Journal of Applied and Natural Science 4 (1): 87-91 (2012)

JANS

Citric acid: A potential permeabilizer against multiple drug resistance enteropathogenic *Escherichia coli*

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Abstract: Enteric diseases enter through the mouth and are usually spread by contaminated food, water or contact with contaminated vomit or feces. Enteric infection encompasses all the infections of the intestinal tract. These intestinal infections include organisms like *Escherichia coli, Salmonella, Shigella, Klebsiella, Proteus* etc. Out of these, *E. coli* are one of the common causes of enteric infection. In spite the introduction of a wide variety of antimicrobial agents against enteric diseases, life threatening infections caused by *E. coli* contributes to morbidity and mortality in patients. The present study was conducted to determine the antibiotic sensitivity pattern of *E. coli* obtained from stool samples and potentiation of antibiotic activity by citric acid against multiple drug resistant *E. coli*. Out of the 200 isolates of *E. coli*, 150 were found to be resistant to one or more antibiotics tested. 0.05% and 0.1% citric acid was found to be effective in increasing the potency of the all the antibiotics used in the study.

Keywords: Antimicrobial, Enteric, *Escherichia*, Potentiation, Potency, Resistance

INTRODUCTION

Escherichia is a major cause of enteric infection and urinary tract infection, a common component of microbial intra-abdominal infections and an occasional cause of a variety of other infections including pneumonia, osteomyelitis, cellulitis, myosistis, septic arthritis and sinusitis.

Although most strains of *E. coli* are harmless, several are known to produce toxins that can cause diarrheal side effects. Since the mid 1930s, clinically important bacteria have been subjected to successive onslaughts of antibacterial drugs; however in response the bacterial population has overcome these attacks by the selection of resistant variants or requires novel genetic material.

Although many communicable diseases have been effectively contained, bacterial infections remain a major cause of morbidity and mortality particularly in developing countries. Moreover, in both developed and developing countries, the risk of some serious bacterial infections has increased because of treatments such as chemotherapy for cancer and the emergence of diseases such as human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS), which impair the patient's defenses against infection.

Antimicrobials have reduced the morbidity and improved the survival of patients with bacterial infections and remain essential for the treatment of many kinds of bacterial diseases. However, the increasing prevalence of strains of common pathogenic bacteria resistant to widely available, affordable antimicrobials is, in many cases, dangerously eroding their effectiveness. It is hoped that by encouraging the appropriate use of antimicrobials, the emergence and spread of antimicrobial resistance may be delayed (WHO, 2001).

Inspite of all the efforts made towards the better chemotherapeutic agent, the problem of overcoming the development of resistance has not yet been solved. Russell *et al.*, 1986 studied the sensitivities of some outer membrane protein (omp) and /or lipopolysaccharides (LPS) mutants of *E. coli* to chorhexidine and some quaternary ammonium compounds (QACs). He found that chlorhexidine was highly active against all the strains tested. The concentration of the compound was within the narrow range of 1 to 2mg. He also studied the comparative sensitivity of smooth, rough and deep rough strains of *E. coli* to dibromopropamidine isethionate, chorhexidine diacetate (CHA) and other QACs. CHA was almost equally active in low concentration against all he strains where as QAC like cetypyridinium chloride (CPC), domiphen bromide and benzethoium chloride were most active against the deep rough strains and least against wild type and slightly rough stains (Russell *et al.*, 1986). In the same year he also studied the effect of three phenolics and of a homologous series of esters of para-hydroxybenzoic acid (the parabenes) on *E. coli* with known deletions in outer membrane. Of the phenolics,

ISSN : 0974-9411 (Print), 2231-5209 (Online)

chlorocresol was the most active and phenol the least, with butyl-p-hydroxybenzoate the most effective parabene.

Nagoba *et al.* (2002) treated non healing ulcers in leprosy patients by citric acid. Nagoba *et al.* (2008) employed the citric acid for the treatment of chronic wound infections caused by antibiotic resistant *E. coli.* A total of 34 cases of chronic wound infections yielding MAREC isolates on culture were studied. The antibacterial effect of citric acid against MAREC was evaluated in vitro by broth dilution method. 3% of citric acid gel was applied to each wound once daily until it healed completely. All 34 isolates were inhibited by citric acid with minimum inhibitory concentration in the range of 1500-2000 µg/ml. Topical application of 3% citric acid to wounds resulted in the elimination of MAREC from infected sites and successful healing of wounds in all the 34 patients.

