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Research Article

Isolation and Identification of ESBL producing *Escherichia coli* from diabetic patients

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Abstract

A total of about 25 samples of diabetic foot infection samples were collected from the diabetic patients by using appropriate sterile cotton swabs from Government Hospital, Kanchipuram. Samples were inoculated in sterile peptone water in screw – capped tubes and transported to the laboratory within an hour. The culture was inoculated in the Nutrient agar plates and the culture obtained was further identified by classical cultural methods. The identified isolate was subjected to the antimicrobial suspectibility using standard antibiotic discs. The resistant strains were subjected to screening for Extended Spectrum Beta Lactamase production by Double disk synergy test, Disk replacement test, E-Strip test and Three dimensional test. The ethanolic seed extracts of four condiments Coriander, Cumin, Fenugreek and Pepper was prepared. The filter paper disc impregnated with each ethanolic seed extract was tested against ESBL producing *Escherichia coli.* Zone of inhibition of each extract were recorded. All the seed extracts were found to be effective. Among the four seed extracts, fenugreek was found to be the most effective. As a global scenario is now changing towards the use of nontoxic plant products having traditional medicinal use, development of modern drugs from these seeds should be emphasized for the control of ESBL producing *Escherichia coli*.

Keywords: Diabetic foot infection, antimicrobial suspectibility, ESBL, *Escherichia coli.*

Introduction

Diabetes mellitus is a chronic disorder and affects large segment of population and is a major public health problem. Diabetes and foot problems are almost synchronous. (Frykberg RG, 1998; Blazer K and Heidrich M, 1999; Logerfo FW and Coffman JD, 1984; Shea KW, 1999)

Infection can be described as disease caused by the microbial pathogen that occurs when the presence of replicating organisms in tissue damage. Diabetic foot infections are sores on the feet that often occur in people with diabetes. People with diabetes mellitus, a disorder in which blood sugar levels are abnormally high, are at risk for foot ulcers. The elevated blood sugar levels that occur with diabetes mellitus damage blood vessels, causing them to thicken and leak. Over time this thickening means they are less able to supply the body, especially the skin, with the blood it needs to remain healthy.

Poor blood supply to the skin often leads to ulcers, especially on the feet. Because of the poor circulation, these ulcers are slow to heal and often become deep and infected. A foot ulcer looks like a painful, red sore on the foot. When infected, it will ooze pus and have a foul smelling discharge. Diabetic Foot Infection can be seen by a visual examination of the feet. If an ulcer develops, the ulcer must be kept very clean. This can be done by washing the feet daily with mild soap or a saline solution and keeping the ulcer covered with clean, dry dressings. Oral antibiotics may also be needed if the ulcer becomes infected. Ulcers can become so deep and infected that the foot needs to be surgically amputated.

Mortality is high and healed ulcers often recur. The pathogenesis of foot ulceration is complex, clinical presentation is variable, and its management requires early expert assessment. (Calhoun JH *et al*., 2002).

Interventions should be directed towards infection control, peripheral ischemia management, and abnormal pressure loading management caused by peripheral neuropathy and limited joint mobility. Despite treatment, ulcers readily become chronic wounds. Diabetic foot ulcers have been neglected in health – care research and planning, and clinical practice is based more on opinions than the scientific figures and facts. Furthermore, the pathological processes are poorly understood and poorly taught. Communication between the many specialities involved is disjointed and is insensitive to the needs of the patients. Ischemia, neuropathy, and infection in patient with DM combine to produce tissue necrosis and ulcers. Early recognition of lesions and prompt initiation of appropriate antibiotic therapy, as well as aggressive surgical debridement of necrotic soft tissues and bones, and a modification of host factors i.e., hyperglycemia, concomitant arterial insufficiency are all equally important for successful outcome (Mills JL *et al*., 1991)

Initial therapy of diabetic foot infections is frequently empiric because reliable culture data is lacking. There is variability in prevalence of common bacterial pathogens isolated, as shown in different studies (Robert GF and Aristidis V, 1996).

The trio of problem leading on to the diabetic foot is neuropathy, vascular changes and infections, which constitute the diabetic foot syndrome. (Smith JMB *et al*., 2002; Meade JW and Muller CB, 1968). Infection complicates the pathological picture of diabetic foot and plays a main role in the development of moist gangrene. *Pseudomonas* species, *Enterococcus* species, and *Proteus* species carry a special role and are responsible for continuing and extensive tissue destruction with the poor blood circulation of the foot.

