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ORIGINAL ARTICLE **OPEN ACCESS**

Microbial Studies of Pathogenic *Escherichia coli* from Coriander, Mint Leaves and Lettuce Collected From Different Localities of Lahore

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ABSTRACT
Salad vegetables are contaminated with pathogens that can cause food poisoning. Escherichia coli O157:H7 is one of them that cause several diseases in humans and animals. This study aimed to determine the presence of pathogenic E. coli O57:H7 on fresh vegetable salads. A total of 300 samples including 100 samples of coriander, 100 samples of mint leaves and 100 samples of lettuce were collected between November 2013 and April 2014, from different localities of Lahore. E. coli O157 was isolated from collected samples of salad vegetables by using different selective media such as MacCkonkey agar, Eosin Methylene Blue agar, Sorbitol MacConkey agar and EC medium modified with novobiocin. Presence of pathogenic E. coli O157:H7 was confirmed by using kit containing antiserum against this bacterial specie.Results indicate that surfaces of salad vegetables (coriander, lettuce and mint leaves) showed 100% contamination.It was found that even after first wash, this pathogenic bacterium remains present on the surface of salad vegetables. So, it is recommended to give two or three washes with purified water before eating.

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INTRODUCTION

Salad vegetables are key components of a healthy diet. Despite the nutritional and health benefits of fresh produce, fruit and vegetables are recently recognized as vehicles for foodborne illness in humans. The consumption of fresh produce has now been linked, both epidemiologically and microbiologically, to infectious intestinal disease. Contamination of fresh produce with enteric pathogens may occur in the field during production, harvesting, and post-harvest processing or at any point from farm to fork.
According to data from the Centers for Disease Control and Prevention in the US (CDC), foodborne illness due to contaminated vegetables is on the rise, with products implicated including baby spinach, lettuce, seed sprouts and green onion [1]. The majority of microorganisms associated with raw vegetables are non-pathogenic and gram negative organisms tend to dominate the bacterial population including Enterobacter spp. and other coliforms. [2]. At the field stage many possibilities for contamination exist in the environment, which include wildlife intrusion, animal manure, soil amendments, water, and cross-contamination from sanitized equipment or workers [3]. During the last two decades, food-borne outbreaks associated with fresh produce have rapidly increased [4]. Low sanitation standards were always reported in such outbreaks. The other potential hazards that increase the chance of food-borne diseases associated with fresh produce include applications of chemical fertilizers, hormones, pesticides, and their residues in food [5]. Minimally processed vegetables and fruits may be contaminated with food-borne pathogens (such as *Listeria monocytogenes*, *Salmonella* and *Escherichia coli* O157:H7) [6,7]. Plant roots are in constant contact with the soil and are often irrigated with polluted water [8]. *E. coli* strains can be differentiated using serological techniques based on three major cell surface antigens: O (somatic), H (flagella) and K (capsular) [9]. .*E. coli* O157:H7 has the ability to survive at refrigeration temperatures and under harsh environmental conditions. Some strains were reported to tolerate low pHs [10]. *E. coli* O157:H7 secretes a very powerful toxin called verotoxin (Vtx). The ability of these bacteria to secrete this toxin was identified in 1982. It binds to the receptors on human kidney, brain and gut cells bringing them to death [11]. *E. coli* O157:H7 cause acute hemorrhagic diarrhea with abdominal cramps that in its severe cases result in hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) and bloody diarrhea [12,13].

The aim of present research was to determine the presence of *E. coli* O157:H7 on the surface on coriander, mint leaves and lettuce.

MATERIALS AND METHODS

SAMPLE COLLECTION

Total 300 samples were collected from different localities of Lahore to test the presence of pathogenic *E. coli* bacteria. First group consists of 100 samples of coriander, second group consists of 100 samples of mint and third group consists of 100 samples of lettuce collected from November 2013 to April 2014.

