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Designing of New Culture Media for Detection and Enumeration of Coli forms in water samples

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Abstract
Ensuring the safety of drinking water and food plays critical role in protection of public health from water borne and food borne microbial pathogens. Coliforms, including *Escherichia coli*, *Enterobacter aerogenes* serves as key indicators of water contamination and indicate the presence of harmful intestinal pathogens. Error free detection and enumeration of coliforms in drinking water free from false positive tests are essential for monitoring sanitary quality of water. Our study showed that CMBB broth can be used as a single direct test for detection of coliforms in water within 12-18 hours. A new selective medium using methylene blue (MB) and Congo red (CR) is described and compared with EMB, MacConkey agar. ***Escherichia coli*** on newly designed culture medium –CMBB agar produced distinct colony morphology, nucleated colonies with blue centre and white periphery. Newly formulated CMBB agar medium was acting as a selective and indicator medium for detection of *E.coli* in water.
Key words – coliforms, E.coli, new culture media, quality of water, selective media, enrichment media, TTC reagent

1. INTRODUCTION

Coliform test is the process of detecting and identifying coliform bacteria to determine sanitary quality of water and foods. Coliforms are facultatively anaerobic, gram negative, non spore forming bacilli that ferment lactose with the production of acid and gas within 48 hrs of incubation at 35°C [1, 2,3]. Coliforms make up approximately 10 % of intestinal microbial flora of humans and animals [4, 5]. The presence of coliform bacteria and E.coli in water, food, and other products is an indicator of possible fecal contamination and can be used to assess the overall hygiene and safety of the water and foods [1, 6, 7, 8].

Coliform identification is crucial in various industries, such as, (i) Food, dairy and beverage industry, ensuring the safety and quality of food and beverages by detecting and preventing contamination[1,9].(ii)Water treatment facilities, for monitoring the effectiveness of water treatment processes and ensuring the safety of drinking water.[10] (iii)Environmental testing: Assessing the impact of human activities on the environment and monitoring water quality in lakes, rivers, and other bodies of water[11]. (iii) Pharmaceutical industry, verifying the cleanliness and sterility of pharmaceutical products[12]. The traditional liquid enrichment media like Lactose broth, Lauryl sulphate tryptose broth, Mac Conkey broth, Brilliant green lactose bile broth, solid selective media such as EMB Agar, Endo Agar, Mac Conkey agar media etc are used for detection of coliforms in water and food samples[6]. These traditional media such as lactose broth have few drawbacks; these include false positive tests due to growth and gas production of some gram-positive bacteria, *Enterococci, Staphylococci,* and yeasts. Besides Enterobacteriaceae, other gram-negative bacteria may also grow. So we have designed a new liquid enrichment indicator medium and solid selective indicator media which particularly allowing growth and identification of coliforms.

2. MATERIALS AND METHODS

2.1. Design of New Culture media:

Literature survey is carried out to find out different liquid enrichment and selective media used for detection of coliforms in water and food samples. Lactose broth, Lauryl Sulphate tryptose broth, Mac Conkey Broth, Brilliant Green Lactose bile broth are liquid media employed in presumptive coliform test and in enumeration of coliforms by MPN method and Mac Conkey agar, Violet red bile agar, Eosin Methylene Blue agar(EMB), Endo agar are selective and indicator media for detection of coliforms in water and food samples for confirmed test. We identified key components present in such media for enrichment (Enrichment media) and selection (selective media) of coliforms in water and food samples. After many trails we have successfully designed an ideal new culture media which allows the growth and detection of coliforms with no flaws. We designed new culture media, Congo red, Methylene blue bile salt broth (CMBB) and Congo red, Methylene blue bile salt (CMBB) agar for detection and identification of coliforms in water, food and pharmaceutical samples.

