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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF *E. COLI* FROM URINE SAMPLE AND THEIR ANTIBIOGRAM PATTERN AND EFFECT OF SPICE EXTRACTS AND NATURAL OILS AGAINST ISOLATED *E. COLI*

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ABSTRACT

Urinary tract infections is the most common type of infection in the body. *E. coli* is the most frequent causative agent of UTI affecting both male and female individuals. In this study, total 151 urine samples were collected from suspected UTI patients from 2 hospitals to isolate and identify uropathogenic *Escherichia coli.* From this study, it was revealed that out of 151 samples, 28 samples were positive for *E. coli* and these showed indole and methyl red tests positive and voges-prauskar and citrate utilization tests negative. Out of twelve antibiotics, chloramphenicol, kanamycin, and ciprofloxacin were found to be the most effective antibiotics against *E. coli.* Among the five spice extracts, *Trachyspermum ammi* (ajwain), *Allium sativum* (garlic), and *Zingiber officinale* (ginger) showed maximum antimicrobial activity against *E. coli*. Among the six natural oils, Eucalyptus oil showed maximum antimicrobial activity against *E. coli.*

INTRODUCTION

The urinary tract comprises the kidneys, ureters, bladder and urethra, is the body's filtering system for removing waste liquid, or urine (Ramadan, 2003). Urinary tract infection is one of the most common and life threatening infection present in community medical practice. Worldwide, about 150 million people are diagnosed with UTI each year (Stamm and Norrby, 2001).

It is one of the important causes of morbidity and mortality in population, affecting different sex and all age groups across the life span but women are more susceptible than men, due to short urethra, absence of prostatic secretion, pregnancy and easy contamination of the urinary tract with faecal flora (Schappert., 2008; Sharma and Bidwai., 2013). UTIs are classified on the basis of type of infection (upper or lower UTI), presence or absence of symptoms (symptomatic or asymptomatic UTI), tendency to recur (single episode or recurrent UTI), and presence or absence of complicating factors (uncomplicated or complicated UTI) (Akram *et al*., 2007; Unchu *et al*., 2002; Gupta, 2011; Nicolle. 2001). UTIs have different names, depending on what part of the tract is infected which include cystitis (bladder), urethritis (urethra), and pyelonephritis (kidneys) (Brusch, 2010).

The symptoms of UTI patients depend on the age which may include fever, burning and painful urination, nausea, vomiting, dysuria, urgency, frequency, abdominal or lower back pain, weakness, and dark, bloody, cloudy or bad smelling urine (Mandell *et al*., 2005). The pathogenesis of UTIs involves complex interaction between an organism, the environment and the potential host (Ouno *et al*., 2013). Most of UTI are caused by gram-negative bacteria like *Escherichia coli, Proteus mirabilis, Proteus vulgaris, Klebsiella spp., Pseudomonas areuginosa, Acinetobacter , Serrati,* and *Morganella morganii* and caused by gram-positive bacteria include *Enterococcus, Staphylococcus* especially coagulase-negative staphylococci, and *Streptococcus agalacticae.* Rare pathogens such as *Corynebacterium urealyticum* or *Mycobacterium tuberculosis* can also involved in UTIs (Tangho and Mcaninch. 2004; Brusch, 2010).

Among these *E. coli* is one of the most common bacteria capable of causing about 80-90% of UTIs in humans (Iroha *et al*., 2009). Theodor Escherich first described *E. coli* in 1885, as *Bacterium coli commune*, isolated from the feces of newborns. It was later renamed *Escherichia coli*. It is the normal microflora of large intestine (Haudault, 2001; Reid *et al*., 2001). Depending upon the particular strain that is present, it can provide resistance against pathogenic organisms or can it self be pathogenic, causing disease at intestinal and extra-intestinal sites (Baily *et al*., 2006; Salyers *et al*., 2002).

E. coli strains that cause disease within the intestinal tract are referred to as *Diarrheagenic E. coli*, and the major pathotypes are *Enterotoxigenic E. coli (ETEC), Enteropathogenic E. coli (EPEC), Enteroinvasive E. coli (EIEC), Enterohemorrhagic E. coli (EHEC), Enteroag gregative E. coli (EAggEC), Diffusely adherent E. coli (DAEC),* and *Adherent-invasive E. coli (AIEC)* (Nataro and kaper., 1998). Strains of *E. coli* that cause disease outside of the gastrointestinal tract are referred to as *Extraintestinal pathogenic E. coli (ExPEC)* and are

divided into *Uropathogenic E. coli (UPEC)*, and *Neonatal meningitis E. coli (NMEC)* (Nicolle., 2008). *Uropathogenic E. coli (UPEC)* is responsible for approximately 90% of urinary tract infections (Todar, 2007). *UPEC* utilizes P fimbrine to bind specifically to the P blood group antigen which contains a D-galactose-Dgalactose residue.

