Pak. J. Bot., 38(3): 779-789, 2006.

MANIPULATION OF DIFFERENT MEDIA AND METHODS FOR COST-EFFECTIVE CHARACTERIZATION OF *ESCHERICHIA COLI* STRAINS COLLECTED FROM DIFFERENT HABITATS

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Abstract

Rapid and reliable identification of about 400 isolates of *Escherichia coli* collected from soil, water, plants and animal faeces was made by membrane filtration (MF), culture media and biochemical methods. Utilization of three types of selective and differential agar media (MacConkey, Eosin Methylene Blue: EBM and Endo agar) rather than one increased the chances of successful isolation/identification. The identified bacteria were re-confirmed through the use of biochemical (IMViC) tests, and diagnostic kits made for this purpose. The results obtained after comparative studies indicated that isolation media and methods used in the present study are not only simple and reliable for large-scale bacterial identification but at the same time are more cost-effective compared to commercially available diagnostic kits. It is anticipated that by using such methods, isolation and identification of *E. coli* can be done effectively without importing expensive diagnostic kits, which is most often difficult especially in the developing countries and thus becomes limiting factor for microbiological investigations.

Introduction

Escherichia coli, an important bacterial species belonging to the family Enterobacteriaceae, is the most frequently encountered microorganism in the food industry. Its presence has also been detected in soil, plants and animal faeces and in water where it could serve as one of the factors affecting animal and human health (Cundell, 1981). Detection and accurate identification of *E. coli* is therefore, enormously important, for which different media and methods are often used depending upon the source of bacterial collection. For example, for the recovery of *E. coli* and other coliforms from drinking water, membrane filtration (MF) method with an agar medium containing a chromagen and a fluorogen, is usually considered superior to MF method with a modified Endo (mEndo) agar and nutrient agar medium (Brenner *et al*., 1993; Brenner *et al*., 1996). These media contain chemicals that produce some characteristic change in the colonies or medium around the colonies of specific bacteria within the group. Selective media often contain inhibitors, which prevent the growth of unwanted and allowing the growth of the desired bacteria, while differential media are used to distinguish between bio-chemically and morphologically related groups of bacteria. Selective and differential agars are used for isolation of *E. coli* from faeces of dairy herds (Wallace & Jones, 1996) because isolation from faeces requires a medium that must comprise bile salt and crystal violet in addition to lactose sugar, which differentiates pathogenic Entero-bacteria (*Salmonella* and *Shigella*) that are usually lactose negative and can therefore, be easily distinguished from non-pathogenic (*E. coli)* Entero-bacterial species (Orskov, 1986). In addition to these components, there are many other tests that are required for accurate identification of *E. coli*, which complicate the task further.

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In order to avoid such difficulties, pharmaceutical companies e.g. BBL have introduced biochemical kits, which can be effectively used without any confusion. In developing countries however, availability of such kits is beset with several factors. For example, importing the kits takes very long time and thus hinders their timely availability. Provision of foreign exchange is another limiting factor. Storage process with the local supplier (in most of the cases) is not very effective and cost of identification is also very high. Such limitations hinder active and efficient bacterial collection particularly in underdeveloped and developing countries like Pakistan, which are otherwise diversity rich countries. In the present paper, comparative identification and characterization made with three types of selective and differential agar media (MacConkey, EBM, and Endo), biochemical test like iMViC (Stanier *et al.*, 1989) and diagnostic kits (Feng & Hartman, 1982) of *E. coli* collected from different local habitats is being presented in order to demonstrate the cost-effectiveness and reliability of isolation, identification and characterization of bacteria with some other methods in case the kits are not available.

Materials and Methods

Sources of microorganisms: Bacterial populations to be examined were collected from various sources such as water, soil, plants and animal faeces at random and over many months.

