MColiPAT kit for early detection of coliforms in water

Ashish Tambi, Urmila Brighu and A. B. Gupta

ABSTRACT

Determining the microbial quality of drinking water by assessing the presence/absence (P/A) or enumeration of indicator bacteria continues to be widely practiced worldwide. However, rapid tests are required for microbiological water quality assessment so that the information is available in the shortest possible time for initiating a timely intervention. Traditional methods for the enumeration of indicator bacteria are not only expensive but also need trained personnel. We have developed a low-cost kit, MColiPAT, and have validated its application for detection of coliforms in drinking water using the IDEXX Colilert-18 Quanti tray method. MColiPAT kit medium was able to detect coliforms down to a level of 3.1 MPN/100 ml within 10.5 hours. The sensitivity and specificity of the kit were 95.45% and 100% respectively. MColiPAT is found to be reliable and accurate for the detection of coliforms in drinking water.

Key words | coliforms, Colilert, indicator, most probable number, rapid detection, water

Ashish Tambi (corresponding author)
Urmila Brighu
A. B. Gupta
Department of Civil Engineering,
Malaviya National Institute of Technology,
Jaipur, 302017 Rajasthan,
India
E-mail: *ashishtambi006@gmail.com*

INTRODUCTION

Microbiological quality monitoring of source and treated drinking water is essential for the timely control of water-borne diseases. The monitoring of water quality should be rapid and reliable to protect consumers against the spread of water-borne diseases (Fiksdal & Tryland 2008). Ideally, the occurrence and number of all pathogens in drinking water should be monitored, however, it is not feasible to assess the levels of pathogens in drinking water in routine analysis. The presence of pathogens is relatively low as compared to that of other microorganisms and these are present only under specific environmental conditions (Wildeboer *et al.* 2010). Indicator organisms have been widely used to assess the microbiological quality of drinking water rapidly (Landre *et al.* 1998; Edberg *et al.* 2000). The indicator organisms most commonly used are coliform bacteria, faecal coliform bacteria, and enterococci. Coliforms include members of the family *Enterobacteriaceae,* e.g. *Escherichia* spp., *Enterobacter* spp., *Klebsiella* spp., and *Citrobacter* spp. Members of the coliform group are aerobic and facultative anaerobic, Gram-negative, non-spore forming rod-shaped bacteria that ferment lactose with gas production within 48 hours at 35 °C (APHA 1998). Coliforms are present in large numbers in faeces of humans and other warm-blooded animals and therefore accepted as an indicator of faecal contamination.

The standard tests employed for the assessment of the coliform group include: multiple-tube fermentation technique (MTF), membrane filter (MF) technique or the enzymatic substrate coliform test (Kodaka *et al.* 2008), which are expensive and require trained laboratory personnel. Conventional methods used for the assessment of the bacteriological quality of drinking water require 18–24 hours to detect the contamination. Confirmation of these results requires another 1–2 days or longer (Ashbolt *et al.* 2001). Further, lack of access to laboratories is an obstacle to the provision of microbiologically safe drinking water to many communities and people worldwide (Bain *et al.* 2015).

Rapid methods based on detection and quantification of waterborne pathogenic bacteria in water have been developed such as real time or quantitative polymerase chain

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reaction (qPCR), DNA microarray and fluorescence in-situ hybridization (FISH) (Deshmukh *et al.* 2016). However, there are some limitations of these methods such as low sensitivity, the need for purified DNA, cross-reactivity, their sensitivity to PCR inhibitors and high cost. There is a common need for rapid analysis, whether testing pathogens directly or testing for indicator organisms such as coliforms for the routine examination of water quality. Apart from being significantly faster, rapid methods should have higher sensitivity and specificity than those of the standard techniques used routinely.

There are presence/absence field test kits (FTKs) such as Colilert-18, Aquagenx and H_2S kits developed for on-site testing of the microbial quality of drinking water. However, like the standard methods mentioned earlier, some of these kits are also relatively expensive and require trained personnel. Although the H_2S field test kit is easy to use and relatively cheap, it often gives false positive results and has low sensitivity and specificity (Mosley & Sharp 2005).

