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# Isolation and Detection of *Escherichia coli* O157 From Potable Water System of Lahore, Pakistan

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**Abstract.-** This study was conducted to carry out the qualitative analysis of drinking water system of Lahore, Pakistan to detect the presence of *Escherichia coli* O157. Potable water samples were collected from different localities and analyzed by the most probable number tests (MPN). Initially, 42 strains that showed morphological resemble with *E. coli* were characterized by growing on Eosine Methylene Blue (EMB) agar. Finally, 13 *E. coli* strains that were non-sorbitol fermenters were selected and their taxonomic status was confirmed by using RapID™ ONE identification system. Antibiotic sensitivity profiling of selected bacterial strains was also analyzed and majority were found to be resistant against ampicillin, erythromycin and carbenicillin. All sorbitol negative and biochmically identified *E. coli* strains were then subjected to serological testing using Prolex™ Latex agglutination kit for detection of somatic antigen *i.e.*, O157. Positive agglutination was recorded in all sorbitol non-fermenting strains except S7d and S7e indicating the presence of this virulent strain in drinking water supply system. Analysis of biofilm formation revealed that S7d was the most effective either as single or in co-cultures (S2a+S7d) in the presence or absence of $FeSO_4$. In conclusion, the detection of *E. coli* O157 from drinking water suggests a possible health threat in terms of diarrheal diseases and renal syndromes to the local community.

**Key words:** *Escherichia coli* O157, Most probable number, Non-sorbitol fermenters, Antibiotic sensitivity pattern, Biofilm formation

## INTRODUCTION

Potable or drinking water is defined as having acceptable quality in terms of its physical, chemical and bacteriological parameters so that it can be safely used for drinking and cooking. However, safe drinking water still remains inaccessible for about 1.2 billion people in the world.

Water is normally disinfected before being distributed to the different end point users and the microbial levels of the water, when leaving the treatment plant, have to be within limits. By the time it reaches the tap in the house of the consumer, water quality may differ dramatically from the quality at the time of treatment. Human illnesses associated with water and food-borne pathogens are as old as the universe. Globally, waves of water and food borne diseases outbreaks have been reported (Reynolds *et al*., 2008; Wood *et al*., 2010).

There are a variety of bacteria, parasites and viruses which can cause health problems when humans ingest them in drinking water. Testing water for each of these germs would be difficult and expensive. Instead, water quality and public health workers measure for the presence of bacteria in drinking water using coliform bacteria as an indicator. The presence of any coliform in drinking water suggests that there may be other disease-causing agents in water (Cappuccino and Sherman, 2002). *E. coli* has been suggested as an indicator of fecal pollution as it can be easily distinguished from the other members of coliforms. To date, several strains of pathogenic *E. coli* have been reported universally, most of which have caused water and food borne disease outbreaks (Rompré *et al*., 2002; Sharma *et al*., 2003; Boubetra *et al*., 2011).

The pathogenic *E. coli* strain include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC) of which *E. coli* O157 is a member. Healthy domestic animals, in particular ruminants like cattle, sheep and goats are regarded as the principal reservoir of *E. coli* O157. *E. coli* O157 is now recognized as an important human pathogen that cuased infection ranging from self-limited watery

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diarrhea to life threating manifestations such as hemolytic uremic syndrome (Padhye and Doyle, 1992).

The main objective of this research work was to carry out the qualitative analysis of drinking water system of Lahore, Pakistan in terms of total coliforms that may indicate the suitability of water for consumption. Recently, a few studies reported the presence of coliforms in potable water supply system of Lahore (Qazi *et al*., 2006; Sulehria *et al*., 2012). However, very little is known about the presence of enterohemorrhagic *E. coli* (EHEC) O157 and its antibiotic susceptibility pattern. Therefore, in present study, the isolation and EHEC was targeted from the water samples collected from different localities of Lahore.

## MATERIALS AND METHODS

*Sample collection*

Potable water samples were collected from distribution system of different areas of Lahore, Pakistan. Before sample collection, tap nozzle was sterilized with 70% ethanol and sufficient time was given for adequate flushing of the pipelines. Sample collection was done in sterile borosilicate bottles of appropriate volume by holding it within the stream of water. The sampling bottles were labeled and sealed with paper tape and kept in an ice box. The samples were transported to the laboratory for testing within 2 h.

