Short communication: Coliform Petrifilm as an alternative method for detecting total gram-negative bacteria in fluid milk

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ABSTRACT

Postpasteurization contamination (PPC) of fluid milk remains a challenge for some dairy processors. *Pseudomonas* is the most common contaminant of fluid milk after pasteurization, and therefore methods to detect PPC should be inclusive of *Pseudomonas* and other gram-negative contaminants (e.g., coliform bacteria). Our objective was to compare the ability of 3M (St. Paul, MN) coliform and *Enterobacteriaceae* (EB) Petrifilm to detect total gram-negative bacteria with that of the standard method, crystal violet tetrazolium agar. To that end, we evaluated coliform Petrifilm, EB Petrifilm, and crystal violet tetrazolium agar to detect gram-negative bacteria in naturally contaminated samples of fluid milk. A total of 92 observations derived from shelf-life testing of 33 milk samples from 5 different processing facilities were evaluated for (1) presence of coliforms on coliform Petrifilm at both 24 and 48 h of incubation; (2) presence of any growth, regardless of gas production, on coliform Petrifilm at both 24 and 48 h of incubation; (3) presence of EB on EB Petrifilm at both 24 and 48 h of incubation; (4) presence of any growth, regardless of gas or acid production, on EB Petrifilm at both 24 and 48 h of incubation; and (5) presence of gram-negative bacteria on crystal violet tetrazolium agar after 48 h of incubation. Sensitivity and specificity analysis of results indicated that compared with the standard method (i.e., crystal violet tetrazolium agar), the method that performed the best, based on balanced accuracy (i.e., the average of sensitivity and specificity), was coliform Petrifilm evaluated for the presence of any growth after 48 h of incubation (sensitivity = 0.787; specificity = 0.839). This method can be easily adopted by the dairy industry as many processing facilities already test for coliforms using coliform Petrifilm. Improving the ability of processors to detect PPC will improve the quality of the fluid milk supply.

Key words: *Pseudomonas*, fluid milk, postpasteurization contamination

Short Communication

Recontamination of fluid milk after pasteurization, or postpasteurization contamination (PPC), is a major hurdle to achieving high-quality fluid milk (Martin et al., 2018). Products that have PPC typically reach the Pasteurized Milk Ordinance (PMO) bacterial limit of 20,000 cfu/mL (FDA, 2017) within 7 to 10 d of pasteurization when stored at 6°C (Ranieri and Boor, 2009). Studies suggest that approximately 50% of fluid milk is contaminated with gram-negative bacteria (e.g., *Pseudomonas*), which is evidence of PPC (Alles et al., 2018; Reichler et al., 2018). This contamination often occurs sporadically at low levels and has frequently been associated with filling equipment (Martin et al., 2018). Further, the types of bacteria that are commonly reintroduced into fluid milk after pasteurization typically grow rapidly at refrigeration temperatures and can produce several enzymes that are deleterious to fluid milk quality, including proteases and lipases (Hayes et al., 2002). Indeed, fluid milk products with evidence of PPC have significantly lower sensory scores than samples showing no evidence of PPC (Reichler et al., 2018). Taken together, PPC is responsible for accelerated product quality deterioration and shorter shelf lives for fluid milk. To reduce the incidence of PPC, processors must detect the contamination quickly and reliably to eliminate the source of contamination.

For decades, the dairy industry has relied on coliform bacteria as indicator organisms for detecting unsanitary conditions and PPC (Martin et al., 2016). Coliforms are gram-negative, facultatively anaerobic, rod-shaped bacteria capable of fermenting lactose to produce gas and acid within 48 h at 32 to 35°C (Davidson et al., 2004) and are limited to 10 cfu/mL in grade A fluid milk by the PMO (FDA, 2017). Many methods have been developed to rapidly detect coliform bacteria in dairy products, yet contemporary studies of postpasteurization contaminants in fluid milk indicate that coliforms represent only a minor proportion of the