Table.1: Designed New Enrichment indicator media and selective media

MBB Broth	**IBB Broth**	**B Broth**
Lactose - 5 g Peptone - 10g Sucrose - 5 g K2HPO4 - 3g Bile Salt - 1.53 g Methylene Blue - 0.0606g Congo Red - 0.0206g Deionized Water - 1000ml pH 7.2	Lactose - 5 g Peptone - 10g Sucrose - 5 g K2HPO4 - 3g Bile Salt - 1.53 g Methylene Blue -0.0606g Deionized Water - 1000ml pH 7.2	Lactose - 5 g Peptone - 10g Sucrose - 5 g K2HPO4 - 3g Bile Salt - 1.53 g Congo Red - 0.0206g Deionized Water - 1000ml pH 7.2
CMBB agar	**MBB agar**	**CB Agar**

Lactose - 5 g Peptone - 10g Sucrose - 5 g K2HPO4 - 3g Bile Salt - 1.53 g Methylene Blue - 0.0606 g Congo Red - 0.0206g Deionized Water - 1000ml Agar agar - 20g Deionized Water – 1000mL pH 7.2	Lactose - 5 g Peptone - 10g Sucrose - 5 g K2HPO4 - 3g Bile Salt - 1.53 g Methylene Blue - 0.0606 g Agar agar - 20g Deionized Water (1000ml) pH 7.2 .	Lactose - 5 g Peptone - 10g Sucrose - 5g K2HPO4 - 3g Bile Salt - 1.53 g Congo Red - 0.0206g Deionized water - 1000mL Agar agar - 20g pH 7.2

2.2. Inoculation of polluted and sterile water samples into liquid enrichment media

Water samples (IDL lake water, tap water and packaged drinking water) in triplicates are inoculated into sterile double strength (DS) and single strength (SS) CMBB broth in tubes containing Durham's tube in inverted manner. For one sample, 15 tubes of which five double strength and 10 single strength CMBB media are used. To the 10 mL of DS CMBB, 10 mL of water sample was inoculated with a sterile pipette in the Laminar flow. To the five tubes of 10 mL of SS CMBB, 1.0 mL of water sample was added to each tube and to the remaining five tubes of SS CMBB, 0.1 mL of water sample was added to each tube. All tubes are incubated at 37℃ for overnight. After incubation tubes were verified for gas formation, bacterial growth and color change. From MPN table number of coliforms was calculated for each sample. This Experiment is carried three times in duplicates.

2.3. Inoculation of suspension from positive presumptive tubes into selective media

Loopful of suspension from tubes of positive presumptive test were inoculated into CMBB agar in Petri dishes in triplicates. Petri dishes were incubated in incubator at 37℃ in incubator for overnight. Petri dishes were observed, photographed and colonycharacteriscs were studied.

2.4. Verification of blue nucleated colonies by IMViC Test and Gram staining

Blue nucleated colonies were selected and streaked on nutrient agar and after growth, microscopic examination of isolate is carried after Gram staining. Nucleated colonies with blue center and white periphery were also subjected to IMVic tests for identification.

2.5. Micro broth test for detection of coliforms

To the 2.5mL of CMBB in screw capped vial, 0.5 mL of *E.coli* culture was inoculated. To other set of tubes 0.5 mL of *Staphylococcus aureus* was inoculated. Tubes were incubated at in incubator at 37℃ for overnight. Tubes were observed for color change. Optical density (OD) was measured against a blank (uninoculated medium) at 600 nm in a colorimeter (Elico). Experiment is repeated thrice in duplicates.

2.6. Study of growth Characteristics of pure cultures in new designed media, EMB agar and MacConkey agar

Pure cultures of *E.coli* and *Staphylococcus aureus* were inoculated into petri dishes containing EMB agar, MacConkey agar, CMBB agar, CB agar and MBB agar. Petri plates were incubated in incubator at 37℃ in incubator for overnight. Petri plates were observed, photographed and colony morphologies were studied. Experiment is repeated thrice in duplicates.

2.7. Microbroth assay by using Triphenyl Tetrazolium chloride (TTC) for detection of coliforms:

To the 3.0 mL of sterile Lactose bile broth medium 0.5 mL of water sample is added and incubated for 12 hours at 37ºC. After 12 hours 100µL of 1% Triphenyl Tetrazolium chloride (TTC) reagent (Himedia,FD057-1VL, Lot No. 000505844) is added and incubated at 37 ºC for one hour. Experiment is repeated thrice in duplicates.

