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Research Article

A Preliminary study on the isolation and identification of ESBL-producing Salmonella and its Antibiogram using certain seed extracts

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Keywords

Extended Spectrum Beta Lactamase,
Double disk synergy test,
Disk replacement test,
E-Strip test and Three dimensional test.

Abstract

A total of about 25 samples including urine, stool and blood were collected from the typhoid patients by using appropriate sterile containers from Government Hospital, Kanchipuram. Samples were inoculated in sterile Selenite F-broth in screw – capped tubes and transported to the laboratory within an hour. The enriched 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 *Salmonella.* 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 *Salmonella*.

Introduction

With the extensive use of third and fourth generation cephalosporins as an important component of empirical therapy in intensive care units and high risk wards, resistance to these drugs has become a major problem all over the world. Resistance has developed in bacteria by possessing extended spectrum beta-lactamases (ESBLs) capable of hydrolyzing these newer cephalosporin. Beta-lactamase mediated resistance may be overcome by combining beta-lactam antibiotics with beta-lactamase inhibitors which bind irreversibly to the beta-lactamase inhibitors (tazobactam, sulbactam and clavulanic acid) are in clinical use, and in combination with beta-lactam antibiotics represent a successful strategy to combat a specific resistance mechanism. With a high prevalence of infections due to ESBL positive bacteria in our hospital, a parallel increase in the use of these combinations is being observed. Hence there is a need to determine the susceptibility pattern of different microorganisms against the commercially available combination agents, knowledge of which is essential to guide empiric as well as appropriate therapy of severe infections in hospitalized patients.

β-lactamases are enzymes produced by some bacteria and are responsible for their resistance to β-lactam antibiotics like Penicillins, Cephalosporins, Cephamycins and Carbapenems. These antibiotics have a common element in their molecular structure: a four-atom ring known as a beta-lactam. The lactamase enzyme breaks that ring open deactivating the molecules antibacterial properties.

Extended spectrum β-lactamase (ESBL) producing organism are among the growing problems in the area of infectious diseases such as ***Salmonellosis***.

β -lactamases

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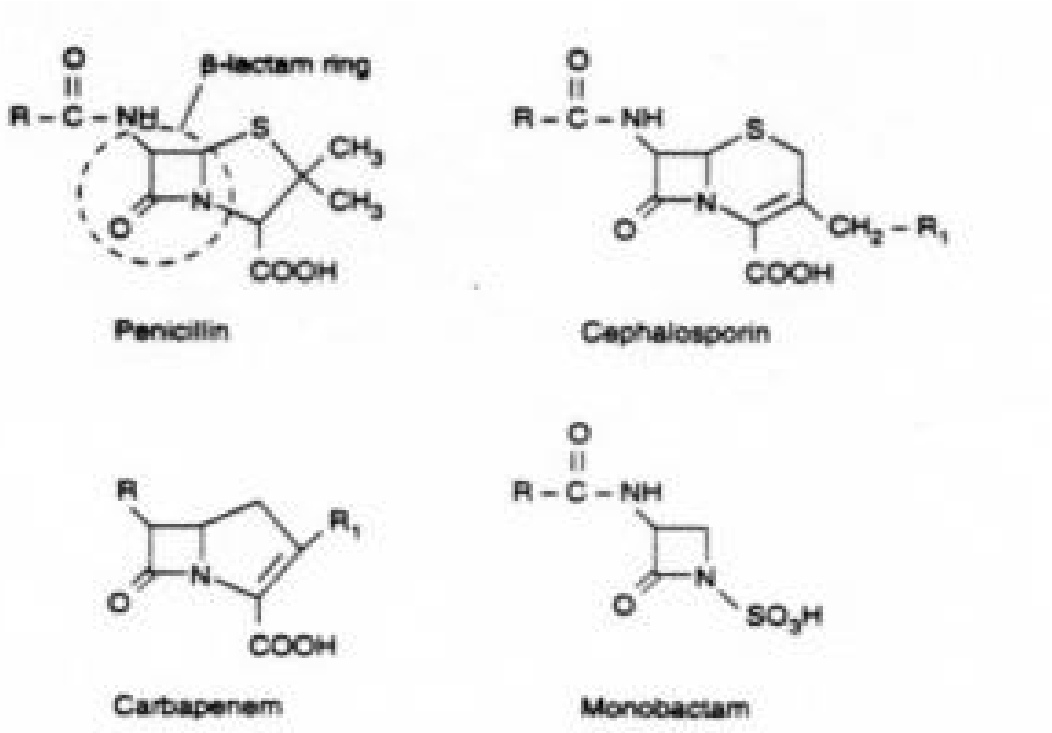
Structure of beta-lactams



Extended spectrum β-lactamases (ESBLs):

ESBLs constitute a growing class of plasmid-mediated β-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 morality 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.

Definition

ESBLs are known as extended spectrum because they are able to hydrolyze a broader spectrum of beta-lactam antibiotics then the simple parent β-lactamases from which they are derived. They are acquired plasmid-mediated β-lactamases. They have the ability to inactivate β-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.

ESBL producing organisms

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.

The origin and genetic determination of ESBLs

ESBLs activity is demonstrated by enzymes with substantial diversity in terms of structure and evolutional origin. The most prevalent ESBL types have evolved through point mutations of key amino acid substitutions in the present TEM and SHV enzymes.