To address these limitations of the field tests, Tambi *et al.* (2016) developed the ColiPAT kit for a presence/absence test, which was able to detect coliforms up to 2 MPN/100 ml within 18 hours of the incubation period.

This paper presents a modified field portable kit (called ^{M}ColiPAT) which is specifically designed to detect coliforms in water with a significantly reduced incubation time period. The kit was prepared by modifying the composition of the ColiPAT kit (a low-cost medium for detection of coliforms in water) developed by Tambi *et al.* (2016). The ColiPAT kit medium detects coliforms based on the principle that coliforms ferment lactose and in turn will produce acid end products that can be detected by using a pH indicator. The pH indicator used in the ColiPAT kit is bromocresol purple which changes its color from purple to yellow at pH 5.2 or less. The presence of coliforms using the ColiPAT kit can be detected when the pH of the medium falls below 5.2 after production of acid end products by coliforms that require almost 18 hours of incubation time, as reported by Tambi *et al.* (2016). However, the amount of acid production that will change the pH is a function of the type of coliform present, i.e one type carries out mixed acid fermentation and the other type carries out 2,3-butanediol fermentation (Guentzel 1996). The source of coliforms in drinking water is usually faeces which may have mixed types of coliforms. Therefore, it is necessary to design a medium for field test kits in such a manner that it is able to detect all the members of the coliform group. The concept behind developing the modified ColiPAT kit (^{M}ColiPAT) is to use an indicator that can detect a pH change as soon as it decreases below 7.0 so that results may be available in the shortest time possible. The pH indicator used in the ^{M}ColiPAT kit medium is phenol red which changes color from red to yellow at pH<6.8. It enables the detection of a pH drop at an early stage which occurs due to a biochemical reaction as the bacteria (coliforms) produce acid. Phenol red indicator is used widely in tissue culture media (Berthois *et al.* 1986), microbiological culture media such as Triple sugar iron agar and Mannitol salt agar, and in fermentation broths for differentiation of members of *Enterobacteriaceae* (Leboffe & Pierce 2011). This pH indicator itself is not reduced by bacteria and it does not have an inhibitory effect on bacterial growth (Chesney 1922).

MATERIALS AND METHODS

^{M}ColiPAT kit

The ^{M}ColiPAT kit consists of 5 ml of liquid medium in a screw-capped sterile glass bottle. The medium was specifically designed for rapid growth of coliform bacteria. The ^{M}ColiPAT medium was prepared by replacement of the pH indicator (bromocresol purple) used in the ColiPAT kit with phenol red. It contains the following ingredients (in g/l): 40 g proteose peptone A (serves as a protein, vitamin and nitrogen source), 20 g lactose (carbon source), 10 g NaCl (for maintaining osmotic balance), 0.02 g phenol red (as pH indicator) and 0.002 g crystal violet (for inhibiting the growth of Gram-positive bacteria), and a pH of 7.4 ± 0.2. Analytical grade reagents of recognized companies were used in the preparation of the medium for the ^{M}ColiPAT kit. Proteose peptone A and lactose were obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India, whereas sodium chloride, phenol red and crystal violet were obtained from Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India. The medium was sterilized by autoclaving at 121 °C for 15 minutes and 5 ml of autoclaved medium was added to screw-capped sterilized glass bottles. The presence of coliform bacteria in

the water sample using the MColiPAT kit can be indicated by a change in colour of the medium from red to yellow.

Sample preparation for analysis of coliforms

Controlled ten-fold serial dilutions of water contaminated with faecal bacteria were prepared from secondary treated sewage obtained from the sewage treatment plant (STP) based on moving bed biofilm reactor (MBBR) technology at MNIT Jaipur. All the samples were prepared in sterile deionized water. Sterile deionized water was used as a negative control for all the experiments. All the experiments were carried out in duplicate (APHA 1998).

