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Comparison of Standardized X-gal, ONPG and MUG Assay Methods with IS Methods by Analyzing Milk Samples

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Food Safety Standards Authority of India (FSSAI) in 2011 has adopted a conventional IS-5887 (Part-I) 1976 and IS-5401 Part-1 (2012) protocol for monitoring of *E. coli* and coliforms in dairy products respectively. These methods are time consuming and sometimes requires further isolation and confirmation to finalize the true contaminant. The current investigation was carried out to compare these methods with developed chromogenic and fluorogenic assay methods to access their suitability in actual analysis in terms of time saving, reliability and reproducibility.
Place and Duration of Study: Department of Dairy Microbiology, SMC College of Dairy Science, Anand Agricultural University, Anand, June 2019 to June 2020.
Methods: Ten samples of raw and pasteurized milks were inoculated with formulated selective broth. After incubation for 10 hrs crude enzymes were extracted to detect the presence of coliforms. Similarly 0.1 ml 4-Methylumbelliferyl-β-D-Glucuronide (MUG) solution was added in Formulated

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selective broth for the detection of *E. coli*. Enzyme extract procedure was not required after incubation of sample for detection of *E. coli*. Then after presence of coliforms were detection by the X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) impregnated strips and quantification was done by the chart developed using optical density measurement in o-nitrophenyl-ß-d-galactopyranoside (ONGP) assay. For *E. coli* detection, blue fluorescence generated was measured under UV light at 350 nm and quantification was done by measuring relative light unit (RLU) generated by Fluorescence spectrophotometer. Same sample was also analyzed by standard IS 5401: part-1 (2012) and IS 5887: Part - I (1976; reaffirmed 2005) procedure and compared with standardized methods for enumeration of coliforms and *E. coli* respectively.

Results: This study made clear that the results of analysis of raw and pasteurized milk samples by developed chromogenic and fluorogenic assay method were in accordance with the conventional IS methods.

Conclusion: Developed chromogenic and fluorogenic assay method indicating their suitability, ease in operation, time saving and preciseness over the conventional methods and can be opt as a suitable alternative for monitoring presence of *E. coli* and coliforms in fluid milks.

Keywords: Coliforms, selective broth; E. coli; validation; IS methods; ONPG assay; X-Gal assay; MUG assay.

1. INTRODUCTION

To develop a rapid and accurate method which enumerates coliforms and *Escherichia coli* in a wide variety of food products remains a challenge in the food industry. Many methods for determining and quantifying the presence of indicator organism particularly for coliforms are exist. It is classified into (1) cultural or traditional, (2) molecular and (3) enzymatic methods [1]. Among these methods, molecular and enzymatic methods are more precise and rarely need any confirmation nevertheless cultural methods purely depend on confirmation of *E. coli* and Coliforms. Most of the approved methods either by Indian Standards or ISO (The International Organization for Standardization) are cultural based and need confirmation step. Present-day demand of the sector is to have such a selective broth which would allow the growth of maximum genera of coliforms group while inhibition of non-lactose fermenting species. Such a broth can reduce the time of incubation and improve the preciseness of the test [2].

Among the traditional methods, non-selective Violet Red Bile Agar (VRBA) is used for enumeration of coliforms in food and dairy products which confirms the recommendation given by American Public Health Association [3]. The VRBA culture medium allows coliform detection and enumeration in 24-48 h; however, it does not allow differentiation of *E. coli* from there rest of the coliforms [4]. If typical coliform colonies appear, it requires further testing confirmation to label it as coliforms [5].

Indian dairy industry is mostly following IS 5401 Part-1/ISO: 4832 (2006), ISO: 16649 -1 (2018) and IS 5401: part-1 (2012) standard procedure for enumeration of coliforms and *E. coli* respectively. Main concern is with the interpretation of the results as it is not mentioned which types of colonies have to be counted and which need not be. Gazette notification of Government of India in 2016, has specified limits of coliforms in dairy products indicating its strong concern towards the presence faecal contaminants. Conventional enrichment and isolation methods for detecting coliforms in foods are generally very reliable, but they are expensive, laborious and time consuming, requiring at least 3-4 days protocol for presumptive identification [6]. Alternative methods based on nucleic acid, fluorescent antibody or immunology based techniques need additional equipments and expensive devices as well as enrichment steps for identification.

