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# EXTENDED SPECTRAL BETA LACTAMASE ACTIVITY OF SELECTED BACTERIA AND ANALYSIS ON THE WAYS OF OVERCOMING IT

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**ABSTRACT**
Extended-spectrum betalactamase enzymes (ESBL), producing bacterial infection is an increasing problem in and around hospital environment. The present study was carried out to isolate ESBL producing bacteria from samples such as soil, water and air collected from different environment such as hospital areas surrounded by more houses with thick population and sewage. The nature of bacterial isolates was identified to include in species level using standard morphological, biochemical and cultural characteristics, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aerugnosa*. Susceptibility to various antibacterial agents was studied to know the effective therapeutic agent as well as to know the resistant pattern. Particularly to penicillin derivatives, Amoxycillin Ampicillin and Cephalosporin derivatives such as ceftriaxone. Sulbactam and Clavulanic acid was used to overcome the penicillin and cephalosporin derivative resistance problem. Different susceptibility pattern was identified for the penicillin and cephalosporin derivative, and sulbactam and clavulanic acid was found to be better to cope up with beta lactamase problem.

**KEY WORDS**: Extended-spectrum beta-lactamases, sulbactam, clavulanic.

## INTRODUCTION

Beta lactam antibiotic resistance infections are common in hospitals and community it continues to be a major cause of morbidity and mortality rate in worldwide with serious consequences on the treatment of infectious diseases (Alekshun and Levy, 2007). These antibiotics are most frequently prescribed antimicrobial agents in treating Gram positive and Gram negative infections in human medicine (Bradford, 2001). ESBLs are beta-lactamases that hydrolyze extended-spectrum cephalosporins with an oxyamino side chain such as, ceftriaxone, cefotaxime and ceftazidime, as well as the oxyimino monobactam aztreonam. A broader set of β-lactam antibiotics are susceptible to hydrolysis by these enzymes. Therefore, antibiotic options in treatment of ESBL producing bacteria are extremely narrow. Carbapenems are the treatment of choice for serious infections due to ESBL producing bacteria, so far carbapenem resistant isolates have recently been reported. ESBL producing bacteria may look susceptible to some extended spectrum cephalosporins. However, treatment with such antibiotics has been associated with high failure rates. A novel approach to countering bacterial β-lactamases is the delivery of a combination with a β-lactamase inhibitor with β-lactam antibiotic. Several such combinations are inhibitors clavulanic acid, sulbactam and tazobactam (Maiti *et al*., 1998). These inhibitors are biological origin so it is nontoxic, although this compound has only weak antibacterial properties; it is a potent inhibitor of many β-lactamases found in clinical isolates (Wise *et al*., 1978). Ampicillin-sulbactam is a parenteral formulation that expands the spectrum of ampicillin to include most of beta-lactamase producing strains (Bush and Johnson 2000).The in vitro activity of this compound in combination with other penicillins is described.

## MATERIALS & METHODS

### Collection of samples

The soil, water and air samples were collected from different areas in Arcot, Vellore district.

### Cleaning of glassware

The glassware of borosil grade was used in all the experiments. The glassware was cleaned by soaking in chromic acid solution (100g potassium dichromate dissolved in one liter water with 500ml concentration sulphuric acid) for 2hours and washed in water.

### Chemicals used

All the chemicals were used of high purity analysis specifications and whenever necessary sigma grade chemicals were used.

### Sterilization techniques

All the glassware was sterilized in a hot air oven at $180^0$C for 2hours. All the prepared media and water blanks were sterilized in an autoclave at 1 atm for 95 minutes. All the antibiotics were filter sterilized using sintered glass filter the isolation, purification, inoculation and other microbiologic lab works were carried out in a laminar airflow chamber (Air Flow, India).

### Isolation of ESBL producing bacteria

Bacteria from the water and soil were isolated using the spread plate technique. Four media were used; Nutrient agar, Blood agar, Mac conkey agar and Mannitol salt agar plates were incubated at 37ºC for 24hrs for bacteria. After 24hrs the colonies grown on the plates were examined. The Air-O-Cell cassette device is used to isolate bacteria from air sample.