Evaluation procedure for analyzing the coliform detection limit of MColiPAT

The Colilert 18/Quanti-Tray 2000 MPN (most probable number) test (IDEXX Laboratories, Inc., Westbrook, ME, USA), was used as a reference method to assess the coliform detection limit of the MColiPAT kit. This is a standard method prescribed by USEPA for the quantitative analysis of coliforms (USEPA 2003). A total of 50 samples along with 50 replicates were prepared in different sets of experiments using diluted sewage and were analyzed using MColiPAT medium and the Colilert-18 test. Secondary treated sewage was diluted with sterile, deionized water to result in a final total-coliform count in the range of 1–2,400 total coliforms per 100 ml so that meaningful comparisons could be made. Sterile deionized water was used as a negative control for all the experiments. Serially diluted samples (100 ml) were added into Quanti trays with Colilert 18 medium, and 20 ml of the same sample was poured into MColiPAT kits. All the samples were incubated at 35 °C in an incubator for 24 hours and were observed at regular time intervals for detection of a change in the colour of the medium. MColiPAT kits showing a change in colour of medium to yellow indicated the presence of coliform bacteria in the sample. The number of coliforms per 100 ml was estimated from the Colilert-18 test. The coliform detection limit of MColiPAT media obtained using diluted sewage was further verified using drinking water samples collected from various sources from rural areas of Jaipur city, Rajasthan, India.

Sensitivity and specificity analysis of the MColiPAT kit

The sensitivity (true positive rate) and specificity (true negative rate) of the MColiPAT kit were analyzed using drinking water samples that were randomly collected from various sources in the rural areas of Jaipur District (Rajasthan, India). A total of 150 drinking water samples were collected from various sources such as handpumps, boreholes, dug wells, tanker supply systems and source reservoirs (Municipal or Community service providers). The results of coliform detection using MColiPAT kits were compared with the results of the standard Colilert-18 h test. All the samples were collected in the MColiPAT kits and in sterilized sample bottles for the Colilert-18 h test. The Colilert-18 h test was conducted simultaneously on the same sample in the laboratory for quantitative determination of coliforms in the drinking water samples. All the experiments were carried out in duplicate (APHA 1998).

Identification and confirmation of coliforms in the MColiPAT kit

The presence of coliforms in water can be detected by a change in colour of the MColiPAT medium from red to yellow. The streak plate method was used to confirm that the change in colour from red to yellow (positive test) in MColiPAT kits was due to the presence of coliforms only. The Eosin Methylene Blue (EMB) agar medium was used for the streak plate method, which is a selective and differential medium for the isolation and differentiation of gram-negative enteric bacilli. *Escherichia coli* can be identified with EMB agar based on the occurrence of a metallic green sheen that appears on the surface of the bacterial colonies, whereas other coliforms appear as purple coloured, dark centred mucoid colonies. A loopful of culture was taken from the positive MColiPAT kits and then streaked over the surface of the EMB agar plate. Petri-plates were kept at 35 °C in an incubator and observed after 24 hours of incubation. Colonies of coliforms on EMB plates were identified morphologically.

Quantitative analysis of coliforms using MColiPAT medium (MColiPAT versus Colilert)

The MColiPAT medium was designed primarily for detecting the presence/absence of coliforms in water samples.

However, another experimental study was carried out to compare ^{M}ColiPAT kit medium with standard Colilert media for more direct quantitative comparison. For this purpose, 96-well semi-automated Quanti trays supplied with the Colilert media were used and 100 ml of drinking water samples were mixed with ^{M}ColiPAT medium and poured into the Quanti trays. Then 100 ml of the same sample was poured into another Quanti tray with one sachet of Colilert medium. Both the trays were sealed and incubated at 35 °C for about 18 hours. Trays with ^{M}ColiPAT medium were observed after 10 hours and those with Colilert medium were observed after 18 hours of incubation time for the color change as per the prescribed protocol. The number of coliforms was calculated by recording the number of yellow colored wells in each Quanti tray and the number of coliforms was determined using the MPN table.

