

Characterization and Differentiation of Some Clinical Isolates from Urine

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Abstract – *E. coli* is mostly a non-harmful bacterium which lives in the intestines of humans and animals. Some strains of this bacterium cause diseases to their hosts that require medical attention. Children are the most vulnerable group and urine samples from those attended the outpatient clinics at Mansoura University, Egypt were used to isolate *E. coli*. The seventeen isolates were subjected to primary identification by Gram staining, growth on MacConkey agar media, and then followed by biochemical identification using catalase test, indole, methyl red, Vogas-Proskauer, and citrate tests. Comparison of the protein profiles helped in the process of differentiation and characterization of the seventeen isolates. Some of the selected isolates were subjected to the three tests of coliform and *E. coli* test (Multiple-tube method (MPN), confirmed test and completed test). All the *E. coli* isolates were Gram-negative, short-rod, produced bright pink colonies on MacConkey agar, catalase positive, indole and methyl red positives, while, they were negatives to Vogas-Proskauer and citrate tests. After the protein profile, six isolates were selected for further studies. All six isolates indicated a positive MPN test, characteristic metallic sheen colonies on the Eosine Methylene Blue (EMB) agar within the confirmed test.

Keywords – *E. coli*, *Enterobacteriaceae*, urine, biochemical identification, and coliform test.

I. INTRODUCTION

The *Enterobacteriaceae* contains a large and diverse number of bacterial genera. All members of it are Gram-negative rods, which are found living freely in the environment and constitute a part of the microbiome of humans and animals. Member of this large family grow quickly under aerobic or anaerobic environments and are metabolically active. They are one of the most commonly urinary tract infections (UTIs), and a limited number of species also are important etiologic agents of diarrhea. Spread to the bloodstream causes Gram-negative endotoxic shock a dreaded and sometimes fatal complication (Ryan *et al*, 2010).

E. coli, generally referred to as Bacterium coli commune, was identified in (1885) by the German pediatrician, **Theodor Escherich**. It is a member of *Enterobacteriaceae*, which include many genera, including many known pathogens such as *Salmonella*, *Shigella*, and *Yersinia*. Although most strains of *E. coli* aren't pathogens, they will be opportunistic

pathogens that cause infections in immunocompromised hosts (Feng *et al*, 2002). One of the foremost notable features of *E. coli* is the broad diversity of disease-causing genotypes. The diseases can encompass different symptoms and gastrointestinal tract pathologies, but there also are diseases at extraintestinal sites. These different genotypes and their disease-causing abilities cause categories of *E. coli* often mentioned as pathotypes. There are six intestinal and two extraintestinal pathotypes currently recognized (Kaper *et al*, 2004).

Isolation and characterization of some clinical isolates from urine samples were achieved by classical bacteriological, biochemical and protein banding patterns. These simple techniques were sufficient for diagnosis of the local isolates and facilitated the treatments of associated illnesses.

II. MATERIALS AND METHODS:

Samples collection:

A total of seventeen urine specimens were collected from patient kids, at outpatient clinic, Faculty of Medicine Mansoura, University, Egypt.

Morphological characteristics:

Gram staining was performed to determine the type, shape, and arrangement of bacteria. A single colony of each isolate was fixed in a clean glass slide. The smear was flooded with crystal violet for one minute and leave to stand. The dye was washed off briefly with distilled water and excess water was drained. After being washed with water, the slide was covered with iodine for another minute and let to stand. The slide was decolorized with 95% Ethanol till no violet color comes off. The smear was carefully washed off with distilled water and stained with safranin for 30 seconds. The color was washed off with water and dried with a filter paper. After air drying, one drop of cedar oil was added to the smear and examined under oil immersion lens (Collee *et al.*, 1996).

Cultural characteristics of *E. coli* isolates:

All the samples were inoculated primarily into nutrient broth and incubated at 37°C for 12 h, then subcultured onto MacConkey agar plates by the streak plate method (Cheesbrough, 1981) to observe the colony morphology (shape, size, surface texture, edge and elevation, color, opacity, etc.).