3. RESULTS

3.1. Design of new culture media

We designed three formulations CMBB broth CB broth and MBB broth for selection and enumeration of coliforms from water. Amongst the three media, CMBB broth was proved as most superior enrichment, selective and indicator media for coliforms and shown best results**.**

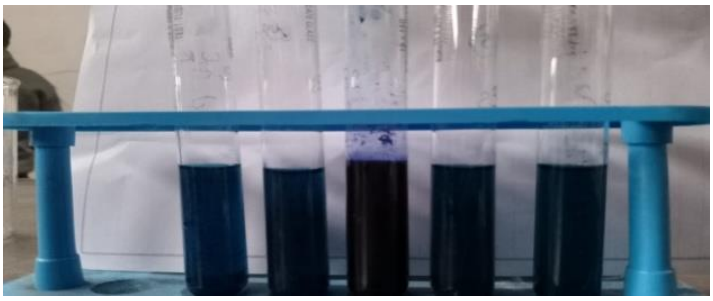


Figure.1 : CMBB broth before inoculation of water samples

3.2. Inoculation of polluted and sterile water samples into liquid enrichment media

The CMBB broth tubes inoculated with pond water, tap water samples collected from kukatpally shown turbidity, color change, gas bubble formation in Durham's tube. Whereas CMBB tubes inoculated with packaged drinking water has not shown color change and gas formation in Durham's tube. Number of coliforms in pond water, tap water, and packaged drinking water were calculated from standard MPN table. The results of water samples are shown in **table.2.**

Figure.2: CMBB broth inoculated with water sample collected from IDL lake , Kukatpally.

Table.2 : Enumeration of coliforms by Most probable number technique employing newly designed CMBB broth

/ater samples	**)mL**	**0mL**	**1 mL**	**o of coliforms/100mL**	**ıality of water**
Pond Water				500	or, Polluted
Tap water				40	or, polluted
Packaged Drinking water					cellent

3.3. Inoculation of suspension from positive presumptive tubes into selective media

CMBB agar inoculated with positive presumptive tubes of CMBB broth clearly indicated the formation of blue centered colonies surrounded by white periphery. Formation of nucleated colonies with blue centre and whitish periphery indicated the presence of *E.coli.*

Figure.3. CMBB agar inoculated with a loopful of inoculum from positive presumptive tube of CMBB broth showing nucleated colonies with whitish periphery.

3.4. Verification of blue nucleated colonies by IMViC Test and Gram staining

Microscopic examination of blue nucleated colonies after Gram's staining clearly showed that these are Gram negative bacilli. These bacilli are positive to indole test, Methyl red (MR) test and negative to Voges Proskeur (VP) test and Citrate utilization test.

