Demonstration of Transmission of Antibiotic Resistance Plasmid from Resistant to Sensitive Strain of Escherichia Coli and Klebsiella Pneumoniae at 37 ° C

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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. Escherichia 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

I. 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

Fig: 1 E.coli on MacConkey

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_2 and by preventing the establishment of pathogenic bacteria within the intestine.

Klebsiella pneumoniae

Fig: 2 K. pneumoniae on MacConkey agar

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 ^{o}C.

OBJECTIVE-

To demonstrate the transmission of antibiotic resistance plasmid from resistance to sensitive strain of Escherichia coli and Klebsiella pneumoniae at 37 ^{o}C.

II. REVIEW OF LITERATURE

According to R. Podschun and U. Ullmann (1992), bacteria belonging to the genus Klebsiella frequently cause human nosocomial infections. In particular, the medically most important Klebsiella species, Klebsiella pneumoniae, accounts for a significant proportion of hospital-acquired urinary tract infections, pneumonia, septicemias, and soft tissue infections. The principal pathogenic reservoirs for transmission of Klebsiella are the gastrointestinal tract and the hands of hospital personnel. Because of their ability to spread rapidly in the hospital environment, these bacteria tend to cause nosocomial outbreaks. Hospital outbreaks of multidrug-resistant Klebsiella spp., especially those in neonatal wards, are often caused by new types of strains, the so-called extended-spectrum-β-lactamase (ESBL) producers. The incidence of ESBL-producing strains among clinical Klebsiella isolates has been steadily increasing over the past years. The resulting limitations on the therapeutic options demand new measures for the management of Klebsiella hospital infections. While the different typing methods are useful epidemiological tools for infection control, recent findings about Klebsiella virulence factors have provided new insights into the pathogenic strategies of these bacteria. Klebsiella pathogenicity factors such as capsules or lipopolysaccharides are presently considered to be promising candidates for vaccination efforts that may serve as immunological infection control measures.

Nabeela Noor and Sheikh Azad Rasool (2004) described that Urinary tract infections (UTIs) are among the most commonly prevalent infections in clinical practice. Escherichia coli is the causative agent in about 85% of community acquired UTIs, followed by Klebsiella that accounts for 6 to 17% of such infections. Present study is based on the isolation-identification and antibiotic resistance pattern of about 60 indigenous bacterial isolates from UTI patients. Prevalence rates were consistent with those from major recent studies reported in the literature, i.e. 73% isolates were identified as E. coli, 16% as K. pneumoniae and 11% as Proteus sp. Bases of identification included morpho-cultural and biochemical characteristics. To assess the breadth of multidrug resistance among these isolates, culture medium incorporation method was employed using ampicillin, fosfomycin, chloramphenicol, tetracycline, and three aminoglycosides (kanamycin, gentamicin, and streptomycin). Of these isolates, 30% offered multidrug resistance to three or more agents. Among multidrug resistant isolates, 100% were resistant to ampicillin, 47% to streptomycin, 41% to chloramphenicol, gentamicin and tetracycline, 35% offered resistance to kanamycin while only 6% showed resistance to fosfomycin. After curing treatment with acridine orange, some of the isolates lost their resistance, thereby indicating the extrachromosomal location of the resistance determinants. Plasmid DNA (bearing multidrug resistant genes) was isolated from the uncured cells, and was stably transformed into the competent cured recipient cells.

According to Ari Robicsek, George A Jacoby, David C Hooper (2006) fluoroquinolones resistance is emerging in Gram-negative pathogens worldwide. The traditional understanding that quinolone resistance is acquired only through mutation and transmitted only vertically does not entirely account for the relative ease with which resistance develops in exquisitely susceptible organisms, or for the very

strong association between resistance to quinolones and to other agents. The recent discovery of plasmid-mediated horizontally transferable genes encoding quinolone resistance might shed light on these phenomena. The Qnr proteins, capable of protecting DNA gyrase from quinolones, have homologues in water-dwelling bacteria, and seem to have been in circulation for some time, having achieved global distribution in a variety of plasmid environments and bacterial genera. AAC (6')-Ib-cr, a variant aminoglycoside acetyltransferase capable of modifying ciprofloxacin and reducing its activity, seems to have emerged more recently, but might be even more prevalent than the Qnr proteins. Both mechanisms provide low-level quinolone resistance that facilitates the emergence of higher-level resistance in the presence of quinolones at therapeutic levels. Much remains to be understood about these genes, but their insidious promotion of substantial resistance, their horizontal spread, and their co-selection with other resistance elements indicate that a more cautious approach to quinolone use and a reconsideration of clinical breakpoints are needed.