Binding of this P fimbriae not only specific to red blood cell but to a specific galactose disaccharide that is found on the surface of uroepithelial cells in approximately 99% of the population (Akram *et al*., 2007). The treatment of UTIs depends upon sensitivity of bacteria towards a variety of antibiotics. So treatment of UTI with the appropriate antibiotic can minimize mortality, morbidity and any renal damage from acute UTI. Choosing the appropriate antimicrobial agents sounds difficult, but advances in the understanding of the pathogenesis of UTI, the development of new diagnostic tests, and the introduction of new antimicrobial agents have allowed physicians to appropriately tailor specific treatment for each patient (Schlager, 2001). Down the ages different spice extracts and natural oils have evoked interest as source of natural products.

They have been screened for their potential uses as alternative remedies for the treatment of urinary tract infections (Tepe *et al.,* 2004).The sequence of steps in performing a complete study of *E. coli* in urine sample includes: Aseptic collection of urine samples*,* Analysis of urine samples, Isolation of *E. coli*, Characterization of *E. coli*, Antimicrobial susceptibility testing, and Effect of different spice extracts and natural oils against *E. coli*.

MATERIALS AND METHODS

Culture media and chemicals

MacConkey agar w/o bile salts, crystal violet and Nacl, Levin Eosin-Methylene Blue agar medium 10, Simmons Citrate agar, [Trachyspermum ammi (ajwain), and Mentha piperita (mint leaves)] and different natural oil [Coconut oil (Cocos nucifera), Amla oil (Ohyllantus emblica), Sarson oil (Brassica nigra), Mixed herb oil (Aloe vera), Nilgiri oil (Eucalyptus spp.), and Olive oil (Olea europaea)] and all the other chemicals and media were of analytical grade.

Collection and transport of urine sample

Total 151 urine samples were collected from suspected urinary tract infection (UTI) patients with or without the case history of diabetic mellitus (DM) and non-diabetic mellitus (NDM) from in-patient (IPD) and out-patient (OPD) department of Haria L. G. Rotary Hospital and Nadkarni's 21st Century Hospital, Vapi, Gujarat, India from period June 2015 to March 2016. Samples of early morning, mid-stream, and clean-catch urine were collected in sterile, wide-necked, leak-proof plastic universal containers (25-30 ml) and they should be transported to laboratory as soon as possible in an ice cold condition (coolant pack). If there should be a chance of delay, to examine the urine sample, it was refrigerated at 4°C to avoid multiplication of bacteria.

Urinalysis

Urinalysis was performed using SD UroColor^{TM}10 multi strips which provide tests for blood, urobilinogen, ketone, protein, nitrite, glucose, pH, specific gravity, and leucocytes in urine. A routine direct microscopy of a centrifuged sample was performed for the examination of white blood cells, red blood cells, casts, crystals, bacteria, yeasts, and parasites.

Isolation and identification of Escherichia coli

Urine samples were inoculated on MacConkey agar and incubated at 37°C for 24 hours. Pink colonies from the Mac Conkey agar were further sub-cultured on eosin methylene blue (EMB) agar and incubated at 37°C for 24 hours. Colonies that had greenish metallic sheen on EMB were presumptively taken as *E. coli* and further characterization was based on gram staining, morphology, motility testing, and biochemical reactions (as Indole (+), Methyl red(+), Voges-prauskar (-), and Citrate (-)). The pink colonies from MacConkey agar that did not produce greenish metallic sheen on EMB were designated as unclassified coliforms. Well isolated organisms were maintained on nutrient agar prior to antimicrobial susceptibility testing.

Antimicrobial susceptibility of Escherichia coli

Antimicrobial susceptibility of *E. coli* isolates was performed on Mueller-Hinton agar using the Kirby Bauer disc diffusion method according to CLSI standards, determining sensitive and resistant bacteria to antibiotics. This testing was carried out using 12 antibiotic discs (vancomycin (30µg), chloramphenicol (30µg), ciprofloxacin (5µg), methicillin (5µg), oxacillin (1µg), kanamycin (30µg), rifampin (5µg), amoxicillin (10µg), erythromycin (15µg), gentamycin (10µg), tetracycline (30µg), and trimethoprim-sulphamethoxazole (23.7µg)) on *E. coli* isolates. The diameter of the zone of inhibition produced by each antibiotic disc was measured, recorded and the isolates were classified as "resistant", "intermediate" or "sensitive" based on the standard interpretation chart.