Biochemical identification:

The isolates were subjected to the IMViC and catalase tests as representative of biochemical identification (Macfadden, 2000).

Catalase test:

A colony of each bacterial isolate was smeared on a clean glass slide, and then covered by a drop of 3% hydrogen peroxide. The results were scored as air bubbles formation (Chelikani *et al.*, 2004).

IMViC biochemical tests:

IMViC is a group of biochemical tests (Abdallah *et al.*, 2014; and Saadi Al-Baer and Hussein, 2017) detailed below.

Indole test:

A single colony of each bacterial isolate was grown shaking in 5 ml tryptone broth (contains per liter: tryptone 10 g, sodium chloride 8 g) at 37°C for 18-24 h. After incubation,

few drops of Kovac's reagent (dissolving 10 gm of p-dimethyl aminobenzaldehyde in 150 ml of isoamyl alcohol and then slowly adding 50 ml of concentrated hydrochloric acid) were added down the inner wall of the tube.

Methyl-red test:

A single colony was inoculated in 5 ml MR-VP broth media (contains per liter: Peptone 7 g, Dextrose 5 g, Potassium phosphate 5 g, and distilled water 1 L) and incubated at 35°C for 48-72 h. After incubation, 3-4 drops of methyl red reagent were added. The appearance of red color reveals a positive result; a yellow color reveals a negative result.

Voges-Proskauer test:

A single colony was inoculated in MR-VP broth media and incubated at 37°C, and after 24 h incubation, a few drops of 15% alpha – naphthol in alcohol was added, then drops of 40% KOH was also added followed with shaken gently. The formation of a red color within 15 min. indicates a positive result but if the color didn't change indicates a negative result.

Citrate utilization test:

A single colony was streaked onto a simmon-citrate agar (contains per liter; Ammonium Dihydrogen Phosphate 1 g Dipotassium Phosphate 1 g, Sodium chloride 5 g, Sodium Citrate 2 g, Magnesium Sulphate 0.2 g, Bromothymol blue 0.08 g, Agar 15 g, and Distilled water 1 L) and then incubated for 24 to 48 h at 37°C. if there is a blue color, this reveals a positive result and if not, it indicates a negative one.

SDS-PAGE

The seventeen *E. coli* isolates were inoculated in LB broth (contains per liter: 10g NaCl, 10g Peptone, and 5g Yeast Extract) at 37°C, 150 rpm for 12 h. One milliliter of each *E. coli* cultures were centrifuged at 13000 rpm for 10 min. A 50 µl of cell suspension was mixed with an equal volume of 2 x treatment buffer (0.125M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% β-M.E.). Cellular proteins were totally dissolved and denatured by boiling the samples for 3 min and transferred immediately to an ice-water bath. SDS-PAGE was performed, as described by, with 4% stacking on top of 12% separating gel. The protein samples were mixed with 2-3 µl of loading dye (Bromophenol blue) then loaded into the wells and therefore the protein standard marker was loaded into a separate well The electrophoresis was run at 80 volts until protein samples emerged from stacking gel, then the voltage was elevated to 100 volts until the loading dye was on the brink of escape rock bottom of the gel. the facility supply was turned off and therefore the glass plate sandwich was detached, the gel was carefully placed into the staining

solution and kept shaken (40rpm) for a minimum of 6 h, then the gel was far away from the staining solution, washed within the destaining solution I for a minimum of 2 h and transferred to destaining solution II for 1 h. At now, protein bands are often visualized by naked eyes under light (Laemmli, 1970).

E. coli Coliform diagnosis tests:

The chosen isolates from the protein profile were further examined to ensure the identity of *E. coli* by three Coliform diagnostic tests (Multiple-tube method, confirmed and completed tests) (Bumadian, 2013) as follows:

Presumptive test

Double strength and single strength MacConkey broth were prepared. The double strength medium was distributed in 3 tubes (5mL in each tube) and single strength medium in 7 tubes (5 mL in each tube) and Durham tubes were added in each tube in an inverted position. five ml of bacterial isolates were inoculated in the 3 tubes of double strength media, while one ml, and 0.1 ml of were inoculated each into three tubes of the single strength ones and one tube left as control. All tubes were incubated at 37°C for 24-48 h. After incubation for twenty-four h, each tube was examined for acid and gas production (Alexander, 1983).