### Identification of colony morphology

The morphology of each type of colony was examined and the results were noted.

**Subculture**

The same type of colony was simultaneously taken from the plate aseptically and streaked on the prepared nutrient agar plate. Then the plates were incubated at 37ºC for 24 hours. After one day, the results were noted for their colony morphology and pigment production and also the colonies growth on the plate were tests and antibiotic sensitivity tests.

**Bacterial identification**

Isolated bacteria were identified using conventional biochemical tests described by MacFadden, 1976.

**Microscopic examination**

**Gram Staining**

According to the method of Hucker *et al*., 1923. The gram staining technique was followed. To prepare a thin smear was prepared on a grease free slide using the individual colony grown on the medium. It was flooded with crystal violet solution and allowed to stand for 1minute. Then it was washed with water and then flooded with gram's iodine solution. It was drained and decolorized with 95% ethanol, which then washed gently in running water. Then the same was stained with a counter stain called safranine for 30seconds. After drying the stained smear was observed under microscope.

**Motility test**

According to the method of Harley and John 2004. The hanging drop technique was followed to observe the motility of the bacteria with which they are classified as motile and non-motile organisms. A drop of suspension of culture was placed at the center of a cover slip and placed in an inverted position over a cavity slide so that the drop is hanging over the cavity. The edge of the drop was observed under low power lens of a microscope.

**Test for Staphylococci**

**Coagulase test**

**Tube test**

According to the modified method of Fisk 1940. To 0.5ml of diluted (1:4) citrated human plasma in a small sterile tube was inoculated with heavy saline suspension of the organism and was incubated at 37ºC for 1-4 hours.

**Selective media**

The suspected colony was taken aseptically and streaked on Baird Parker agar medium and were incubated at $37^0$C for 24hrs.

**Biochemical Tests**

According to the method of (Clark *et al*., 1984, Gilardi 1985) sugar fermentation test (glucose, xylose, and maltose), Indole production, Methyl red, Voges Proskauer, citrate utilization, catalase, oxidase, gelatin liquefaction, Urease, and oxidative-fermentative test. These tests were considered conventional identification methods for identification of bacteria.

**Sugar fermentation test**

The term sugar in microbiology denoted any fermentable substance. They may be monosaccharides, disaccharides, polysaccharides, trisaccharides, alcohols, glycosides and non-carbohydrate substances such as inositol. The usual sugar media consist of 1% of the sugar concerned in peptone water (or Himedia discs) with an appropriate indicator. A Durham's tube was kept inverted in the sugar tube to detect gas production. The incubation period was 24hrs and the temperature was generally 37ºC. The organisms grown in the plates were inoculated in the prepared different sugar media containing glucose, sucrose, lactose and mannitol and were incubated for 24hrs at $37^0$C

**Indole Test**

The colony from the plate was inoculated into the indole medium in a tube and then incubated at $37^0$C for 24hrs

**Methyl Red Test**

The colony from the plate was inoculated into MR-VP broth tubes and incubated at $37^0$C for 24hrs.

**Voges-prosaur test**

The same type colony was inoculated into the MR-VP broth tubes and incubated at $37^0$C for 24hrs.

**Citrate utilization test**

Slant of Simmon's citrate agar medium was inoculated with the organism grown on the plate and incubated at $37^0$C for 24hrs.

**Oxidase test**

The organisms taken from the plate was streaked on filter paper incorporated with oxidase reagent (1% tetramethyl paraphenylene diamine dihydrochloric acid in water).

**Urease test**

This test done in urease medium. Inoculate the slope heavily and incubate at $37^0$C.

**Catalase production**

Add a loopful of 10% $H_2O_2$ on colonies on nutrient agar.

**Gelatin Hydrolysis**

The colony to be tested was stabbed into gelatin deep tubes. The tubes were incubated at $37^0$C for 24hrs. Then the tubes were kept in the refrigerator for ½ an hour at $4^0$C.