Tumane *et al.* (2006) studied the amtimicrobial activity of citrus fruit juices against pathogenic bacteria. The study showed that the growth of *Salmonella typhimurium, S. paratyphi, Shigella flexneri, Proteus mirabilis* and *P. vulgaris* was inhibited by 2% citric acid. Owing to this fact an attempt was made to potentiate the antibiotic activity by citric acid against drug resistant isolates of *E. coli* isolated from the patients suffering from enteric infections.

MATERIALS AND METHODS

Isolation and identification: Total of 200 *E. coli* isolates extracted from the clinical samples of stool and urine from both outdoor and indoor patients were aseptically collected from different wards of GMC and IGMC during the period of Jan. 2010-Dec. 2010. Organisms were identified on the basis of colony morphology and cultural characteristics according to Bergey's manual of determinative bacteriology, 9th edition. For identification at molecular level, genomic DNA was extracted from the isolates using DNA extraction kit (Bangalore Genie, India). The 16s rRNA gene was amplified by PCR. PCR amplification was performed followed by standard procedure (30cycles of 30S at 94^{0}C, 30S at 55^{0}C and 2 min at 72^{0}C) with Taq DNA polymerase. Then sequence analysis was performed with the BLAST program.

Antibiotic susceptibility testing: Antibiotic susceptibility testing was carried out by disk diffusion method of Bauer *et al.* (1996) using Muller Hinton agar plates (Hi-media, India). Well isolated colonies of *E. coli* isolates from selective media plates were inoculated in the tube containing 5ml tryptone soya broth (Hi-media, India) and incubated at 37^0 C until it achieved or exceeded the turbidity of 0.5 McFarland standards which approximately corresponds to 10^8 CFU/ml. From the 5ml broth, 1ml of *E. coli* culture was uniformly spread on MHA plates and antibiotic disks were placed over it. The antimicrobial discs used were Cefuroxim (CU), Cephalexin (CP), Cefepime (CPM), Amikacin (AK), Carbenicillin (CB), Ofloxacin (OF), Gatifloxacin (GF), Levofloxacin (LE) and Sparfloxacin (SC). *E. coli* ATCC 25922 was used as a control strain. The diameter of zone of inhibition was measured using the calibrated ruler and the results were interpreted according to the CLSI standards.

Determination of minimum inhibitory concentration (MIC) of citric acid: Before going to see the potentiating effect of citric acid the present section deals with the study of 'in vitro' antimicrobial effect of citric acid against multiple drug resistant isolates of *E. coli* by micro broth dilution and macro broth dilution methods. Here out of 200 isolates, 150 were found to be multiple drugs resistant. For MIC of citric acid, all the 150 multiple drugs resistant isolates of *E. coli* were used. For this Hi- sensitivity test broth [M486] was procured from Hi-media, Mumbai, India.

MIC procedure for broth macro dilution method (NCCLS dilution method M7-A2): This test was performed in round bottom sterile glass test tubes (Borosil made). A working citric acid solution of 20 mg/ml was prepared by dissolving 200 mg of citric acid in 10 ml of sterile distilled water, two fold dilutions were made in test tube containing sterile distilled water. Then equal amount of double strength sterile Hi-sensitivity broth was mixed in each test tube and inoculated with 0.1ml inoculum. The set was incubated at 37 ^{0} C overnight. MIC was recorded accordingly.

MIC procedure for broth micro dilution method (NCCLS dilution method M7-A2): Two fold citric acid dilutions were made in 96 well micro titer plates and each well inoculated with 10µl of inoculums with micropipette. The plates were incubated at 37^{0}C overnight. The MIC was recorded accordingly.

Potentiation of antibiotic activity by citric acid: The isolates which were found to be resistant to one or more antibiotics were further studied for potentiation purpose. Out of 200 isolates of *E. coli*, 150 were found to be multiple drug resistant (MDR). For potentiation purpose different concentrations of citric acid (Blank, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 1% and 2%) were used. All the 150 MDR were grown overnight. Within 15min after adjusting the turbidity (0.5 McFarland Standards) of inoculums suspension, a sterile cotton swab was dipped into it; the swab was streaked over the entire surface of Muller Hinton agar (MHA) medium containing different concentrations of citric acid (0% bank to 2%). The antimicrobial discs were applied over the plates as soon as possible. All the plates were incubated at 37^{0}C overnight and results were reported according to CLSL guidelines.