Collection of samples and their processing: Water samples were collected from irrigation channels and processed within 30 minutes after collection. Density of *E. coli* was determined by adding 2 ml of freshly collected channel water to 10 ml of sterile distilled water and analyzed immediately. Soil samples were collected in sterile Petri plates from different irrigated fields, processed and analyzed immediately after they were received in the laboratory. One gram of fresh soil was shaken manually in 10 ml of sterile distilled water for few minutes. Serial dilutions (1:10) were prepared in sterile distilled water and allowed to settle for some time. Plant material (leaves, petals and stem pieces) were also collected and examined immediately after washing different organs with sterile distilled water separately. Faecal samples were obtained from buffalo, cow, goat, horse, donkey, mule and humans. For each animal, samples were collected from fresh faeces at different localities around Faisalabad. All faecal samples were placed in separate sterile Petri plates in a refrigerator and processed within 4 hours. One gram of faecal sample was suspended in 10 ml of sterile phosphate buffer (pH 7.2). The buffer comprised 1.25 ml of solution A [3.4 g KH_2PO_4 + 50 ml reagent grade water (pH 7.2 with 1N NaOH) and diluted to 100 ml with reagent grade water] and 5.0 ml of solution B [3.8 g $MgCl_2$ + 100 ml reagent grade water diluted to one litre with reagent grade water]. Serial dilutions were prepared in the same buffer.

Direct isolation: Plant samples collected from various sources were spread across the surface of nutrient agar plates. These plates were then incubated for 18 hours at 37ºC. After overnight growth, bacterial colonies were picked and maintained on nutrient agar slants for biochemical tests.

Membrane filtration (MF) method: Approximately 5 ml of water, soil and faecal samples were passed through millipore pre-filtration pad to remove dust particles. The pre-filtrate was then filtered through 0.45 µm-pore-size cellulose nitrate sterile membrane filter (MF). The membrane filter funnel and flask (filtration assembly fabricated locally) were autoclaved before each experiment.

Lactose fermentation test: Culture media containing lactose (a fermentable carbohydrate), and a dye/pH indicator (capable of detecting changes in pH occurring during growth), were used for differentiating the non-pathogenic coliforms (lactose positive) from the potentially pathogenic bacteria (lactose negative).

Selective and differential media: Three types of media were used for isolation and identification of *E. coli*, which included moderately selective and differential medium (MacConkey agar), differential medium (Eosin Methylene Blue or EMB agar), and selective medium (Endo agar).

Bacterial culture: The filter was placed on a plate containing 10 ml of MacConkey agar followed by incubation at 44ºC for 24 hours. The presumptive positive colonies of *E. coli* were again transferred on to MacConkey agar and incubated for another 18 hours at 37ºC. Two to three well-grown colonies were picked up randomly and streaked on the surface of EMB agar to obtain well-isolated single colony. Plates were incubated overnight at 37ºC and each single colony was subsequently isolated, maintained on nutrient agar slants. All these colonies were further confirmed by inoculating on the Endo agar plates by streak-plate method. These plates were incubated for 24 hours at 35ºC. Further confirmation was made through conventional biochemical tests (IMViC) that is Indole (I), Methyle red (M), Voges-Proskauer (Vi), and Citrate (C) as described by Stanier *et al.*, (1989) and were used primarily to distinguish between *Escherichia coli* and *Enterobacter aerogenes*.

Test Kit: Bactident® *E. coli* rapid identification kit (E.C Merck) was also used along with conventional identification methods based on series of biochemical tests. The principle of the test is based on the enzyme profile of the *E. coli*, particularly on the detection of the enzyme β -D-glucuronidase and tryptophanase (indole formation). One pack of test kit contained 50 strips, 50 reaction vessels and reagents required to perform 50 tests. Bacterial strain was suspended in a reaction cuvette containing 200 µl of deionized water. The Bactident® *E. coli* test strip was inserted in the reaction vessel and was incubated at 37ºC for 30-120 min. The presence of β-D-glucuronidase was assessed under a long wavelength (360 nm) UV lamp. One drop of KOVAC's reagent was added to the cuvette for detecting indole formation.