A high frequency of anaerobic infection has also been reported. (Bailey TS *et al.,* 1985; Miler RS and Amyes SGB, 1996; Forbes BF *et al.,* 1998). The infection leads to the early development of complication even after a trivial trauma, the disease progresses and becomes refractory to antibacterial therapy. (Pittet D *et al.,* 1999). It is essential to assess the magnitude of bacterial infection of the lesions to avoid further complications and save the diabetic foot. Early diagnosis of microbial infections is aimed to institute the appropriate antibacterial therapy and to avoid further complications

The presence of microorganisms in a wound, however, does not itself define a clinical infection. It is important to recognize that there is a spectrum of disease. All the wounds are exposed to the skin commensals and their microflora will represent the surrounding environment. These contaminating microbes can quickly become established with in a wound. Diabetic foot ulcers are commonly colonized with multiple species of microorganisms that do not normally interfering with healing. Multiplication of bacteria within the wound can reach the stage of critical colonization, in which the host defenses are unable to maintain a balance, thus resulting in delaying healing.

Extended spectrum -lactamase (ESBL) producing organism are among the growing problems in the diabetic foot infections.

-lactamases are enzymes produced by some bacteria and are responsible for their resistance to -lactam antibiotics. The -lactam antibiotics have a common element in their molecular structure; a four atom ring known as -lactam. The lactamase enzyme breaks that ring open, deactivating the molecule's antibacterial properties.

ESBLs constitute a growing class of plasmid-mediated -lactamases which confer resistance to broad spectrum beta-lactam antibiotic. They are commonly expressed by ***Enterobacteriaceae*** but the species of organisms producing these enzymes are increasing and this is a cause for great concern.

The prevalence of ESBL-producing organisms is increasing worldwide and several outbreaks have been reported. Serious infections with these organisms are associated with high mortality rates as therapeutic options are limited.

The emergence of ESBLs creates a real challenge for both clinical microbiology laboratories and clinicians because of their dynamic evolution and epidemiology, wide substrate specificity with its therapeutic implications, their significant diagnostic challenges and their prevention and infection control issues.

ESBLs are known as extended spectrum because they are able to hydrolyze a broader spectrum of beta-lactam antibiotics then the simple parent -lactamases from which they are derived. They are acquired plasmid-mediated -lactamases. They have the ability to inactivate -lactam antibiotic containing an Oxyimine-group such as Oxyimino-Cephalosporins (e.g.Ceftazidime, Ceftriaxone, Cefotaxime) as well as Oxyimino-monobactam (aztreonam). They are not active against Cephamycins and Carbapenems.

ESBLs have been found in a wide range of Gram-negative rods. However, the rest majority of strains expressing these enzymes belong to the family ***Enterobacteriaceae.*** *Klebsiella pneumoniae* seems to remain the major ESBL producer. Another very important organism is *Escherichia coli*. It is important to note the growing incidence of ESBLs in *Salmonella species*. Non - Enterobacteriaceae ESBL producer are relatively rare with *Pseudomonas aeruginosa* being the most important organism.

Aim and objectives

Isolation and identification of *Escherichia coli* species from diabetic foot infection swabs
Screening for the ESBL producing *Escherichia coli* by

Double Disk Synergy test
Disc replacement test
E-test strip
Three dimensional test
Preparation of ethanolic extracts of seeds
Antibiogram of ESBL producing *Escherichia coli* using certain seed extracts**.**

Materials and Methods

Collection of samples

A total of about 25 samples of diabetic foot infection samples were collected from the diabetic patients by using appropriate sterile cotton swabs from Government Hospital, Kanchipuram.

Transportation of sample

Samples were inoculated in sterile peptone water in screw – capped tubes and transported to the laboratory within an hour.

Processing of sample

Microscopic Examination

Staining Method

The collected specimen was subjected to differential staining by Gram's Staining techniques and observed for the presence of Gram

negative rod under oil - immersion lens of light Microscope.

Hanging Drop Method

The specimen was subjected to hanging drop method and observed for the presence of motile rods.