For the conventional methods biochemical tests used for bacterial identification and enumeration in classical cultural methods are generally based on metabolic reactions [7]. Hence, conventional methods are not completely specific and requires many additional tests to obtain precise confirmation. The use of microbial enzyme profiles to detect indicator bacteria is an attractive alternative to the classical methods. Enzymatic reactions can be group-, genus- or species-specific, depending on the enzymes targeted. Moreover, reactions are rapid and sensitive. Thus, the possibility of detecting and enumerating coliforms through specific

enzymatic activities has been under investigations since long time.

Till today many enzyme based methods either chromogenic or fluorogenic have been developed and certified. These methods have rendered rapid and much easier measurement of *E. coli* and coliforms than the methods approved in the past, hence these are attracting greater interest from researchers and industries [8]. These methods concurrently detect the total coliforms and *E. coli* which increasingly make possible the quantification of *E. coli*, rather than simply 'thermotolerant coliforms'. In the past decade, diverse methods using chromogenic and/or fluorogenic substrates to reveal β-d-glucuronidase and β-d-galactosidase activity on culture media have been reported to determine whether a strain belongs to the coliforms group and/or *E. coli*. Major advantage of using such media is that they are able to give results in less than 24 hrs [9].

Overall, traditional methods used in detection and enumeration of *E. coli* and coliforms bacteria are time, space consuming, require confirmation while alternative methods with incorporation of chromogens and fluorogens are much needed to fasten the process of overall evaluation of results and confirmation.

2. MATERIALS AND METHODS

The study was planned to develop a lateral flow enzyme substrate assay strip and a MUG assay strip for qualitative and quantitative estimation of coliforms and *E. coli* respectively. In the later stages, these developed tests were plan to compare with conventional IS methods for estimation of coliforms and *E. coli* counts. This work was conducted in the Department of Dairy Microbiology, SMC College of Dairy Science, Kamdhenu University, Anand. It was planned to use Formulated selective enrichment broth to test the performance of coliforms detection strip and MUG assay for *E. coli* from raw and pasteurized milk samples.

The mentioned Formulated selective coliforms broth was developed with addition of Sodium lauryl sulphate salt @ 0.2g, Gentamicin sulphate + Amoxycillin (1:1 ratio) @ 10 µl and Cefsulodin @ 312.5 µl per 100 ml which exhibited strong inhibition of targeted organisms like *Salmonella typhi* ATCC 14028, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 while promoted the growth of coliforms and *Escherichia coli* ATCC 25922 [2,10,11]. This Formulated selective broth was used to inoculate spike coliforms and *Escherichia coli* ATCC 25922 in later and was used to develop enzyme substrate and fluorescence assay. To spike specific population of coliforms and *E. coli*, protocol described by Gawai et al. [12] was used.

2.1 Preparation of X-gal Substrate strip

Preparation of X-gal substrate strip started with sample processing. Sample either milk or coliforms cocktail or *E. coli* spiked broth processing protocol was standardized after slight modification in the method described by Prasad et al. [13] and Makwana [14]. β-galactosidase and other enzymes present in coliforms are intracellular type, hence to extract these ultra-sonicator was used. Amplitude and time for ultra sonication were standardized using a statistical program software Response Surface methodology [2].

2.1.1 Protocol for crude enzyme extraction

Milk sample were added in 9 ml formulated selective broth and incubated at 37 °C up to 10 h. After incubation, test tube was removed and mixed carefully. Detailed flow chart for crude enzyme extraction for coliform testing using X-gal strip is given in Fig. 1.

2.1.2 Preparation of enzyme substrate assay strip test

A strip was used to make interaction of enzymes extracted from the sample and impregnated dried substrate. For preparation of a strip, an absorbent pad (Axiva Chemicals Limited, New Delhi) was used. It was cut in size of ≈8 cm x 0.8 cm. On the strip, X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) (100 mg/4 ml Dimethyl sulfoxide) solution was added @ 20 ul using 2.5 ml medical grade syringe and allowed it to dry for 4 h. These strips were stored in cool and dry place till onset of experiment.