Results

Samples processed through standard membrane filtration technique (Fig. 1a-c) were tested through lactose fermentation test. The identification and differentiation of *E. coli* colonies on MacConkey agar is shown in Fig. 2a. Three types of colonies appeared on MacConeky agar that are bright pink colonies with red halo (*E. coli*), yellow colonies with red border and pinkish red colonies with yellow border, which indicated the presence of *Klebsiella and Enterobacter*, respectively in the test sample (Fig. 3).

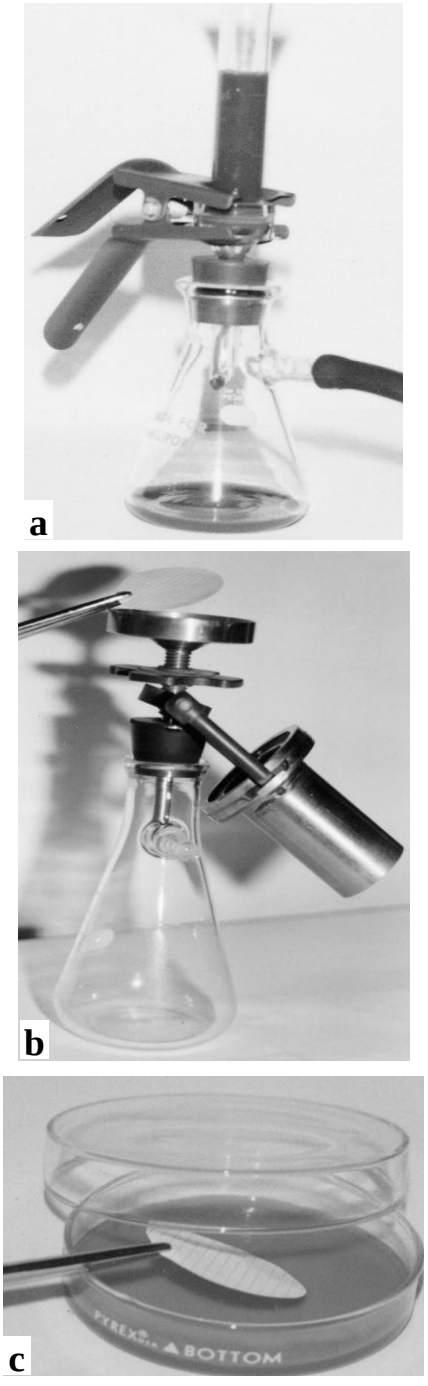

Fig. 1. Membrane filtration technique for the isolation of *E. coli* (a) pre-filtration of sample; (b) filtration; (c) membrane filter on MacConkey agar.

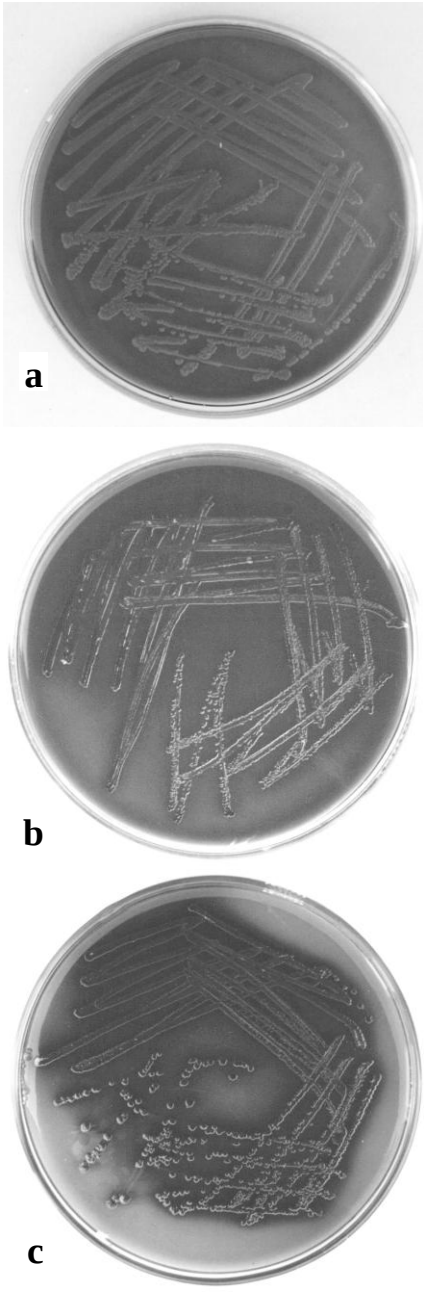

Fig. 2. *E. coli* colonies on (a) MacConkey agar; (b) EMB agar (c) Endo agar.