Culture
Media used
Basal medium
Nutrient Agar

The above ingredients were weighed accurately and mixed well with sterile distilled water in a conical flask.
The pH of the medium was adjusted to 7.4.
Then the medium was sterilized by autoclaving at 121^0C for 15 minutes.
20ml of the sterilized medium was poured into the Petri plates.
After, the sample was inoculated and incubated at 37^0C for 24 hours.
The above mentioned ingredients were weighed accurately and mixed well with sterile distilled water in a conical flask.
The PH of the medium was adjusted to 7.4.
Then the medium was sterilized and transferred to the test tubes.
After, the sample was inoculated and incubated at 37^0C for 24 hrs.

Eosin Methylene Blue Agar were weighed accurately and mixed well with sterile distilled water in a conical flask.
The PH of the medium was adjusted to 7.0.
The medium was sterilized by autoclaving at 121^0 C at 15lbs pressure for 15 minutes.
20 ml of sterilized medium was poured in sterile Petri plates.
Appropriate amount of sample was inoculated and incubated at 37^0C for 24 hours.

Biochemical Characteristics
Catalase test

A loop full of culture was introduced into hydrogen peroxide, formation of bubbles were observed.

Oxidase test

The oxidase disc (Tetramethyl – Paraphenylene - diamine dihydrochloride) was taken and the overnight culture of the test organism was streaked on the disc using a sterile loop.
The colour change was observed.

Indole test

Tryptone broth was prepared and dispensed in the test tubes.
After sterilization, the culture was inoculated and incubated at 37^0C for 24 hours.
After incubation, the Kovac's reagent was added in drops and the colour change was observed.

Methyl red test

MR broth was prepared and dispensed in the test tubes.
After sterilization, the culture was inoculated and incubated at 37^0C for 24 hrs.
After incubation, Methyl red reagent was added and gently mixed.
The result was observed after 15 minutes.

Voges – Proskauer Test

VP broth was prepared and dispensed in the test tubes.
After sterilization, the culture was inoculated and incubated at 37^0 C for 24 hrs.
After incubation, 0.5 ml of VP reagent Aand 0.5 ml of VP reagent B was added and gently mixed.
The result was observed after 15 minutes.

Citrate utilization test

Slants of Simmon's Citrate agar was prepared.
After sterilization, the overnight culture was streaked in the slants and incubated at 37^0C for 24 hrs.
After incubation, the change was observed .

Triple sugar Iron Test:

The TSI agar slants were prepared. Then, the test organism was inoculated and incubated for 24 hrs at 37^0C. After incubation, the result was observed.

Urease test
Christensen's Urea agar

Christensen's urea agar slants were prepared and sterilized.
The test organism was inoculated and incubated at 37^0C for 24 hrs.
After incubation, the result was observed.

Antimicrobial susceptibility of Salmonella species against standard antibiotics

The sterilized Mueller Hinton Agar medium was poured into a sterile Petri plate.
After solidification, a lawn culture of the organism was made and it is allowed to dry for 5 minutes.
The standard antibiotic discs were placed on to the surface of the inoculated plates (Ampicillin, Ceftazidime, Chloramphenicol, Ciprofloxacin, Gentamicin, Ceftozidime) and gently pressed in order to adhere the discs.
Then the plates were incubated at 37^0C for 18 - 24 hours.

Screening for Beta lactamase production

The beta lactamase producing Salmonella was identified by
performing the following tests

Double Disk Synergy test
Disc replacement test
E-test strip
Three dimensional test

Double disk synergy test

The earlier double disk approximation or double disk synergy (DDS) was the first detection test described in 1980’s.
DDST is a disk diffusion test in which 30 µg antibiotic disks of ceftazidime, ceftriaxone, cefotaxime and aztreonam are placed on the plate, 30 mm (center to center) from the amoxicillin/clavulanate (20µg/10µg) disk.

A clear extension of the edge of the antibiotic’s inhibition zone toward the disk containing clavulanate is interpreted as synergy, indicating the presence of an ESBL.

Disk replacement test

Three amoxicillin/ clavulanate disks are applied to a Muller-Hinton plate inoculated with the test organism.
After one hour at room temperature, these antibiotic disks are removed and replaced on the same spot by disks containing ceftazidime, cefotaxime and aztreonam.
Control disks of these three antibiotics are simultaneously placed at least 30 mm from these locations.
A positive test is indicated by a zone increase of 0.05 mm for the disks which have replaced the amoxicillin/ clavulanate disks compared to the control disks.