2.2 Testing of Crude Enzyme Extracted from Milk Sample using ONPG and X-gal Assay

A properly dried dip strip aseptically added in sterilized empty test tube. To this, 1000 µl of prepared crude extract was added and incubated the test tube at 37°C for 15 min in an incubator and observed for change in colour of a strip from white to blue.

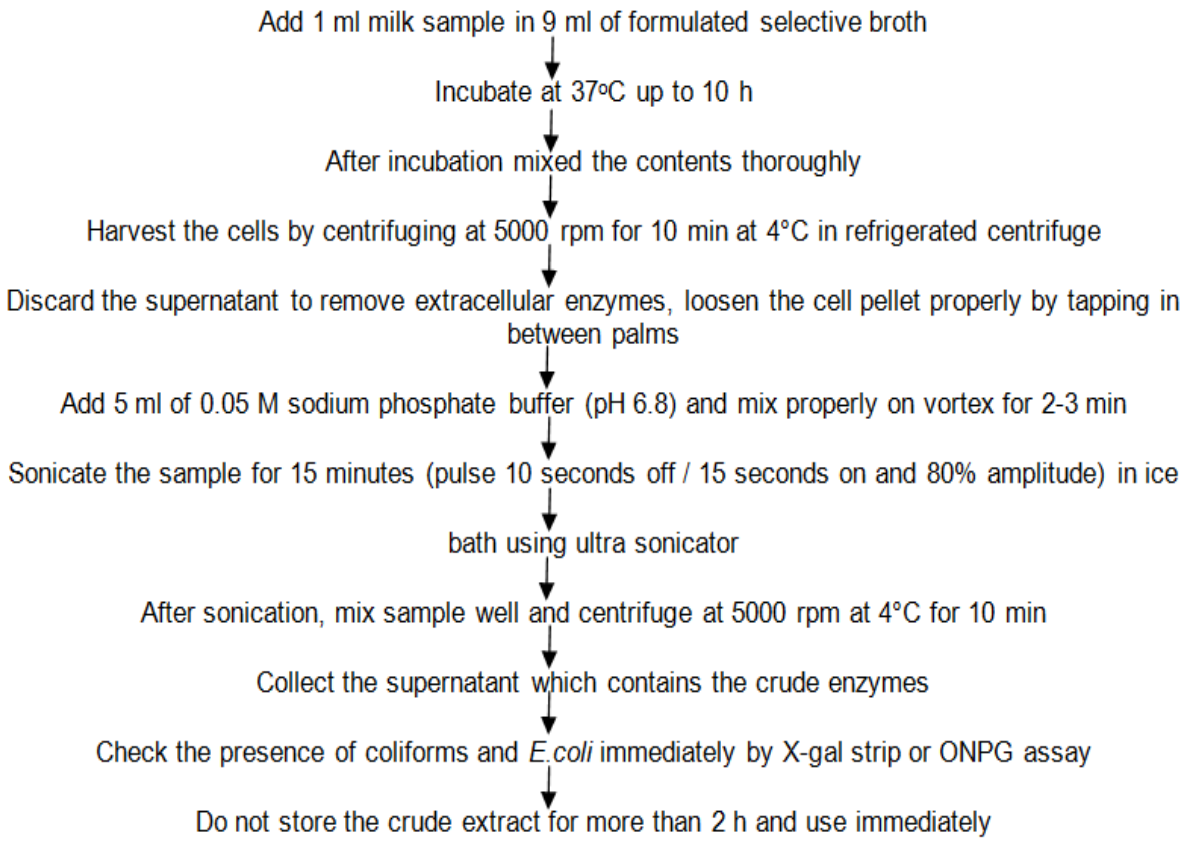


Fig. 1. Flow chart for crude enzyme extraction for coliform testing using X-gal strip or ONPG assay

Similarly, 500 ul of ONPG (O-nitrophenly-β-D-galactopyranoside) solution was added in sterilized empty test tube. In the same test tube added 500 ul of extracted solution and incubated the test tube at 37°C for 15 min and observe for change in colour in test tube. For quantification of coliforms, interpretation chart was prepared for different ranges of *E. coli* spiked cells against optical density changes in ONPG assay generated in different time intervals [2]. Colour changes from opaque white to yellow based on the intensity of reaction. Colour developed was then check by optical density by taking 100 ul of samples in micro titre plate at an absorption wavelength of 690 nm. This chart was used to make decision about possible coliforms population could present in the tested sample.