*Water analysis*

The three basic tests to detect coliform bacteria in water *i.e.* presumptive, confirmed and completed were performed sequentially for each water sample by means of the most probable number tests (MPN). Measured aliquots of the water to be tested were added to a lactose broth (LB) in double or single strength with slight modifications as mentioned in Cappuccino Sherman (2002). These tests detect the presence of coliforms, Gram negative, non-spore forming bacilli that ferment lactose broth with the production of acid and gas that is detectable following a 24 h incubation period at 37$^{o}$C. After the presumptive analysis, 42 bacterial strains that showed close resemblance with *E. coli* were selected and analyzed for colony and cell morphology. Strains that differ in morphology were selected and streaked on their respective medium such as Eosin Methylene Blue Agar (EMB) or nutrient agar. Finally, 13 bacterial strains that were sorbitol non-fermenting *E. coli* were selected for further study.

*Biochemical identification of* E. coli

Strains of *E. coli* were identified by using RapID™ ONE identification system (Remel). RapID™ ONE System is a qualitative micromethod employing conventional and chromogenic substrates for the manual identification of medically important *Enterobacteriaceae* and other selected oxidase-negative, Gram-negative bacilli. This system is comprised of RapID™ ONE panels and reagents. Each RapID™ ONE panel has 18 different reaction cavities molded in the plastic tray, containing 19 biochemical tests. A suspension of the test organism in RapID™ inoculation fluid was used to inoculate the panel according to manufacturer's instruction. After incubation of the panel, each test cavity is examined for reactivity by noting the development of the color. In some cases, reagents were added to the test cavities to provide a color change. The resulting pattern of positive and negative test scores was used as the basis for the identification of strains by computer-generated code compendium.

*Antibiotic profiling of coliforms*

This assay was performed with 13 strains that showed their affiliations with *E. coli*. Antimicrobial susceptibility test discs (Bioanalyse Co., Ltd., Turkey) were used to evaluate the sensitivity pattern of strains. Four different antibiotic discs of specified concentrations were used namely ampicillin (10 µg), erythromycin (15 µg), oxytetracycline (30 µg) and carbenicillin (100 µg). This assay allows rapid determination of the efficacy of this drug against microorganisms isolated from different environmental or clinical samples. Muller-Hinton agar was heavily inoculated with bacterial inoculum by means of a cotton swab to ensure the confluent growth of microorganisms. The antibiotic discs were aseptically applied to the surface of the agar plate at well spaced intervals. The plates were then incubated at 37$^{o}$C for 24 h. After incubation, plates were examined for the presence of growth of

inhibition for each disc. The susceptibility of strains to specific drug was determined by the size of its zones of inhibition in millimeters and the diameter of each zone was then compared with the standardized chart (Cappuccino and Sherman, 2002).

*Enrichment of* E. coli *O157*

Sorbitol MacConkey medium is a variant of MacConkey agar in which lactose is replaced by sucrose. Differences in sugar fermentation are used to differentiate *E. coli* O157 from other coliforms. It is based on the formula by Rappaport and Henig (1952). Originally developed for isolating EPEC serotypes, this medium is recommended for the isolation and differentiation of EHEC O157. Pink color colonies indicate positive sorbitol fermentation while colorless colonies were considered to be of non-sorbitol fermenters. All 13 identified strains of *E. coli* were streaked on sorbitol MacConkey agar and examined for growth at 37°C after 24 h.

*Prolex™ latex agglutination test*

Sorbitol non-fermenting *E. coli* strains were further tested for the presence of somatic antigen *i.e.* O157 using Prolex™ Latex agglutination kit. The test involves mixing the suspected organisms with the antiserum containing *E. coli* O157 antibodies. The bacteria will agglutinate (clump) in the presence of homologous antiserum. This kit contained *E. coli* Latex reagent (blue polystyrene latex particles coated with an antibody against *E. coli* O157 somatic antigen.), *E. coli* O157 positive control (*E. coli* O157 antigen) and *E. coli* O157 negative control (latex particle purified with rabbit IgG that does not react with *E. coli* serogroup O157). Before running the test, quality control procedure was performed according to Manufacturer's instruction in order to ensure validity of the results. For test strain, an overnight fresh culture of test strain was used. Using a sterile pipette, 0.2 ml of normal saline was transferred into a 5 ml test tube. Then with the help of a sterile loop, sufficient colonies were picked up from the plate and suspended in saline in order to achieve tubidity equivalent to 2 McFarland standard. One drop of *E. coli* O157 latex reagent was placed on one of the test circle of the provided test card, onto which one drop of test suspension was added, which were then mixed with sterile mixing sticks. Test card was gently rocked and examined for agglutination for 2 min. Isolates that gave positive test result were then tested with negative control. Positive test isolate showing no agglutination with negative control were considered to be conclusively positive result. For further confirmation of positive test results, clump formation was observed under low power objective.

