

**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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**ABSTRACT**

*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. Antibiotic therapy is useful in resolving signs and symptoms and can be considered in an attempt to eliminate the carrier state. The choice of antibiotics is dependent on the susceptibility of the organism.

Multidrug-resistant isolates of *Salmonella typhi* are common the results obtained in the present investigation 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. *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.  $\beta$ -lactamases are enzymes produced by some bacteria and are responsible for their resistance to  $\beta$ -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. Medicinal plant extract antimicrobial susceptibility on the isolates against ethanolic extract in coriander, cumin, Fenugreek and papper was given in anti microbial activity.

**Key words:** *Salmonellosis, Enteric fever, coriander, cumin, Fenugreek, papper.*

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**INTRODUCTION**

*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. (Aitmand *et al.*, 2002).

Typhoid fever is caused by *Salmonella typhi* (properly speaking, *S. enterica* subsp. *enterica* serovar Typhi) – a gram negative bacillus. It is group D *Salmonella*, as are many of those that cause non-typhoidal *Salmonellosis*. Paratyphoid fever, a milder form of typhoid-like illness, can be caused by *Salmonella paratyphi* (*S. enterica* subsp. *enterica* serovar Paratyphi) serotypes A,B and C. *Salmonella paratyphi* B is the most common serotype. *Salmonella paratyphi* A is less frequent and *Salmonella paratyphi* C is rare (Anna Baraniak *et al.*, 2002).

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**Typhoid** (“enteric”) **fever** has a very different presentation than the more common kinds of Salmonellosis. Vomiting and diarrhea are typically absent indeed, constipation is frequently reported. Initial symptoms typically include fever, anorexia, lethargy, malaise, headache, non-productive cough, abdominal pain and constipation. As the illness progresses, there is protracted fever and mental dullness (stupor). Diarrhea may develop, particularly in children less than one year old. Many patients develop hepatosplenomegaly. After the first week, approximately 30% of cases develop a salmon-colored maculopapular rash (rose spots) on the trunk. Mild infections are common, particularly in endemic areas. As many as 10-20% of untreated infections may be fatal and relapses are not uncommon. **Paratyphoid fever** is a similar but usually milder illness and is reported as Salmonellosis (Antunes *et al.*, 2006).

Antibiotic therapy is useful in resolving signs and symptoms and can be considered in an attempt to eliminate the carrier state. The choice of antibiotics is dependent on the susceptibility of the organism. Multidrug-resistant isolates of *Salmonella typhi* are common. 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 (Armand-Lefevre *et al.*, 2003).

Resistance has developed in bacteria by possessing extended spectrum beta-lactamases (ESBLs) capable of hydrolyzing these newer cephalosporins. 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 (Asma, and Jasser., 2006).

### **β-lactamases**

β-lactamases are enzymes produced by some bacteria and are responsible for their resistance to β-lactam antibiotics. The β-lactam antibiotics have a common element in their molecular structure; a four atom ring known as β-lactam. The lactamase enzyme breaks that ring open, deactivating the molecule's antibacterial properties (Babypadmini, and Appalaju., 2004).

### **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. (Basudha Khanal *et al.*, 2007).

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 (Bhatia *et al.*, 2007).

### **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 (Catherine Llanes *et al.*, 1999).

### **The origin and genetic determination of ESBLs**

ESBLs activity is demonstrated by enzymes with substantial diversity in terms of structure and evolutionary origin. The most prevalent ESBL types have evolved through point mutations of key amino acid substitutions in the present TEM and SHV enzymes (Charlotte pars *et al.*, 1996).

### **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 hemodialysis, 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 aztreonam administration and prior residence in a long term care facility (David *et al.*, 2005).

### **Medicinal Plant Seed Coriander**

Coriander seed preparations were used as a digestive aid and to treat stomach disorders in traditional Chinese, Indian, and European medicine; often in combination with other seeds such as cardamom, fennel, anise, and caraway. The

seeds can be used for making medicinal teas to soothe an upset stomach, treat indigestion, and relieve intestinal gas. In Germany, it's approved for the treatment of dyspeptic complaints, mild gastrointestinal upsets, flatulence, and to help stimulate the appetite. Coriander sometimes appears as a component in laxative remedies and medications for diarrhea. The seeds can sweeten bad breath and act as a mouthwash. In Asia, they treat colic (Eddy Vercauteren *et al.*, 1997).