2.3 Protocol for Testing a Sample using Fluorescence Emission

To detect the presence of *E. coli* in the milk sample, 0.1 ml of 4-methylumbelliferyl-β-D-glucuronidetrihydrate in the formulated selective broth just before addition of sample. After that, inoculated 1 ml of the milk sample added in 9 ml of selective formulated broth. Further test tube was incubated for 10 h at 37°C. After incubation, the test tube was observed under the UV light at 350 nm for development of blue color. For quantification of *E. coli,* interpretation chart was prepared for different ranges of *E. coli* spiked cells against the relative light unit generated in different time intervals [2,15]. This chart was used to make decision about possible *E. coli* population could present in the tested sample.

2.4 Comparison of Developed X-gal/ONPG and Mug Assay Test with IS Methods

Ten samples each of raw and pasteurized milk were collected in sterile 100 ml sample bottles from cattle yard and local retails market of Anand, Gujarat (details given in Table 1) were screened for the presence of Coliforms, *E. coli* using developed methods as well as IS 5887 (Part - I) 1976 method.

Table 1. Description of milk samples tested for coliforms and *E. coli*

Sr. No	Sample Source/Type	No of samples	Analytical methods
1.	Raw milk: cattle yard, Local vendors and dairies of Anand, Gujarat	10	Developed enzyme assay / *E. coli* detection IS 5887- (Part-I) 1976 method [16] and
2.	Pasteurized milk: Purchased from local market (retail suppliers)	10	Coliform detection by IS, 5401 Part-1 (2012) method [17]

2.4.1 Analysis of milk samples by developed methods

One ml sample was added in the 9 ml of coliforms and *E. coli* formulated broth tube respectively. 0.1 ml of MUG was added just before addition of sample and mixed. The test tubes were incubated for 10 h at 37°C. After incubation, crude extracts of enzyme was prepared and sample was tested by X-gal, ONPG and MUG assay.

2.4.2 Analysis of milk samples by IS 5401 (Part 1): 2012 method

Eleven ml of the well mixed milk sample was diluted in 99 ml of 0.1% Peptone water to make a 10^{-1} dilution. This blend was used to make required serial dilutions with the buffer. Transferred 1 ml of the sample into a duplicate petri dish. Poured 15 ml of melted and cooled Violet Red Bile Agar medium into each Petri dish. After mixing the inoculum with the media, allowed the mixture to solidify, with the petri dishes standing on a cool horizontal surface. Prepared a sterile control plate with same VRBA media. After complete solidification, created an overlay by pouring another 4ml of the VRBA medium onto the surface of the inoculated medium. Solidified plates were incubated at 37°C for 24 h ± 2 h and after that results were expressed as cfu/ml.

2.4.3 Analysis of milk samples by IS 5887 (Part - I) 1976 method

Similar protocol was followed for estimation of *E. coli* as mentioned for coliforms by IS 5401 (Part 1): 2012 standard. Here bacteriological media used was Eosin Methylene Blue agar. It is differential media for coliforms and *E. coli*.

3. RESULTS AND DISCUSSION

As mentioned samples from raw and pasteurized milks were prepared and analyzed by assay methods developed and by standard IS methods. Results obtained are resented herewith.

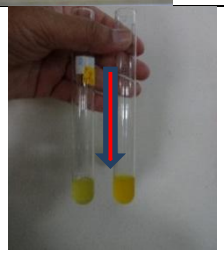
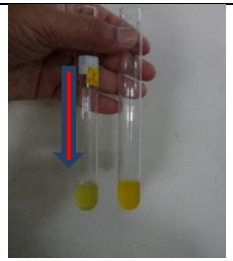
3.1 Analysis of Raw Milk and Pasteurized Milk Samples by Developed Coliform Detection Method

Details of analysis of raw milk samples are presented in Table 2 and in Fig. 2 and details of analysis of pasteurized milk samples are presented in Table 3 and in Fig. 3.