Laboratory Detection

The detection methods can be divided into:

- Phenotypic methods
- Molecular methods

Phenotypic methods

They are based upon the resistance that ESBLs confer to oxyimino-beta-lactams (e.g. ceftriaxone, cefotaxime, ceftazidime and aztreonam) and the ability of a beta-lactamase inhibitor, usually clavulanate, to block this resistance.
Several tests have been proposed. They are as follows

- **Double disk diffusion test**
- **Cephalosporin/clavulanate combination**
- **Agar supplemented with clavulante**
- **Disk replacement method**
- **Three dimensional test**
- **E-test for ESBL**

Epidemiology of ESBL producing organisms

Infection and colonization with ESBL producing organisms are usually hospital-acquired especially in intensive care units. Other hospital units that are at increased risk include surgical wards, pediatrics and neonatology, rehabilitation units and oncology wards. Community clinics and nursing homes have also been identified as a potential reservoir. Risk factors for infection or colonization with ESBL - producing organisms include: length of hospital or ICU stay, presence of vascular or urinary catheters, undergoing hem dialysis, emergency abdominal surgery, gut colonization, low birth weight, prior exposure to any antibiotic (*e.g.*, quinolones, trimethoprim-sulfamethoxazole, amino glycoside and metronidazole), prior ceftazidime or azteronam administration and prior residence in a long term care facility. Very often the exact source of outbreak is never identified. However, the lower digestive tract of colonized patients has been recognized as the major source of ESBL-producing organisms and their cross-transmission among patients has been attributed to the hands of medical and nursing personnel. Environmental foci have also been reported but they are rare. They include ultrasound gel, thermometers, blood pressure cuffs and contaminated bronchoscopes.

Spectrum of clinical disease:

ESBLs-producing organisms cause a wide spectrum of clinical diseases ranging from colonization to serious infections. The common types of infections include urinary tract infections, peritonitis, cholangitis and intra abdominal abscess. They are a common cause of nosocomial pneumonia and central venous line related bacteremia hospitalized patients undergoing neurosurgical procedures, ESBL producers may also cause meningitis.

Microbial resistance through (ESBL) was first reported in the early 1980s in Europe and subsequently in the United States soon after the introduction of third generation Cephalosporin in clinical practice. Today this resistance mechanism has emerged globally and ESBL producing ***Enterobacteriaceae*** are recognized worldwide as nosocomial pathogens of major importance.

Early determination of ESBL-mediated resistance is clinically crucial in serious infections in order to start appropriate therapy as soon as possible. Even in less severe cases, early determination of antibacterial susceptibility is important to select the appropriate treatment regimens and increase the success rate of therapy, lower the rate of side effects of antibiotics and decrease the health care costs.

In this study, we isolated and identified the ESBL-producing *Esherchiae coli* from the diabetic foot lesions by using double disk synergy test. The isolates were also subjected to anti susceptibility testing against four different plant seed extracts.

Aim and objectives

Isolation and identification of *Escherichia coli* species from clinical specimens (urine, stool and blood etc).

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.

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 tubs. 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.

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 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 Jarlier 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. 5 mm for the disks which have replaced the amoxicillin/ clavulanate disks compared to the control disks.

E – strip test

β – 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 pipet with a sterile pipet 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 37^0C 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 *Salmonella* by disc diffusion method.

Antibiogram of ESBL *Salmonella* 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 37^0C for 18 - 24 hours.

After incubation the zone of inhibition around the disc were measured.

Results

Out of 25 samples collected all the isolates were identified as *Salmonella typhi* as they are enriched with selenite F broth and based on their morphology, cultural and biochemical characteristics.

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- White 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	
Bismuth Sulphite Agar	Black coloured colonies
Deoxycholate Citrate Agar	Colourless colonies
Salmonella Shigella Agar	Jet black colonies

From the above mentioned biochemical characteristics and cultural characteristics on various selective media the isolate was identified as *Salmonella typhi*.

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 Salmonella species 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 25 positive *Salmonella typhi*, 5 were found to be ESBL producing *Salmonella*.

Double disk synergy test

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

Disk replacement test

The results for the screening of Beta lactamase producing *Salmonella typhi* by Disk replacement test.

E-Strip Test

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

Three dimensional test

The results for the screening of Beta lactamase producing *Salmonella typhi* by Three dimensional test

Antibiogram of ESBL *Salmonella* 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 *Salmonella* 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

Salmonellosis ranges clinically from the *Salmonella gastroenteritis* to *enteric fever* which are life-threatening febrile systemic illness requiring prompt antibiotic therapy. It is endemic in many developing countries with poor sanitary conditions, but emerges sporadically as a serious public health threat in developed countries.

Enteric fever is a growing concern worldwide. World Health Organization (WHO) estimates that there are about 22 million cases of typhoid fever worldwide every year(Bhatia *et al*., 2007).

Unlike other Salmonellae, *Salmonella typhi* infect only humans. Chronic carriers are important reservoirs for *Salmonella typhi*. About 2-5% of cases become chronic carriers, some after asymptomatic infection, but the risk is highest for persons infected in middle age, particularly women with gall bladder abnormalities. Chronic carriage is customarily defined as carriage extending beyond one year. 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 β-lactamase (ESBL) producing organism are among the growing problems in the area of infectious diseases such as *Salmonellosis* (Asma M Al-Jasser., 2006).

In this present study an attempt is made to isolate and identify the ESBL producing *Salmonella* in chronic carriers of Enteric fever by Double disk synergy test, Disk replacement test, E-Strip test and Three dimensional test (Kenneth *et al*., 1992).

In addition the ESBL producing *Salmonella* 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 *Salmonella*. Among the four, the fenugreek was found to be very effective as it shows maximum zone of inhibition.

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