Confirmed test:

Ten µl from the positive presumptive tubes were transferred and spread on Eosine Methylene Blue (EMB) agar (contains per liter: Peptone 10 g, Lactose 5 g, Sucrose 5 g, Dipotassium phosphate 2 g, Eosin Y 0.4 g, Methylene blue 0.065 g, Agar 13.5 g, and Distilled water 1 L) and incubated at 37°C for 24-48 hours.

III. RESULTS AND DISCUSSIONS:

Morphological characteristics:

Gram stain is made from organisms on the MacConkey agar. All bacterial isolates were Gram-negative short rod as shown in Fig.1. (Kravitz *et al.*, 1999).



Fig.1. Gram staining for two of the bacterial isolates no. (4 and 9). The two isolates were G-ve, short rod.

Cultural characteristics of E. coli isolates:

All *E. coli* colonies grown onto MacConkey agar fermented lactose and appeared pink in color (Fig.2). These cultural characteristic of *E. coli* agreed with the description of such bacteria (Mohammed and Abd AL-Muhsin AL-Khyat, 2008). Also, Van Belkum *et al.*, (2012) stated that rapid identification of *E. coli* and processing of *Enterobacteriaceae*, colonies were first examined for their abilities to ferment lactose (direct examination on MacConkey agar).

Biochemical tests:

Bacterial isolates suspected to be *E. coli* according to morphological characteristics were subjected to the related biochemical tests. Results illustrated in Table (1). Results showed that all the seventeen isolates were catalase, indole, and methyl red positive, but citrate and Vogas-Proskauer negative. Such characteristics are distinct to *E. coli* (Kithar *et al.*, 2016) and Forbes *et al.*, 2007).

Table (1) biochemical tests for E. coli isolates.

Bacterial isolates	Indole test	MR test	VP test	Citrate utilization	Catalase test
1	+	+	-	-	+
2	+	+	-	-	+
3	+	+	-	-	+
4	+	+	-	-	+
5	+	+	-	-	+
6	+	+	-	-	+
7	+	+	-	-	+
8	+	+	-	-	+
9	+	+	-	-	+
10	+	+	-	-	+

11	+	+	-	-	+
12	+	+	-	-	+
13	+	+	-	-	+
14	+	+	-	-	+
15	+	+	-	-	+
16	+	+	-	-	+
17	+	+	-	-	+

Catalase test

All bacterial isolates were catalase positive due to the production of air bubbles by the addition of hydrogen peroxide (Fig.2.) Two catalase enzymes are found in *E. coli* which are, hydroperoxidase I (HPI) and HPII, which catalyze the degradation of peroxide to water and oxygen (Loewen *et al.*, 1985).

IMViC biochemical tests:

All isolates showed a pattern of biochemical reactions typical for *E. coli* (Cheesbrough, 2000).

Indole test:

This test was performed to recognize the capability of *E. coli* isolates for indole production. The degradation of tryptone produces indole, pyruvic acid, ammonia, and energy. The presence of indole can be detected by the addition of Kovac's reagent into the growth culture. All *E. coli* isolates produced indole which reacted with Kovac's reagent and gave a red ring at the top of the tube (Fig.2.) (Abdallah *et al.*, 2014; and Saadi Al-Baer and Hussein, 2017).

Methyl- red test:

This test indicated MR color change upon pH Production of acids shifted PH down to four at which MR turned to red. *E. coli* is a mixed acid fermenter where the end products were a mixture of fermentation acids. All the 17 tested isolates gave a positive result by converting the color of the tube into the red (Fig.2.) (Abdallah *et al.*, 2014, and Saadi Al-Baer and Hussein, 2017).