Effect of spice extracts on growth of Escherichia coli

The spices namely Zingiber officinale (ginger), Allium sativum (garlic), Murraya koenigii (curry leaves), Trachy spermum ammi (ajwain), and Mentha piperita (mint leaves) were used for the present study collected from the local market.

Extraction of spices

Extract of each spice was prepared by 10 gm of dry spice in 50 ml of methanol at 30°C for 48 hours in shaking condition at 120 r.p.m. and then was filtered using mesh cloth. The extract was collected, dried and stored at 4°C until further use.

Antimicrobial activity of spice extracts

DMSO (Di Methyl Sulfoxide) solvent is the common solvent and the spice extracts were dissolved in this solvent. Extracts with 100% concentration were prepared. Antibacterial assay of spice extracts were performed using agar well diffusion method. About 0.1 ml of 24 hours old culture (0.2 O.D.) was inoculated in melted top agar previously cooled to 50°C. It was mixed well and poured it over the nutrient agar (base agar) and allow it to solidify. With the help of sterile cup borer four wells were made per plate. About 0.2 ml of different spice extracts were added to these wells and plates were incubated at 37°C for 24 hours. After incubation, diameter of clear zone produced surrounding the wells were measured to the nearest mm with the help of scale.

Effect of different natural oil on growth of E. coli Isolates

The six natural oils such as Coconut oil (Cocos nucifera), Amla oil (Ohyllantus emblica), Sarson oil (Brassica nigra), Mixed herb oil (Aloe vera), Nilgiri oil (Eucalyptus spp.), and Olive oil (Olea europaea) were tested for their antibacterial effects. The test was performed by agar well diffusion method.

About 0.1 ml of 24 hours old culture (0.2 O.D. culture) was inoculated in melted top agar previously cooled to 50°C. It was mixed well and poured it over the nutrient agar (base agar) and allow it to solidify. Four wells were made per plate with the help of sterile cup borer and 0.2 ml of different oil samples were added to these wells and plates were incubated at 37°C for 24 hours. After incubation, diameter of growth inhibition zones produced surrounding the wells were measured to the nearest mm with the help of scale.

RESULTS AND DISCUSSION

Sampling design and handling

During June 2015 to March 2016, 151 urine samples were collected from Haria L. G. Rotary Hospital, and Nadkarni's 21st Century Hospital, Vapi, Gujarat, India and these were analyzed in the laboratory. In the present study the urine samples were collected from diabetic patients (51) and non-diabetic patients (100) who were infected with UTI (Table 1).

The samples were from different age group ranging from 1-60 years and above, out of which 64 (42.38%) were females and 87 (57.62%) males (Figure 1 and 2).It was found that out of 151 samples 28 (18.5%) isolates were identified as *E. coli*. The rate of positive culture in diabetic patient was 28.57% and 71.43% in non- diabetic patients. Hence report suggests that the majority of E. coli was isolated from UTI of non-diabetic patients.

Table 1 Clinical samples (urine)

Sr. No.	Specimen	DM	NDM	Total	No. of positive	Percentage (%)
1.	Urine	51	100	151	28	18.5

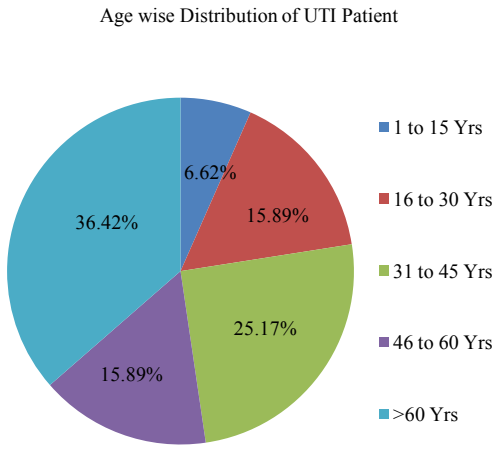

Figure 1 Interpret the age wise distribution of UTI patients involved In this study. The majority of the samples were obtained from UTI patients above 60 years