E – strip test
– lactamase filter Paper Method

Cut whatman No. 1 filter paper into 6 ck strips and sterile in hot air oven. Mix equal amount of buffered penicillin G and 1% starch solution.
Dry the filter paper at 37^0C for overnight and store the strips in airtight brown bottles at 4^0C.
Add 1-2 drops of Iodine Solution to the filter paper to spread evenly.A loop full of organism was spread into corresponding sector using separate loop or applicator sticks.Kept in a moist chamber for 1.5 min and observe the change.

Three dimensional disk diffusion:

The three dimensional test is a modification of the disk diffusion procedure.
It comprises an additional step which involves the application of bacterial inoculum into a circular slit in the agar 3mm from the antibiotic disks, towards the interior of the plate.

After the surface of the susceptibility plate was inoculated by the method of the disk diffusion procedure, the agar was stabbed vertically with a sterile no. 11 scalpel blade so that the point of the blade passed to the bottom of the agar at a predetermined point 3 mm inside the position at which the antibiotic disk were to be placed.
The blade was oriented perpendicular to the radius of the plate so that when the plate was rotated on a turntable, a circular slit was cut in the agar concentric with the margin of the plate.
After completion of the circular cut, the blade was withdrawn and sterilized. The plate was then rotated again on the turntable and the three-dimensional inoculum was dispensed into the circular slit by using a 200 µl Pipetman pipette with a sterile pipette tip.
The inoculum was dispensed so that the slit was filled but there was no overflow onto the agar surface.
After inoculation, the antibiotic disks were placed on the agar 3 mm outside of the inoculated circular slit, and the plate was incubated at 37^{0}C for 24 hours.

Collection of selected seeds

The dried seeds of selected plant (Cumin, Coriander, Fenugreek and pepper) were collected and were grind into a fine powder.

Preparation of ethanolic extract

A known quantity of each seed powder (50 gm) was taken in a
250 ml beaker and added with 100 ml of ethanol.
The preparation was kept at room temperature for 48 hrs and rapidly stirred using glass rod every 4 hrs.
After 48 hrs, the individual seed extracts were filtered through Whatmann No. 1 fitter paper to exclude the leaf powder.
Each seed extract was taken in separate beaker and kept in a water bath at 40 – 50 ^{0}C until the solvent gets evaporated.
A greasy final material (ethanolic extract) obtained from the plant was transferred to sterile screw capped bottles and stored under refrigerated condition till use.

Preparation of filter paper disc impregnated with ethanolic seed extracts

Filter paper disc of 6mm diameter were cut using a punching machine in Whatmann No.1 filter paper.
The discs were sterilized by dry heat sterilization.
20µl of each ethanolic seed extracts were added to the separate discs.
The dried extract impregnated discs were used for testing antibacterial activity against ESBL producing *Escherichiae coli* by disc diffusion method.

Antibiogram of ESBL *Escherichiae coli* using ethanolic seed extracts

The sterilized Muller Hinton Agar medium was poured into a sterile Petri plate.
After solidification, a lawn culture of the organism was made and it is allowed to dry for 5 minutes.
The filter paper discs impregnated with ethanolic seed extracts were placed on to the surface of the medium 3mm apart and gently pressed in order to adhere the discs.
Then the plates were incubated at 37^{0}C for 18 - 24 hours.
After incubation the zone of inhibition around the disc were measured.

Results

Out of 25 samples collected, 15 were found be positive for the prevalence of *Escherichiae coli* based on their morphology, cultural and biochemical characteristics and the results are presented in Table – 1.

Identification of isolates

Morphology - Gram negative, long, slender rods.
Motility - Actively motile

Endospore staining - Negative

Cultural characteristics - Aerobic and facultative anaerobe.

Colony morphology

Nutrient agar- Large, 2-3mm in diameter circular, low convex opaque, grey white colonies

MacConkey agar - Lactose Fermenting pink coloured colonies

The biochemical characterization of the isolate was given in the Table 1.

