Agriculture (SUA) for storage in the Laboratory (-20°C) before analyses. The temperature of the cool box was maintained by the addition of reserve ice blocks and transportation to the Laboratory was achieved within maximum of 2 hours after sampling.

Table 1. *Types of milk sampled for laboratory analysis.*

Type of milk	Source	No. of samples
Raw milk	Smallholder farms	30
Raw milk	Dairy farms (Magadu, Mazimbu)	10
Boiled milk	Restaurants	8
Locally cultured milk	Retails shop	8
Industrial Vanilla yogurt and cultured milk (Brand X)	Supermarket	2
Industrial vanilla yogurt and cultured milk (Brand Y)	Supermarket	2
Total		60

2.2. Total Bacteria Count (TBC)

The total bacterial count (TBC) was analyzed using both conventional and rapid methods. Prior to analysis, frozen milk samples were removed from the deep freezer and left to thaw at room temperature for 2 hours. After thawing, 1 ml of each sample was dispensed into a new sterilized falcon tube, followed by the addition of 9 mls of sterilized buffered peptone water (BPW) (Oxoid® Ltd., Basingstoke, Hampshire, England). This was followed by serial dilution tenfold (10^0 to 10^9).

2.2.1. The Conventional Method

About 1 mL of diluted test sample (starting with 10^9) was inoculated in a prepared plate count agar (PCA). Then, the plates were allowed to dry for about 15 minutes. Thereafter, the plates were inverted and incubated at 37°C under aerobic conditions for 24 hours to allow the bacterial growth. The procedure was repeated for the remaining samples. After the incubation period, bacteria colonies on the culture plates were counted manually. A plate was divided into quarters using a marker pen, and Colonies forming unit were counted on at least two critical dilution plates with the aid of a colony counter. Two consecutive plates with less than 300 colonies were considered for record [18]. The countable bacteria colonies from two consecutive plates of each sample were converted into colony forming unit per milliliter (cfu/mL) using a formula provided by [19]:

Total bacterial count (CFU/mL) = (Number of colonies counted)/ (Dilution factor x volume plated)

2.2.2. Rapid Methods

About 1 mL of the diluted test sample was inoculated onto a petrifilm plate (3M, St paul, USA). A petrifilm spreader was used to distribute the inoculum over the circular area of the petrifilm plate and was left for 1 minute to solidify. Thereafter, the plates were incubated for 24 hours at 37°C under aerobic conditions according to the instructions of the manufacturer, followed by colony counting with the aid of a colony counter in the selected plates. The Petrifilm plates were gridded into 20 squares, comprising fewer colonies (less than 300) and large colonies (more than 300). During counting, the colonies were counted in all squares (for squares with fewer colonies and in representative squares (at

least 3) for the large colonies. Colonies in counted squares were averaged and multiplied by 20 to obtain the total (estimate) number of colonies to express in cfu/mL.

2.3. Isolation and Identification of Selected Bacteria in the Samples

Two priority bacteria of the food industry were targeted for isolation and identification by culture and biochemical tests representing the Conventional method and identification by PCR representing the Rapid methods.

2.3.1. Biochemical Tests to Detect Escherichia Coli and Staphylococcus Aureus

To detect *S. aureus*, [20] protocols were used. Prepared mannitol salt agar (Oxoid® Ltd., Basingstoke, Hampshire, England) was used as a differential medium. By using a sterile wire loop, samples were cultured on top of the media in a petri dish. The plates were incubated at 37°C for 24 hours, followed by examination of the colonies, whereby *S. aureus* in mannitol salt agar produced small colonies surrounded by yellow zones. The selected *S. aureus* colonies were again sub-cultured onto blood agar (E&O Laboratories Ltd., Scotland) for 24 hours to obtain pure cultures. Well isolated colonies were selected and further sub cultured onto nutrient agar, followed by biochemical identification using catalase test.