*Biofilm forming potential of* E. coli

The microtiter plate test of Stepanović *et al*. (2000) was modified to determine the biofilm forming potential of single (S2a, S2b, S6a, S7d, S7a) and mixed cultures (S2a+S6a, S2a+S6a, S2a+S7a). Biofilm formation was evaluated by growing *E. coli* strains in R2A broth in the absence and presence of 10 μg $l^{-I}$ $FeSO_4$. The wells of plate were filled with 200 μl of cell suspension adjusted to $10^7$ cells per ml in R2A broth or R2A+$FeSO_4$. To promote biofilm formation, the plates were incubated aerobically on a shaker at 150 rpm for 72 h at 37°C. After 72 h, the content of each well was discarded and washed three times with 250 μl of sterile distilled water to remove any non-adherent and weakly adherent bacteria. Negative controls were prepared by incubating the wells only with R2A broth and all treatments were performed in triplicate. The bacterial biofilms in the 96-well microtiter plates were fixed with 250 μl/well of 98% methanol for 15 min. Afterwards, the plates were emptied and allowed to dry and then fixed bacteria were stained for 5 min with 200 μl of crystal violet. Excessive stain was rinsed out by placing the plate under low-running tap water and then allow to air dry. Crytal violet bound to the adherent cells was resolubilized by 200 μl/well of 33% (vol/vol) glacial acetic acid. To measure the formation of biofilms, optical density of the solution was measured at 570 nm using microplate spectrophotometer (Epoch, BioTek).

*Statistical analysis*

For biofilm formation, data was subjected to analysis of variance (ANOVA) and means separated by using Duncan's multiple range test ($P = 0.05$).

## RESULTS

*Coliforms detected in potable water*

In presumptive test, following incubation at 37°C, all tubes were examined for positive gas production. Positive gas producing tubes from all three sets were counted to compare with MPN index for determination of total coliforms in 100 ml water sample. Results revealed that samples collected from Bagarian, Multan road, Burdwood road, Green town and Band road were contaminated with fecal coliforms that showed, respectively, 1600, 920, 540, 240 and 240 MNP per 100 ml (Fig. 1). All positive tubes from presumptive test were streaked on Eosin methylene blue (EMB) agar for the detection of coliform colonies especially *E. coli.* Positive confirmed samples that showed typical coliform colonies *i.e.* metallic green sheen colonies of *E. coli* on EMB agar were then tested individually for the production of acid and gas in MacConkey broth. A total of 42 *E. coli* strains were isolated and all were positive for gas and acid production. All positive strains from completed test were streaked on N-Agar for analysis of cell and colony morphology. Strains that showed morphological resemble with *E. coli* were characterized by growing on sorbitol MacConkey agar. Out of 42, 13 strains that were non-sorbitol fermenters were selected for further screening for O157 antigen and potential for biofilm formation.

*Taxonomic status of* E. coli

Finally, taxonomic status of *E. coli* was confirmed by using RapID™ ONE identification system (Remel). In this test the colors of the test substrates were changed after 4 h of incubation at 37°C. Biochemical analysis indicated that majority of the strains gave positive results for Ornithine Lysine, Sorbitol, ρ-Nitrophenyl-ß,D-galactoside and Indol. A few strains also showed variation for Urea, Aliphatic thiol, Glucose, Malonate and γ-glutamyl-ß-naphthylamide γ-glutamyl-ß-naphthylamide. However, for rest of the biochemical tests, none of the strains recorded positive result. The 7-digit microcode obtained from the scoring of positive tests before and after the addition of kit reagents, was checked in the electronic RapID™ compendium ERIC® (http://www.remel.com/ERIC/IdentificationSingle.aspx) for the identification of the test organisms. All the test strains showed more than 99% similarity with *E. coli* and the test results are given in the Table I.