Table -1 Biochemical characterization

Biochemical Test	Result
Catalase	**Positive**
Oxidase	**Negative**
Indole	**Negative**
Methyl red	**Positive**
Voges-Proskauer	**Negative**
Citrate utilization	**Positive**
Triple Sugar Iron test	**Acid butt, Alkaline slant, Gas +,H_2+**
Urease	**Negative**
Growth characteristics on selective media	
Eosin Methylene Blue Agar	**Metallic Sheen colonies**

From the above mentioned biochemical characteristics and cultural characteristics on various selective media the isolate was identified as *Escherichiae coli*

Antimicrobial susceptibility of Salmonella species against standard antibiotics

The antimicrobial susceptibility of the isolates against the standard antibiotic was given in **Table 2.**

Table 2 Antimicrobial susceptibility of *Escherichiae coli* against standard antibiotics

S. no	Standard antibiotic	Zone of inhibition (diameter in cm)
1.	Ampicillin	**0.8**
2.	Chloramphenicol	**2.2**
3.	Ciprofloxacin	**1.4**
4.	Ceftaxime	**1.2**
5.	Ceftozidime	**1.6**
6.	**Gentamicin**	**2.0**

Screening for Beta lactamase production

All the isolates were subjected for screening for Beta lactamase production. Among the 15 positive *Escherichiae coli,* 5 were found to be ESBL producing *Escherichiae coli.*

Double disk synergy test

The results for the screening of Beta lactamase producing *Escherichiae coli* by Double disk synergy test.

Disk replacement test

The results for the screening of Beta lactamase producing *Escherichiae coli* by Disk replacement test .

E-Strip Test

The results for the screening of Beta lactamase producing *Escherichiae coli* by E-Strip test.

Three dimensional test

The results for the screening of Beta lactamase producing *Escherichiae coli* by Three dimensional test.

Antibiogram of ESBL *Escherichiae coli* using ethanolic seed extracts

The antimicrobial susceptibility of the isolates against the ethanolic seed extracts was given in **Table 3.**

Table 3. Antibiogram of ESBL *Escherichiae coli* using ethanolic seed extracts

S. no	Ethanolic seed extract	Zone of inhibition (diameter in mm)
1.	Coriander	**1.0**
2.	Cumin	**1.6**
3.	Fenugreek	**2.0**
4.	**Pepper**	**1.8**

Discussion

Urbancic Roven V and Gubina M, 1997, confirmed the polymicrobial etiology of diabetic foot infections of *Staphylococcus aureus* (26.7%) *Staphylococcus epidermidis* (22%), *Escherichia coli* (3.2%) *Klebsiella* species (5.4%).

McLigeyo and Otieno LS, 1991 reported that *Staphylococcus aureus* was 100% sensitive to augmentin, clindamycin, amikacin, while gram negative bacilli were sensitive to amikacin, cefotaxime, clindamycin, chloramphenicol.

Michael Rossini JR 1992, stated that 52.8% and 34.8% were aerobic gram negative and gram positive bacteria respectively.

Feleke Y *et al.,* 2002, reported the most frequent bacterial isolates were *Staphylococcus aureus* (30%), *Klebsiella pneumoniae* (23.4%), *Escherichia coli (19%) Pseudomonas* species (15%).

Ako – Nai AK *et al.,* 2006, reported that gram negative rods accounted for 57.9%, *Escherichia coli(15.1%), Staphylococcus aureus* (13.2%), *Pseudomonas aeruginosa* (12.5%*), Proteus* species (32.9%).

The prevalence of ESBL-producing organisms is increasing worldwide and several outbreaks have been reported. Serious infections with these organisms are associated with high morality rates as therapeutic options are limited (Asma M Al-Jasser., 2006).

Extended spectrum -lactamase (ESBL) producing organism are among the growing problems in the area of diabetic foot inections. (Asma M Al-Jasser., 2006).

In this present study an attempt is made to isolate and identify the ESBL producing *Escherichiae coli* in chronic diabetic patients with diabetic foot infection by Double disk synergy test, Disk replacement test, E-Strip test and Three dimensional test (Kenneth *et al*., 1992).

In addition the ESBL producing *Escherichiae coli* was subjected to antibiogram using ethanolic extracts of four seed extracts that are used in our Indian diet (Sabahat *et al*.,2007).

They showed reasonable zone of inhibition against the ESBL producing *Escherichiae coli*. Among the four, the fenugreek was found to be very effective as it shows maximum zone of inhibition.

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