(*&**) Pink colonies were further confirmed through streaking on EMB agar and IMViC tests

Fig. 3. Differentiation of gram-negative bacilli based on lactose fermentation test.

Table 1. The IMViC reactions for distinguishing *E. coli* and *Enterobacter aerogenes*.

Strain	IMViC Tests			
	Indole	**Methyl red**	**Voges-proskauer**	**Citrate**
Escherichia coli	Dark red ring (+)	Magenta red colour (+)	Brown colour (-)	No visible growth (-)
Enterobacter aerogenes	No red ring (-)	Yellow colour (-)	Pink/crimson colour (+)	Visible growth (+)

As described by Stanier *et al.*, 1989.

On EMB agar, *E. coli* appeared as 2-3 mm diameter colonies with fluorescent blue-black color reflecting greenish metallic sheen when exposed to light (Fig. 2b) and a dark or black center in transmitted light, while *Enterobacter* appeared as 4-6 mm diameter colonies with gray-brown center (or sometimes black) without any metallic sheen in transmitted light. On Endo agar plates, presumptive *E. coli* appeared as red colonies with a permanent metallic sheen (Fig. 2c). Lactose-negative and weakly lactose-positive *E. coli* does not show any fuchsin sheen.

The results of IMViC reactions for distinguishing *E. coli* and *Enterobacter aerogenes* are shown in Table 1. A dark red colour in the amyl alcohol surface layer constituted a positive indole test and confirmed the presence of *E. coli*. In Methyl Red (MR) test, a magenta red color indicated the presence of *E. coli* (positive test) and yellow color its absence (negative test). The bacterial strains producing red color were referred to as MR positive (confirmed *E. coli*). Likewise strains producing a yellowish color were termed MR negative (non-*E. coli*) and were identified as *Enterobacter aerogenes*. The Voges-Proskauer (VP) reaction was a qualitative test performed to detect the presence of acetyl methyl carbinol: the end products of glucose fermentation. Since the bacterial strains that were tested did not produce a pink color in the presence of alkali, therefore, these were considered as VP negative (*E. coli* confirmed).

A total of 50 selected strains with diagnostic characteristics of *E. coli* were re-confirmed with the test kit by determining the profile of enzyme β-D-glucuronidase and tryptophanase (indole formation), which is characteristic of *E. coli*. The results show that 100% of the strains were positive for these enzymes. The presence of β-D-glucuronidase was indicated by the appearance of light blue fluorescence in UV light of long wavelength (360 nm) and indole formation was indicated by the appearance of red colour on addition of KOVAC's reagent (positive reaction for *E. coli*).

Table 2 shows the distribution pattern of 474 bacterial strains isolated from 90 samples collected from different sources including water channels, soil from irrigated fields, different types of plants and feces of animals and human. Among them, 424 *E. coli* strains were confirmed through manipulation of different media/method and biochemical methods described above. Of the 90 samples collected, 41 (45%) samples were from fecal matter of animals, from where about 159 *E. coli* strains were isolated (Table 3). Remaining samples were from soil (13), plants (16) and water (20), which helped in isolation of an additional 100, 50 and 115 *E. coli* strains, respectively. Of the 424 *E. coli*, 215 strains (37% of the total) were collected only from irrigation channels and soil from irrigated fields (Fig. 4) whereas the recovery rate from water, soil and plants was 27%, 24% and 12%, respectively.

Table 2. Bacterial strains isolated from various sources.

