

# Original Research Article

## **Comparison of X-gal, ONPG and MUG Assay developed methods with IS methods by Analyzing Milk Samples**

### **ABSTRACT**

#### **Background:**

FSSAI 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 assess their suitability in actual analysis by time saving, reliability and reproducibility.

#### **Methods:**

Formulated selective broth was used to inoculate 10 samples raw and pasteurized milks. After incubation of 10 hrs crude enzymes were extracted to detect presence of coliforms. Similarly 0.1 ml MUG solution was added in Formulated selective broth for the detection of *E.coli*. Enzyme extract procedure was not required for detection of *E.coli*. Then after presence of coliforms were detected by the X-Gal impregnated strips and quantification was done by chart developed using ONPG assay and optical density measured by micro titre plate. For *E.coli* detection blue fluorescence generated was measured under UV light at 350 nm and quantification was done by measuring RLU generated by Fluorescence spectrophotometer. Same sample was also analyzed by standard IS 5401: part-1 (2012) and IS 5887 (Part - I) 1976) procedure for enumeration of coliforms and *E.coli* respectively.

#### **Results:**

In the present study it was clear that the results of analysis of raw and pasteurized milk samples by the developed methods were in accordance with the conventional IS methods. Out of 10 samples of raw milk tested, all of the samples showed positive results for coliforms by X-gal and ONPG assay method. While 8 samples showed positive results for *E.coli* by MUG assay. Similarly results of all ten pasteurized milk samples tested for the presence of

coliforms by developed methods with IS methods were alike, indicating suitability with conventional methods.

**Key words:** Coliforms, selective broth, *E.coli*, validation, IS methods, ONPG assay, X-Gal Assay, MUG Assay

## **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 mainly for coliforms exist. It is classified into (1) cultural or traditional, (2) molecular and (3) enzymatic methods (Rompre *et al.* 2002). Among these methods, molecular and enzymatic methods are more precise and rarely need any confirmation nevertheless cultural methods purely depends on confirmation of *E.coli* and Coliforms. Most of the approved methods either by Indian Standards or ISO are cultural based and need confirmation step. Present-day demand of the sector is to have such a selective broth which would maximum genera of coliforms and *E.coli* while inhibition of non-coliforms i.e. non lactose fermenter. Such a broth can reduce the time and improve precision of the test (Gawai, 2020).

Among the traditional methods, non-selective Violet Red Bile Agar is used for enumeration of coliforms in food and dairy products which confirms the recommendation given by American Public Health Association (APHA, 1992). 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 (APHA, 2001). If typical coliform colonies appear, it requires further to confirmation to designate it as coliforms (APHA, 2001; Begley *et al.* 2005).

Indian dairy industry is mostly following IS 5401 Part 1/ISO: 4832 and IS 5887: Part 1 or ISO: 16649-2IS 5401: part-1 (2012) standard procedure for enumeration of coliforms and *E.coli* respectively. Main concern is with the interpretation of 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 like pasteurized butter, milk powders, ice cream cheese, fermented milk and traditional Indian dairy products indicating strong concern towards their presence. 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 (Teramura *et al.* 2019). Alternative methods based on nucleic acid, fluorescent antibody or immunology based techniques needed 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 (Tavakoli *et al.*, 2008). Due to this, these 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 classical methods. Enzymatic reactions can be group-, genus- or species-specific, depending on the enzyme 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 (Kanangire, 2013). These methods concurrently detect the total coliforms and *E.coli* which increasingly make possible the quantification of *E.coli*, rather than simply 'thermotolerant coliforms' (Siegrist, 2020). In the past decade, diverse methods using chromogenic and/or fluorogenic substrates to reveal  $\beta$ -d-glucuronidase and  $\beta$ -d-galactosidase activity on culture media have been reported to determine whether a strain belongs to the coliform group and/or *E. coli* (Siegrist, 2020). Various commercial media like Chromo-agars (Alonso *et al.*, 1999) and Chromocults (Geissler *et al.*, 2000) which contents chromogenic substrates have been available for the identification and enumeration of *E. coli* and Total Coliforms (TC). Major advantage of using such media is that they are able to give results in less than 24 hrs.