**Oxidation Fermentation Test**

O-F base was prepared and sterilized at $121^0$C for 15mins. 1 gram of carbohydrate in 10ml of distilled water was sterilized by filtration. After sterilization O-F medium base was cooled to $55^0$C and 10ml of sugar solution was added to the medium. Then the medium was distributed in 5ml quantities in 12x100mm tubes and allowed to set in an upright to get the solid butt. Then the test organism was stabbed 3 times and petroleum jelly was poured over the butt and then incubated at $35^0$-$37^0$C for 4 days. Controls were prepared with and without inoculums.

**Antibiotic Sensitivity Test**

**Test for the isolates**

After confirming the quality of medium and discs using standard strain antibiotic sensitivity test was performed in Muller Hinton agar medium. A lawn culture was prepared on the media with the swab from the culture in nutrient broth. Antibiotic disc like Amoxicillin with clavulnic acid disc were placed on the media using sterile forceps. After 24hrs of incubation the clear zone of inhibition around the disc was measured and the results were noted (Modified Kirby- Bauer technique)

**Test for the isolates resistant to Penicillin and Cephalosporin Derivatives**

Muller Hinton agar was prepared and sterilized at $121^0$C for 15mins, after sterilization, pinch of Ampicillin was added to the medium and poured in to the sterile petri plates. After solidification different types of environmental samples were streaked in to the medium. Incubate at $37^0$C for 24hrs, on the next day bacteria resistant organisms were isolated and the bacteria resistant organisms were

streaked in to different combinations of antibiotics sulbactam with ampicillin incorporate medium, Clavulanic acid with ampicillin incorporate medium and Sulbactam with Ceftrioxone incorporate medium. Similar test was carried out for amoxicillin also separately.

## RESULTS & DISCUSSION

**TABLE 1:** ESBL-producing bacteria isolated from soil, water and air samples of different environment

| S.No | Place of sample collected | Type of sample (numbers) | Name of the organisms | Percentage of organisms in collected sample |
|---|---|---|---|---|
| 1. | Hospital | Soil | *S. aureus* | 55 |
| | 100 | 32 | *E. coli* | 23 |
| | | | *B. subtilis* | 12 |
| | | | *P. aeruginosa* | 10 |
| | | Water | *S. aureus* | 46 |
| | | 43 | *E. coli* | 32 |
| | | | *B. subtilis* | 12 |
| | | | *P. aeruginosa* | 10 |
| | | Air | *B. subtilis* | 65 |
| | | 25 | *P. aeruginosa* | 35 |

Table 1 shows the total number of 100 independent isolates of different organisms belonging to *S. aureus, E. coli, B. subtilis,* and *P. aeruginosa* were collected. The ESBL-producing bacteria isolated were isolated from soil sample *S.aureus* (55% of the isolates), *E. coli* (23%), *B. subtilis* (12%) and *P. aeruginosa* (10%). Water sample *S.aureus* (46% of the isolates), *E. coli* (32%), *B. subtilis* (12%) and *P. aeruginosa* (10%). and air sample *B. subtilis* (65%) and *P. aeruginosa* (35%). All these ESBL-Eb were obtained in samples taken from different environment of soil, water and air. Among the isolated from soil, water and air sample *S. aureus* are more in number followed by *E.coli, B. subtilis* and *P. aeruginosa*. Veldman *et al*., 2014 previous studies have reported the isolation of ESBL bacteria in fresh culinary herbs from Southeast Asia, highlighting the potential human health risk. Reuland *et al*., 2014 a recent report carried out in raw vegetables from the Netherlands refers the detection of the same ESBL bacteria detected in our study ESBL producing *E.hormaechei* isolates are unfrequently reports related with the detection of ESBL *E. hormaechei* isolates at the hospital level Carvalho Assef *et al*., 2014 survey a high prevalence of ESBL producing *P. aeruginosa* with important antimicrobial resistance rates to commonly used antibiotics.