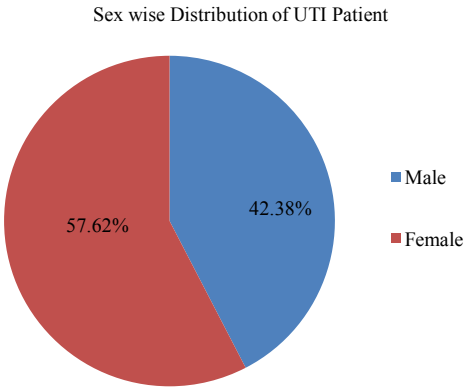

Figure 2 Showed sex wise distribution of UTI patients used in this study. Urinary Tract Infection is more prevalent in female Than male

URINALYSIS RESULTS

Urine specimens may vary in color from pale yellow to dark amber depending on the presence of urochrome, uroerythrin, and urobilin pigments. The color may be the result of pathogenic conditions (nephrosis, bacterial infections, etc.) but in many instances is due to the presence of a drug or its metabolite or food. In the present study, out of 151 urine samples, 141 (93.38%) samples were pale yellow, 7 (4.63%) samples were yellow, and 3 (1.99%) samples were reddish in color. Normal urine is clear when freshly voided but cloudy or turbid urine is seen in presence of bacteria, proteins, crystals or leucocytes. Cloudiness may also develop due to precipitation of urates (acids) or phosphates and carbonates (alkaline). Out of 151 urine samples, 46 (30.46%) samples were clear, 57 (37.75%) samples were turbid, and 48 (31.79%) samples were cloudy in appearance.

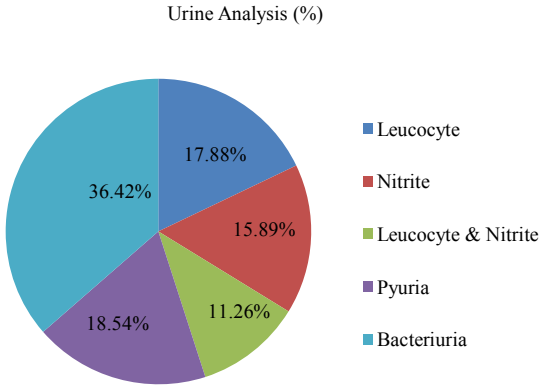

Figure 3 Urinalysis (%)

Investigations of blood, bilirubin, urobilinogen, ketone, protein, nitrite, glucose, pH, specific gravity, and leucocytes of 151 urine samples were examined by SD UroColor10 reagent strips. These strips provided information regarding the status of carbohydrate metabolism, kidney and liver function, acid-base balance, and urinary tract infection. Proteinuria is associated often with bacteriuric urine. Results in Figure 3 show 27 cases (17.88%) of samples were positive for leucocytes, 24 cases (15.89%) of samples were positive for nitrite, 17 cases (11%) of samples were positive for both leucocyte and nitrite, and 28 cases (18.5%) of samples were

positive for pyuria, and 55 cases (36.4%) of samples were positive for bacteriuria of microscopy test.

Isolation and identification of Escherichia coli

E. coli was isolated from urine samples from UTI patients. Total 151 urine samples were streaked on MacConkey's agar plates. After incubation, typical circular lactose fermenting pink color colonies were selected for further confirmation of *E. coli*. Out of 151 samples, 89 (58.94%) samples showed pink color colonies on MacConkey's agar plates. Eosin methylene blue (EMB) agar medium contained lactose and the dyes eosin and methylene blue that permitted differentiation between enteric lactose fermenters and non-fermenters as well as identification of the colon bacillus *E. coli*. Colonies were small, dark centered with greenish metallic sheen caused by the large quantities of acid that was produced and precipitated out the dyes onto the growth surface on EMB. These isolates were presumptively taken as *E. coli*. A well isolated single colony from MacConkey's agar plate was streaked onto EMB agar plate for presumptive confirmation as *E. coli* of each isolate. After incubation, out of 89 isolates, 53 (35.09%) isolates showed greenish metallic sheen which were selected for further identification. Rest of isolates that did not produce greenish metallic sheen on EMB were designed as unclassified coliforms.

Further identification of 53 *E. coli* isolates was carried out on the basis of Gram staining, morphology, motility testing, and biochemical tests. Typically *E. coli* is a gram-negative short rods stained pink in color arranged singly or in pairs. 53 isolates which gave greenish metallic sheen on EMB agar plates were further tested by different biochemical tests such as IMViC tests (Indole test, Methyl red test, Voges-Prausker test and Citrate utilization test). Out of which 28 (18.54%) isolates were confirmed as *E. coli.* These 28 isolates showed Indole and M-R test positive whereas V-P and Citrate utilization test negative.