Overall, traditional methods used in detection and enumeration of *E.coli* and coliform bacteria are time, space consuming, require confirmation while alternative methods incorporation of chromogens and fluorogens are need of the present time to fasten the process of overall evaluation of results and confirmation. These keys points are tired to address in this research work.

## **Material 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 from raw and pasteurized milk samples.

Formulated selective coliforms broth was developed with addition of Sodium lauryl sulphate salt @ 0.2g, Gentamicin sulphate + Amoxicillin (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 (Gawai, 2020). This formulated selective broth was used to inoculate spike coliforms and later its presence was detected using developed enzyme substrate assay. The cocktail of coliform culture was prepared by mixing equal contents from three positive tubes of MacConkey's broth. This culture was propagated in nutrient broth medium and incubated at 37°C for 24 h and then stored at  $5 \pm 2^\circ\text{C}$ . Sub culturing was done at an interval of 7 days during the course of the study. (METHODOLOGY IS NOT CLEAR. PLEASE USE SCIENTIFIC TERMS)

#### **Preparation of X-gal substrate strip**

Preparation of X-gal substrate strip started with sample processing. Sample whether milk of spiked broth processing protocol was standardized after slight modification in the method described by Prasad *et al.* (2013) and Makwana (2017). B-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.

#### **Protocol for crude enzyme extraction**

Milk samples 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.

#### **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  $\approx 8 \text{ cm} \times .8 \text{ cm}$ . On the strip, X-gal (5-bromo-4-chloro-3-indolyl  $\beta$ -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.

#### **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 the colour of a strip from white to blue.

Similarly, 500 ul of ONPG (O-nitrophenyl-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 (Gawai, 2020). 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.

#### **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 (Gawai, 2020). This chart was used to make decision about possible *E.coli* population could present in the tested sample.

#### **Comparison of developed X-gal/ONPG and Mug Assay test with IS methods**

Twenty samples of raw and pasteurized milk were procured from cattle yard, local market of Anand, Gujarat (Table 1) were screened for the presence of Coliforms, *E. coli* using developed methods as well as IS 5887 ( Part - I) 1976 method.

Ten samples of each of raw and pasteurized milk were collected in sterile 100.0 mL sample bottles, packets and cups from local cattle yard, dairies of local market and retail milk selling shops. Samples were kept under refrigerated conditions until processed and were analyzed on

the day of collection. All the samples were analyzed by developed detection method and simultaneously analyzed by enumerating for Coliform count by IS 5401 (Part 1): 2012.

#### **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 4-methylumbelliferyl- $\beta$ -D-glucuronidetrihydrate 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.

#### **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 1ml of the sample into a duplicate petri dish. Poured 15ml 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. Once the overlay has solidified, incubated the plates at 37°C for 24 h  $\pm$  2 h. After the specified period of incubation, petri dishes with 10 or more colonies and fewer than 150 colonies were counted. Counted the purplish red colonies with a diameter of at least 0.5 mm or greater (sometimes surrounded by a reddish zone of precipitated bile), which are considered as typical colonies of coliforms and do not require further confirmation. Atypical colonies (*e.g.* of smaller size), were confirmed further. For confirmation of such atypical colonies, five colonies were inoculated into five tubes of brilliant green lactose bile broth (BGBLB). Incubated these tubes at 37°C for 24 h  $\pm$  2 h and formation of gas in the Durham's tube were considered as confirmed for the presence of coliforms.

#### **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*.

#### **Results and Discussions**

As mentioned samples from raw and pasteurized milks were prepared and analyzed by assay methods developed and by standard IS methods.

### **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 Plate no. 1 and details of analysis of pasteurized milk samples are presented in Table. 3 and in Plate no. 2.

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*.

Similarly all the samples of raw milk analyzed for coliforms by using Violet red bile agar. Simultaneously they were analyzed for *E.coli* by Eosin methylene blue lactose agar. The results obtained are given in plate no. 1 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 Plate no. 2 indicating that they are in accordance with the results obtained from developed methods.