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total population of bacteria causing PPC (Alles et al., 2018; Reichler et al., 2018). Instead, studies indicate that *Pseudomonas* is the major organism responsible for PPC in contemporary fluid milk (Alles et al., 2018; Reichler et al., 2018). *Pseudomonas* is not a coliform and not a member of the *Enterobacteriaceae* (EB) family; therefore, it is not detected through the use of common methods used by the dairy industry to detect PPC (e.g., different methods that detect coliform and EB), and therefore such contamination may go undetected. Several studies have suggested the use of a medium that is capable of detecting all gram-negative bacteria, such as crystal violet tetrazolium agar (CVTA), as a better indicator of PPC in fluid milk (Van Tassell et al., 2012; Alles et al., 2018). *Pseudomonas*, coliform bacteria, and EB are all detected using CVTA.

Despite the demonstrated benefits of using CVTA for detection of PPC in fluid milk, industry adoption of this method has been slow. We hypothesize that this is due to (1) the lack of regulatory requirements for total gram-negative testing and (2) the fact that CVTA requires the use of traditional agar plates, which can be costly, labor intensive, and space inefficient. To address these issues, our goal for this study was to identify an alternative method to CVTA for detecting total gram-negative bacteria in fluid milk. Coliform Petrifilm (3M, St. Paul, MN), used frequently in the dairy industry to detect coliforms, is easy to use, saves space, and anecdotally has been known to allow for the growth of *Pseudomonas* and other non-coliform gram-negative bacteria after 48 h of incubation (personal communication, Kevin McGoldrick, 3M, St. Paul, MN). Previous studies have evaluated the ability of coliform Petrifilm and EB Petrifilm (3M) to enumerate select *Pseudomonas* species in experimentally contaminated fluid milk (Van Tassell et al., 2012); however, to our knowledge no published studies have evaluated the ability of these media to detect total gram-negative bacteria in naturally contaminated fluid milk products. Therefore, the goal was to evaluate the ability of coliform Petrifilm and EB Petrifilm to detect PPC in fluid milk compared with CVTA.

To that end, samples of fluid milk ($n = 33$) were collected from 5 fluid milk processors in the northeastern United States in August 2018. Samples represented whole, 2%, 1%, skim, and chocolate milk and were commercially packaged in various sizes (e.g., half gallon, quart, 8 oz). Samples were transported to the Milk Quality Improvement Program laboratory at Cornell University (Ithaca, NY) on ice and were aseptically distributed into individual sterile bottles for shelf-life testing at 6°C as previously described (Martin et al., 2012). Microbiological testing was performed on d 10, 14, 17, and 21 after pasteurization and included spiral plating

on CVTA and plating undiluted fluid milk on both coliform Petrifilm and EB Petrifilm (3M) according to the manufacturer's instructions. All microbiological tests were conducted in duplicate. The CVTA plates were incubated at 21°C for 48 h (Frank and Yousef, 2004) before evaluation for the presence or absence of typical colonies (i.e., red colonies). Coliform and EB Petrifilm were incubated at 32°C and evaluated for the presence or absence of typical colonies (i.e., colonies with acid production and associated gas bubbles on coliform Petrifilm, and colonies with either acid production or associated gas bubbles on EB Petrifilm) as well as presence or absence of atypical growth at both 24 and 48 h of incubation.

Data were managed in Microsoft Excel (Professional Plus 2016; Microsoft Corp., Redmond, WA), and statistical analysis was performed in R (Kuhn, 2008). Microbiological testing data for the 33 samples were compiled and parsed such that the final data set included only sample results from a given shelf-life day (d 10, 14, 17, or 21) if all 8 tests were performed. Data were recorded as either negative (0) or positive (1) for detection of typical or any growth depending on the test. In total, 92 observations (1 per unique sample and shelf-life day) were retained, including 9 samples tested on 4 d, 8 on 3 d, and 16 on 2 d. Coliform and EB Petrifilm results from those 92 observations were compared with results from CVTA, and sensitivity, specificity, positive predictive value, negative predictive value, balanced accuracy, and accuracy P -value (i.e., accuracy of each test compared with the "no information rate," which is taken to be the largest class percentage in the data) were calculated using the caret package (Kuhn, 2008) in R. Data and code used for this study are available on GitHub (<https://github.com/FSL-MQIP/Alternative-Gram-negative>). Sensitivity describes the ability of each test to correctly identify a positive sample compared with the gold standard (i.e., CVTA), also known as the true-positive rate, whereas specificity describes the ability of each test to correctly identify a negative sample compared with the gold standard, also known as the true-negative rate.