Statistical analysis

The McNemar chi-square test was used for homogeneity analysis of presence/absence results of water samples using the Colilert 18 h test and the ^{M}ColiPAT kit respectively. All statistical analyses were performed with a 5% level of significance. Evaluation of the equivalence of Colilert and ^{M}ColiPAT for quantitative analysis of coliforms was done using a paired t-test for the MPN data. All the MPN data per 100 ml were converted to $\log_{10}$ MPN/100 ml before statistical analysis. Statistical calculations were carried out with Microsoft Excel 2000 statistics package.

RESULTS AND DISCUSSION

The coliform detection limit of the ^{M}ColiPAT kit, prepared by replacing the pH indicator of the ColiPAT kit medium, was tested against the Colilert-18 method. A total of 50 replicate samples prepared using diluted sewage were analyzed using the ^{M}ColiPAT kit and the Colilert-18 method and the time required to detect coliforms was recorded. Table 1 summarizes the results of coliform detection limits of the ^{M}ColiPAT kit for various concentrations of coliforms using diluted sewage. The data shown below were compiled using mean results of different sets of experiments.

Table 1 | Coliform detection limit of ^{M}ColiPAT using controlled samples

Coliforms (MPN/100 ml) (based on Colilert Quanti-tray data)	No. of samples	Average incubation time required using ^{M}ColiPAT for diluted sewage (in hours)
2,419.6	15	5.0 ± 0.16
1,732.5	9	6.0 ± 0.22
1,011.2	15	6.0 ± 0.34
629.4	8	6.25 ± 0.27
285.1	12	7.5 ± 0.35
146.7	8	7.5 ± 0.23
85.7	13	8.25 ± 0.23
25.9	6	10 ± 0.32
8.6	10	10.5 ± 0.20
3.1	14	10.5 ± 0.28
1.0	7	Not Detected (ND)

It can be observed that the ^{M}ColiPAT kit was able to detect coliforms within 5 hours of incubation for samples prepared using diluted sewage at a coliform concentration of 2,419.6 MPN/100 ml. The lowest concentration of coliforms detected by the ^{M}ColiPAT kit was found to be 3.1 MPN/100 ml within 10.5 hours of the incubation time period.

The coliform detection limit of ^{M}ColiPAT was also analyzed using drinking water samples. A total of 150 drinking water samples obtained from sources such as handpumps, boreholes, dug wells and municipal taps were analyzed in duplicate for the same. The results of the incubation time required to detect coliforms using drinking water samples are summarized in Table 2.

Table 2 | Coliform detection limit of ^{M}ColiPAT using drinking water samples

Coliform concentration (MPN/100 ml) (based on Colilert Quanti-tray data)	No. of samples	Average incubation time required by ^{M}ColiPAT using drinking water (in hours)
<1.0	40	Not Detected (ND)
1–3	05	Not Detected (ND)
3–10	17	10.5 ± 0.25
10–50	13	10 ± 0.35
50–100	8	10 ± 0.19
100–500	21	9.5 ± 0.45
500–1,000	14	9.0 ± 0.48
1,000–2,000	18	9.0 ± 0.48
2,000–2,500	14	8.5 ± 0.62

The coliform detection limit of MColiPAT using drinking water samples was also found to be 3.1 MPN/100 within 10.5 hours of the incubation time period. The kit was not able to detect samples having coliform counts of <3.0 MPN/100 ml. The non-detects of MColiPAT for MPN <3.0/100 ml may reflect a Poisson process as it is difficult to confirm that a 100 ml sample has a discrete number of coliforms (particularly less than 5), and so the non-detects may not necessarily reflect that the method is less accurate than Colilert. A slight variation was also observed in the incubation time required by MColiPAT using controlled diluted sewage and drinking water samples. This may be due to the fact that the bacterial growth state in sewage may be very different from drinking water due to nutrient enrichment in sewage as compared to drinking water. Therefore, a reduced time for detection was observed for sewage samples in contrast to drinking water samples.