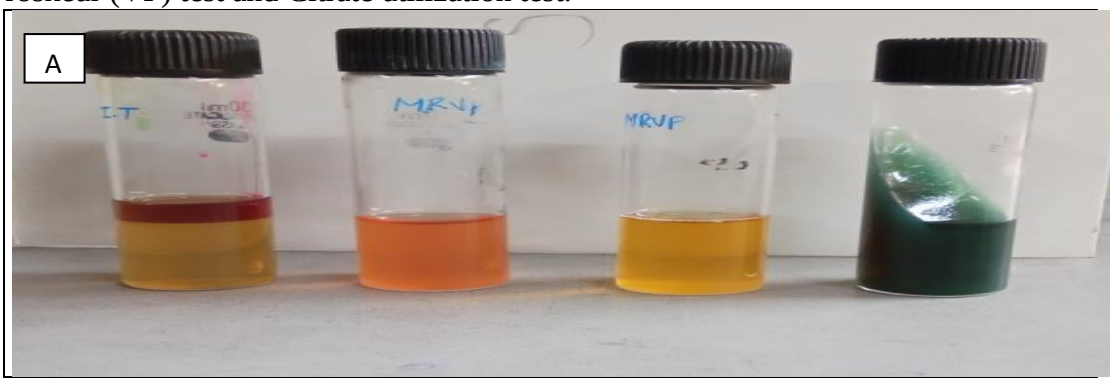
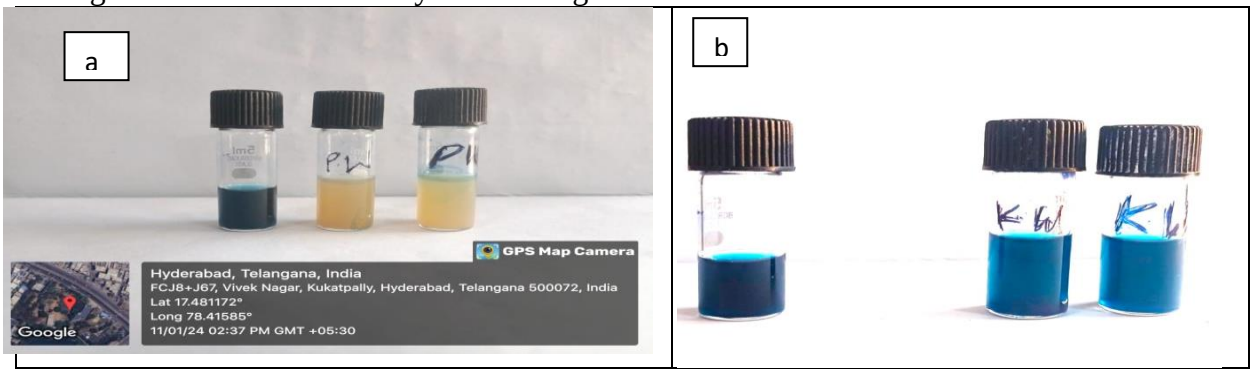


Figure.4. IMViC test of Blue nucleated colonies. Indole test positive, MR test Positive, Voges Proskauer test negative and Citrate utilization test Negative.

3.5. Micro broth test for detection of coliforms

CMBB micro broth tubes inoculated with water samples collected from IDL lake, Kukatpally shown color change and turbidity. Tap water samples collected at Government degree college, Kukatpally also showed color change and turbidity. Whereas CMBB micro broth tubes inoculated with packaged drinking water has not shown any color change and are free from coliforms.



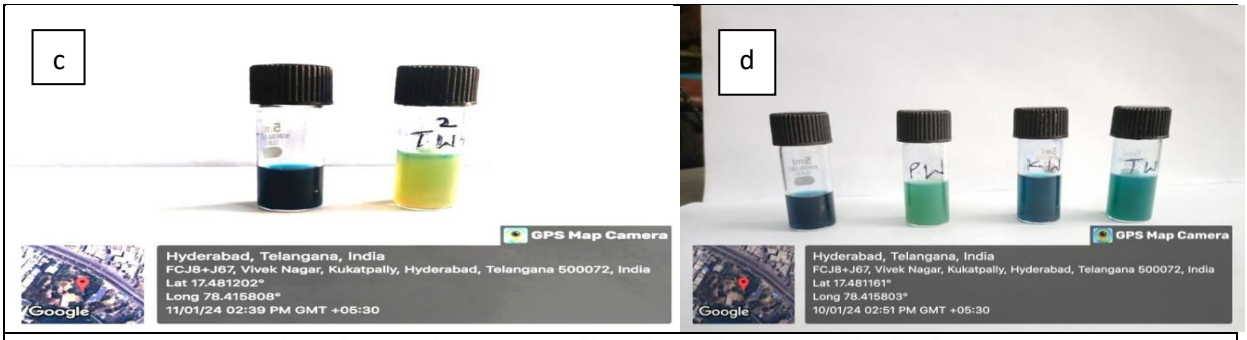


Figure: 5. Micro-broth rapid test (a) Polluted pond water (b) kinley water (c) tap wate sample (d) pond water, kinley water, tap water