Vogas-Proskauer test:

This test was done to detect the production of 2,3-butanediol in the medium from glucose fermentation. The addition of 40% and 50% solution of α - naphthol in absolute ethanol (Barritt's reagent) indicated the presence of acetoin which is a precursor in the synthesis of 2,3- butanediol. In case of all tested isolates, no red color developed which indicated a negative result (Fig.2.) (Abdallah *et al.*, 2014; and Saadi Al-Baer and Hussein, 2017).

The citrate test:

This test was done to find out the capacity of selected bacteria to consume citrate as the carbon source. This ability based on the presence of citrate permease which simplifies the transference of citrate into the bacterium. Inside the bacterium cell, citrate is converted to pyruvic acid and carbon dioxide. The carbon dioxide would react with sodium in Simmon's citrate agar and water to produce sodium carbonate. Sodium carbonate is an alkaline product so that, it raised the pH and converted the pH indicator to a blue color. All tested isolates the color of the media stayed green which was a negative result (Fig.2.) (Abdallah *et al.*, 2014; and Saadi Al-Baer and Hussein, 2017).

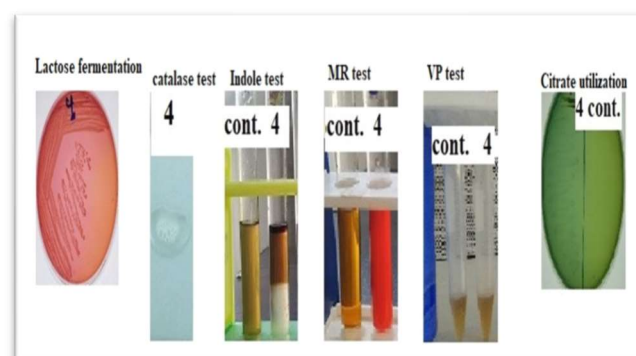


Fig.2. Biochemical positive results of a representative isolate (no.4). Isolate 4 was positive on MacConkey, indole, methyl red, and catalase. However, it produced negative result with Vogas-Proskauer and citrate utilization tests.

SDS-PAGE:

Significant differences between the total cellular protein patterns of *E. coli* isolates. Few cell types showed distinctive proteins that did not appear in the other strains as shown in Fig.3(a) and (b). Depending on protein profile 6 isolates (4, 9, 10, 11, 14, and 17) were chosen for upcoming work because they showed the presence of bands of molecular mass 45.8, 49.66, 52.44, 58.776, and 63 KDa and were found in 17, 9, 11, (4, 14), and 10 *E. coli* isolates, respectively as shown in Fig.3. (a) and (b).

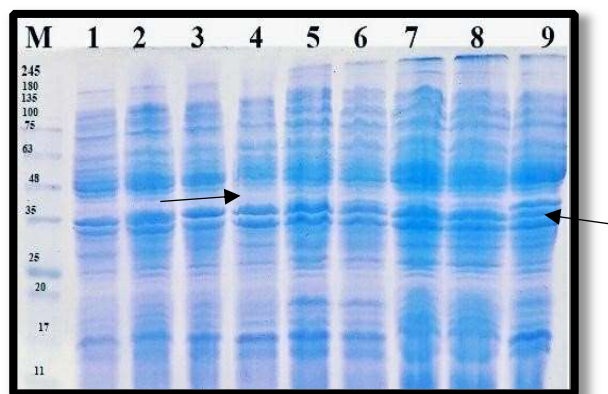


Fig.3(a). The protein profile for some *E. coli* isolates. Lane M: molecular weight marker, lanes from 1 to 9 contain the profiles of isolates 1 to 9, respectively.

Fig.3(b). The protein profile for some *E. coli* isolates. Lane M: molecular weight marker, lanes from 10 to 17 contain the profiles of isolates 10 to 17, respectively. Lane S: standard *E. coli* ATCC 10536.

E. coli Coliform diagnosis tests:

Results of Coliform tests were illustrated in **Table (2)**. Results showed that all the selected six isolates were presumptive, confirmed tests positive and G-ve, short rod.