MATERIALS

The following materials were utilized for the current research work: Nutrient agar (NA), Nutrient broth (NB), MacConkey agar, Eosine methylene blue agar (EMB), Sorbitol MacConkey agar medium (SMAC), EC medium modified with novobiocin (m EC), confirmation kit.(Media and confirmation kit was prepared by Lab M Ltd Company, Heywood, UK)

CHEMICALS

The chemicals used in research work were NaCl, distilled water, ethanol, crystal violet, safranin, iodine solution and acetone. (All chemicals were prepared by Merck (Pvt.) Ltd Company, Karachi, Pakistan).

CULTURING OF MICROBIAL PATHOGENS

PREPARATION OF NUTRIENT BROTH MEDIUM

3.25g of nutrient broth medium was added into the 250ml of distilled water and mixed well with stirrer. After complete mixing poured into the conical flask. Mouth of the conical flask was covered with a cotton plug and aluminum foil and autoclaved it at 15psi pressure and temperature of 121 ºC for at least 30 minutes.

PREPARATION OF NUTRIENT AGAR MEDIUM

6.8 g of nutrient agar were added in the 250ml of distilled water and mix these ingredients by using the stirrer. When the components of the media are completely dissolved, poured the media in the conical flask. Covered the mouth of the conical flask with cotton plug and aluminum foil and autoclaved it at 15psi pressure and temperature of 121 ºC for at least 30 minutes.

POURING OF MEDIA

Autoclaved nutrient agar media was poured into the sterile Petri plates by filling them less than half. After solidifying, sealed them with parafilm and placed as inverted in an incubator for 24 hours at 37 ºC.

INOCULATION OF LIQUID MEDIA

After 24 hours the collected samples of salad vegetables on were dipped into the nutrient broth by using the autoclaved forceps. And then covered the mouth of the test tubes again with the cotton plug and aluminum foil and placed them in the incubator-shaker for 16 - 18 hours at 37 ºC (120 rpm).

STREAKING OF PLATES

After 18 hours of incubation of test tubes, clear turbidity ensured the bacterial presence. After overnight incubation of poured plates, samples from the liquid media is inoculated into the pour plate by using the sterile inoculating loop is called streaking. Covered and sealed the plates with parafilm and label it. It was then placed in the incubator at 37 ºC for 16-18 hours.

DETECTION OF MICROBIAL PATHOGENS ON SELECTIVE MEDIA

PREPARATION OF MACCONKEY AGAR

50g of MacConkey agar was added into the one liter of distilled water in a beaker and mix the media with the help of stirrer. After the complete dissolve of media into the water, poured the media into the conical flask. Covered the mouth of the conical flask with cotton plug and aluminum foil and autoclaved it. Pouringand streaking is done and cultured plates were incubated at 37ºC for 24 hours.

PREPARATION OF EOSINE METHYLENE BLUE AGAR (EMB)

37.7g of EMB was added into the one litre of distilled water in a beaker and mix the media with the help of stirrer. After the complete dissolve of media into the water, poured the media into the conical flask. Covered the mouth of the conical flask with cotton plug and aluminum foil and autoclaved it. Pouringand streaking is done and cultured plates were incubated at 37ºC for 24 hours.

PREPARATION OF SORBITOL MACCONKEY AGAR MEDIUM

Suspend 50 g of the medium in one liter of purified water. After the complete mixing of media into water, poured the media into the conical flask. Covered the mouth of the conical flask with cotton plug and aluminum foil and autoclaved it for 15 minutes.Pouringand streaking is done and cultured plates were incubated at 37ºC for 24 hours.

EC MEDIA MODIFIED WITH NOVOBIOCIN

5g of novobiocin containing media was dissolved in 150ml of distilled water in a beaker. It was mixed on magnetic stirrer, autoclaved for 2-3 hours and then cooled at room temperature. 1ml of autoclaved media was poured in each eppendroff and was incubated for 16-18 hrs.

INOCULATION OF EC MEDIA MODIFIED WITH NOVOBIOCIN

10µl of previously prepared nutrient broth culture was added in each eppendrof having 1ml of novobiocin containing media. It was tightly sealed with Para film and was placed in the shaker at 37°C and at 150rpm (revolution per minutes) for overnight. The novobiocin containing media became turbid showing that the growth had been occurred. Pouring and streaking is done and cultured plates were incubated at 37ºC for 24 hours.