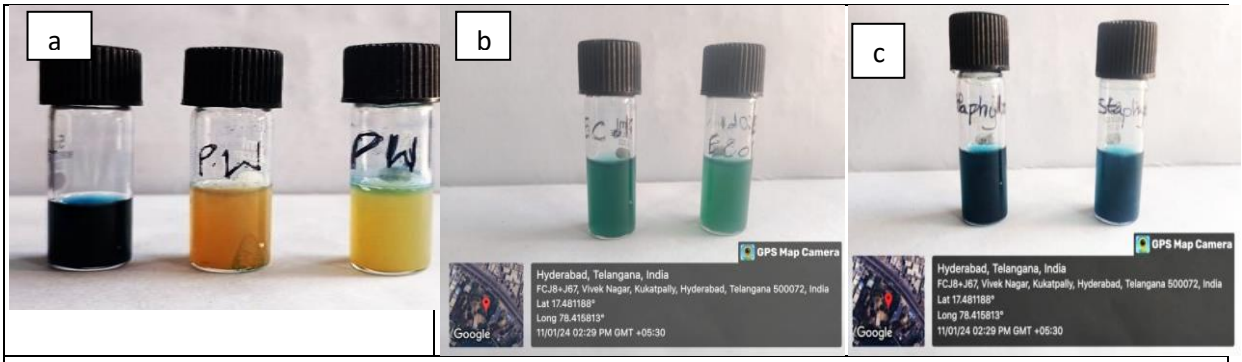


Figure.6. Micro-broth rapid test : (a)CMBB broth inoculated with IDL lake water showir colour change. (b)CMBB Broth inoculated with *E.Coli* showing colour change *(*c) CMBB Broth inoculated with *Staphylococcus aureus* showing no change in colour.

Table.3.Micro

Sample	**ptical Density (OD 600nm**
Escherichia coli	1.69 ± 0.05
Staphylococcus aureus	0 ± 0.01
IDL Lake Kukatpally	1.52 ± 0.03
Tap water	1.22 ± 0.22
Packaged drinking water	0 ± 0.01

broth rapid test

3.6. Study of growth Characteristics of Pure cultures in Designed media and EMB agar and MacConkey agar

On CMBB agar, *E.coli* produced nucleated colonies with blue centre and whitish periphery after 18 hours incubation at 37^{o}C. On the EMB agar, *E.coli* produced nucleated colonies with translucent periphery and metallic sheen. It produced small pink colonies on MacConkey agar medium. Whereas Gram positive bacteria, *Staphylococcus aureus* failed to grow on MacConkey agar, EMB agar and CMBB agar (New media).

New Selective Culture media CMBB agar compared and validated with EMB and MacConkey agar

