

Comparative Evaluation of EMB Agar and Hicrome *E. coli* Agar for Differentiation of Green Metallic Sheen Producing Non *E. coli* and Typical *E. coli* Colonies from Food and Environmental Samples

Ally C. Antony^{1,2*}, Mini K. Paul², Reshma Silvester¹, Aneesa P.A.¹, Suresh K.¹, Divya P.S.¹, Simmy Paul³, Fathima P.A.⁴ and Mohamed Hatha Abdulla¹

¹Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Lakeside Campus, Cochin - 682 016, India.

² M.E.S. College, Marampally, Ernakulam - 683 107, India.

³School of Medical Education, Mahatma Gandhi University, Kottayam, India.

⁴State Council of Educational Research and Training, Trivandrum, India.

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The performance of Hicrome *E. coli* agar (Himedia) versus EMB agar (Himedia) was compared for the detection of *E. coli* from food and environmental sources. Four hundred bacterial isolates producing typical *E. coli* like green metallic sheen on EMB agar were subjected to biochemical analysis by IMViC tests and they exhibited 6 different types of reactions. Isolates exhibiting only two distinct reaction types were proved to be typical *E. coli*, which were reconfirmed by molecular detection of species-specific *uid A* gene as well as 16S rRNA sequencing. Isolates exhibiting the remaining four reaction types comprised of *Enterobacter asburiae*, *Kluyvera georgiana*, *Pantoea agglomerans* and *Phytobacter diazotrophicus* respectively, all belonging to the family Enterobacteriaceae. The *E. coli* strains recovered from Hicrome *E. coli* agar comprised of 20 different serogroups including glucuronidase positive atypical O157: H7. Hicrome *E. coli* agar had better sensitivity (100%), specificity (99%) and efficiency (99.5%) than EMB agar in differentiating the false positives and false negatives and does not require any further confirmatory tests.

Keywords: *Escherichia coli*; EMB agar; Hicrome *E. coli* agar, metallic sheen.

E. coli is the widely accepted faecal indicator, since its presence does not occur in other niches, unless and until there is faecal contamination. It is easily detectable and its presence in food, water and other sources is indicative of recent faecal contamination and possible presence of other pathogens^(1, 2). Even though most *E. coli* are non-pathogenic, there are several potentially pathogenic strains with virulent factors capable of causing diarrheal disease in

humans^(3, 4). The concentration of faecal coliforms or *E. coli* is the universally accepted microbiological quality parameter, determining the sanitary quality of food commodities meant for domestic as well as export purposes. In order to ensure public safety, it is strictly monitored in case of water bodies used for recreation, drinking water purposes or shellfish growing. Certification of shellfish harvesting areas based on *E. coli* levels has been made mandatory^(5, 6), failing of which stringent actions like closing of the harvesting areas are implemented until the required standards are met with. For all these evaluations, isolation and identification of *E. coli* is a prerequisite which is often complicated in practice, due to the co-

* To whom all correspondence should be addressed.
E-mail : allyjoemon@gmail.com

occurrence of other interfering enteric bacteria such as *Citrobacter*, *Klebsiella* and *Enterobacter*, which also ferment lactose and exhibit similar colony morphology on Levine's Eosin Methylene Blue (EMB) agar selective for *E. coli*.^(7, 8, 9) These coliforms, unlike faecal coliforms, may also be present in other environmental sources and hence their presence need not essentially indicate faecal contamination. Incubation at elevated temperatures (44.5 °C) and use of Brilliant Green Lactose Bile Broth (BGLB) medium are employed, to distinguish these competing microbiota, but is not always possible, since they also exhibit similar reactions.

The problem is more evident especially when these competing flora are present in relatively high numbers, increasing the number of false positives, thus interfering with the isolation of the actual required isolates and reducing the efficiency of this selective medium⁽⁷⁾. Even though many researchers often encounter this problem, there are very few reports suggesting an alternative to this medium and still less on the identification of the interfering microbiota, which may be helpful in avoiding them. There are many reports related to use of different chromogenic media for isolation of enterohaemorrhagic *E. coli* O157:H7, from different sources; but it is not so with other pathogenic or non-pathogenic *E. coli* serotypes^(10, 11). Hence, newer media or methods should be attempted for the rapid and precise isolation of this sanitary indicator which may be helpful in avoiding erroneous interpretations.

