

**A PRELIMINARY STUDY ON THE ISOLATION AND IDENTIFICATION OF ESBL-PRODUCING SALMONELLA AND ITS ANTIBIOGRAM USING CERTAIN SEED EXTRACTS**

**R. Sumathi., G. Radhaselvi., P.K. Nagalakshmi and T. Sivakumar**

Department of Microbiology, Kanchi Shri Krishna College of Arts and Science, Kilambi, Krishnapuram, Kancheepuram -631 551 Tamil Nadu, India.

(E-mail: [renusumathi@rediffmail.co](mailto:renusumathi@rediffmail.co))

**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 susceptibility 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 tests. 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.

**KEY WORDS:** Beta lactamase, ESBL Salmonella- antimicrobial susceptibility, Ethanolic seed extract, Fenugreek.

**INTRODUCTION**

*Salmonellosis* ranges clinically from the *Salmonella gastroenteritis* to enteric fevers 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. 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 (Perez *et al.*, 2003).

Emergence of bacterial antimicrobial resistance has become a serious problem worldwide. While much of the resistance observed in human medicine is attributed to inappropriate use in humans, there is increasing evidence that antimicrobial use in animals selects for resistant foodborne pathogens that may be transmitted to humans as food contaminants (White *et al.*, 2002). To examine the incidence of extended spectrum  $\beta$  lactamase (ESBL) producing strain and multi-drug resistant strains of *Klebsiella pneumonia* isolated from children between 0-5 years of age. (Subha *et al.*, 2002). Antibacterial activity of aqueous infusions and decoctions of Kalonji (*Nigella sativa* L., *Ranunculaceae*), Cumin (*Cuminum cyminum* L., *Umbelliferae*), and Poppy seed (*Papaver somni ferum* L., *Papaveraceae*) were investigated against 188 bacterial isolates belonging to 11 different genera of Gram-positive and Gram-negative microorganisms isolated from oral cavity of apparently healthy individuals( Hosseini, 2008).

The emergence of resistance to antimicrobial agents within the *Salmonella* is a worldwide and server problem. A case of treatment failure due to the emergence of resistance to ceftriaxone is *Salmonella enterica* serotype to ceftriaxone is *Salmonella enterica* serotype Anatum and *Escherichia coli* strains were isolated concomitantly 2 weeks after the initiation of ceftriaxone therapy. The patient eventually died of a sepsis caused by the ceftriaxone –resistant *Salmonella*. The emergence of resistance to ceftriaxone in *Salmonella enterica* serotype Anatum was due to the invivo acquisition of a plasmid containing the bla<sub>CTX</sub> –M-3 gene and was the cause for treatment failure in this patient. (Lin- Hui Su *et al.*, 2002). The aim of the present study was to evaluate antimicrobial susceptibility patterns with special reference isolation and identification of *Salmonella*, Screening for the ESBL producing *Salmonella* by Antibioqram of ESBL-*Salmonella* using certain seed extracts.

**MATERIALS AND METHODS**

**Collection and transportation of samples**

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.

## Processing of collected samples

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

### Culture of collected samples by using Enrichment medium (Selenite F-broth)

Suspend 4 gram of part B in 1000 ml of distilled water. Add 19 gram of part A. Mix well and warm to dissolve the medium completely. Distribute in sterile test tubes. Sterilize in a boiling water bath or a free flowing steam for 10 minutes.

### Basal medium

The media 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<sup>0</sup>C for 15 minutes. 20ml of the sterilized medium was poured into the Petri plates. After, the sample was inoculated and incubated at 37<sup>0</sup>C for 24 hours.

### Nutrient broth

The media 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<sup>0</sup>C for 24 hrs.

### Deoxycholate citrate agar

The media were weighed accurately and mixed well with sterile distilled water in a conical flask. The PH of the medium was adjusted to 7.5. The medium was sterilized by autoclaving at 121<sup>0</sup> 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<sup>0</sup>C for 24 hours.

### Salmonella Shigella Agar

The media 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<sup>0</sup> 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<sup>0</sup>C for 24 hours.

### Bismuth sulphite agar

The media 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<sup>0</sup> 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<sup>0</sup>C for 24 hours.

### Biochemical Characteristics of isolated bacteria

Biochemical tests such as Catalase test, Oxidase test, Indole test, Methyl red test, Voges – Proskauer Test, Citrate utilization test, Triple sugar Iron Test and Urease test were performed.

### 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<sup>0</sup>C for 18 - 24 hours.

### Screening for Beta lactamase production

#### 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.

### $\beta$ – Lactamase filter Paper Method

Cut whatman No. 1 filter paper into 6 strips and sterilize in hot air oven. Mix equal amount of buffered penicillin G and 1% starch solution. Dry the filter paper at 37°C for overnight and store the strips in airtight brown bottles at 4°C. 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  $\mu$ l Pipetman pipet 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°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 filter paper to exclude the leaf powder. Each seed extract was taken in separate beaker and kept in a water bath at 40 – 50 °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. 20 $\mu$ l 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°C 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 (Table.1).

**Identification of isolates**

- Morphology - Gram negative, long, slender rods.
- Motility - Actively motile
- Endospore staining- Negative
- Cultural characteristics - Aerobic and facultative anaerobe.
- Nutrient agar - White coloured colonies.

**Table -1. Biochemical characterization of isolates**

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 <sub>2</sub> +
Urease	Negative
<b>Growth characteristics on selective media</b>	
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*. 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 typhi* by Double disk synergy test. The results for the screening of Beta lactamase producing *Salmonella typhi* by Disk replacement test. The results for the screening of Beta lactamase producing *Salmonella typhi* by E-Strip 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 of fenugreek was found to be more effective against Salmonella with compared with other type of seed extracts (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 fevers* 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 mortality rates as therapeutic options are limited (Asma M Al-Jasser, 2006). Extended spectrum  $\beta$ -lactamase (ESBL) producing organisms 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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