*Antibiotic profiling of coliforms*

Antibiotic sensitivity pattern of *E. coli* was analyzed by disc diffusion method. All bacterial strains were resistant to erythromycin and carbenicillin (Table II). Similarly, majority of the strains were also resistant against ampicillin; nevertheless, S2a and S7a showed sensitivity with zone of inhibition 18 and 17 mm, respectively. For oxytetracyclin, all strains except S7h and S7d were sensitive.

*Screening for* E. coli O157

A total of 42 presumptively identified *E. coli* were screened for their ability to ferment sorbitol. On the basis of sorbitol fermentation, they were either characterized as sorbitol fermenters (SF) or sorbitol non-fermenters (SNF). Strains of *E. coli* that gave pink color growth on sorbitol MacConkey medium after 24 h of incubation were considered to be sorbitol fermenters while those who gave colorless growth were known to be non sorbitol fermenter. All strains except S2a, S2b, S2c, S7a, S7b, S7c, S7d, S7e, S7f, S7g, S7h, S7i and S7j were sorbitol fermenters. All biochemically characterized sorbitol negative *E. coli* strains were subjected to pre-enrichment in tryptic soy broth supplemented with novobiocin. Afterwards, each strain was plated on cefixime-tellurite sorbiotl MacConkey (CT-SMAC) medium. Appearance of colorless colonies on CT-SMAC was considered as presumptive positive test for *E. coli* O157. All presumptively positive *E. coli* O157 strains were then tested for the presence of somatic antigen *i.e.,* O157 by Prolex™ latex agglutination kit. When tested against Latex *E. coli* O157 reagent, all strains except S7d and S7e were agglutination positive. Strains giving positive agglutination results were then tested against latex *E. coli* O157 negative control which showed no clump formation (Fig. 2).

*Biofilm formation*

Biofilm forming potential of 5 selected bacterial strains (S2a, S2b, S6a, S7d, S7a) was

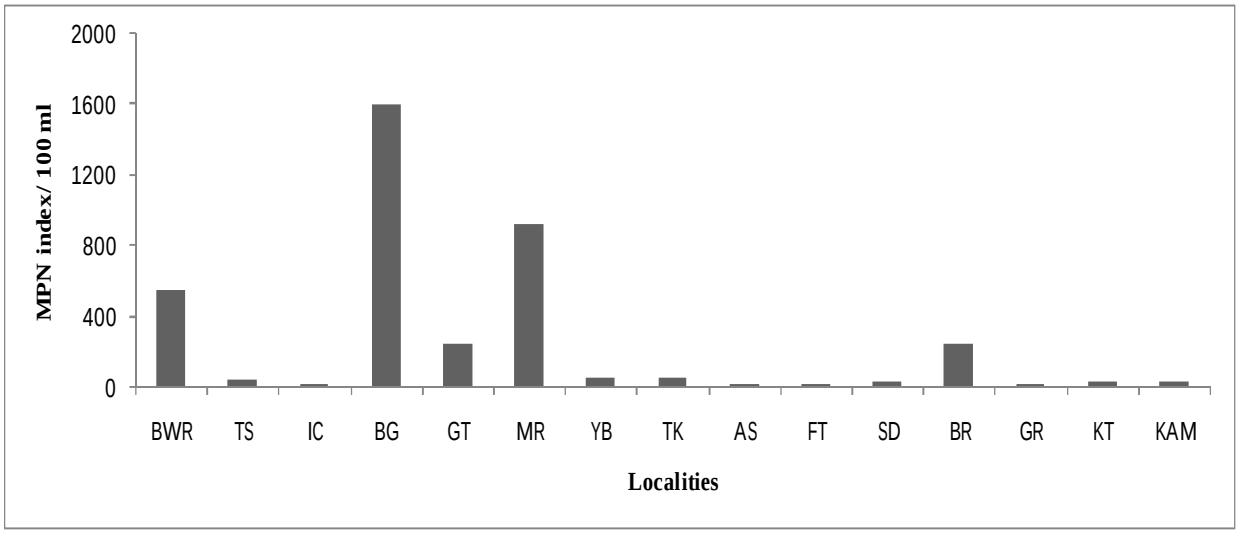


Fig. 1. MPN index of coliform bacteria per 100 ml of water sample from different localities of Lahore, Pakistan. Affiliations: BWR, Burdwood Road; TS, Township; IC, Ichara; BG, Bagariyan; GT, Green Town; MR; Multan Road; YB, Yohannabad; TK, Thokar; AS, Amar Sadu; FT, Faisal Town; SD, Shadman; BR, Band Road; GR, Ghazi Road; KT, Khokar Town; KAM, Kot Abdul Malik