GLYCEROL STOCKS

After 18 hours of incubation of cultured test tubes, clear turbidity ensured the bacterial presence. For making glycerol stocks of these cultures put 0.7ml of bacterial culture in the sterile eppendrof and then add 0.3ml of sterile glycerol solution. Each eppendrof tube was covered with parafilm and stored at -20 ºC, for future research purposes.

GRAM STAINING OF PATHOGENIC BACTERIA

PROCEDURE OF GRAM STAINING

First the slide was washed with water and dried. Bacterial colony was picked with sterilized inoculating loop and placed on slide having a drop of water on it. Prepared smear of colony and passed it over flame to fix it. To smeared area add 2-3 drops of Crystal violet dye and waited for 1 minute. The primary stain renders all the bacteria uniformly violet. Rinse gently. Then 2-3 drops of mordantIodine solution were added and waited for 1minute. This results in formation of a dye-iodine complex in the cytoplasm. Rinse gently. Decolorize with ethyl alcohol or acetone. A mixture of acetone-ethyl alcohol (1:1) can also be used for decolorization. The process of decolorization is fairly quick and should not exceed 30 seconds for thin smears. Acetone is a potent decolorizer and when used alone can decolorize the smear in 2-3 seconds. A mixture of ethanol and acetone acts more slowly than pure acetone. After decolorization, the Gram-positive cell remains purple and the Gram-negative cell loses its purple color. Decolorization is the most crucial part of Gram staining and errors can occur here. Prolonged decolorization can lead to over-decolorized smear and a very short decolorization period may lead to under-decolorized smear. After the smear is decolorized, it is washed in water without any delay. 2-4 drops of Counter stain Safranin dye is added on it and waited for 1minute and rinse it with water. Allowed the smear to air dry and slides were observed under microscope.

USE OF KIT FOR CONFIRMATION OF *E. coli* O157

Materials Required: Standard microbiological supplies and equipment such as loops, pasteur pipettes, Saline, timer, other culture media, swabs, applicator sticks, incinerators, and incubators, etc.

Materials Supplied:

Prolex™ O157 Latex Reagent	Blue latex particles coated with purified rabbit IgG which react with *E. coli* serotype O157, 3.1ml
Prolex™ O157 Positive Control Suspension	*E. coli* serotype O157:H7 colonies grown on agar medium, harvested and inactivated, 1.5ml
Prolex™ O157 Negative Control Latex Reagent	Blue latex particles coated with purified rabbit IgG which does not react with *E. coli* serotype O157, 1.5ml
Latex Test Cards	Disposable white cards with 10 reaction circles
Mixing Sticks	Disposable wooden sticks

Procedure:

Allow reagents to come to room temperature for at least 10 minutes prior to use. One drop of sterile Saline was placed within the circle on the test card. 1 to 4 well-isolated colonies were selected from the Sobitol MacConkey Agar, Eosin Methylene Blue Agar, Tryptic Soy Agar and EC media. An emulsion of the colonies was created by mixing the Saline on the test card with mixing sticks. Mix the Latex Reagents, by inverting the tubes, prior to use. 1 drop of E. coliPRO™ O157 Latex Reagent was dispensed onto a test circle on the test card Mixed the Latex Reagent and the organism suspension with the wooden sticks provided, using the complete area of the circle. A new stick should be used for each reagent. Gently hand shaked the entire card, allowing the mixture to flow slowly over the ring area. For up to 2 minutes, under normal lighting conditions, observation was done for agglutination (strong clumping) of the latex particles. All organisms yielding a positive agglutination reaction should be retested with the Negative Control Latex Reagent. Steps 4-8 were repeated with the Negative Control Latex Reagent. A rapid and significantly strong clumping of the latex particles in under 2 minutes requires additional testing with the Negative Control Reagent to confirm that the observed agglutination reaction was specific for the *E. coli* O157 serotype. If the organism agglutinates with the Latex Test Reagent and fails to agglutinate with the Negative Control Latex Reagent, this indicates the identification of *E. coli* serotype O157. If weak clumping or a non-specific reaction (stringiness) is present in the test circle after 20 seconds, the test should be

repeated using a fresh subculture. If the same result is seen after retesting, biochemical testing should be used to identify the isolate. If the organism agglutinates with the Latex Test Reagent and also agglutinates with the Negative Control Latex Reagent, this is considered a false reaction due to auto agglutination or cross reaction of the strain.

