DEMONSTRATION OF TRANSMISSION OF ANTIBIOTIC RESISTANT PLASMID FROM RESISTANT TO SENSITIVE STRAINS OF ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE AT 37°C

Tarneesh Soni* and Dr. Shweta Sao**

Department of Life Sciences, Dr. C.V. Raman University Kargi Road Kota Bilaspur (C.G.) 495013.

*Research Scholer, Department of Life Sciences, Dr. C.V. Raman University Kargi Road Kota Bilaspur (C.G.)

**Head and Associate Prof, Department of Life Sciences, Dr. C.V. Raman University Kargi Road Kota Bilaspur (C.G.)

ABSTRACT

In human body numerous bacteria are present in the form of normal bacterial flora. As many of them doesn’t came in contact of antibiotics shows sensitivity at the time of contact. When the patient is in continuous contact with hospital or community acquired infection, his normal flora becomes resistant to those antibiotics which he or she is taking during treatment. Escheria coli and Klebsiella pneumoniae are the most common hospital acquired pathogen which secretes Extended Spectrum Beta Lactamase enzyme to hydrolyze the beta lactam ring most of the antibiotics due to which it becomes resistant to that particular antibiotic. The resistant bacteria transfer its plasmid to sensitive bacteria and thus the sensitive one become resistant. So for the diagnosis of these microbes we apply Disc Diffusion Method in which strains of both sensitive and resistant bacteria is inoculated and then mixed in fixed proportion and then sensitivity test is done.

KEYWORDS: Extended Spectrum Beta Lactamase Enzyme, Disc Diffusion Method.

INTRODUCTION

Hospital-acquired bacterial infections may dominate the headlines, but most infections occur in the community. Indeed, 80% of the antibiotic prescribing takes place in the community –
in local practices, daycare centers and long-term care facilities such as nursing homes and rehabilitation centers. Most patients hospitalized in the Intensive Care Units after being discharged continue to carry Extended Spectrum β-lactamase (ESBL) producing Enterobacteriaceae over prolonged periods.

Extended-spectrum β-lactamases (ESBLs) are a rapidly evolving group of β-lactamases which share the ability to hydrolyze third-generation cephalosporins and momobactams such as aztreonam yet are inhibited by clavulanic acid (CLSI, 2010). Typically, they derive from genes for TEM-1, TEM-2, or SHV-1 by mutations that alter the amino acid configuration around the active site of these β-lactamases. This extends the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes. The first plasmid mediated β-lactamase in Gram-negative bacteria, TEM-1, was described in the early 1960s (Datta 1965).

A plasmid is a small DNA molecule that is physically separate from, and can replicate independently of, chromosomal DNA within a cell. Most commonly found as small circular, double-stranded DNA molecules in bacteria (Wikipedia 2010).

Plasmid-mediated transfer of drug-resistance genes among bacterial strains was considered one of the most important mechanisms for the spread of multidrug resistance. Characterizing plasmids from different bacterial species or strains is a key step towards understanding the mechanism of virulence and their evolution, and the design of more effective drugs against resistant pathogens (P.Chakraborty 2007)

**Escherichia coli**

*Escherichia coli* commonly abbreviated (*E. coli*) is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in
humans, and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K\textsubscript{2} and by preventing the establishment of pathogenic bacteria within the intestine.

**Klebsiella pneumoniae**
Morphologically *Klebsiella* species simulate *E.coli* except that they are nonmotile and possess a polysaccharide capsule. The capsule is responsible for the Mucoid appearance of the bacterial colonies and the enhanced virulence of the organism in vivo. They grow on ordinary media; produce pink colonies in MacConkey agar and Mucoid colonies of varying stickiness. They are widely distributed in nature, occurring both as commensal in human and animal intestines as well as saprophytes in soil, water and vegetation. These Gram-negative, encapsulated, non-motile, short, plump, straight rods measure 1-2 x 0.5-0.8 µm (P. Chakraborty 2007).

**TOPIC OF STUDY**
Demonstration of transmission of antibiotic resistance plasmid from resistance to sensitive strain of *Escherichia coli* and *Klebsiella pneumoniae* at 37°C.

**OBJECTIVE**
To demonstrate the transmission of antibiotic resistance plasmid from resistance to sensitive strain of *Escherichia coli* and *Klebsiella pneumoniae* at 37°C.

**MATERIALS**
For the completion of this research work I am using a no. of materials and these materials are as follows: Samples, Glassware’s, Laboratory Equipments, Culture Medias, Biochemical Reagents, Different Types of Antibiotic Discs.

**A. Samples:** Urine

**B. Glasswares:** Test Tubes, Petri Dishes, Microscope Slides, Glass Rod

**C. Laboratory Equipments:** Autoclave, Incubator, Biosafety Cabinet, Microscope

**D. Culture Media:** Blood Agar, MacConkey Agar, Mueller Hinton Agar

**E. Biochemical Reagents:** The term biochemical refers to something relating to biochemistry, the application of the tools and concepts of chemistry to living systems:- Citrate Test, Urease Test, Indole Test, Catalase Test, TSI Test.
Different Types of Antibiotic Discs
Amikacin, Amoxicillin/Clavulanic acid, Cefazolin, Cefuroxime, Cefepime, Cefoxitin, Cefoprarazone/Sulbactam, Ciprofloxacin, Gentamicin, Netilmicin.