Source	Samples examined (n)	Strains (n)		Total
		E. coli	Non-*E. coli*	
Water	20	115	-	115
Soil	13	100	-	100
Plants	16	50	50	100
Animal feces	41	159	-	159
Total	**90**	**424**	**50**	**474**

Table 3. *Escherichia coli* isolated from faeces of different animals.

Source	Samples examined (n)	*E. coli* isolated (n)
Buffalo	9	27
Cow	5	25
Goat	7	25
Horse	3	15
Donkey	10	35
Mule	4	15
Human	3	17
Total	**41**	**159**

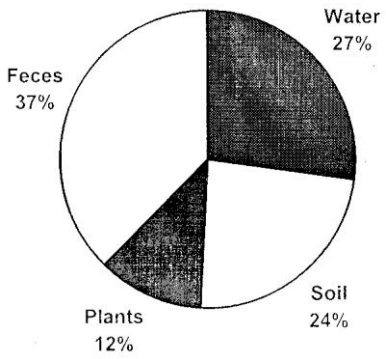

Fig. 4. The percent distribution of *E. coli* strains isolated from different sources.

Discussion

In the present study, membrane filtration (MF), culture media, biochemical methods and diagnostic kits were used for identification of *E. coli*. Lactose-fermenting intense pink colonies (presumptive *E. coli*) with red halo: a characteristics of *E. coli* (MacConkey. 1905) were observed on MacConkey agar. In addition to *E. coli*, other colonies such as *Enterobacter*, *Klebsiella* were also present that were eliminated in EBM agar as it provides favorable conditions for growth of *E. coli* and improves its proliferation in particular compared to other lactose-positive bacteria. Since, *E. coli* produced large amounts of acid from lactose and gave the colonies a very dark, metallic sheen, which *Klebsiella* and *Enterobacter* were lacking may be due to accumulation of less acid, and gave the colonies a purple center and pink periphery which helped

discriminating *E. coli* from the accompanying *Klebsiella* and *Enterobacter*. The growth of other Gram-positive microorganisms was inhibited by the dyes (Eosin and Methylene Blue) contained in EMB agar (Levine, 1921). Despite this, confusion can be created in identifying *E. coli* and *Enterobacter*, both of which appeared with dark/black and gray/brown (or gray/black), respectively upon viewing under transmitted light. Presumptive positive *E. coli* were therefore, confirmed again both culturally through the use of Endo agar and bio-chemically through IMViC tests.

On Endo agar plates (Endo, 1904), presumptive *E. coli* appeared as red colonies with a permanent metallic sheen. This is because Endo agar contains Sodium sulfite, which after reacting with aldehyde and acid (liberated by *E. coli* through metabolizing lactose) produced fuchsin-sulfite, which turns the colonies red. In the case of *E. coli*, this reaction is so intense that the fuchsin crystallizes out giving the colonies a permanent greenish metallic sheen (fuchsin sheen). Lactose-negative and weakly lactose-positive bacteria do not show any fuchsin sheen. The Indole test, which confirmed the presence of *E. coli* is based on the enzyme tryptophanase (indole formation), which is a characteristic of *E. coli* only, and is not found in *Enterobacter aerogenes* (Stanier *et al.*, 1989) and thus served as confirmatory test for *E. coli.* In Methyl-red test, the red color was an indication of substantial acid production: a characteristic of mixed acid type fermentation in *E. coli*. The purpose of using Methyl red was to use it as pH indicator to determine the pH of dextrose broth culture after 2-4 days' incubation. This indicator is yellow at pH 4.5 or higher (negative test) and red at lower pH values. Test is said to be positive when the accumulation of acidity is sufficient to turn the indicator red: a characteristic of only the *E. coli*.

In Voges-proskauer test, in the presence of alkali, the acetyl methyl carbinol is oxidized to di-acetyl, which in turn reacts with some constituents of the peptone to give it a pink color. When acetyl methyl carbinol is produced, the bacterial strain is said to be VP positive. Since *E. coli* lacks this ability hence it appeared negative.