Overall, of the 92 observations on the 33 samples evaluated here, 61 were positive for gram-negative bacteria on CVTA. Sensitivity, or true-positive rate, ranged from a low of 0.098 for both coliform and EB Petrifilm evaluated at 24 h for only typical growth to a high of 0.787 for coliform Petrifilm evaluated at 48 h for all growth (Table 1). Specificity, or true-negative rate, ranged from a low of 0.839 for coliform Petrifilm evaluated at 48 h for all growth to a high of 1.000 for coliform Petrifilm evaluated at 24 and 48 h for typical growth, EB Petrifilm evaluated at 24 and 48 h for typical growth, and EB Petrifilm evaluated at 24 h for

all growth (Table 1). Taken together, the balanced accuracy, or the average between sensitivity and specificity, ranged from a low of 0.402 for both coliform and EB Petrifilm evaluated at 24 h for typical growth to a high of 0.804 for coliform Petrifilm evaluated at 48 h for all growth (Table 1). Coliform Petrifilm evaluated at 48 h for all growth was the only test combination that had a significant P -value ($P = 0.002$) for balanced accuracy out of the 8 tests evaluated (Table 1) compared with the “no information rate.” It is unsurprising that coliform and EB Petrifilm evaluated for typical growth after 24 h of incubation resulted in the lowest balanced accuracy because these methods do not detect important PPC bacteria (namely *Pseudomonas*) that are detected using the reference method (CVTA). Importantly, 48-h coliform Petrifilm, which performed well, does allow for detection of *Pseudomonas*. For example, in a study by Alles et al. (2018), only 7% of fluid milk samples with evidence of PPC showed evidence of non-coliform EB and 20% of fluid milk samples with PPC showed evidence of contamination with coliform bacteria, whereas 100% of fluid milk samples with evidence of PPC showed evidence of contamination with non-coliform, non-EB bacteria (e.g., *Pseudomonas*). In another study, Reichler et al. (2018) found that in samples with spoilage due to PPC ($n = 132$), *Pseudomonas* were the predominant causative organisms (found in 101 samples with evidence of PPC), whereas only 27 of the 132 samples with PPC showed evidence of coliform bacteria contamination.

Our study indicated that coliform Petrifilm evaluated at 48 h for all growth had the highest balanced accuracy (0.804) of all 8 test combinations compared with the current standard method for detecting total gram-negative bacteria, CVTA (Table 1). Anecdotally, coliform Petrifilm has been known to allow for breakthrough growth of non-coliform gram-negative bacteria after 48 h of incubation (personal communication, Kevin McGoldrick, 3M, St. Paul, MN). This was supported in our study by the improved sensitivity of coliform Petrifilm evaluated at 48 h for all growth (0.787) compared with coliform Petrifilm evaluated at 24 h for all growth (0.410). Importantly, even coliform Petrifilm evaluated at 24 h for all growth had a higher sensitivity than coliform Petrifilm evaluated at 24 h for typical growth (0.098), which is the current method used by the US dairy industry as an indicator of unsanitary conditions. Indeed, the coliform Petrifilm literature indicates that the growth of lactose non-fermenting bacteria after 24 h of incubation is typically due to non-coliform bacteria such as *Pseudomonas*, yet our data suggest that not all samples contaminated with *Pseudomonas* will be positive after 24 h, necessitating the additional 24-h incubation period. Although CVTA has been shown