Paul D. Stapleton, Kevin P. Shannon, and Gary L.enmerate that French (2008) three cefoxitin-resistant *Escherichia coli* isolates from stool specimens of a patient with leukemia were either resistant, intermediate, or sensitive to imipenem. Conjugation experiments showed that cefoxitin resistance, but not imipenem resistance, was transferable. All isolates were shown by isoelectric focusing to produce two β-lactamases with isoelectric points of 5.4 (TEM-1, confirmed by sequencing of a PCR product) and >8.5 (consistent with a class C β-lactamase). The gene coding for the unknown β-lactamase was cloned and sequenced and revealed an enzyme which had 99.9% sequence identity with the plasmid-determined class C β-lactamase CMY-2. The cloned β-lactamase gene differed from $bla_{\text{CMY-2}}$ at one nucleotide position that resulted in an amino acid change, tryptophan to arginine at position 221. We propose that this enzyme be designated CMY-4. Both the imipenem-resistant and -intermediate isolates lacked a 38-kDa outer membrane protein (OMP) that was present in the imipenem-sensitive isolate. The lack of an OMP alone did not explain the difference in carbapenem susceptibilities observed. However, measurement of β-lactamase activities (including measurements under conditions where TEM-1 β-lactamase was inhibited) indicated that the imipenem-intermediate isolate expressed six to eight folds less β-lactamase than did the other isolates. This study illustrates that carbapenem resistance in *E. coli* can arise from high-level expression of plasmid-mediated class C β-lactamase combined with an OMP deficiency. Furthermore, in the presence of an OMP deficiency, the level of expression of a plasmid-mediated class C β-lactamase is an important factor in determining whether *E. coli* isolates are fully resistant to carbapenems.

III. 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:

I am using following glassware's in my research work. They are as follows:

- Test Tubes
- Petri Dishes
- Microscope Slides
- Glass Rod

C. Laboratory Equipments:

In our research work we are using equipments like:

- Autoclave
- Incubator
- Biosafety Cabinet
- Microscope

D. Culture Media:

The following Medias are used by me in my research work:

- 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

We are doing a no. of biochemical tests for which we need biochemical reagents, the tests are:

- Citrate Test

- Urease Test
- Indole Test
- Catalase Test
- TSI Test

Different Types of Antibiotic Discs:

In my research work I am using following antibiotics:

1. Amikacin
2. Amoxycillin/Clavulanic acid
3. Cefazolin
4. Cefuroxime
5. Cefepime
6. Cefoxitin
7. Cefoprerazone/Sulbactam
8. Ciprofloxacin
9. Gentamicin
10. Netilmicin

IV. 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 37^0C aerobically and after overnight incubation, they were checked for bacterial growth.

Culture media showing growth for E.coli identification

Fig: 4 After overnight incubation in MacConkey Agar Plate

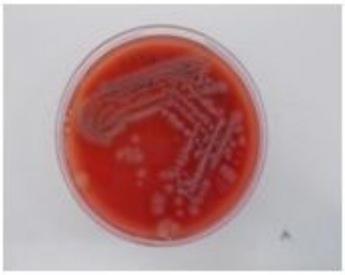

Fig: 5 After overnight incubation in Blood Agar Plate

Culture media showing growth for Klebsiella pneumoniae identification

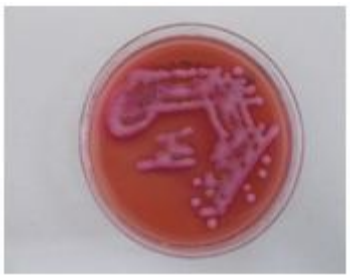

Fig: 6 After overnight incubation in MacConkey Agar Plate

Fig: 7 After 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_2S production, sugar fermentation.

Phenotypic Characteristics

Morphology

1. Microscopical morphology
2. Cultural characteristics

(a) Colonial morphology
(b) 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_2O_2) into H_2O 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 micro organisms 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^{O} 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

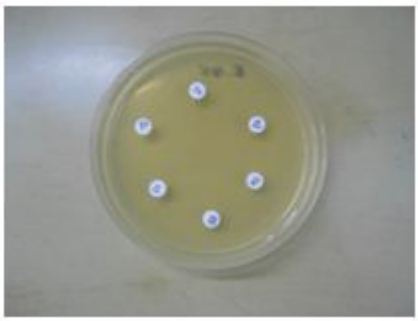

Fig: 15 AST of E. coli

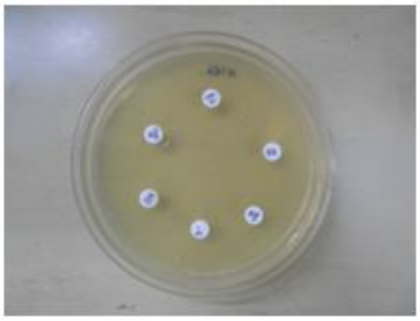

Fig: 16 AST of K. pneumonia

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: I have taken 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 (20/10 mcg), 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).