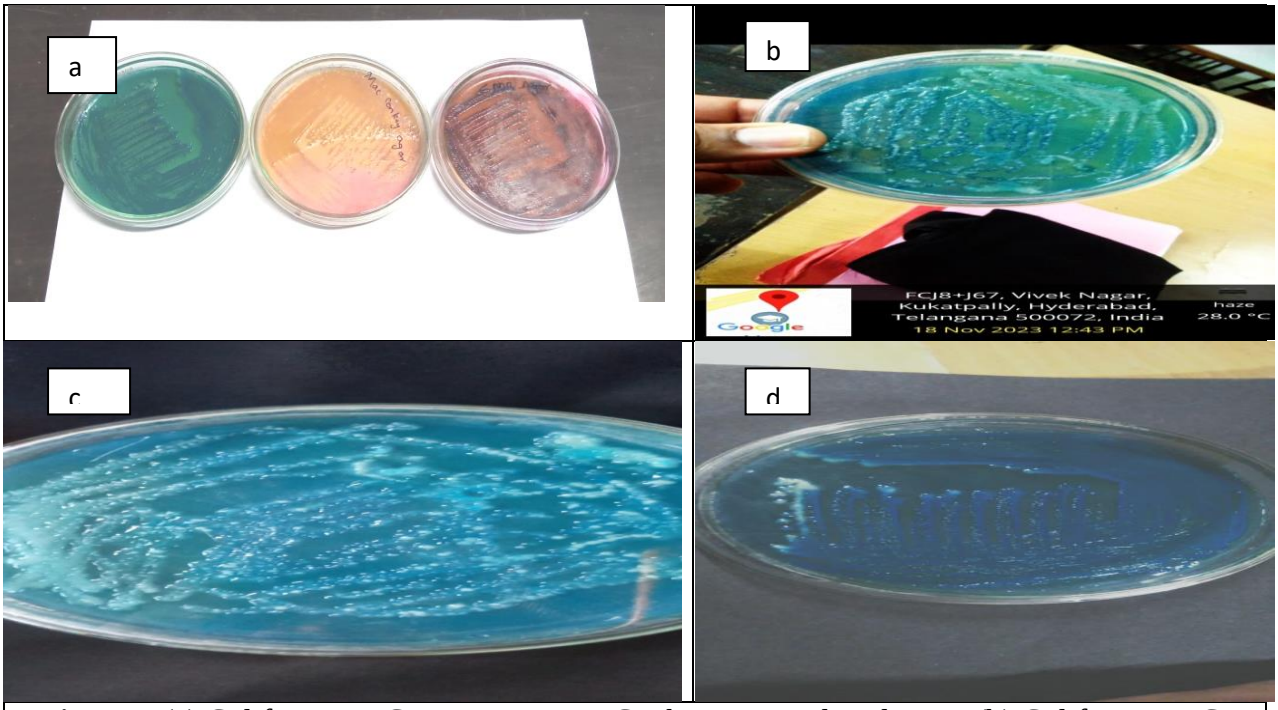


Figure.7.(a) Coliforms on CMBB agar, MacConkey agar and Endo agar (b) Coliforms on CMB agar (c,d) Coliform colonies on CMBB agar with blue center surrounded by white periphery

3.7. Micro broth assay by using Triphenyl Tetrazolium chloride (TTC) for detection of coliforms

In Lactose bile broth, only coliforms present in water samples multiplied and other bacterial species were died or their growth was inhibited. Coliforms growing in these media were detected by addition of 1% TTC reagent. The red formazan crystals formed in tubes indicated the presence of coliforms in water samples. Results are shown below in figure.8

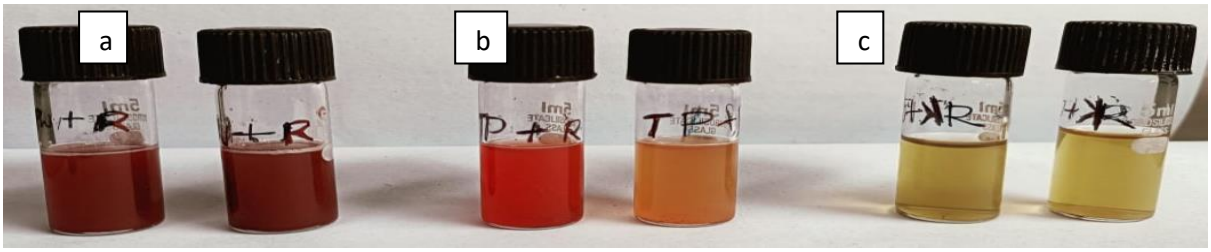


Figure.8. Lactose bile broth inoculated with (a) IDL lake water, (b) tap water , (c) kinley packaged drinking water. Packaged drinking water (Kinley) showing negative test. IDL lake water and tap water showing positive test to coliforms.

4. DISCUSSION

The lactose broth prescribed by the committee of the American Public Health Association (1917) contains 0.3% beef extract, 0.5%o peptone, and 1% lactose [13]. Alfred MacConkey in 1900 formulated MacConkey broth and MacConkey agar for detection of coliforms in treated waters of sewage treatment plants [14]. In 1938, Cowls demonstrated that the addition of Sodium Lauryl sulphate to lactose broth gave a medium selective for the coliform group. Darby and Mallmann showed in a laboratory study that the use of a new broth, Lauryl Sulfate Tryptose Broth for the Detection of Coliform Organisms [15] . Brilliant green lactose bile (BGB) broth is a modification of MacConkey's liquid medium for the isolation of Enterobacteriaceae and has been formulated by Dunham and Schoenlein to attain maximum recovery of bacteria of the coli-Aerogenes group,

while inhibiting most gram-positive organisms that might hinder the development of the bacteria sought[16]. Eosin-methylene blue (EMB) agar was initially formulated in 1916 by Holt-Harris and Teague, to visibly differentiate between the lactose fermenting and non-fermenting microorganisms. through the use of eosin and methylene blue dyes[17]. Endo agar medium was originally developed by Endo for the isolation of Bacillus typhosa (*Salmonella typhi)* from fecal samples, it is now used mostly as a coliform medium [18,19].