For the identification of *E. coli*, [21] protocols were used, prepared MacConkey agar (Hi media laboratories pvt ltd., Mumbai, India) was used as a differential media. Samples were inoculated into Mac Conkey agar plates by using a sterile wire loop. The plates were then incubated at 37°C for 24 hours, followed by examination of colonies, whereby *E. coli* in Macconkey agar appeared in small colonies with a pinkish colour indicating lactose fermenters. The well selected colonies were again sub cultured onto Macconkey agar under the same conditions in order to get pure isolated cultures. After the next 24 hours of incubation, the well isolated colonies were selected and sub cultured further onto nutrient agar followed by biochemical identification using Tripple sugar iron (TSI) and IMVIC tests.

2.3.2. Polymerase Chain Reaction

The genomic DNA was extracted from milk samples using the Quick-DNA Universal Kit as per the instructions of the manufacturer (The Epigenetics Company, Virginia, USA). The target bacteria in these experiments were *S. aureus* and *E. coli*. The reaction was performed using a PCR Master Mix premix (Bioneers, Daejeon, Korea). Specifically, the reaction mixture consisted of 8.5 µL of nuclease free water, 12.5 µL ready-made Master Mix, 0.5 µL of each forward and reverse primer (Table 2), as well as 3 µL of DNA template together at a final volume of 25 µL. The reaction was run in a thermal cycler (Biosystems Waltham, Massachusetts, USA) for 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds, and elongation of DNA strands at 72°C for 30 seconds. The initial denaturation (pre-heating) and final extension (cooling) stages were run at 95°C for 5 minutes and for 10 minutes respectively. After the reaction, the temperature in the thermo cycler was maintained at 4°C to enable cooling before further analyses. Thereafter, the PCR products were loaded into the wells of the agarose gel (1.5%) machine along with a 100 bp DNA marker followed by running the horizontal gel electrophoresis at 80V for 40 minutes. The bands were visualized under ultraviolet (UV) trans-illumination machine (Figures 2a and b).

Table 2. *Primers targeting genomes of two priority bacteria in the study.*

Bacteria	Primer name	Primer sequence	Size of the PCR product	Reference
E. coli	16s Forward	5′GACCTCGGTTTAGTTCACAGA 3′	585-bp	[22]
	16s Reverse	5′CACACGCTGACGCTGACCA 3′		
S. aureus	16s Forward	5′AACTCTGTTATTAGGGAAGAACA3′	756-bp	[23]
	16s Reverse	5′CCACCTTCCTCCGGTTTGTCACC3′		

3. Data Management and Analyses

Statistical analyses were conducted using R software version 4.2.3, followed by a paired t-test, which was performed to compare the two methods. When statistical differences between samples and the ISO standards were found, least squares differences (LSD) were used to determine values that were statistically different ($p<0.05$). All analyses were carried out in duplicate the results are expressed as means ± standard deviation (SD). In addition, the Pearson correlation coefficient was run in the software to measure and quantify the strength of the relationship between the results obtained by the two methods, whereby the values were between −1 and +1. A value of 0 would suggest that there was no relationship between the two methods; a value of +1 meant a perfect positive or direct linear relationship, −1 a perfect negative or indirect linear relationship [24].

4. Results

The distribution of microorganisms from different sampled milk products analyzed by both conventional and rapid methods (Table 3). It became clear that, both methods, at different levels, could detect microorganisms in the milk samples. For both methods, higher bacteria counts were observed in the raw milk analysed. The different in values of bacterial count in raw milk (similar samples) was significantly different ($p<0.05$) between the two methods. For local fermented milk and industrial processed products, the differences in bacterial count were not significant ($p>0.05$) between conventional and rapid methods.

Table 3. *Mean bacterial count in different types of milk samples analysed by conventional and 3M Petrifilms methods.*

No	Type of Milk	No of sample	Conventional method (cfu/ml)	3M petrifilm method (cfu/ml)	P-value
1	Raw milk	40	1.3×10^{9}	1.5×10^{9}	0.012^{S}
2	Boiled milk	8	2.5×10^{8}	2.5×10^{8}	0.982^{NS}
3	Local fermented milk	8	2.3×10^{7}	1.9×10^{7}	0.9^{NS}
4	Industrial Processed products	4	2.7×10^{7}	4.7×10^{7}	0.4^{NS}