RESULTS

Results indicate that surfaces of salad vegetables (coriander, lettuce and mint leaves)showed 100% contamination. Potential pathogens observed from these samples were: *E.coli*, *Salmonella, Shigella, Staphylococcus, Micrococcus and Streptococcus species* on nutrient agar medium. Presence of pathogenic bacteria *E.coli* 0157:H7was analyzed by using general, selective and differential methodsin the microbiology research laboratory located in zoology department of Lahore College for Women University.*E. coli* 0157:H7was observed in all 300 samples.

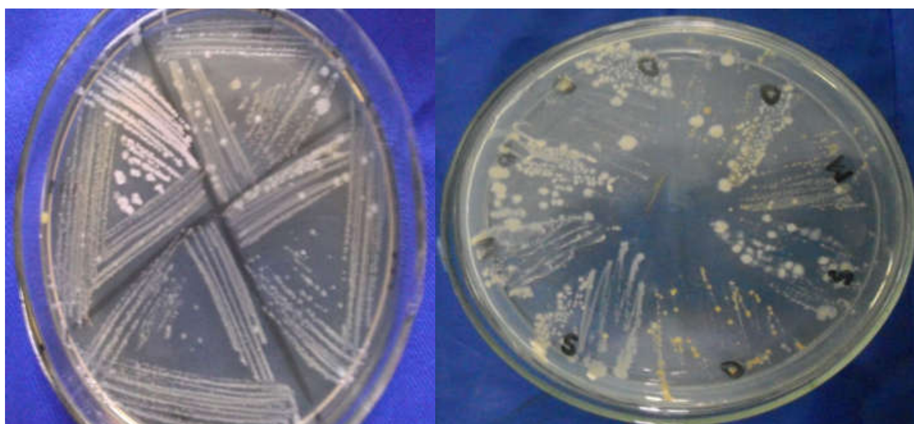

Fig 1: Growth of different pathogenic bacteria on Nutrient agar.

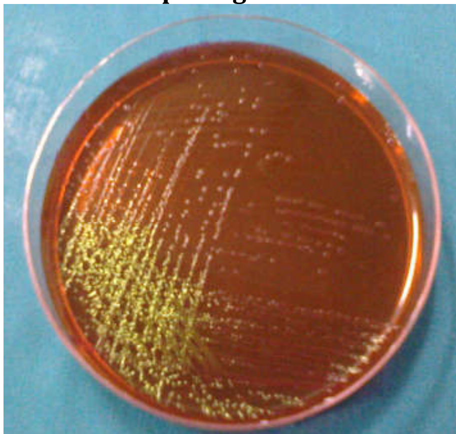

Fig 2: EMB media showing greenish golden colonies of lactose fermenting pathogenic *E. coli* while colorless colonies are of non-lactose fermenting *E. coli*.

Fig 3: Sorbitol MacConkey Agar plates showing growth of pathogenic *E.coli* O157:H7.

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CONFIRMATION OF *E. coli* O157 BY KIT