METHODS
1. Sample Collection
A. Collection of Urine Sample
Patients included in the study were given a sterile, dry, test tube and request for 10-20 ml specimen. The first urine passed by the patient at the beginning of the day was collected for examination (clean catch, mid stream).

B. Collection of Wound Swab
A sterile technique was applied to aspirate or collect pus or wound swab from abscess or wound infection, either by disposable syringe or by sterile swab stick.

2. Inoculation of Samples
All samples were routinely cultured on MacConkey and blood agar plates. These plates were routinely incubated at 37°C aerobically and after overnight incubation, they were checked for bacterial growth.

Culture media showing growth for E.coli identification

Fig: 4 overnight incubation in MacConkey Agar Plate
Fig: 5 overnight incubation in Blood Agar Plate

Culture media showing growth for Klebsiella pneumoniae identification

Fig: 6 overnight incubation in MacConkey Agar Plate
Fig: 7 overnight incubation in Blood Agar Plate
3. Isolation and Identification of Organisms

Suspected Gram negative organisms were identified by colony characteristics, motility, oxidase reaction, citrate utilization, indole and gas production and sugar fermentation reactions. Triple sugar iron agar was used for H$_2$S production, sugar fermentation.

**Phenotypic Characteristics**

**Morphology:** Microscopical morphology.

Cultural characteristics:- Colonial morphology Growth in liquid media.

**Grams Staining**

There are two types of micro-organism seen: first gram positive cocci shows violet colour and gram negative bacilli shows pink colour under microscope.

**Catalase Test**

Catalase is the enzyme that breaks hydrogen peroxide (H$_2$O$_2$) into H$_2$O and O$_2$. Hydrogen peroxide is often used as a topical disinfectant in wounds, and the bubbling that is seen is due to the evolution of O$_2$ gas.

**Motility Test**

Motility test medium was used to test the motility of the bacteria. Semisolid media (0.5%) was used for this purpose.

**Test for Indole Production**

Indole production was tested for some bacteria, which has the ability to degrade tryptophan to indole. Indole production was detected by Kovac’s reagent (4-dimethyl amino benzaldehyde, isoamyl alcohol, hydrochloric acid).

**Citrate Utilization Test**

Simon’s citrate agar media was used for differentiating the intestinal bacteria and other microorganisms on the basis of citrate utilization. Citrate utilization is followed by alkaline reaction e.g., change of color from light green to blue.

**Triple Sugar Iron Agar (TSI)**

This media was used for initial identification of Gram negative bacilli, particularly members of *enterobacteriaceae*. Three primary characteristics of a bacterium was detected by this
media, include ability to ferment carbohydrate (lactose, sucrose, glucose,), ability to produce gas, and the production of hydrogen sulfide gas.

**Urease Agar Test**
Urea Agar was devised by Christensen for use as a solid medium for the differentiation of enteric bacilli. It differentiates between rapid urease-positive *Proteus* organisms. Some bacteria produce the enzyme Urease, which catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. Organisms that do not produce this enzyme cannot metabolize urea.

**Spot Oxidase Test (Cytochrome Oxidase)**
This test was used to identify the organisms, which produce the enzyme oxidase. A positive reaction indicates by a deep purple blue within 5-10 seconds.

**Maintenance and Preservation of Culture Strains**
Organisms grown in appropriate media for 18 hours were preserved in a nutrient agar slant at 2-8°C in a refrigerator and this culture was used within two weeks for routine laboratory works.

**Antimicrobial Susceptibility Test Done by Modified Kirby-Bauer Sensitivity Testing or Disc Diffusion Method**
Kirby-Bauer antibiotic testing (KB testing or disk diffusion antibiotic sensitivity testing) is a test which uses antibiotic-impregnated wafers to test whether particular bacteria are susceptible to specific antibiotics. A known quantity of bacteria is grown on agar plates in the presence of thin wafers containing relevant antibiotics. If the bacteria are susceptible to a particular antibiotic, an area of clearing surrounds the wafer where bacteria are not capable of growing (called a zone of inhibition).

**Inoculation of Isolated Bacteria and Placement of Discs**

![Fig: 15 AST of *E. coli*](image1)

![Fig: 16 AST of *K. pneumoniae*](image2)
Modified Kirby-Bauer sensitivity testing method was used for this purpose. Muller Hinton agar media was used, which has PH 7.2-7.4.

After AST we found sensitive as well as resistant strains of *E. coli* and *Klebsiella pneumoniae* by which we have to perform inoculum preparation.