The current study compares the performance of a chromogenic medium, Hicrome *E. coli* agar (Himedia, India), over Eosin Methylene Blue (EMB, Himedia, India) agar, for isolation of *E. coli* from various sources. The interfering microbiota was also identified and a phylogenetic comparison of the same with *E. coli* was being undertaken.

MATERIALS AND METHODS

Bacterial cultures and standard strains

Four hundred presumptive *E. coli* isolates obtained from various sources such as, live clam (n=140), bivalve shellfish meat available in market (n=45), water (n=100) and sediments from a tropical estuary (n=100) and drinking water (n=15) were used for the study.

E. coli ATCC 25922 and 4 other strains CUSMBES1, CUSMBES 9, CUSMBES10 and CUSMBES 11a maintained in our laboratory with GenBank accession numbers JX183939.1, JX183940.1, JX183941.1, and JX183942.1 respectively were used as the standard reference strains.

Sample processing, isolation and biochemical analysis

Samples were aseptically collected and processed as per standard guidelines^(12, 13 14). Approximately 25 g of shellfish meat was aseptically transferred to a sterile stomacher bag and blended with 225 ml of sterile peptone water in a stomacher (IUL Instruments, Spain). Five tube MPN test using lactose broth was performed for the isolation of *E. coli*. Similarly, sediment and water samples were also diluted (1:9 ratio) and examined for the presence of *E. coli* by the same method. The tubes were examined for evidence of lactose fermentation after incubation at 44.5 °C for 24 - 48 hours. From the tubes showing growth and gas production, loopful was streaked on to conventional Levine's Eosin-methylene blue agar (L-EMB, Himedia, India) and alternatively to Hicrome *E. coli* agar⁽¹⁴⁾. The plates were observed following 24 hours of incubation at 37 °C. On EMB, strong acid producers like *E. coli* usually form colonies with green metallic sheen, showing a dark nucleated centre. Under acidic conditions, the dye eosin Y is precipitated, producing dark coloured colonies usually with a green metallic sheen due to the formation of an amide bond between eosin Y and methylene blue in the medium^(15,9). On the other hand, Hicrome *E. coli* agar detects *E. coli* based on the presence of the glucuronidase enzyme. The chromogenic substrate p-nitrophenyl-p-D-glucopyranosiduronic acid (PNPG) in the medium is absorbed by *E. coli* and the intracellular β -Glucuronidase enzyme present in most serotypes other than O157:H7 cleaves the bond between chromophore and the glucuronide, releasing the former, producing a blue-green coloured colony⁽¹⁶⁾. Prior to testing samples, the efficiency of the two media was assessed, under controlled conditions using five standard strains mentioned above and was found to be 100% for both EMB agar and Hicrome *E. coli* agar alike. Typical colonies were subjected to biochemical confirmation by IMViC tests, which included Indole

production test, Methyl red test, Voges-Proskauer and Citrate utilization tests^(17,14).

Molecular confirmation by detection of *uid A* gene

Genomic DNA was extracted by boiling method and molecular confirmation was done by PCR amplification of *uid A* gene which codes for β -D-Glucuronidase enzyme of *E. coli* using the primer set UAL- 754 (5'-AAAACGGCAAGAAA AAGCAG-3') and UAR- 900 (5' ACGCGTGG TTACAGTCTTGCG-3')^(18,19). The PCR products were resolved by agarose (1.5% w/v) gel electrophoresis in 1X TBE buffer, containing 0.5 μ g/ml of ethidium bromide and visualized by Gel Documentation System (Bio-Rad Gel Doc EZ Imager, USA). Amplicon sizes were compared with 100 bp DNA ladder; 147 bp PCR products confirmed the presence of *E. coli*.

Serotyping of *E. coli*

The confirmed *E. coli* strains were serotyped at the National *Salmonella* and *Escherichia* centre, Central Research Institute (Kasauli, Himachal Pradesh, India).