Table.4. Enrichment-Selective media developed for detection of coliforms and determination of sanitary quality of water

S.No	Enrichment – Selective media Coliforms/ Enteric bacteria	Year	Author	Reference
1	MacConkey Broth, MacCon agar	1900	Alfred Theoc MacConkey	[20,21]
2	Endo Agar	1904	Endo	[18,19]
3	Violet red bile Agar	1905	Alfred Theoc MacConkey	[22]
4	EMB agar	1916	Holt-Harris and Teague	[16,23]
5	Lactose Broth	1961	North.Jr. W.R	[24]
6	Brilliant Green Lactose Bile Bro	1926	Dunham and Schoenlein	[25]
7	Lauryl Sulphate Tryptose Broth	1940	Darby and Mallmann	[26]
8	Coliforms broth	2022	Gawai Kunal et al.,	[27]
9	Colilert defined substrate mediu	1991	Park, S. J., Lee, etal	[13]

In our study in CMBB broth, only coliforms- *E.coli* produced gas and turbidity and color change where as a Gram positive *Staphylococcus aureus* fails to grow. Congo red and methylene blue probably inhibited growth of Gram positive bacteria. Bile salts in the medium acted as a selective component for selective growth of *E.coli* and *Enterobacter* from water samples. Thus newly formulated CMBB broth acts as selective and indicator medium for detection of coliforms. CMBB micro broth assay carried in this study showed that CMBB broth can be used as a single direct test for detection of coliforms in water within 12-18 hours**.** CMBB broth can also be used in presumptive coliform test as an alternative medium to lactose broth, Lauryl sulphate tryptose broth, MacConkey broth. Newly formulated CMBB agar medium allows only growth of coliforms and do not allow Gram positive bacteria such as *Staphylococcus aureus*. More over fecal coliform, *Escherichia coli* produced nucleated colonies with blue centre and white periphery **(Figure.7).** CMBB agar acting as a selective and indicator medium for *E.coli.* It can be used as an alternative medium for EMB agar and Endo agar in confirmatory test of coliform test. TTC test using lactose bile broth medium can be used as a rapid test to determine sanitary quality of water **(figure.8)**. In our study IDL lake water of kukatpally showed the presence of coliforms. Packaged drinking water samples are excellent and are free from coliforms.

5. Conclusion

The newly formulated CMBB broth was proved as an efficient enrichment and indicator medium for detection of coliforms in water to determine sanitary quality of water. The CMBB micro broth tubes can also be used for rapid tests for detection of coliforms and evaluation of quality of water, food and pharmaceuticals etc. More over Coliform, *Escherichia coli* produced blue nucleated colonies with translucent periphery on Congo red Methylene blue bile salt agar (CMBB) medium. Our study demonstrated that Gram positive bacteria do not grow in CMBB broth and CMBB agar which are selective and indicator media for coliforms. CMBB broth is an alternative new culture medium to lactose broth, Lauryl sulphate tryptose broth and Brilliant green lactose bile broth. CMBB agar was a new alternate selective and indicator media to EMB agar, Endo agar for detection and identification of coliforms for analysis of water, food and pharmaceuticals.

6. Declaration

The author hereby declare that the work done by him was original and done at Department of Microbiology, Government Degree College, Kukatpally, Medchal district, Telangana state, India.

7. Acknowledgements

I would like to thank the Government Degree College, Kukatpally, Medchal district, Telangana state, India for providing the necessary requirements, infrastructure and laboratory facilities for carrying this research work.

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