"Bactident® *E. coli* rapid identification kit was used to compare the validity, reliability and cost-effectiveness of conventional screening procedures based on different agars and series of biochemical tests and to reconfirm the identified *E. coli*. Test kit that takes only 2-3 minutes to set up reaction and 30-120 minutes to produce results detected the presence of β-D-glucuronidase and tryptophanase (indole formation) in all the 50 strains selected for this test thus confirming them pure *E. coli* cultures. This comparative study showed that the selective and differential agars, used in the present study for the isolation and identification of *E. coli*, are economical and reliable.

Bactident® *E. coli* kit serves as a screening test for *E. coli* therefore, such kits are usually desirable in the clinical microbiological laboratory for rapid diagnosis. In contrast for screening of a large number of samples for culture collection, this kit is not economical because one kit, containing 50 strips and 50 reaction vessels, costs about US$ 135 which is enough for only a maximum of 50 tests. With differential and selective media, hundreds of isolates were screened for the identification of *E. coli*. Thus the use of kits for this purpose was not considered cost-effective. Compared to short shelf life of the kits, media used in the present study were technically simple and relatively inexpensive and were also devoid of problems related with shelf-life. The use of agars also reduced the cost; e.g. 500 g of EMB, MacConkey and Endo agar (BBL) cost about US$ 80 each or less than that and can be used to perform hundreds of tests. Moreover compared to these expensive kits, the agars were within the range of most of the laboratories in

Pakistan and other countries like Pakistan, where such items suffer inadequate supply and/or storage facilities. In addition to these kits, Immuno-Magnetic Separation (IMS), visual immuno-precipitate assay (VIP) and visual immunoassay (VIA) are also being used in food testing, clinical, veterinary and environmental sciences (Chapman, 2000; Chapman *et al.*, 1994; Warburton, 1996; Warburton, 1997) and for isolation of pathogenic and non pathogenic bacteria from foods, vegetables, and dairy products (Warburton, 2001). Although these assays and kits are more sensitive, easy to use and rapid in the detection of *E. coli*, nevertheless, they are expensive and may not prove economical when used for testing large number of samples. Compared to this, membrane filtration method used in the present study is easier than isolating and identifying *E. coli* by diagnostic kits (Fig. 5) and is also cheaper. For example, ONPG Discs (BBL) for detecting lactose fermenters is commercially available and a pack of six discs costs US$ 278. Identification of *E. coli* by performing indole and glucuronidase test through "ColiScreen" kits (Hardy Diagnostics, online data) is a rapid and reliable method of *E. coli* identification. Nevertheless, one test kit costs about US$ 19, which is sufficient for only 20 tests and should be refrigerated on arrival. Sim Plates used for total coliforms and *E. coli* count are also very expensive and their shelf life is only six months (BioControl, online data).

The present study has been conducted stepwise and highlighted the importance of cost-effectiveness in identification of large number of samples. We believe that since this area is continuously developing and a universally accepted method is yet to be developed, hence media and methods used in the present study will encourage resource deficient laboratories especially in the third world countries, which are reluctant to undertake extensive microbiological tasks only due to inadequate and untimely supply of expensive diagnostic kits.

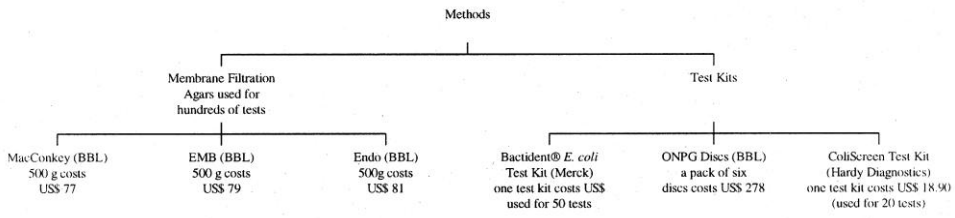

Fig. 5. Cost-effectiveness of methods used for identification of *E. coli*.

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(Received for publication 29 March 2004)