Table 1. Comparison of 8 methods using coliform and *Enterobacteriaceae* (EB) Petrifilm (3M, St. Paul, MN) to detect gram-negative bacteria in naturally contaminated fluid milk samples compared with the gold standard, crystal violet tetrastolium agar

Medium	Incubation period at 32°C	Type of growth enumerated ¹	Sensitivity ²	Specificity ³	Positive predictive value ⁴	Negative predictive value ⁵	Balanced accuracy ⁶	Accuracy P -value ⁷
Coliform Petrifilm	24 h	Typical coliform	0.098	1.000	1.000	0.360	0.402	1.000
	48 h	Typical coliform	0.115	1.000	1.000	0.365	0.413	1.000
	24 h	Any growth	0.410	0.935	0.926	0.446	0.587	0.949
EB Petrifilm	48 h	Any growth	0.787	0.839	0.906	0.667	0.804	0.002
	24 h	Typical EB	0.098	1.000	1.000	0.360	0.402	1.000
	48 h	Typical EB	0.164	1.000	1.000	0.378	0.446	1.000
	24 h	Any growth	0.295	1.000	1.000	0.419	0.533	0.997
	48 h	Any growth	0.656	0.903	0.930	0.571	0.739	0.074

¹Typical growth on coliform Petrifilm was defined as red colonies with associated gas. Typical growth on EB Petrifilm was defined as red colonies associated with gas or yellow zone.

²Sensitivity = $TP/(TP + FN)$, where TP = true positive and FN = false negative.

³Specificity = $TN/(TN + FP)$, where TN = true negative and FP = false positive.

⁴Positive predictive value = $TP/(TP + FP)$, where TP = true positive and FP = false positive.

⁵Negative predictive value = $TN/(TN + FN)$, where TN = true negative and FN = false negative.

⁶Balanced accuracy = $(\text{sensitivity} + \text{specificity})/2$.

⁷Accuracy P -value is a 1-sided test to determine whether accuracy of each test is better than the “no information rate,” which is taken to be the largest class percentage in the data.

in multiple studies to outperform other methods for detection of *Pseudomonas* and other common fluid milk contaminants that cause PPC (Van Tassell et al., 2012; Alles et al., 2018), it is a traditional culture-based method that is inaccessible for many industry laboratories. Conversely, Petrifilms are used widely in the US dairy industry, specifically coliform Petrifilms. Leveraging this existing and common test, which is used to confirm that fluid milk is within the PMO limit of 10 cfu/mL coliform, to test for a larger group of gram-negative contaminants including *Pseudomonas*, is a low-input way of improving the dairy industry's ability to detect PPC. It is notable that resolving PPC events often requires additional follow-up testing beyond the initial detection of gram-negative contaminants to identify the source of the contamination. We anticipate that the method used in our study will provide a useful tool for this follow-up testing but caution against using this method to specifically isolate *Pseudomonas* because there likely are variations in the individual bacterial strains capable of growing on coliform Petrifilm versus a non-selective medium or a medium specifically designed for total gram-negative bacteria.

Addressing PPC in fluid milk greatly depends on the ability of processors to detect contamination. Studies show that the primary bacterial organisms responsible for fluid milk PPC are not detected by coliform testing, the indicator group currently required by the PMO. To improve processors' ability to detect relevant bacterial contaminants in fluid milk, tests should be used that detect coliforms as well as *Pseudomonas* and other gram-negative bacteria. This study indicates that the coliform Petrifilm method, a test already in use by many processors, can be modified (i.e., incubation for 48 h and evaluation of all growth) to allow for growth of non-coliform gram-negative bacteria, improving the ability of coliform Petrifilm to detect total gram-negative bacteria compared with the standard method, CVTA. Although this method has reduced sensitivity and specificity relative to plating on CVTA, the ease of use and accessibility of coliform Petrifilm overall improves the ability of the dairy industry to detect PPC. Implementing this method, which is easily adoptable and uses pre-existing resources, will allow fluid milk processors to better respond to contamination events, leading to less PPC and improved fluid milk quality.

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