RESULTS AND DISCUSSION

Diseases classified as enteric enter through the mouth and intestinal tract are usually spread by contaminated

Table 1. Morphological, biochemical characteristics of *E. coli.*

Gram staining	Motility	I	MR	VP	C	Oxidase	H_2S	Catalase	Glucose (Gas production)	Lac	Man
Gram negative coccobacillus	Sluggishly motile	+	+	-	-	-	-	+	+	+	+

I-Indole, MR-Methyl red, VP-Vogues Proskauer,C-Citrate, + Positive, - : Negative

food, water or contact with contaminated vomit or feces. The topic of enteric infection encompasses all the infections of the intestinal tract. Symptoms similar to those caused by pathogens may be produced by chemical toxins in ingested foods and by allergic reactions to certain food substances. The bacteria commonly involved in enteric infections are *E. coli, V. cholera, Salmonella sp., Klebsiella pneumoniae,* several species of *Shigella* etc. Enteric infections are characterized by diarrhea, abdominal discomfort, nausea, vomiting and anorexia. A significant loss of fluid and electrolytes may result from severe vomiting and diarrhea (Mosby's Medical Dictionary, 2009). In addition to acute risk of disease, long term complications of enteric disease include malnutrition, malabsorption of vital drugs and immunological complications.

Food borne diseases affect people's health and well being as well as have an economic impact on individuals and Nations. Diarrheal diseases have been a major public health problem causing high morbidity and mortality among the children (Bureau of Epidemiology, 2004). Approximately one million cases of acute diarrhea and more than 1,20,000 cases of food poisoning are reported each year (Bureau of Epidemiology, 2004). Food is considered as the main source of microorganism causing diarrheal diseases (Rabbani and Greenough, 1999; Jay, 2000; Fang *et al.*, 2003).

In the current investigation total of 200 *E. coli* isolates were isolated from clinical samples of stool. A series of biochemical, morphological and cultural tests (Table 1) were conducted for the identification and characterization of *E. coli* as outlined in Bergey's manual of Determinative bacteriology (9th edition). For confirm identification 16s rRNA sequencing was carried out according to the standard procedures. BLAST analysis revealed that the given isolates are of *E. coli.*

Selection of antibiotics against any type of infection should be based on antimicrobial susceptibility testing. Periodic evaluation of antimicrobial activity of different antibiotics is essential as the pattern of antibiotic sensitivity may vary over short periods (Jones and Thornsberry, 1982). Choice of treatment should be made