#### **Medicinal Plant Cumin Seed**

The dried fruit used both as whole cumin seed and or as powdered form. Cumin seeds have also been used in ancient medicine of Ayurveda as an ingredient for many digestive formularies. Indian cumin finds world - wide use in foods, beverages, liquors, medicines, toiletries and perfumery. The spice is of particular value in the blending of Indian curry powder (George *et al.*, 1991).

#### **Medicinal Plant Fenugreek Seed**

During the early stages of any of the respiratory tract infection such as bronchitis, influenza, sinusitis, catarrh and suspected pneumonia, fenugreek tea helps to perspire dispel toxicity and shorten gestation period of fever. some extent they help in improving functions of liver. The herb is rich in vitamins and minerals. Leaves and seeds contain calcium, phosphorous, few vitamins from  $\beta$ -complex group in addition to vitamin C. It is also rich source of iron. They have haematinic value (Keiteda Silva Nogueira *et al.*, 2006).

Fenugreek has mild laxative property which also helps cleaning *ama* choked in body systems thus helping restoration from illness. A paste prepared from fenugreek and few other substances helps soothing irritation of skin and in addition reduce swelling of the part. Fenugreek helps in improving digestive tract functions and should be regularly used as a cleansing agent. Fenugreek is also useful in healing of different ulcers in digestive tract, in chronic problems like ulcerative colitis etc. It has also shown results in controlling diabetes mellitus (Kenneth *et al.*, 1992).

#### **Medicinal Plant Pepper Seed**

During a spell of fever which is accompanied by chills, taking a decoction prepared by boiling water with pepper corns is found to reduce rigors and lower temperature. It is folk herbal remedy and it is claimed that frequent use of this combination helps to cure skin problems like scabies, allergy rash and eczema kind of skin disorders (Khan *et al.*, 2008).

### **MATERIALS AND METHODS**

#### **Collection 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, cheyyar.

#### **Transportation of sample**

Samples were inoculated in sterile Selenite F-broth 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**

Different culture isolation and identification on the Enrichment medium Selenite F-broth, Basal medium Nutrient Agar, Nutrient broth, Deoxycholate citrate agar, Salmonella Shigella Agar, Bismuth sulphite agar (Ki Eun Lee and Yeonhee Lee., 2007).

##### **Biochemical Characteristics**

Different biochemical special characteristic identification test Catalase test, Oxidase test, IMVC test, Triple sugar Iron Test, Urease test, Antimicrobial susceptibility of Salmonella species against standard antibiotics test, Screening for Beta lactamase production test, Double disk synergy test, Disk replacement test, E – strip test  $\beta$  – lactamase filter Paper Method, Three dimensional disk diffusion test, (Laurent Poirel *et al.*, 2005, Reena Randhir *et al.*, 2004).

##### **Collection of selected seeds**

The dried seeds of selected plant (Cumin, Coriander, Fenugreek and pepper) were collected and were grind into a fine powder (Mallinson *et al.*, 2000).

### Preparation of ethanol 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.
- 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 (Mammeri *et al.*, 2007).

### Antibiogram of ESBL *Salmonella* using ethanolic seed extracts

#### Muller Hinton Agar

Beef heart infusion	-	300.00 gm
Casein acid hydrolysate	-	17.50 gm
Starch	-	1.50 gm
Agar	-	17.00
Distilled water	-	1000 ml
pH	-	7.3 ± 0.2

- 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 (Yusha *et al.*, 2007).
- 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 (Maria Isabel Morosini *et al.*, 1995).

## RESULTS AND DISCUSSION

Tables 1-3, Fig 1-2 indicate the results obtained in the present investigation 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. *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 (Michael *et al.*, 2007).

### 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*. The results for the screening of Beta lactamase producing *Salmonella typhi* by Double disk synergy test, Disk replacement test, E-Strip Test and Three dimensional test, also determine production of ESBL (Olivier Gaillot *et al.*, 1999).

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 (Olonisakin, *et al.*, 2006).