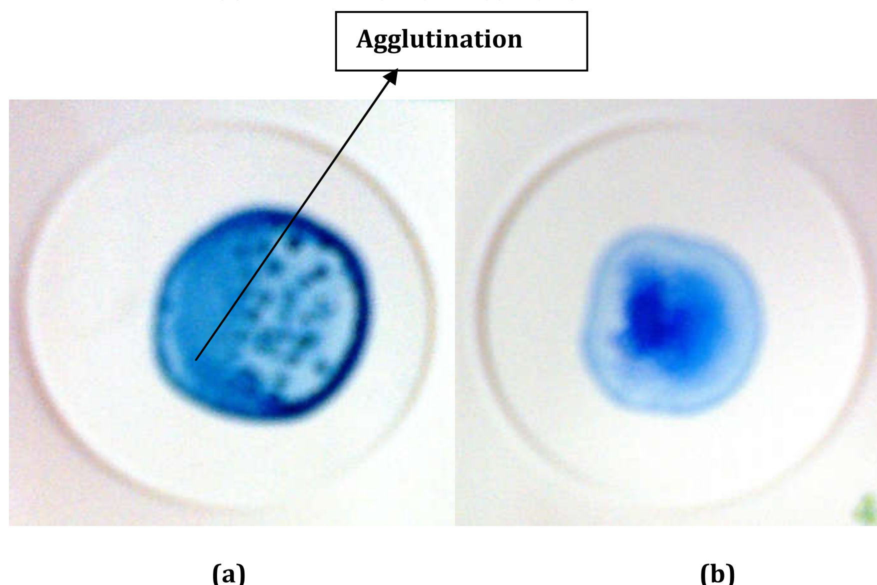

Fig 4 (a): Positive Control (agglutination). Agglutination showed the presence of E. coli O157:H7. (b): Negative Control (no agglutination).

Table1: Results showing growth of ***E. coli*** on different media isolated from mint

NO.	SAMPLE NAME	LOCALITY	GROWTH OF *E.coli* ON DIFFERENT MEDIA					GRAM STAINING
			MAC	TSA	EMB	EC	SMAC	
1	10 samples of mint	SAMANABAD	++	+++	++	+++	+++	-
2	10 samples of mint	JOHAR TOWN	+++	++	++	+++	+++	-
3	10 samples of mint	ICHRA BAZAR	++	+++	++	+++	+++	-
4	10 samples of mint	FAISAL TOWN	++	++	++	+++	++	-
5	10 samples of mint	SADR BAZAR	+++	+++	++	+++	+++	-
6	10 samples of mint	MODEL TOWN	++	+++	++	+++	++	-
7	10 samples of mint	PECO ROAD	++	+++	++	+++	+++	-
8	10 samples of mint	WAPDA TOWN	++	+++	+++	+++	+++	-
9	10 samples of mint	IQBAL TOWN	++	+++	+++	+++	++	-
10	10 samples of mint	MODEL BAZAR	+++	++	+++	+++	+++	-

MINIMUM GROWTH = + MAXIMUM GROWTH= +++
MODERATE GROWTH = ++ NO GROWTH = -

Table 2: Results showing growth of ***E. coli*** on different media isolated from lettuce

NO.	SAMPLE NAME	LOCALITY	GROWTH OF *E.coli* ON DIFFERENT MEDIA					GRAM STAINING
			MAC	TSA	EMB	EC	SMAC	
1	10 samples of lettuce	FAISAL TOWN	++	+++	++	+++	+++	-
2	10 samples of lettuce	VALENCIA	+++	++	++	+++	+++	-
3	10 samples of lettuce	USMANI ROAD	++	+++	++	+++	+++	-
4	10 samples of lettuce	KALMA CHOWK	++	++	++	+++	++	-
5	10 samples of lettuce	SHAHEED ROAD	+++	+++	++	+++	+++	-
6	10 samples of lettuce	BAGHARIAN	++	+++	++	+++	++	-
7	10 samples of lettuce	SHADMAN	++	+++	++	+++	+++	-
8	10 samples of lettuce	ICHRA BAZAR	++	+++	+++	+++	+++	-
9	10 samples of lettuce	MODEL BAZAR	++	+++	+++	+++	++	-
10	10 samples of lettuce	SADR BAZAR	+++	++	+++	+++	+++	-

MINIMUM GROWTH = + MAXIMUM GROWTH= +++
MODERATE GROWTH = ++ NO GROWTH = -

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Table 3: Results showing growth of ***E. coli*** on different media isolated from coriander