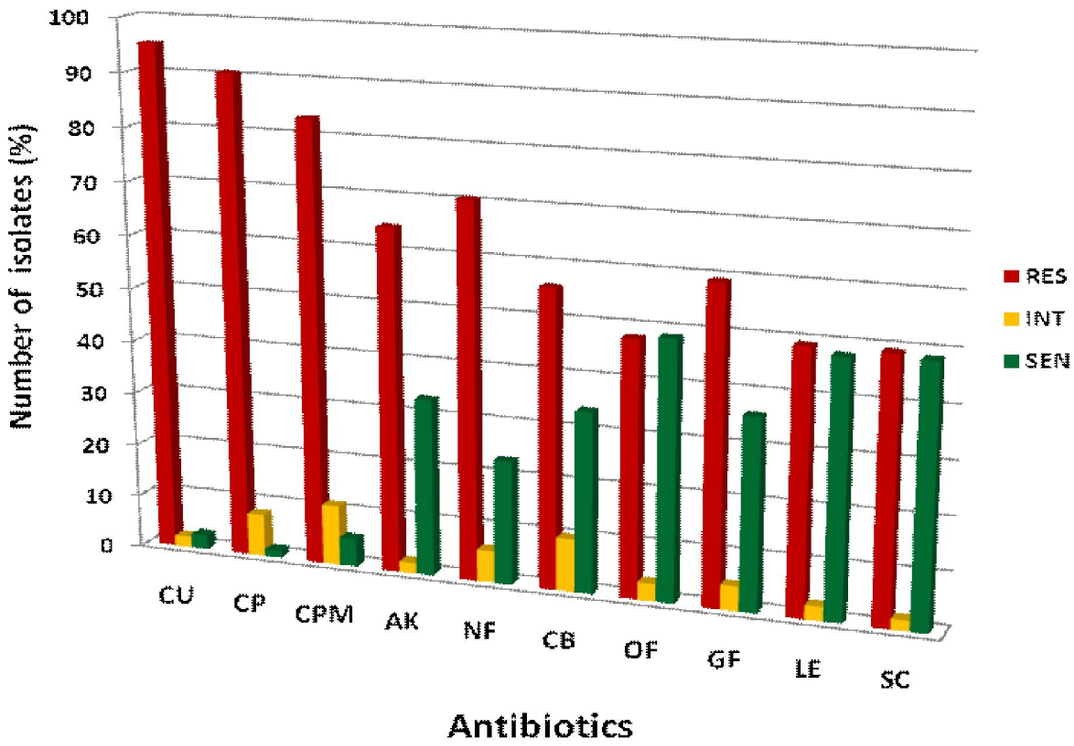

Fig. 1. *Resistance pattern of E. coli before potentiation.*

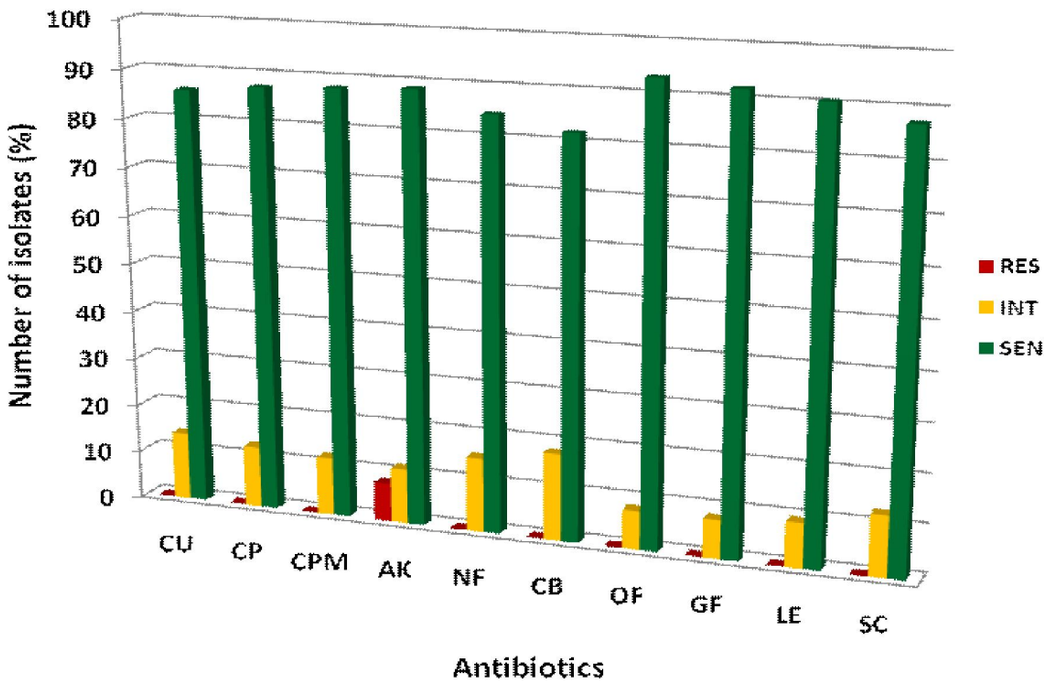

Fig 2. *Sensitivity pattern of E. coli after potentiation with citric acid.*

after the sensitivity of causative organisms has been determined "*in vitro*". Enteric diseases vary from place to place from time to time and so their sensitivity to different antimicrobial drugs also changes. Information concerning the drug resistance pattern of the prevailing pathogenic bacteria and the appearance of new resistant characteristic is of utmost value for a proper selection of antimicrobial agents for therapeutic purposes. Unawareness of local drug resistant pattern in pathogens may foster it use and often over use of antibiotic with their all harmful consequences.

With regards to the antibiotic sensitivity pattern of isolates in the present investigation, *E. coli* was found to be highly resistant to first and second generation cephalosporins like cefuroxime (95.33%), Cephalexin (90.66%) and Cefepime (83.33%). Nitrofurantoin showed 70.66% resistance against *E.coli.*

The development of antimicrobial resistance is a natural process which cannot be stopped but can be minimized or reduced by some or the other way. So an attempt was made to potentiate the activity of antibiotics by citric acid against multiple drug resistant enteropathogenic *E. coli*.

For this purpose we thought to systematically study the effective concentration of citric acid against multiple drug resistant *E. coli* "*in vitro*". In the present investigation, citric acid was tested against all the 150 multiple drug resistant *E. coli* using micro broth dilution and macro broth dilution methods. We found that citric acid alone at a concentration of 1000 µg/ml (Micro broth dilution) and 2500 µg/ml (Macro broth dilution) is highly active against these enteric pathogens. Similar findings were observed by Gomashe and Tumane, 2006.

On the basis of these findings we proceeded for potentiation of preselected antibiotics with different concentrations of citric acid (0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 1% and 2%). we found that an effective concentration of citric acid to potentiate the activity of antibiotics was 0.05% and 0.1%. Combination of above 0.1% citric acid and antibiotic resulted in the complete inhibition (Figs. 1 and 2).

After potentiation it has been found that all the *E. coli* strains which were resistant or moderately sensitive (intermediate) towards previously tested antibiotics became sensitive when tested with 0.05% and 0.1% citric acid. From the given study one may conclude that, a small percentage of potentiating agents like citric acid when used in combination with conventional drugs can make a big impact on their sensitivity.

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