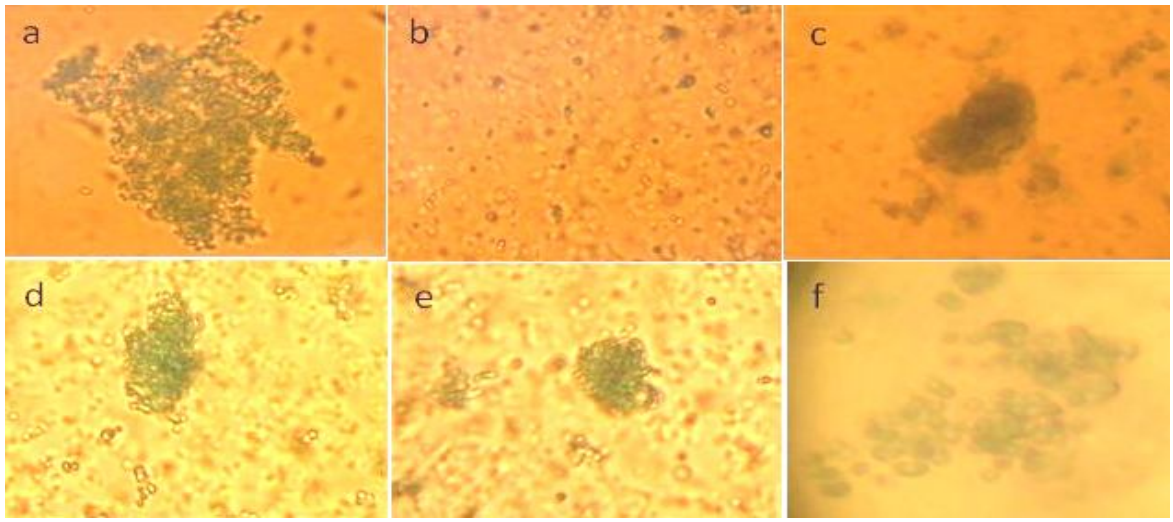


Fig. 2. Prolex$^{TM}$ latex agglutination test confirmed by microscope. (a) Positive control; (b) Negative control; (c) S7c; (d) S7j; (e) S7a; (f) S7g and (g) S7c.

analyzed in terms of monoculture and co-cultures in the absence and presence of $FeSO_4$. In single cultures, S7d was found to be the most potent biofilm producer, while remaining bacterial strains showed growth trend in the following order S6a> S2a> S7a> S6a (Fig. 3). In co-cultures, S2a+S7d combination was the most effective in biofilm formation that showed statistically similar response in the presence and absence of $FeSO_4$. However, other two combinations of mixed culture showed similar response as compared to control.

## DISCUSSION

In present study, potable water samples were collected from 15 different localities of Lahore,