16S rRNA amplification and sequencing

Representative isolates from the confirmed and suspected groups were subjected to 16S rRNA gene amplification using the universal primer set (27F: 5'-AGA GTT TGA TCC TGG CTC AG-3', 1492R: 5'-GGTTACCTT GTTACG ACTT-3') as described elsewhere⁽²⁰⁾. PCR products were sequenced by Sangers dideoxy method (SciGenome, India) and the sequences edited using Gene Tool lite version 1.0 software. The percentage of homology was determined against known sequences in the NCBI GenBank database, using the Basic Local Alignment Search Tool algorithm (BLAST; <http://www.ncbi.nlm.nih.gov/BLAST>) and the sequences were submitted in the GenBank and allotted with an accession number.

Phylogenetic analysis

The partial 16S rRNA gene sequences of the *E. coli* and other false positive isolates obtained in this study, together with 16S rRNA gene sequences of similar isolates from previous reports and other related genera obtained from GenBank, were aligned using the CLUSTAL W program and the phylogenetic tree was constructed using Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test with 1000 replicates (Boot strap value) is shown below the

branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 software.

Statistical analysis

Statistical comparison of the performances of the two media, EMB agar and Hicrome *E. coli* agar was done by calculating percentages of sensitivity, specificity and efficiency using the equations below²¹.

% Sensitivity = True positives x 100/ (True positives + False Negatives)

% Specificity = True negatives x 100/ (True negatives + False positives)

% Efficiency = (True positives + True negatives) x 100/ Total

RESULTS AND DISCUSSION

EMB agar and Hicrome *E. coli* agar showed 100% efficiency in identification of the five standard strains used in the study (data not shown). However, problem arose when both metallic sheen producing non *E. coli* and true *E. coli* were present together, as in the natural samples tested. The results of the isolation and identification of *E. coli* from the sources mentioned above using the two media under comparison are summarised in Tables 1 and 2. False positive results due to competing flora were found to be very high on EMB agar (40 %) whereas on Hicrome it was found to be very low (0.5%). False negatives were also high on EMB agar (15.75%), due to the sheen of true *E. coli* masked by the highly mucoid background flora. No false negatives were detected on Hicrome *E. coli* agar (Table 1). Similarly the sensitivity, specificity and efficiency of Hicrome *E. coli* agar were far above those of the Levine's EMB agar (Table 2).

Appearance of *E. coli* and Non-*E. coli* on Hicrome *E. coli* agar vs EMB agar

True *E. coli* produced colonies with typical high metallic sheen on EMB agar and blue-green colonies on Hicrome *E. coli* agar respectively (Fig. 1). On the contrary, a large number of typical high metallic sheen *E. coli* like colonies isolated from EMB agar, produced white colonies on Hicrome *E. coli* agar and when tested

biochemically, were proved to be Non *E. coli* (Fig. 2). It was also observed that, if the *E. coli* concentration is comparatively less in the sample, the sheen is often masked by the highly mucoid interfering background flora such as *Klebsiella* (Fig. 3), resulting in false negative results, which

could also be effectively differentiated by the use of Hicrome *E. coli* agar.

Biochemical characterisation of the isolates

The isolates with high metallic sheen isolated from EMB agar exhibited 6 different types of IMViC reactions (Table 3). The first two reaction

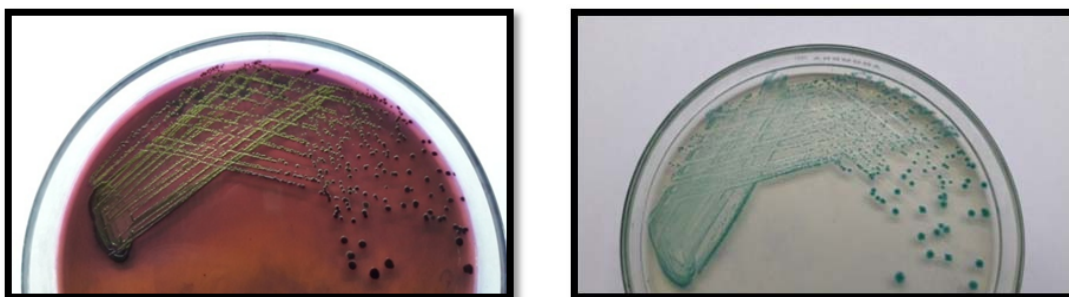


Fig. 1. (a) High metallic sheen producing *E. coli* colonies on EMB agar and (b) Blue-green *E. coli* colonies on Hicrome *E. coli* agar