Not Significant (NS) p>0.05, Significant (S) p<0.05

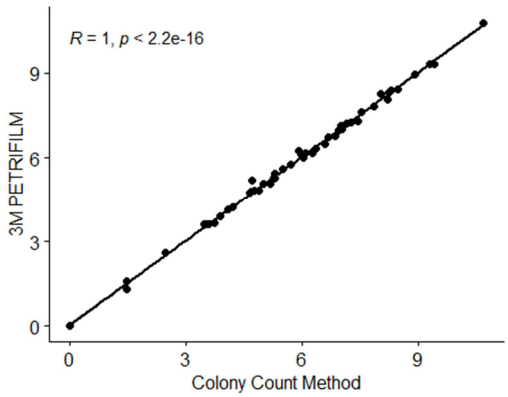


Figure 1. *Correlation between 3M Petrifilm and conventional culture (colony count method) in the detection of bacterial in milk.*

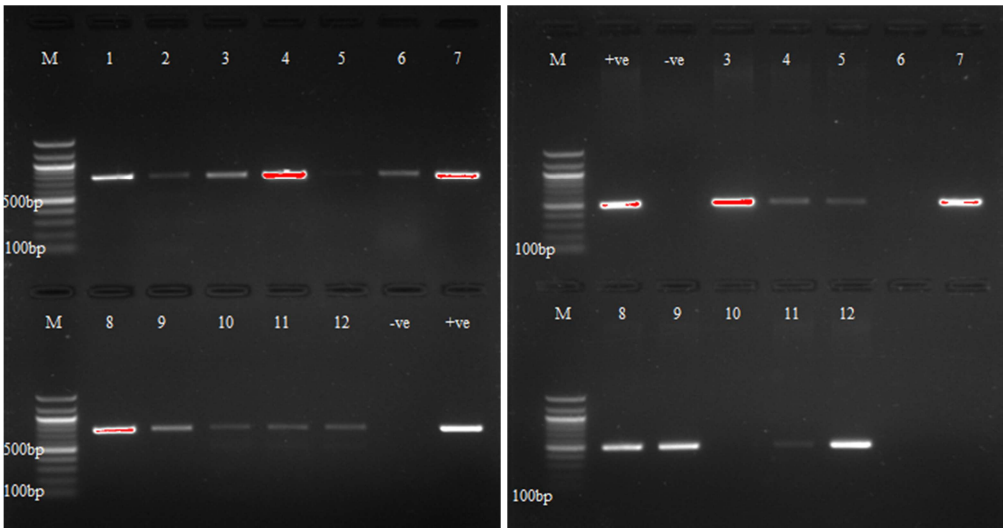
Further, we performed correlation analyses to assess the strength of the relationship between conventional and rapid methods. The analysis provided insights into the reliability and efficiency of a rapid method. Regardless of the milk type, the 3M petrifilm and conventional method showed a strong positive correlation, indicated by R, and signifying that the two methods are strongly correlated (Figure 1).

With regard to identification, our study showed that the PCR obtained more positive samples than conventional culture (Table 4). In total, the PCR detected more *S. aureus* in the samples than those detected by the conventional confirmatory tests (Table 4). A similar trend was also observed for *E. coli* detection.

Table 4. *Number of positive samples of S. aureus and E. coli from various types of milk analysed by both PCR and Conventional Methods.*

S/N	Type of milk	No of samples	*S. aureus*		*E. coli*	
			Conventional method	PCR method	Conventional method	PCR method
1	Raw milk	40	5 (12.5%)	2 (10%)	1 (2.5%)	3 (7.5%)
2	Boiled milk	8	0 (0%)	7 (87.5%)	0 (0%)	4 (50%)
3	Local fermented milk	8	0 (0%)	3 (37.5%)	0 (0%)	1 (12.5%)
4	Processed milk (vanilla-flavored yogurt, cultured milk)	4	0 (0%)	0 (0%)	1 (25%)	0 (0%)
5	Total	60	5	12	2	8