**Table I.- Identification of *E. coli* by RapID$^{TM}$ ONE identification system.**

| Tests | Strains | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **S2a** | **S2b** | **S2c** | **S7a** | **S7b** | **S7c** | **S7d** | **S7e** | **S7f** | **S7g** | **S7h** | **S7i** | **S7j** |
| URE | + | - | - | - | - | - | - | - | + | - | - | - | - |
| ADH | + | - | + | + | - | - | + | + | + | - | - | - | - |
| ODC | + | + | + | + | + | + | + | + | + | + | - | + | + |
| LDC | - | + | - | - | + | + | + | + | - | + | + | + | + |
| TET | - | - | - | - | - | - | - | - | - | - | + | - | - |
| LIP | - | - | - | - | - | - | - | - | - | - | - | - | - |
| KSF | - | - | - | - | - | - | - | - | - | - | - | - | - |
| SBL | + | + | + | + | + | + | + | + | + | + | - | + | + |
| GUR | - | - | - | - | - | - | + | + | - | - | + | - | - |
| ONPG | + | + | + | + | + | + | + | + | + | - | - | + | + |
| GLU | - | - | - | - | - | - | - | - | - | - | + | - | - |
| XYL | - | - | - | - | - | - | - | - | - | - | - | - | - |
| NAG | - | - | - | - | - | - | - | - | - | - | - | - | - |
| MAL | - | - | - | - | - | + | - | - | - | - | + | - | - |
| PRO | - | - | - | - | - | - | - | - | - | - | - | - | - |
| GGT | + | - | - | - | - | - | - | - | + | - | + | - | - |
| PYR | - | - | - | - | - | - | - | - | - | - | - | - | - |
| ADON | - | - | - | - | - | - | - | - | - | - | - | - | - |
| IND | + | + | + | + | + | + | + | + | + | + | + | + | + |
| RapID$^{TM}$ Code | 6121001 | 4121001 | 6021001 | 6021001 | 4121001 | 4121201 | 6161001 | 6161001 | 6121001 | 4120001 | 4121111 | 4121001 | 4121001 |
| Similarity (>99%) | | | | | | | *E. coli* | | | | | | |

Abbreviations: URE, Urea; ADH, Arginine; ODC, Ornithine; LDC, Lysine; TET, Aliphatic thiol; LIP, Fatty acid ester; KSF, Sugar aldehyde; SBL, Sorbitol; GUR, ρ-Nitrophenyl-ß,D-glucuronide; ONPG, ρ-Nitrophenyl-ß,D-galactoside; GUL, ρ-Nitrophenyl-ß,D-glucoside; XYL, ρ-Nitrophenyl-ß,D-xyloside; NAG, ρ-Nitrophenyl-n-acetyl-ß,D-glucosaminide; MAL, Malonate; PRO, Proline-ß-naphthylamide; GGT,γ-glutamyl-ß-naphthylamide; PRY, Pyrrolidonyl-ß-naphthylamide; ADON, Adonitol; IND, Trytophan

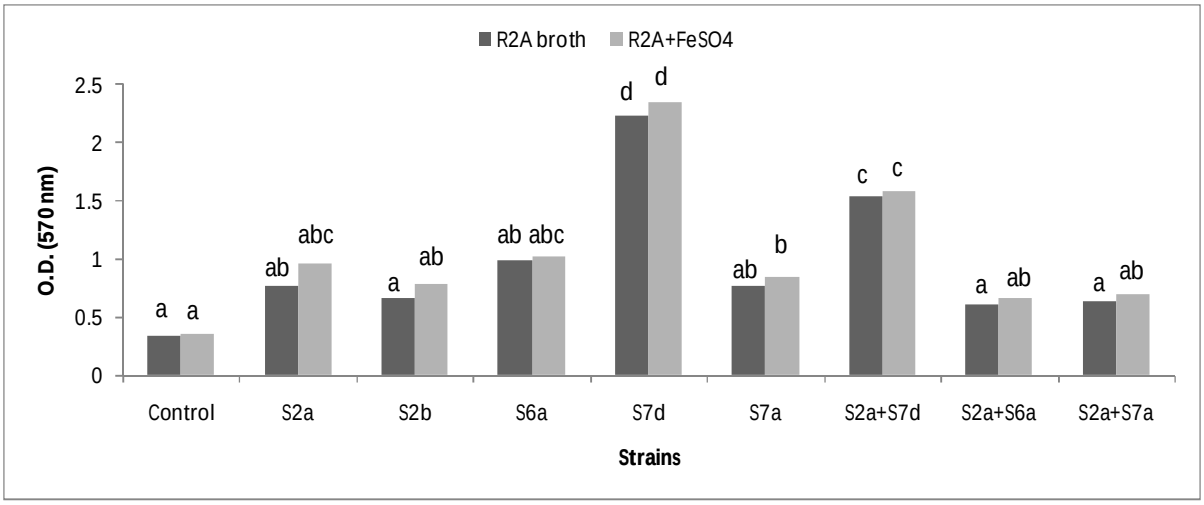


Fig. 3. Biofilm formation potential of different *E. coli* strains in the presence and absence of $FeSO_4$. Mean of three replicates. Different letters on bars indicate significant difference between treatments using Duncan's multiple range test (P = 0.05).