Table (2) Coliform diagnostics tests:

Bacterial isolates	Presumptive test	Confirmed test	Completed test
4	+	+	G-ve, short rod.
9	+	+	G-ve, short rod.
10	+	+	G-ve, short rod.
11	+	+	G-ve, short rod.
14	+	+	G-ve, short rod.
17	+	+	G-ve, short rod.

Presumptive test:

MacConkey broth is both a selective and differential medium. The differential ingredient in the MacConkey medium is lactose. *E. coli* could produce lactase enzymes that ferment lactose and produce acid which will lower the pH of the media resulting in the change of the pH indicator (phenol red) color to yellow when lactose was fermented. Carbon

dioxide was produced as a by-product of the fermentation process and accumulated at the top of the Durham's tube. All six isolates gave a positive presumptive test of yellow color and accumulation of gas at the top of Durham's tubes (**Fig.4**) (Ahmed *et al.*, 2013).

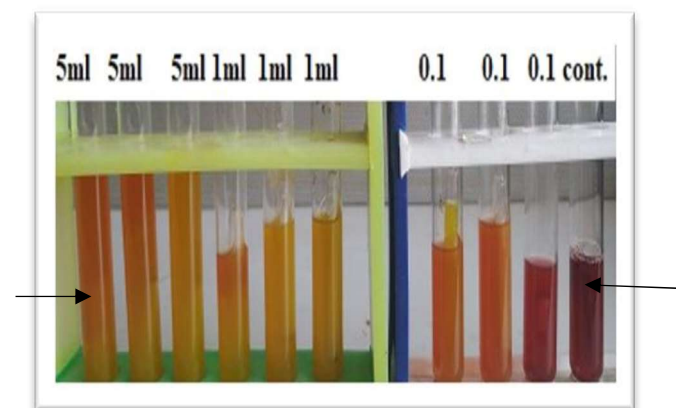
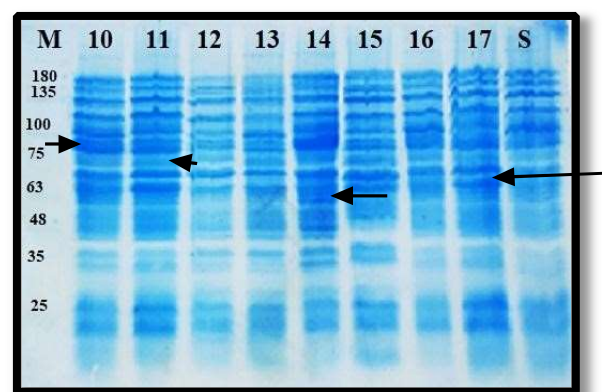


Fig.4. The presumptive test of the *E. coli* isolate no. 4. Color changed to yellow is an indication of acid production and gas collected in Durham's tube suggested growth of *E. coli*.

Confirmed test:

The methylene blue in EMB agar inhibited Gram positive organisms and allowed the Gram negative coliforms to grow. *E. coli* could be distinguished from others by the size and color of the colonies. *E. coli* colonies were small and had a green metallic sheen. All the six bacteria isolates gave green



colonies with metallic sheen (**Fig.5**) (Kravitz *et al.*, 1999).

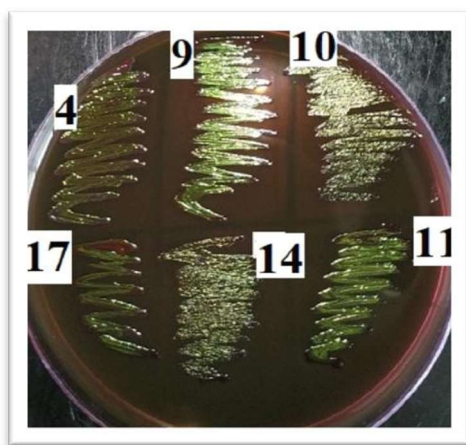


Fig.5. *E. coli* isolates showing green metallic sheen on Eosin-methylene Blue agar.

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