The ColiPAT kit designed by Tambi *et al.* (2016) was based on the fact that coliforms ferment lactose which lowers the pH of the medium and this can be easily visualized using a pH indicator. The MColiPAT kit medium was designed based on the concept that pH indicators with a colour transition range close to neutral conditions can detect coliforms in the shortest incubation time possible. The indicator used in this kit is phenol red which changes its colour from red to yellow at a pH of 6.8 and below. The results obtained supported this selection criterion, which is an important outcome of the study as the incubation period required to detect coliforms using presence/absence field test kits (FTKs) was reduced significantly.

Comparison of MColiPAT kit and Colilert using drinking water samples

One hundred and fifty (150) drinking water samples collected from various sources from rural areas of Jaipur were analyzed using the Colilert-18 h test and the MColiPAT kit. The presence/absence results using MColiPAT and Colilert-18 were compared. For the coliform test, a total of 110 samples were positive with Colilert-18 and 105 samples with MColiPAT (Table 3). All the samples that gave a positive result using the MColiPAT kit were also confirmed by streaking culture on EMB agar plates. No false positive results were observed when cultures from MColiPAT were streaked on EMB agar plates.

Table 3 | Comparative analysis of Colilert-18 and MColiPAT kit

MPN Index (MPN/100 ml) (based on Colilert Quanti-tray data)	No. of samples	Colilert 18		MColiPAT	
		Positive	Negative	Positive	Negative
<1.0	40	0	40	0	40
<4.0	10	10	0	5	5
4–2500	100	100	0	100	0
TOTAL	150	110	40	105	45

The results obtained from the Colilert 18 h method and the MColiPAT kit were analyzed by applying the McNemar test for paired samples at a 95% confidence interval ($\alpha = 0.05$). The two-tailed p-value of the McNemar chi-square test was 0.073. These results indicated no significant difference between the two media using the McNemar chi-square test ($P > 0.05$). The only difference obtained in the two sets of results was for the very low MPN range of 1–3. The detection limit of MColiPAT as analyzed was found to be 3.1 MPN/100 ml and therefore the samples in the MPN range <1–3.0 were not detected by MColiPAT. Thus our kit, with a very low cost associated, could match a standard quantitative method for testing presence/absence of coliforms at an early stage proving the importance of this new medium that has been developed.

A comparison of the results obtained by the analysis of water samples using these two test kits is summarized in Table 3.

Sensitivity and specificity analysis of the MColiPAT kit

Sensitivity (SN) is the proportion of samples contaminated that are correctly identified by the method [true positive (tp)/(true positive (tp) + false negative (fn)]. Specificity (SP) is the proportion of uncontaminated samples that are correctly identified by the method [true negative (tn)/(true negative (tn) + false positive (fp)]. False negative results from a testing method, especially when assessing suitability of drinking water, may lead to health related concerns when contamination is not detected. False positive results can trigger unnecessary corrective actions that may lead to wastage of time and resources. The sensitivity (true positive rate) and specificity (true negative rate) of the MColiPAT kit as against the standard Colilert-18 h test was found to be 95.45% and 100% respectively (Table 4).

Table 4 | Sensitivity and specificity analysis of ^{M}ColiPAT kit

True Positive Rate (TPR), Sensitivity	95.45%
True Negative Rate (TNR), Specificity	100%
Positive Predictive Value (PPV), Precision	100%
Negative Predictive Value (NPV)	88.89%
False Negative Rate (FNR), Miss Rate	4.54%
False Positive Rate (FPR), Fall out	0%
Accuracy (ACC)	96.67%

Quantitative analysis of coliforms using ^{M}ColiPAT medium (^{M}ColiPAT versus Colilert)

A total of 100 drinking water samples were used for quantitative analysis of coliforms using both Colilert and ^{M}ColiPAT. The results obtained were analyzed for testing equivalence between the Colilert and ^{M}ColiPAT methods. The MPN results for coliforms using the two methods were between <1 and 2,419.6 MPN/100 ml. The Spearman's rank correlation coefficient (r_s) between the two methods was 0.98. All the MPN data per 100 ml were converted to $\log_{10}$ MPN/100 ml before statistical analysis. Figure 1 shows a scatter plot of ^{M}ColiPAT compared to Colilert-18 depicting considerable agreement between the two tests. Samples were excluded from calculations when both methods gave zero (0, 0). The means of MPN results for total coliforms with ^{M}ColiPAT and Colilert-18 were not statistically significantly different by the paired t-test ($p = 0.18$).