**Table II.- Antibiotic sensitivity pattern of different strains of *E. coli*.**

| Strains | Antibiotics | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ery (15µg) | | Amp (10µg) | | Oxy (30µg) | | Car (100µg) | |
| | Zone (mm) | Susceptibility | Zone (mm) | Susceptibility | Zone (mm) | Susceptibility | Zone (mm) | Susceptibility |
| S2a | 0 | R | 18 | S | 22 | S | 10 | R |
| S2b | 0 | R | 11 | R | 23 | S | 9 | R |
| S2c | 0 | R | 15 | R | 21 | S | 10 | R |
| S7a | 13 | R | 17 | S | 24 | S | 11 | R |
| S7b | 11 | R | 16 | R | 21 | S | 14 | R |
| S7c | 9 | R | 14 | R | 22 | S | 16 | R |
| S7d | 7 | R | 10 | R | 18 | R | 10 | R |
| S7e | 12 | R | 15 | R | 23 | S | 11 | R |
| S7f | 7 | R | 14 | R | 22 | S | 14 | R |
| S7g | 7 | R | 14 | R | 19 | S | 15 | R |
| S7h | 9 | R | 10 | R | 17 | R | 18 | R |
| S7i | 11 | R | 10 | R | 19 | S | 16 | R |
| S7j | 11 | R | 14 | R | 22 | S | 11 | R |

**Abbreviations:** Ery, Erythromycin; Amp, Ampicillin; Oxy, Oxytetracyclin; Car, Carbenicillin

Pakistan. These samples were analyzed by standard Most Probable Number (MPN) method which provides an indirect estimate of the number of microorganisms present in water. Out of 15, 11 water samples *i.e.*, 73% (Fig. 1) were positive for total coliforms (TC). Majority of the water samples were positive for the presence of typical coliform colonies especially *E. coli*. Finally, 13 strains that were non-sorbitol fermenters were selected for further screening for O157 antigen and potential for biofilm formation. Taxonomic status of selected strains was confirmed by using RapID™ ONE identification system (Table I). Bacterial drug resistance is a major problem in the treatment of infectious diseases around the world. In present study, antibiotic susceptibility pattern showed that majority of the strains were resistant against different drugs except oxytetracyclin (Table II). Similarly, Yilmaz *et al*. (2009) reported the resistance of *E. coli* isolated from clinical samples

against variety of antibiotics.

When tested against Latex *E. coli* O157 reagent, all strains except S7d and S7e were agglutination positive. Further confirmation was done by observing the Ag-Ab reaction under light microscope (Fig. 2). Serotypic analysis is the predominant mean by which pathogenic strains are differentiated. Immunoassays are based on antibody and antigen recognition. Antigens may be present in both viable and dead cells and cross-reaction with matrices may result in false-positive results. The latex test was found to be a simple, highly efficient and reliable test in detecting *E. coli* O157:H7 with 100% sensitivity and specificity (March and Ratnam, 1989). A specific combination of O and H antigens defines the "serotype" of an isolate. That can be associated reproducibly with certain clinical syndromes. According to the modified Kauffman scheme, *E. coli* was serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles (Nataro and Kaper, 1998).

Analysis of biofilm formation showed that in single cultures, S7d was found to be the most potent; whereas, in co-cultures, S2a+S7d combination was the most effective both in presence and absence of $FeSO_4$. The presence of iron corrosion products is reported to favor bacterial activity in drinking water networks, resulting in increases of both suspended and biofilm-associated bacteria (Niquette *et al.*, 2000; Appenzeller *et al.*, 2001). Finally, it can be concluded that isolation and detection of coliforms particularly those of fecal origin from potable water system showed their poor water quality with respect to the standard maximum contamination limits (MCL) set by WHO. High levels of fecal coliforms; especially, *E. coli* suggested sewage mixing with our distribution lines and inadequate disinfection treatment. In addition, detection of *E. coli* O157 from water suggests a possible health threat in terms of diarrheal diseases and renal syndromes to the local community. Biofilms are more resistant to disinfection than suspended microflora and frequently allows the re-growth of microorganism on arrival of favorable conditions. High levels of biofilm formation observed in the presence of iron suggested that these nutrients serves as limiting factors for coliform growth and should be eliminated from water system.

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