**Inoculum Preparation**

**Growth Method**

The growth method is performed as follows:

1. At first we have to take 2 strains of *E. coli* out of which one strain is sensitive and the other one is resistant to antibiotics including that we have to take same strains for *Klebsiella pneumoniae*.
2. At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture of *E. coli* and *Klebsiella pneumoniae* separately and we have to separate them on the basis of sensitive and resistant strains against antibiotics. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of a suitable broth medium, such as peptone broth.
3. The broth cultures are incubated at 37°C at 18 to 24 hours. The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standards. This results in a suspension containing approximately 1 to 2 x 10^8 CFU/ml for *E. coli* as well as *Klebsiella pneumoniae*.

   [Note: 1 sensitive strain of *Escherichia coli* and 20 resistant strains of *Escherichia coli* for inoculation preparation and also done the same thing with *Klebsiella pneumoniae*.]

**Inoculation of Test Plates**

1. In the inoculum suspensions, a sterile cotton swab is dipped into the adjusted suspension.
2. The dried surface of a Mueller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface.
3. The lid may be left agar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

**Placement of Discs to Inoculated Agar Plates**

1. The predetermined battery of antimicrobial discs is dispensed onto the surface of the inoculated agar plate. Each disc must be pressed down to ensure complete contact with the
agar surface. Whether the discs are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from center to center.

2. The plates are inverted and placed in an incubator set to 37°C for 24 hours after the discs are applied.

3. Antimicrobial discs used for Gram negative bacteria were Amoxycillin-clavulanic acid (20/10mcg), Cefazolin (30 mcg), Cefuroxime (30 mcg), Cefoxitin (30 mcg), Cefoprerazone/sulbactam (75/30 mcg), Cefepime (30 mcg), Gentamicin (10 mcg), Amikacin (30 mcg), Netilmicin (30 mcg), Ciprofloxacin (5 mcg), (CLSI 2010).

**OBSERVATION AND RESULT**

On doing Antibiotic Sensitivity Testing of strains of *E. coli* and *Klebsiella pneumoniae* we found following zones after incubation: Table representation of strains of *E. coli*:

<table>
<thead>
<tr>
<th>S. No.</th>
<th>NAME OF ANTIBIOTICS</th>
<th>ANTIBIOTIC SENSITIVE STRAIN (in mm)</th>
<th>ANTIBIOTIC RESISTANT STRAIN (in mm)</th>
<th>FROM ANTIBIOTIC RESISTANT TO SENSITIVE STRAIN (MIXED) (in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Amoxycillin/Clavulanic-acid</td>
<td>22 mm</td>
<td>-</td>
<td>08 mm</td>
</tr>
<tr>
<td>2.</td>
<td>Cefazolin</td>
<td>26 mm</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Cefuroxime</td>
<td>26 mm</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Cefoxitin</td>
<td>27 mm</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>Cefoprerazone/Sulbactam</td>
<td>30 mm</td>
<td>19mm</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>Gentamicin</td>
<td>25 mm</td>
<td>21mm</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: (-) No Zone, (+) Fully Resistant

Fig: 18.1 *E.coli* Antibiotic Sensitive Strain
Fig: 18.2 *E.coli* Antibiotic Resistant Strain
Fig: 18.3 *E.coli* Antibiotic Resistant To Sensitive (Mixed) Strain
Table: 2 *Klebsiella pneumoniae* Strain No. (27)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>NAME OF ANTIBIOTICS</th>
<th>ANTIBIOTIC SENSITIVE STRAIN (in mm)</th>
<th>ANTIBIOTIC RESISTANT STRAIN (in mm)</th>
<th>FROM ANTIBIOTIC RESISTANT TO SENSITIVE STRAIN (MIXED) (in mm)</th>
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<tr>
<td></td>
<td>SEN</td>
<td>SEN</td>
<td>RES</td>
<td>SEN</td>
</tr>
<tr>
<td>1.</td>
<td>Cefazolin</td>
<td>24 mm</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Cefuroxime</td>
<td>23 mm</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Cefepime</td>
<td>27 mm</td>
<td>-</td>
<td>08 mm</td>
</tr>
<tr>
<td>4.</td>
<td>Amikacin</td>
<td>23 mm</td>
<td>20 mm</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>Netilmicin</td>
<td>22 mm</td>
<td>20 mm</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>Ciprofloxacin</td>
<td>22 mm</td>
<td>-</td>
<td>18 mm</td>
</tr>
</tbody>
</table>

**Note:** (-) No Zone, (+) Fully Resistant

**DISCUSSION**

Hospital-acquired bacterial infections may dominate the headlines, but most infections occur in the community. Indeed, 80% of the antibiotic prescribing takes place in the community – in local practices, daycare centers and long-term care facilities such as nursing homes and rehabilitation centers. Most patients hospitalized in the Intensive Care Units after being discharged continue to carry Extended Spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* over prolonged periods. Continued carriage of such strains may contribute to their extra hospital propagation.

These nosocomial pathogens mutant the normal microbial flora of patient’s body and convert it from sensitive to resistant by mutating the plasmid of normal flora. Antibiotics are being used for treating these infections but sometimes the resistant nosocomial pathogen overcomes it and maintains its dignity. So it is our duty to find a positive way so that we could save the life of many patients and thus by doing this research work I put forward a step in achieving that goal of healthy and prosperous life.
REFERENCES