Antimicrobial susceptibility of Escherichia coli

In the present study, 28 isolates obtained from urine samples were subjected to susceptibility testing using twelve antibiotics by agar disc diffusion method which was performed by inoculating Muller-Hinton agar plate. Antimicrobial susceptibility pattern of all 28 *E. coli* isolates is given below in Table 2.

Table 2 Antimicrobial susceptibility pattern of 28 *E. coli* isolates

Sr. No.	Antimicrobial Agents	Symbol	Disc Content (µg)	Susceptibility (n=28)		
				Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
1.	Amoxycillin	AMX	10	24(85.72)	3(10.71)	1(3.57)
2.	Chloramphenicol	C	30	0(0)	0(0)	28(100)
3.	Ciprofloxacin	CIP	5	5(17.86)	3(10.71)	20(71.43)
4.	Methicillin	MET	5	28(100)	0(0)	0(0)
5.	Oxacillin	OX	1	28(100)	0(0)	0(0)
6.	Kanamycin	K	30	1(3.57)	0(0)	27(96.43)
7.	Erythromycin	E	15	28(100)	0(0)	0(0)
8.	Rifampin	RIF	5	28(100)	0(0)	0(0)
9.	Gentamycin	GEN	10	8(28.57)	11(39.29)	9(32.14)
10.	Tetracycline	TE	30	14(50)	7(25)	7(25)
11.	Trimethoprim-sulphamethoxazole	TR	1.25/23.7	15(53.57)	8(28.57)	5(17.86)
12.	Vancomycin	VA	30	28(100)	0(0)	0(0)

The results of AST indicated that all *E. coli* isolates (100%) were found to be sensitive to Chloramphenicol, and 96.43% and 71.43% of the isolates were sensitive to Kanamycin and Ciprofloxacin respectively. In addition, only 32.14% of all isolates were sensitive to Gentamycin. Tetracyclines (25%), Trimethoprim-sulphamethoxazole (17.86%), and Amoxycillin (3.57%) showed less sensitivity to isolates. *E. coli* isolates (100%) were resistant to Methicillin, Oxacillin, Eryth romycin, Rifampin, and Vancomycin. All isolates were also highly resistant to Amoxycillin. In addition, all isolates were also resistant to Trimethoprim-sulphamethoxazole (53.57%), Tetracycline (50%), Gentamycin (28.57%), Ciprofloxacin (17.86%), and Kanamycin (3.57%). This present study showed very sad and alarming results that all isolates were resistant to most of the antibiotics.

Effect of different spice extract on growth of E. coli isolates

Five spice extracts were screened for potential antimicrobial activity against 28 *E. coli* isolates. This was performed by inoculating Nutrient agar plate using agar well diffusion method. Out of 5 extracts, the extracts of ajwain, garlic, and ginger were highly effective against 28 isolates which reduced 100%, 92.86% and 78.57% respectively growth of *E. coli* isolates whereas the extract of mint leaves was less effective compared to ajwain, garlic, and ginger extracts which was 46.43% r.

The extract of curry leaves had negligible effective against *E. coli* isolates.Considering the above results, it can be suggested that addition of spices to the food preparations helps to keep a check on the concentration *Escherichia coli* in the body. Garlic, ginger, and ajwain are used as most important food additives with antimicrobial activities in Indian recipes.

Effect of different natural oil on growth of E. coli isolates

Six natural oils were used to check the antimicrobial activity against 28 isolates using agar well diffusion method. Out of six oils, Eucalyptus oil was observed to inhibit 100% growth of all *E. coli* isolates. Rests of the natural oils were less effective against these isolates.

CONCLUSION

From our study it can be concluded that twenty eight *E. coli* isolates were successfully identified and characterized by biochemical techniques. High level of antibiotic resistance pattern was found among those uropathogens.It is quite alarming to note that almost all of the isolates included in this study were found resistant to five or more antibiotics.

Antibiotic resistance is becoming a big problem for the individuals admitted to health care centers with chronic conditions as well as for medical professionals. All isolates

showed multiple antibiotic resistance property, maxi-mum resistance was found against methicillin, oxacillin, erythromycin, rifampin, and vancomycin. All twenty eight isolates were sensitive to chloramphenicol, kanamycin, and ciprofloxacin and hence those might be the drugs of choice to treat *UPEC*.

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