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 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).

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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.

#### Identification of isolates

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

#### Cultural

characteristics	-	Aerobic and facultative anaerobe.
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#### Colony morphology

Nutrient agar	-	White coloured colonies.
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**Table: 1.** Biochemical characteristics and cultural characteristics on various selective media the isolate was identified as *Salmonella typhi*

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
Growth characteristics on selective media	
Bismuth Sulphite Agar	Black coloured colonies
Deoxycholate Citrate Agar	Colourless colonies
Salmonella Shigella Agar	Jet black colonies

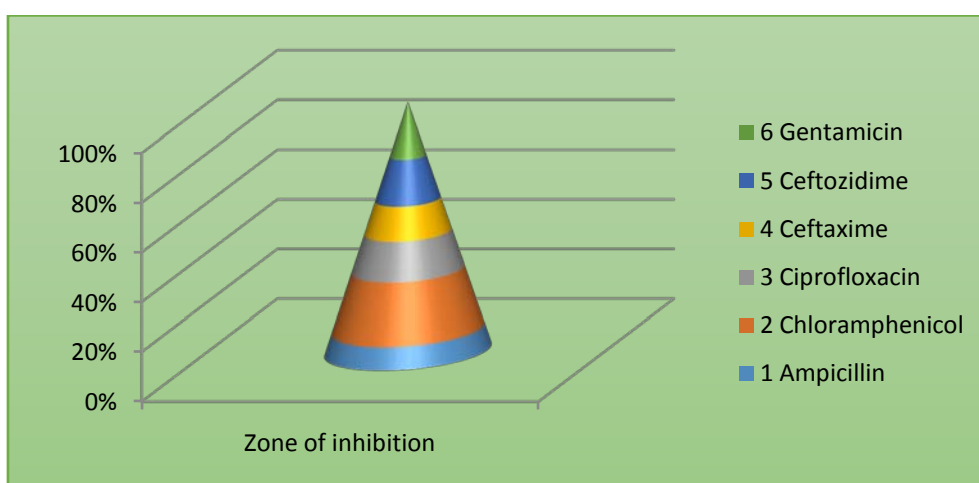
**Table: 2.** Antimicrobial susceptibility of Salmonella species against standard antibiotics

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

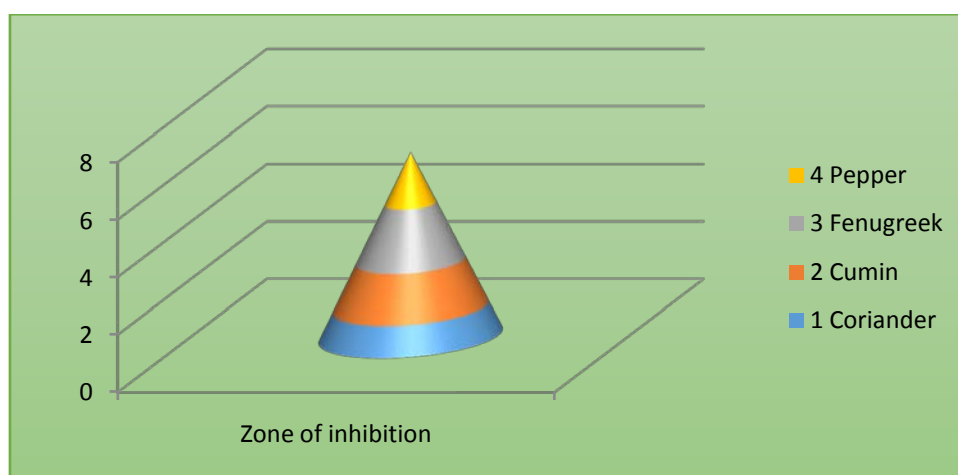
**Table: 3.** The antimicrobial susceptibility of the isolates against the ethanolic seed extracts was given in Antibigram of ESBL *Salmonella* using ethanol + seed extracts.

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

**Fig. – 1.** Antimicrobial susceptibility of Salmonella species against standard antibiotics



**Fig,-2.** The antimicrobial susceptibility of the isolates against the ethanolic seed extracts was given in Antibigram of ESBL *Salmonella* using ethanol + seed extracts.



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