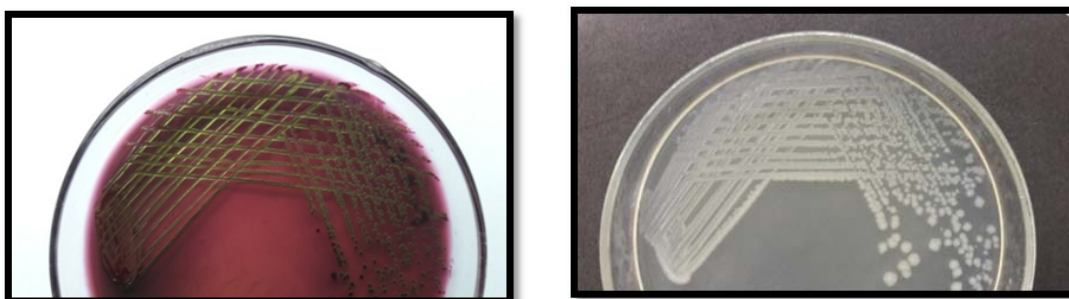


Fig. 2. (a) High metallic sheen producing false positive Non *E. coli* colonies on EMB agar and (b) the corresponding colonies on Hicrome *E. coli* agar

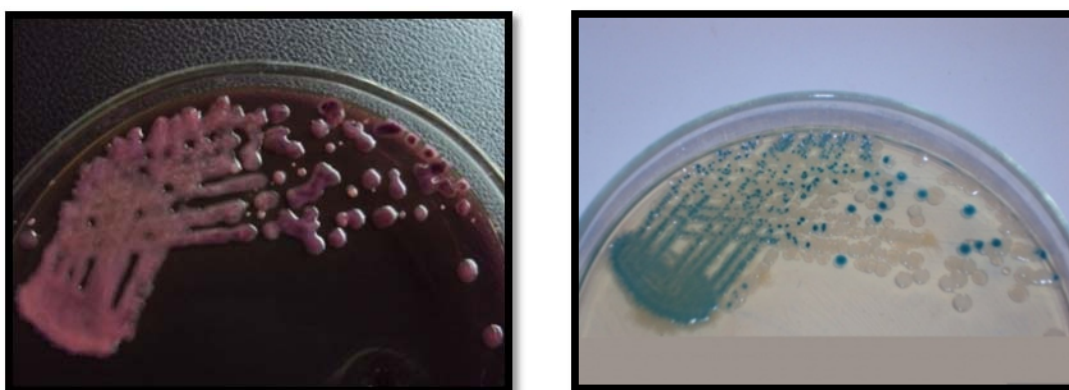


Fig. 3. Interfering microbiota on EMB agar (a) and corresponding Hicrome *E. coli* agar plate (b). On Hicrome *E. coli* agar, true *E. coli* producing blue-green colonies are clearly differentiated from the interfering microbiota which formed white colonies

types comprised true *E. coli* which belonged to biotype I and II respectively⁽¹⁴⁾, whereas all others were proved to be non-*E. coli*.

Molecular confirmation of the isolates

All the biochemically confirmed *E. coli* strains were positive for *uid A* gene, yielding 147 bp specific PCR amplicons(not shown). The remaining four biochemical groups yielded either non specific PCR products or no products at all when tested for *uid A* gene.

16S rRNA amplification and sequencing

When subjected to 16S rRNA sequencing, isolates exhibiting the first two reaction types were reconfirmed to be *E. coli* (EF2p4) and the sequence was submitted in the GenBank with accession number KT804408. Non *E. coli* isolates exhibiting the remaining four reaction types were identified to be *Enterobacter asburiae* (E19), *Kluyvera*

georgiana (E3), *Pantoea agglomerans* (E30) and *Phytobacter diazotrophicus* (2ss1B3) all belonging to the Enterobacteriaceae family and their 16S rRNA sequences were submitted in the GenBank with accession numbers KT804411, KT804412, KT804413 and KU240006 respectively.