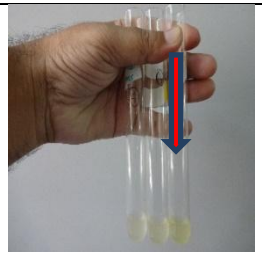
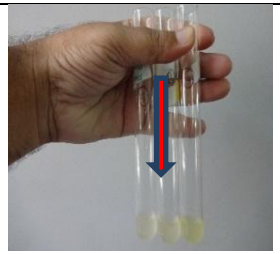
From the analysis of raw and pasteurized milk samples it was clear that the results were accordance with the conventional IS methods. Out of 10 samples of raw milk tested, all of the samples were found positive for the presence of coliforms tested by developed X-gal strip method and by ONPG assay method. Out of the 10 samples, R-9 and R-10 did not show presence of *E. coli* by MUG assay while rest all samples were identified positive for *E. coli.* The results obtained from the analysis of raw milk are given in Fig. 2 indicating that they are in accordance with the results obtained from developed methods.

Ten pasteurized milk samples were tested for the presence of coliforms by developed methods and all the samples except P-5 showed negative results for coliform by X-gal strip and ONPG assay method. From the sample tested for *E. coli*, out of the 10 samples P-5 and P-7 showed presence of *E. coli* by MUG assay while rest all samples were tested negative for *E. coli.* Simultaneously all the samples of pasteurized milks analyzed for coliforms by using Violet red bile agar and for *E. coli* by Eosin methylene blue lactose agar. The results obtained are given in Fig. 3 indicating that they are in accordance with the results obtained from developed methods.

Lawaniya [18] developed an enzyme(s) assay for detection of *E. coli* in milk by targeting enzyme-substrate reactions for specific marker enzymes of targeted bacteria releasing free chromogen which was visualized by color change in novel selective medium. The developed assay was further,

Raw milk sample Code	Results by enzyme substrate assay based strip	Results by enzyme substrate ONPG assay	Quantification of coliforms in the sample	Results by enzyme substrate MUG assay	Quantification of *E. coli* in the sample	Coliform count by IS, 5401 Part-1 (2012)	*E. coli* count by IS-5887 (Part 1) (1976)
R-1			>100000 cells/1 ml		>1000 cells/1 ml	115000 cfu/ml	1100 cfu/ml
R-2			>10000 cells/1 ml		>100 cells/1 ml	11850 cfu/ml	95 cfu/ml
R-3			>1000 cells/1 ml		>10 cells/1 ml	1250 cfu/ml	Absent in 1 ml
Raw milk sample Code	**Results by enzyme substrate assay based strip**	**Results by enzyme substrate ONPG assay**	**Quantification of coliforms in the sample**	**Results by enzyme substrate MUG assay**	**Quantification of *E. coli* in the sample**	**Coliform count by IS, 5401 Part-1 (2012)**	***E. coli* count by IS-5887 (Part 1) (1976)**

R-4			>100000 cells/1 ml		>100 cells/1 ml	105000 cfu/ml	150 cfu/ml
R-5			>10000 cells/1 ml		>10 cells/1 ml	10800 cfu/ml	20 cfu/ml
R-6			>1000 cells/1 ml		>100 cells/1 ml	1350 cfu/ml	140 cfu/ml
Raw milk sample Code	**Results by enzyme substrate assay based strip**	**Results by enzyme substrate ONPG assay**	**Quantification of coliforms in the sample**	**Results by enzyme substrate MUG assay**	**Quantification of *E. coli* in the sample**	**Coliform count by IS, 5401 Part-1 (2012)**	***E. coli* count by IS-5887 (Part 1) (1976)**

R-7			100000 cells/1 ml		>1000 cells/1 ml	110000 cfu/ml	1180 cfu/ml
R-8			>1000 cells/1 ml		>10 cells/1 ml	1240 cfu/ml	Absent in 1 ml
R-9			>10 cells/1 ml		Absent in 1 ml	24 cfu/ml	Absent in 1 ml
Raw milk sample Code	**Results by enzyme substrate assay based strip**	**Results by enzyme substrate ONPG assay**	**Quantification of coliforms in the sample**	**Results by enzyme substrate MUG assay**	**Quantification of *E. coli* in the sample**	**Coliform count by IS, 5401 Part-1 (2012)**	***E. coli* count by IS-5887 (Part 1) (1976)**