**TABLE 2:** Morphology and biochemical characterization of the bacterial isolates

| S.No | Name of the Test | *E. coli* | *P. aeruginosa* | *B. subtilis* | *S. aureus* |
|---|---|---|---|---|---|
| 1. | Gram reaction | - | - | + | + |
| 2. | Shape | Bacilli | Bacilli | Bacilli | Cocci |
| 3. | Arrangement | Single | Single | Single | Cluster |
| 4. | Motility | Motile | Motile | Motile | Non Motile |
| 5. | Nutrient Agar | Large, Round, Opaque colony | Earthy smell, Green colour with diffused pigment | Greysih, yellow large colony | Circular convex, smooth, golden yellow colour colony |
| 6. | Macconkey Agar | LF colony | NLF colony | NLF colony | LF colony |
| 7. | Mannitol salt agar | Large, round, opaque colony | Large, opaque, irregular colony | Dry, flat irregular with lobate margins | Yellow colour colony |
| 8. | Blood agar | γ haemolytic colonies | β haemolytic colonies | β haemolytic colonies | β haemolytic colonies |
| 9. | Catalase | + | + | + | + |
| 10. | Oxidase | + | + | - | - |
| 11. | Coagulase | Not done | Not done | Not done | + |
| 12. | Indole | + | - | - | - |
| 13. | Methyl red | + | + | - | + |
| 14. | Voges Proskauer | - | - | + | + |
| 15. | Citrate | - | + | + | + |
| 16. | Urease | - | + | - | + |
| 17. | Gelatinase | - | + | + | + |
| 18. | Nitrate | + | + | + | + |
| 19. | O/F test | Oxidative | Oxidative | Oxidative | Oxidative |
| 20. | Glucose | + | + | + | - |
| 21. | Lactose | + | - | - | - |
| 22. | Sucrose | + | + | + | + |
| 23. | Mannitol | - | - | - | + |

Key: + (Positive) ; - (Negative)

Brolund *et al*., 2013 reports that the number of ESBL producing *E. coli* is steadily increasing in Sweden. Hamilton-Miller, 1990 reports that emergence and spread of antibiotic resistance bacteria are due to use of antibiotics in hospitals environment. Jenkinson, 1996 Studied bacteria have acquired resistances to specific antibiotics that include antibiotic inactivation, target alteration, or drug exclusion. It is important that new antibiotic are discovered or designed that will poison the pumps or bypass the efflux mechanisms.

Table 2 shows the all isolates were initially identified by the following conventional tests: In gram staining results shows as gram positive rod, gram negative rods or gram positive cocci in cluster. The organisms studied mainly here are gram positive cocci in cluster. Motility test shows where the darting or corkscrew movement of the bacteria is motile or nonmotile. In coagulase test the formation of coagulation and compared with the controls. By studied for their colony morphology and colour on its selective media are used to confirm *S. aureus*. Sugar fermentation the sugars were fermented by various types of bacteria and in that process acid and gas was produced which were indicated by the colour change for acid and gas collected in to the inverted Durham's tube. Indole test the formation of red ring upon the addition of Kovac's reagent indicated the positive reaction. Methyl red test the formation of red colour on the addition of methyl red indicator indicated the positive reaction. In Voges Proskauer test to development of pink to bright red colour on the addition of Barrit's reagent to the medium indicates positive reaction. Citrate utilization test to change the colour from green to blue indicates the positive reaction. In oxidase test the appearance of purple colour indicates positive result. In urease test change of colour from a purple pink colour indicate positive reaction. In gelatin hydrolysis that remain liquefied produce gelatinase and shows positive reaction.

**TABLE 3:** Screening of Antibiotic activity

| S.No | Antibiotic | Diameter of Zone of inhibition (mm) | | | |
|---|---|---|---|---|---|
| | | *E.coli* | *S.aureus* | *P.aeruginosa* | *B.subtilis* |
| 1. | Ampicillin | 12 (R) | 10 (R) | 14 (R) | 12 (R) |
| 2. | Amoxycillin | 08 (R) | 08 (R) | 09 (R) | 07 (R) |
| 3. | Cefotaxime | 17 (I) | 14 (S) | 15 (I) | 17 (S) |
| 4. | Ceftriaxone | 10 (R) | 11 (R) | 11 (R) | 08 (R) |
| 5. | Cephalexin | 19 (S) | 19 (S) | 12 (R) | 19 (S) |
| 6. | Ciprofloxacin | 26 (S) | 26 (S) | 25 (S) | 26 (S) |
| 7. | Cloxacillin | 18 (S) | 18 (S) | Not used | 18 (S) |
| 8. | Co-trimaxazole | 21 (S) | 21 (S) | 14 (I) | 21 (S) |
| 9. | Erythromycin | 08 (R) | 08 (R) | No Zone (R) | 08 (R) |
| 10. | Gentamycin | 14 (I) | 14 (I) | 16 (S) | 14 (I) |
| 11. | Penicillin | 05 (R) | 05 (R) | Not used | 05 (R) |
| 12. | Norfloxacin | 22 (S) | 22 (S) | 20 (S) | 22 (S) |
| 13. | Ofloxcin | 10 (R) | 10 (R) | No Zone (R) | 10 (R) |
| 14. | Methicillin | 12 (R) | 12 (R) | Not used | 12 (R) |
| 15. | Tetracycline | 22 (S) | 22 (S) | No Zone (R) | 22 (S) |