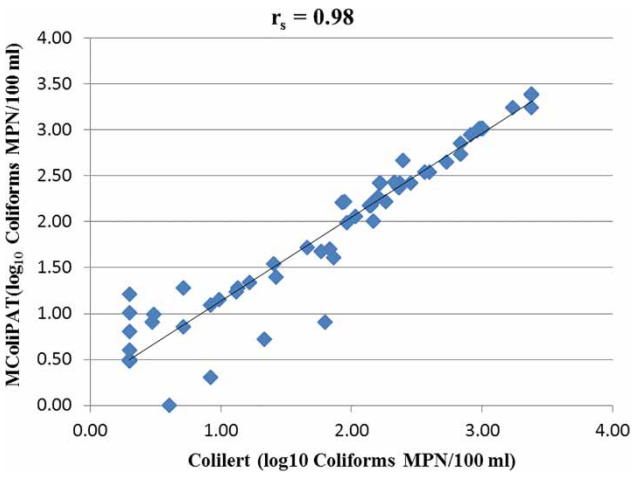

Figure 1 | Scatter plot of ^{M}ColiPAT coliforms estimates versus Colilert-18.

Cost of ^{M}ColiPAT kit

The ^{M}ColiPAT kit is a cost-effective kit for coliform detection when compared to other presence/absence test kits. The cost of the ^{M}ColiPAT kit medium based on materials only is Rs. 1.5. (1.5 Indian rupees = 0.02 US dollars (USD), 1 USD = Rs. 70.86 as on 16th December 2019. The cost per test is based on catalogue prices (2019) from HiMedia Laboratories and ThermoFisher Scientific India Pvt. Ltd, India.) The cost of testing one sample using the ^{M}ColiPAT kit would be around Rs. 25–30 INR (<0.5 USD) per sample (including the cost of chemicals, bottle, sterilization & packaging). The cost per sample of other presence/absence test kits such as Colilert and Aquagenx is about Rs. 800 (~11.29 USD) and Rs. 690 (~9.74 USD) respectively in India (retail price in Jaipur (Rajasthan) India as per the quotation received from authorized supplier in India in 2019).

There are about 60 million reported drinking water sources in India and if these are to be tested twice in a year for bacteriological analysis, 120 million water samples have to be tested in the country (MDWS 2013). The number of water testing laboratories in India is not sufficient to test such a large number of water samples. Using the ^{M}ColiPAT kit for routine water quality testing can overcome these constraints in developing countries such as India. Thus, ^{M}ColiPAT proved to be an economical test kit with high sensitivity and specificity that can be used for rapid detection of coliforms in low resource situations.

CONCLUSION

The aim of this work was to improve the ColiPAT kit for even faster detection of coliforms in water by reducing the incubation time period without losing sensitivity. Such a kit is needed to detect coliforms in the minimum possible time to allow early remedial action in the case of a pollution event. ^{M}ColiPAT offers a sensitive and rapid method for on-site analysis of coliforms in water. Improving the sensitivity through the selection of a pH based indicator proved useful. ^{M}ColiPAT gave results that were almost statistically equivalent to a standard Colilert-18 h test. The relatively high sensitivity and specificity of this kit makes it a highly reliable method for the detection of coliforms. We therefore

conclude that the MColiPAT kit is a low cost option, with ease of operation, and a more suitable alternative presence-absence test kit for field application in the monitoring of microbiological quality of drinking water than the existing kits, particularly during outbreaks of water-borne infectious diseases. This kit can also be used as a screening test for assessing the microbial quality of water if laboratory scale standardized tests are required, thereby reducing the overall expenses of water quality monitoring.

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