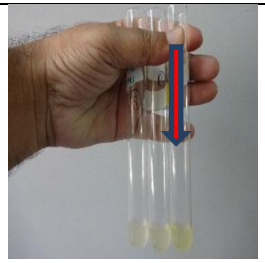
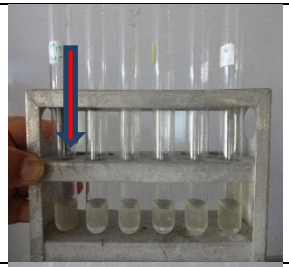
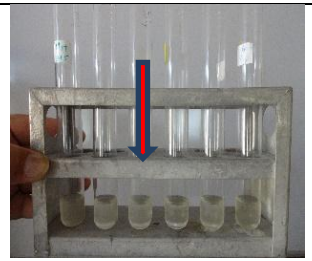
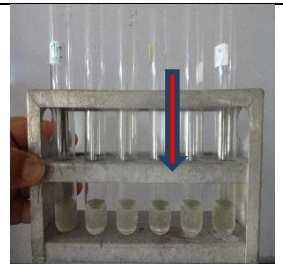
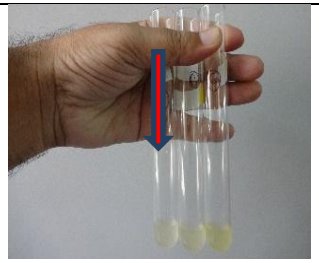
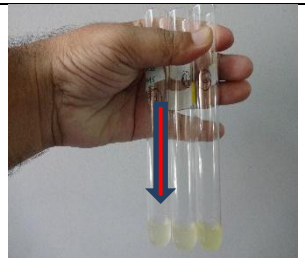
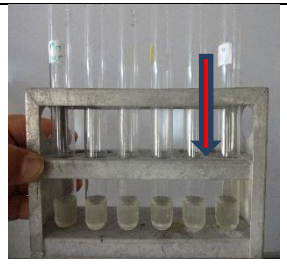
R-10			>10 cells/1 ml		Absent in 1 ml	18 cells/1 ml	Absent in 1 ml

Fig. 2. Results of analysis of raw milk by developed detection methods for coliforms and *E. coli*

Pasteurized milk sample Code	Results by enzyme substrate assay based strip	Results by enzyme substrate ONPG assay	Quantification of coliforms in the sample	Results by enzyme substrate MUG assay	Quantification of *E. coli* in the sample	Coliform count by IS, 5401 Part-1 (2012)	*E. coli* count by IS-5887 (Part 1) (1976)
P-1			Absent in 1 ml		Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
P-2			Absent in 1 ml		Absent in 1 ml	Absent in 1 ml	Absent in 1 ml

P-3			Absent in 1 ml		Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
Pasteurized milk sample Code	**Results by enzyme substrate assay based strip**	**Results by enzyme substrate ONPG assay**	**Quantification of coliforms in the sample**	**Results by enzyme substrate MUG assay**	**Quantification of *E. coli* in the sample**	**Coliform count by IS, 5401 Part-1 (2012)**	***E. coli* count by IS-5887 (Part 1) (1976)**
P-4			Absent in 1 ml		Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
P-5			>10 cells/1 ml		>10 cells/1 ml	22 cfu/1 ml	8 cfu/1 ml

P-6			Absent in 1 ml		Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
Pasteurized milk sample Code	**Results by enzyme substrate assay based strip**	**Results by enzyme substrate ONPG assay**	**Quantification of coliforms in the sample**	**Results by enzyme substrate MUG assay**	**Quantification of *E. coli* in the sample**	**Coliform count by IS, 5401 Part-1 (2012)**	***E. coli* count by IS-5887 (Part 1) (1976)**
P-7			Absent in 1 ml		>10 cells/1 ml	Absent in 1 ml	15 cells/1 ml
P-8			Absent in 1 ml		Absent in 1 ml	Absent in 1 ml	Absent in 1 ml