V. OBSERVATION AND RESULT

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

1. Table representation of strains of *E. coli*:

Table: 8 E.coli Strain No. (8)

S. No.	NAME OF ANTIBIOTICS	ANTIBIOTIC SENSITIVE STRAIN (in mm)	ANTIBIOTIC RESISTANT STRAIN (in mm)		FROM ANTIBIOTIC RESISTANT TO SENSITIVE STRAIN (MIXED) (in mm)	
		SEN	SEN	RES	SEN	RES
1.	Amoxycillin/Clavulanic- acid	22 mm	-	08 mm	-	06 mm
2.	Cefazolin	26 mm	-	+	-	+
3.	Cefuroxime	26 mm	-	+	-	+
4.	Cefoxitin	27 mm	-	+	-	+
5.	Cefoprerazone/Sulbactam	30 mm	19mm	-	21 mm	-
6.	Gentamicin	25 mm	21 mm	-	23 mm	-

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

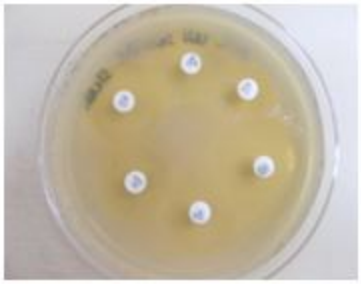

Fig: 18.1 E.coli Antibiotic Sensitive Strain

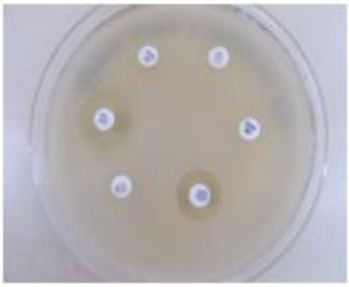

Fig: 18.2 E.coli Antibiotic Resistant Strain

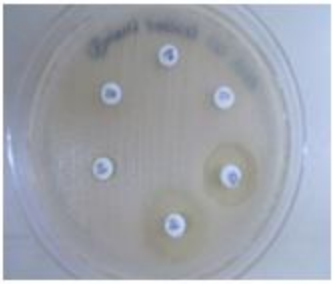

Fig: 18.3 E.coli Antibiotic Resistant To Sensitive (Mixed) Strain

2. Table representation of strains of Klebsiella pneumoniae:

Table: 27 Klebsiella pneumoniae Strain No. (27)

S. No.	NAME OF ANTIBIOTICS	ANTIBIOTIC SENSITIVE STRAIN (in mm)	ANTIBIOTIC RESISTANT STRAIN (in mm)		FROM ANTIBIOTIC RESISTANT TO SENSITIVE STRAIN (MIXED) (in mm)	
		SEN	SEN	RES	SEN	RES
1.	Cefazolin	24 mm	-	+	-	+
2.	Cefuroxime	23 mm	-	+	-	+
3.	Cefepime	27 mm	-	08 mm	-	10 mm
4.	Amikacin	23 mm	20 mm	-	21 mm	-
5.	Netilmicin	22 mm	20 mm	-	22 mm	-
6.	Ciprofloxacin	22 mm	-	18 mm	-	18 mm

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

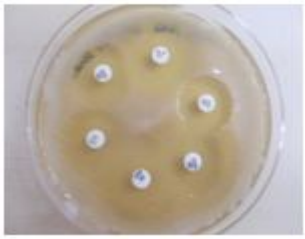

Fig: 36.1 Klebsiella Pneumoniae Antibiotic Sensitive Strain

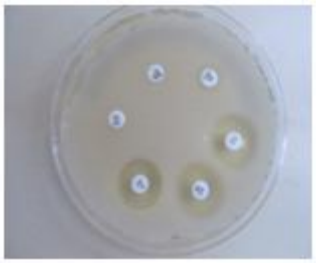

Fig: 36.2 Klebsiella Pneumoniae Antibiotic Resistant Strain

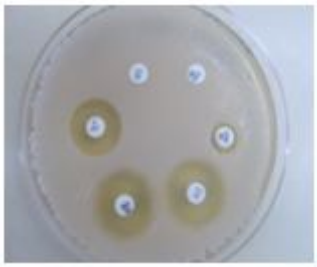

Fig: 36.3 Klebsiella Pneumoniae Antibiotic Resistant To Sensitive (Mixed) Strain

VI. 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.

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