NO.	SAMPLE NAME	LOCALITY	GROWTH OF *E.coli* ON DIFFERENT MEDIA					GRAM STAINING
			MAC	TSA	EMB	EC	SMAC	
1	10 samples of coriander	SHADMAN	++	+++	++	+++	+++	-
2	10 samples of coriander	GULBERG	+++	++	++	+++	+++	-
3	10 samples of coriander	WALTON	++	+++	++	+++	+++	-
4	10 samples of coriander	KAHNA	++	++	++	+++	++	-
5	10 samples of coriander	TOWNSHIP	+++	+++	++	+++	+++	-
6	10 samples of coriander	FAISAL TOWN	++	+++	++	+++	++	-
7	10 samples of coriander	MODEL TOWN	++	+++	++	+++	+++	-
8	10 samples of coriander	IQBAL TOWN	++	+++	+++	+++	+++	-
9	10 samples of coriander	WAPDA TOWN	++	+++	+++	+++	++	-
10	10 samples of coriander	JOHAR TOWN	+++	++	+++	+++	+++	-

MINIMUM GROWTH = +   MAXIMUM GROWTH= +++
MODERATE GROWTH = ++   NO GROWTH = -

DISCUSSION

The main objective of the present microbial study was to isolate and identify the pathogenic *E. coli* O157 on the external surface of salad vegetables such as coriander, lettuce and mint leaves to create public awareness about the health hazards resulting from these pathogenic microorganisms. To conduct this study, total 300 samples were collected from different vegetables shops, located in Lahore, Pakistan. All the samples were first cultured on nutrient broth and nutrient agar, which ensured the presence of certain pathogenic microorganisms. For further confirmation and identification, the culture from the nutrient agar was streaked on different selective media and the presence of pathogenic bacteria like *E.coli, Salmonella, Shigella, Staphylococcus,* and *Streptococcus, Micrococcus.*The selective media which were used for identification of pathogenic *E. coli* O157 include Sorbitol MacConkey Agar,Tryptic Soy Agar,Eosine Methylene Blue Agar, and EC media. All the selective media showed the growth of respective bacteria.

MacConkey agars were used for the confirmation of *E. coli.* MacConkey agars are slightly selective and differential plating media mainly used for the detection and isolation of gram-negative organisms from clinical [11], dairy [14], food [15] and water [16].

Eosin Methylene Blue Agar, abbreviated EMB, was developed by Holt-Harris and Teague (1916). This formula contains lactose and sucrose with two indicator dyes, Eosin Y and Methylene Blue. Levine modified the formula by removing sucrose and doubling the concentration of lactose [17]. EMB Agar (either with or without sucrose) is included in the set of low-selectivity isolation media for *Salmonella* from fecal and other specimens [18]. Gram positive bacteria, such as fecal *streptococci, staphylococci* and yeasts, may either grow on this medium or form pinpoint colonies, or may be inhibited.

By utilizing the lactose available in the medium, Lac+ bacteria such as *Escherichia coli*, *Enterobacter* and *Klebsiella* will produce acid, which lowers the pH of the agar below 6.8 and results in the appearance of red/pink colonies. The bile salts precipitate in the immediate neighborhood of the colony, causing the medium surrounding the colony to become hazy [19].

The last media used was EC media modified with novobiocin. This media is used for identification of *E.coli*. The media was prepared and streaked with the bacterial culture, after the incubation of 24 hours it was observed that yellow gold color of media turned pink. This pink color ensured the presence of *E.coli*.EC Medium, Modified with Novobiocin is used for the selective enrichment of *E. coli* O157:H7.EC Broth (Reduced Bile Salts) can be used for the enrichment of *E. coli* O157 directly from food and environmental samples, especially for use with frozen food samples, where the bacterium may be sub-lethally damaged [20].

Prolex™ O157 Kit contains blue latex particles coated with an antiserum against *E. coli* O157 antigen. When the coated latex particles are mixed with fresh colonies of *E. coli* serotype O157 the bacteria will bind to the antiserum, causing the latex particles to visibly agglutinate, indicative of a positive reaction [21]. Bacteria which do not belong to the O157 serotype will not bind to the antiserum and will not result in agglutination, indicative of a negative reaction [22].

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