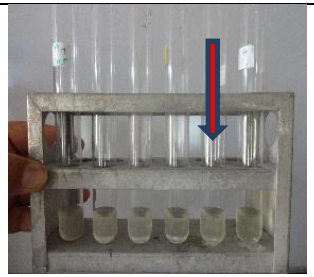
P-9			Absent in 1 ml		Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
Pasteurized milk sample Code	**Results by enzyme substrate assay based strip**	**Results by enzyme substrate ONPG assay**	**Quantification of coliforms in the sample**	**Results by enzyme substrate MUG assay**	**Quantification of *E. coli* in the sample**	**Coliform count by IS, 5401 Part-1 (2012)**	***E. coli* count by IS-5887 (Part 1) (1976)**
P-10			Absent in 1 ml		Absent in 1 ml	Absent in 1 ml	Absent in 1 ml

Fig. 3. Results of analysis of Pasteurized milk by developed detection methods for coliforms and *E. coli*

Table 2. Validation of developed methods by analysis of raw milk samples

Sample code of raw milk	Detection coliform by X-gal assay	Detection coliform by ONPG assay	Detection of *E. coli* Fluorescence by MUG assay
R-1	+ve	+ve	+ve
R-2	+ve	+ve	+ve
R-3	+ve	+ve	+ve
R-4	+ve	+ve	+ve
R-5	+ve	+ve	+ve
R-6	+ve	+ve	+ve
R-7	+ve	+ve	+ve
R-8	+ve	+ve	+ve
R-9	+ve	+ve	-ve
R-10	+ve	+ve	-ve

Table 3. Validation of developed methods by analysis of pasteurized milk samples

Sample code of raw milk	Detection coliform by X-gal assay	Detection coliform by ONPG assay	Detection *E. coli* of Fluorescence by MUG assay
P-1	-ve	-ve	-ve
P-2	-ve	-ve	-ve
P-3	-ve	-ve	-ve
P-4	-ve	-ve	-ve
P-5	+ve	+ve	+ve
P-6	-ve	-ve	-ve
P-7	-ve	-ve	+ve
P-8	-ve	-ve	-ve
P-9	-ve	-ve	-ve
P-10	-ve	-ve	-ve

validated using spiked raw milk as well as IS-5887 (Part-I) 1976 method adopted for testing of *E. coli* in foods including dairy products. The assay was evaluated under field conditions with raw milk, pasteurized milk and ice-cream samples procured from different sources. Seven out of fifty five raw milk samples showed green color in developed assay after 12.25 h of incubation in *E. coli* selective medium (EC-SM), which indicates presence of *E. coli*. None of pasteurized milk and ice-cream samples showed the presence of *E. coli* even after incubation for 12.25 h.

Foschino et al. [19] developed Colifast® Milk, a fluorescence based rapid screening test for the detection of total coliforms in milk. In this, 800 samples of homogenized pasteurized milk, with different fat content (1.5 and 3.5%) and contaminated with various concentrations of coliforms (from 0.03 to > 10000 cfu/ml), were analyzed by Colifast® Milk method and compared with the standard method. They also checked the effect of the incubation temperature (30 and 39 °C) on the results. They reported that incubation at 30 °C improved the recovery of coliforms by Colifast® Milk *i.e.* 72% (r2 = 0.760; P = 0.89) compared when the incubation temperature was 39 °C *i.e.* 56% (r2 = 0.735; P = 0.87). Finally they came up with conclusion that the sensitivity showed by the fluorimeteric method did not sufficient for the detection of coliforms in pasteurized milk and need further testing to make final conclusion.

To reduce the analysis time needed for the enumeration of *Escherichia coli*, a rapid fluorogenic method (MUG) was compared with International Standards Organization (ISO) protocol. Here, 500 food samples which were analysed for *E. coli* enumeration. This study came with results that fluorogenic method is more reliable and shorter to perform than the standard ISO method [20].