Phylogenetic analysis

The phylogenetic analysis showed that the *E. coli* (EF2p4) and *P. diazotrophicus* (2ss1B3) belong to the same cluster with a boot strap value of 37. *E. asburiae* (E19) and *P. agglomerans* (E30) form a cluster with a boot strap value of 100. *K. georgiana* (E3) does not belong to any of these clusters but form a cluster with *K. ascorbata* strain ATCC 33433 with a boot strap value of 82 (Fig. 4).

Serotyping of *E. coli*

The confirmed *E. coli* isolates when serotyped constituted 20 different serogroups,

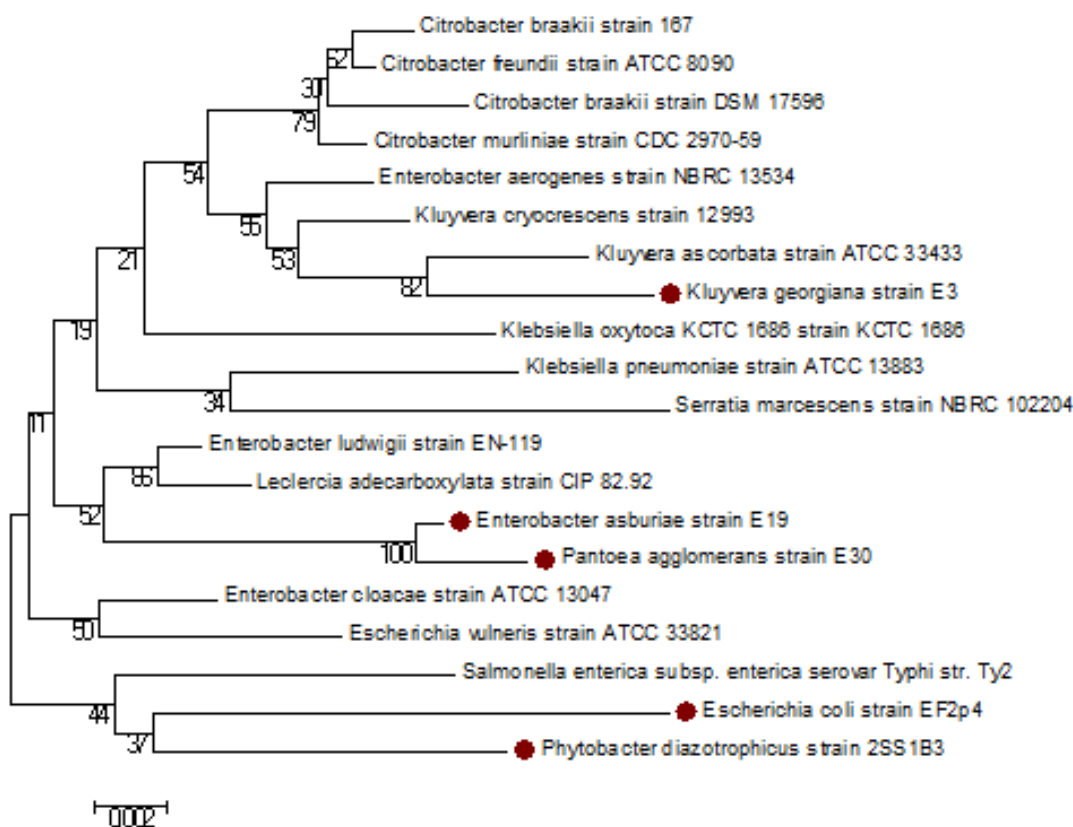


Fig. 4. Phylogenetic tree of 16S rRNA gene sequences, constructed using Neighbour-Joining method, showing relationships between the false positive *E. coli* strains, and typical *E. coli* identified in this study (marked with bullets) and similar false positive isolates in other published reports and other closely related genera is shown. The scale on bottom measures evolutionary distance in substitutions per nucleotide. Boot strap values are shown below the branches

including enterohaemorrhagic O157:H7 (3.5%), enterotoxigenic O8 (8%), shigatoxigenic O121 (4.5%), enteroinvasive O124 (0.5%) and enteropathogenic O91 (0.5%) serogroups. Thus Hicrome *E. coli* agar enabled the isolation of β -D-Glucuronidase positive atypical *E. coli* O157:H7 strains also, which would have been otherwise missed on media specific for the same, since they specifically detects, glucuronidase negative typical isolates only⁽²¹⁾. The other serogroups identified included O2, O4, O9, O17, O27, O33, O34, O35, O84, O118, O119, O120, O126, O141, and O156.