Lawaniya (2014) 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, 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.*, 2003 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 as the correspondence between values obtained with the two methods reached 72% ( $r^2 = 0.760$ ;  $P = 0.89$ ) if compared with 56% ( $r^2 = 0.735$ ;  $P = 0.87$ ) when the incubation temperature was 39 °C. Finally they came up with conclusion that the sensitivity showed by the fluorimetric method did not sufficient for the detection of coliforms in pasteurized milk and need further testing to make final conclusion.

A fluorogenic assay procedure developed with 4-methylumbelliferyl- $\beta$ -D-glucuronide incorporated into lauryl sulfate broth to detect and confirm the presence of *Escherichia coli* in foods by Robinson, (1984). This assay was tested concurrently with standard methods for detection of *E. coli* in 270 samples of raw ingredients and powdered food products. Total correlation between the two methods was 94.8%; there was a false-positive rate of 4.8% and no false-negatives. Researcher mentioned that the 4-methylumbelliferyl- $\beta$ -D-glucuronide assay was rapid, cheaper, accurate and easy to perform (Robinson, 1984).

Moberg *et al.*, 1988 conducted a collaborative study to compare a proposed LST-MUG method with the AOAC official method for *Escherichia coli* detection. Incorporation of the MUG substrate into lauryl tryptose broth (LST) enables a rapid quantitative method for screening *E. coli*. In this study, 5 food samples, 2 frozen (entree sauce/gravy and dairy topping) and 3 chilled (hamburger, pork sausage, and cheese), were tested for *E. coli* detection by 17 collaborating laboratories. Results indicated that the LST-MUG method is equal to or better than the current AOAC method for detecting *E. coli*. In another collaborative study, Twenty-four laboratories participated to validate a hydrophobic grid membrane filter (HGMF) method incorporated with 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) for enumeration of total coliform and *Escherichia coli* bacteria in foods by comparing its performance against the AOAC 3-tube MPN method (46.013-46.016). Raw milk, raw ground poultry, whole egg powder, cheese powder, and ground black pepper were included in the study. The total coliform methods did not differ significantly, except that the

3-tube method detected a significantly higher level of total coliforms than HGMF method in the ground black pepper case. Conversely, the HGMF/MUG *E. coli* method detected significantly higher numbers of *E. coli* present in the egg powder, cheese powder, and ground black pepper samples, while not differing significantly from the 3-tube method for the raw milk and raw ground poultry samples. The overall confirmation rate of MUG-positive colonies isolated using the HGMF method was 99.5% (Entis, 1989).

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 (Dogan *et al.*, 2002).

In a similar method to the present investigation, Gray *et al* (2002) added and mixed equal proportion of modified selective broth with 4-methylumbelliferyl  $\beta$ -D-glucuronide and food sample in a sterile test tube and incubated at 37 °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 log<sub>10</sub> cfu/ml detection time was 10 h while for highly contaminated samples i.e. 8 log<sub>10</sub> cfu/ml detection time was just 4 h incubation. Similarly Kadyan, 2015 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  $0.35 \pm 0.10$  log cfu/ml and  $0.57 \pm 0.15$  log cfu/ml population within  $14.30 \pm 0.45$  h and  $12.15 \pm 0.30$  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 (2001) compared three methods namely AOAC methods using lauryl tryptose broth (LST) medium, LST-4-methylumbelliferyl- $\beta$ -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 contain *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*.

## **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 results of all ten pasteurized milk samples tested for the presence of coliforms by developed methods with IS methods were matching, indicating 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 ISO protocol require 4-5 days. In view of current legislation and changing scenario at global level, where food standards are harmonizing globally and food business operators are looking forward for a novel alternative to clear a batch of production, these developed methods would be useful.