In a similar method to the present investigation, Gray et al. [21] added and mixed equal proportion of modified selective broth with 4-methylumbelliferyl β-D-glucuronide and food sample in a sterile test tube and incubated at

37^{o}C. Time taken to give positive fluorescence reaction was monitored at regular 30 min intervals and results were compared with actual *E. coli* numbers from tested samples. The correlation between *E. coli* counts by the conventional plating method and positive reaction (fluorescence production) times in test tubes was highly significant (r = 0.95). In the case of low *E. coli* numbers *i.e.* 2 log10 cfu/ml detection time was 10 h while for highly contaminated samples *i.e.* 8 log10 cfu/ml detection time was just 4 h incubation. Similarly Kadyan, 2015 [22] developed a two-stage assay for detection of *E. coli* and coliforms. He developed *E. coli* selective medium in lyophilized form which was able to detect 0.35± 0.10 log cfu/ml and 0.57±0.15 log cfu/ml population within 14.30±0.45 h and 12.15±0.30 h of incubation at 37˚C respectively. With this developed assay he evaluated 139 milk samples and random tests were carried out with IS-5887 (Part 1): 1976 Part-I, IS-5401 (Part-2): 2002 protocol. Fifty one out of 96 raw milk and seven out of 43 pasteurized milk samples showed the presence of *E. coli* in first stage and subsequently confirmed in stage using employing markers enzymes. Results obtained in this study was comparable with results of IS methods.

Ekholm and Hirshfield [23] compared three methods namely AOAC methods using lauryl tryptose broth (LST) medium, LST-4-methylumbelliferyl-β-D-glucuronide (MUG) medium, and a proposed method using regular LST in combination with *E. coli* (EC)-MUG medium to enumerate *Escherichia coli* in food. They tested 170 cheeses, 40 frozen processed seafood samples, 210 tree nuts, and 40 other samples and found that a presumptive positive in the LST-MUG medium was highly correlative with the biochemical tests that confirmed a sample contains *E. coli*. In case of spiked samples with *E. coli*, the results from all these 3 methods were identical, and consistent in enumerating *E. coli*.

As per FSSAI, minimum microbiological limit (m) of coliforms for pasteurized milk is stated as <10 cells /ml while limit of *E. coli* is not mentioned. There is not any standards prescribed for acceptance of raw milk for both *i.e.* coliforms and *E. coli*. In present study conducted, all the pasteurized milk samples have fulfilled prescribed microbiological limit specified by the FSSAI [24]. Nine samples out of 10 were having absent of coliforms per ml of samples. Only P-5 samples showed presence of less than 10 coliforms cells per ml but it is within standard range. Thus all the pasteurized milk samples have full filled the basic legal conditions. These results are also confirming by the results obtained by IS methods. In case of raw milk also results obtained by X-Gal and Mug method using interpretation chart as comparable with exact count obtained by IS methods for both coliforms and *E. coli* respectively (Figs. 3 and 4).

Pasteurized milks are highly perishable commodity having shelf life less than 48 hrs. Halt dispatches to get results of coliforms and *E. coli* count is not practically and economically possible. When compared the present work with the time required by conventional IS methods, it observed that these developed methods are rapid and could gave results in less time *i.e.* nearly in 12 hrs [2]. It includes inoculation of sample with formulated broth, incubation up to 10 hrs, processing of sample and till appearance of visible result. Conventional IS methods requires at least 24 hrs to get the results which is nearly closer to 50 % of pasteurized milk's shelf life. In such situation, these proposed methods could be better alternative to industry people to release a lot of products in approximately 50 % less time in comparison of the results obtained by conventional methods. These methods also need lesser capital investment and are affordable.

4. CONCLUSION

From the analysis of raw and pasteurized milk samples it was clear that the results were in accordance with the conventional IS methods. Out of 10 samples of raw milk tested, all of the samples were positive for the presence of coliforms by X-gal and ONPG assay method. Eight samples showed presence of *E. coli* by MUG assay while two samples were identified negative. Similarly for all ten pasteurized milk samples results obtained by developed methods were matching with IS methods, indicating its compatibility with conventional methods. It can be concluded that methods developed for detection of coliforms and *E. coli* can be of immense importance for dairy industry for rapid detection within 10 h time, which otherwise; by conventional ways require 4-5 days. In view of current legislation and changing scenario at global level, where food standards are harmonizing and food business operators are looking forward for a novel alternative to clear a batch of production, these developed methods would be very useful.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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