The study has proved the diagnostic efficiency of Hicrome *E. coli* agar (99.5%) over EMB

agar (44.25%). Similarly, the sensitivity and specificity of Hicrome *E. coli* agar were also far higher than those of EMB agar (Table 2). Increased incidence of false positives on EMB agar make the isolation of this otherwise simple organism cumbersome, involving too many colony picks, finally yielding very few positives or no positives at all, while missing the true ones (Fig. 2 and Fig. 3).

Often it was also observed that *E. coli* isolated from EMB agar, existed in strong association with other flora, which on prolonged storage outgrew the *E. coli*, leading to the loss of the original required isolate. This phenomenon also

Table 1. Characteristic appearance of true positive, false positive, true negative and false negative colonies on EMB and Hicrome *E. coli* agar respectively

Media	True positive (n %)	True negative (n %)	False positive (n %)	False negative (n %)
EMB	Green metallic sheen (34.25)	No sheen (10)	Green metallic sheen (40)	No sheen (15.75)
Hicrome <i>E. coli</i> agar	Blue green (50)	Colourless (49.5)	Blue green (0.5)	Colourless (0)

Table 2. Comparison of EMB agar and Hicrome *E. coli* agar in terms of percentages of sensitivity, specificity and efficiency

Medium	Total isolates (n)	True positive (n)	True negative (n)	False positive (n)	False negative (n)	% Sensitivity	% Specificity	% Efficiency
EMB	400	137	40	160	63	68.5	20	44.25
Hicrome <i>E. coli</i> agar	400	200	198	2	0	100	99	99.5

Table 3. Summary of the biochemical, molecular identifications and appearance of the isolates on EMB as well as Hicrome *E. coli* agar.

IMViC reaction	Appearance on EMB agar	Appearance on Hicrome <i>E. coli</i> agar	<i>uidA</i> gene	Organism
++--	Green metallic sheen	Blue green	+	<i>E. coli</i>
----	Green metallic sheen	Blue green	+	<i>E. coli</i>
---+	Green metallic sheen	Colour less	-	<i>Enterobacter asburiae</i>
+++	Green metallic sheen	Colour less	-	<i>Kluyvera georgiana</i>
++++	Green metallic sheen	Colour less	-	<i>Pantoea agglomerans</i>
++-- (atypical reaction, yellow colour for Citrate utilization test)	Green metallic sheen	Colour less	-	<i>Phytobacter diazotrophicus</i>

could be overcome or rather reduced considerably by the use of chromogenic medium. Most of the false positives isolated from EMB belonged to the family Enterobacteriaceae, which reveals the inefficiency of EMB agar in differentiating *E. coli* from other Enterobacteriaceae members exhibiting similar phenotypic and biochemical characteristics. There have been previous reports regarding *Citrobacter* spp., *Enterobacter* spp., *Escherichia vulneris*, *Kluyvera cryocrescens*, *Hafnia*, *Klebsiella*, *Rahnella* and *Serratia* forming *E. coli* like false positive colonies on EMB agar^(14,7,8). However to our knowledge, this is the first report of *Enterobacter asburiae*, *Kluyvera georgiana* and *Phytobacter diazotrophicus* forming false positive colonies on EMB agar.

CONCLUSION

Even though Hicrome *E. coli* agar is more expensive than EMB agar as any other chromogenic medium, when all the factors are considered, it is much more efficient, rapid and cost effective method and could save time and money. In short, this medium is highly recommended for the analysis of large number of samples, due to the accuracy and simplicity of which, even less experienced lab personnel can confirm the identification without further confirmatory biochemical or molecular tests.

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