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## TABLES

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

Sr. No	Sample Source/Type	Number of sample	Analytical methods
1.	Raw milk: cattle yard, Local vendors and dairies of Anand, Gujarat	10	Developed enzyme assay / <i>E.coli</i> detection IS 5887- (Part-I) 1976 method and
2.	Pasteurized milk: Purchased from local market	10	Coliform detection by IS, 5401 Part-1 (2012) method

**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 <i>E.coli</i> 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 <i>E.coli</i> 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

Add 1 ml milk sample in 9 ml of formulated selective broth

Incubate at 37 °C up to 10 h

After incubation mixed the contents thoroughly

Harvest the cells by centrifuging at 5000 rpm for 10 min at 4°C in refrigerated centrifuge

Discard the supernatant to remove extracellular enzymes, loosen the cell pellet properly by tapping in between palms

Add 5 ml of 0.05 M sodium phosphate buffer (pH 6.8) and mix properly on vortex for 2-3 min

Sonicate the sample for 15 minutes (pulse 10 seconds off / 15 seconds on and 80 % amplitude) in ice bath using ultra sonicator

After sonication, mix sample well and centrifuge at 5000 rpm at 4°C for 10 min










Collect the supernatant which contains the crude enzymes


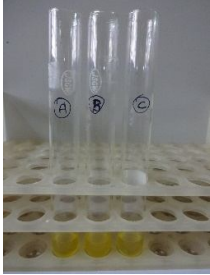







Check the presence of coliforms and *E.coli* immediately by X-gal strip or ONPG assay


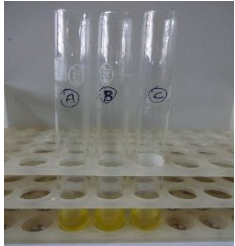







Do not store the crude extract for more than 2 h and use immediately




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

**Plate. 1 Results of analysis of raw milk by developed detection methods for coliforms and *E.coli***










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 <i>E. coli</i> in the sample	Coliform count by IS, 5401 Part-1 (2012)	<i>E. coli</i> 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	Results by	Results by enzyme	Quantification	Results by	Quantification	Coliform	<i>E. coli</i> count










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





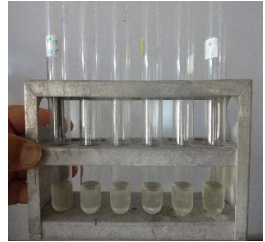




milk sample Code	enzyme substrate assay based strip	substrate ONPG assay	of coliforms in the sample	enzyme substrate MUG assay	of <i>E. coli</i> in the sample	count by IS, 5401 Part-1 (2012)	by IS-5887 (Part 1) (1976)
R-7			100000 cells/1 ml		>1000 cells/1 ml	110000 cfu/ml	1180 cfu/ml
R-8			>1000 cells/1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




sample Code	substrate assay based strip	assay	the sample	substrate MUG assay	sample	5401 Part-1 (2012)	(Part 1) (1976)
R-10			>10 cells/1 ml		Absent in 1 ml	18 cells/1 ml	Absent in 1 ml

**Plate. 2 Results of analysis of Pasteurized 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 <i>E. coli</i> in the sample	Coliform count by IS, 5401 Part-1 (2012)	<i>E. coli</i> 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 <i>E. coli</i> in the sample	Coliform count by IS, 5401 Part-1 (2012)	<i>E. coli</i> 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	Results by enzyme	Results by enzyme substrate ONPG	Quantification of coliforms in	Results by enzyme	Quantification of <i>E. coli</i> in the	Coliform count by IS,	<i>E. coli</i> count by IS-5887 (Part

sample Code	substrate assay based strip	assay	the sample	substrate MUG assay	sample	5401 Part-1 (2012)	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	Results by enzyme substrate assay	Results by enzyme substrate ONPG	Quantification of coliforms in	Results by enzyme substrate	Quantification of <i>E. coli</i> in the	Coliform count by IS, 5401 Part-1	<i>E. coli</i> count by IS-5887 (Part

Code	based strip	assay	the sample	MUG assay	sample	(2012)	1) (1976)
P-10			Absent in 1 ml		Absent in 1 ml	Absent in 1 ml	Absent in 1 ml

(IS THIS A PLATE, TABLE OR FIGURE?)