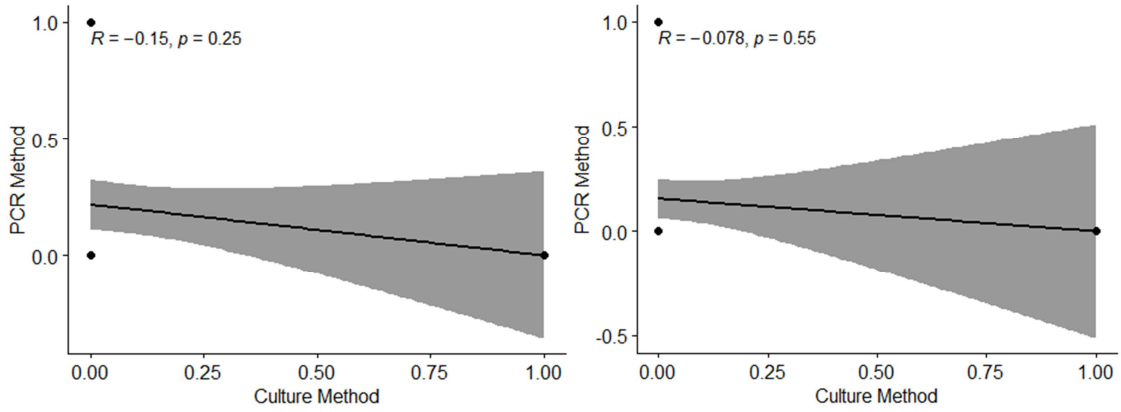
Further evidence of detection of bacteria can be seen in the electrophoresis photographs in Figure 2.



(a) (b)

Figure 2. *Electrophoresis photographs showing detected bacteria in milk sample by PCR (a) Staphylococcus aureus at 756bp. Positive samples are shown in lanes labeled 1 to 12. Lanes 13 and 14 are the negative and positive control samples, respectively, (b) Escherichia coli at 585bp. Positive samples are represented in lanes 3 to 5, 7 to 9 and 11 to 12 whereas lanes 6 and 10 are negative samples. Lanes 1 and 2 are positive and negative control samples respectively. M is a 100bp ladder marker.*

On the other hand, the study showed a strong negative correlation between PCR and conventional methods for *E. coli* and *S. aureus* identification (Figure 3).



(a) (b)

Figure 3. *Correlation between PCR and conventional method in the detection of target bacteria in the samples (a) S. aureus (b) E. coli.*

5. Discussion

The results from 3M petrifilm and conventional methods for total bacteria count in boiled milk, local fermented milk and processed milk products were in line with previous studies [25-29]. Results for raw milk align well with previous study [30], although their study focused on cheese, milk powder, ice cream and other dairy products. The correlation coefficient between petrifilm and the conventional method in this study is in line with previous studies [28, 31]. Furthermore, due to the excellent correlation between petrifilm and conventional methods many studies have concluded that both methods can be used; also, petrifilm can be used as alternative to conventional method [31-33].

The results for *E. coli* identification in all milk types align well with other studies [34-36]. The results for *S. aureus* identification in all milk types are similar to those of other studies [37-40]. These additional positive samples obtained by PCR can be explained by the presence of dead cells and viable cells, which cannot be cultured by conventional methods, and the detection of dead and live cells at the same time by PCR [41, 40]. The PCR and conventional methods correlation coefficient are the same to those of other study [42] and different from [43].

6. Conclusion

Regardless of the milk type, both 3M petrifilm plates and conventional methods showed excellent performance in microbial count in the laboratory. For bacterial identification, the PCR performed better than the conventional method, as samples that are more positive were detected, an indicator or sensitivity combined with automated interpretation of the results through electrophoresis. A positive correlation between rapid and conventional methods in bacterial counting suggests that the rapid method can be used reliably in research and represents a consistent testing method, whereas the negative correlation signals further investigation with the goal of identifying the reasons behind the differences. Therefore, since the performance of rapid methods was better than the conventional methods, the former are highly recommended for routine laboratory analysis to ensure the quality and safety of milk and milk products and hence healthy consumers.

Conflict of Interest

The authors declare that they have no competing interests.

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