(S) – Sensitive, (R) – Resistance, (I) – Intermediate

Table 3 shows the number of antibiotics used for screening of different antibiotic activity of organisms *S. aureus, E. coli, B. subtilis,* and *P.aeruginosa* were identified as Resistance, Sensitive and Intermediate. Out of 15 antibiotics used *E. coli* shows Resistance (n=7), Sensitive (n=6) and Intermediate (n=2), *S. aureus* shows Resistance (n=7), Sensitive (n=7) and Intermediate (n=1), *B. subtilis* shows Resistance (n=7), Sensitive (n=7) and Intermediate (n=1) and *P. aeruginosa* shows Resistance (n=7), Sensitive (n=3) and Intermediate (n=2).

**TABLE 4:** Amoxycillin with Sulbactam Medium and Clavulanic acid

| S.No | Name of the organisms | Amoxycillin Medium | Amoxycillin with Sulbactam | Amoxycillin with Clavulanic acid |
|---|---|---|---|---|
| 1. | *S.aureus* | Resistance | Sensitive | Sensitive |
| 2. | *E.coli* | Resistance | Intermediate | Intermediate |
| 3. | *B.subtilis* | Resistance | Intermediate | Intermediate |
| 4. | *P.aeruginosa* | Resistance | Intermediate | Intermediate |

Table 4, 5 and 6 shows the resistant in Amoxycillin, Ampicillin and Ceftriaxon medium after incorporation of sulbactam and clavulanic acid the organisms shows different results Amoxycillin combined with Sulbactam / Clavulanic acid as sensitive to *S.aureus* and intermediate to *E. coli, B. subtilis, P.aeruginos*a. Ampicillin combined with sulbactam / Clavulanic acid as sensitive to *S.aureus* and intermediate to *E. coli and B. subtilis.* Resistance to *P. aeruginos*a. Ceftriaxone combined with Sulbactam / Clavulanic acid as sensitive to *S.aureus* and intermediate to *E. coli, B. subtilis, P. aeruginos*a. Wise *et al*., 978 reported that Clavulanic acid appears to inhibit a wide range of β lactamases found in clinical isolates of gram positive and gram negative organisms. The concept of therapy with β lactam drugs together with an enzyme inhibitor. Sibhghatulla Shaikh et al 2014 reports that carbapenems are widely used drugs for the treatment of severe infections caused by ESBL-producing Enterobacteriaceae, isolates exhibited co-resistance to an array of antibiotics tested. Reading 1977 studied the MIC

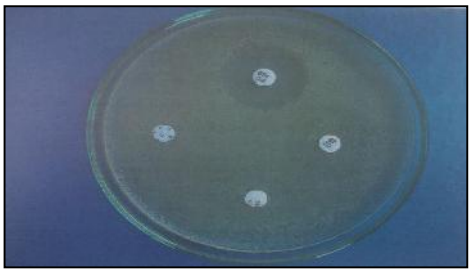
of ampicillin and cephaloridine against, β lactamase producing, penicillin resistant strains of *S. aureus*, *B. cereus*, *P. mirabilis*, *P. aeruginosa* and *E. coli* to be considerably reduced by the addition of low concentrations of clavulanic acid.

**TABLE 5:** Ampicillin with Sulbactam Medium and Clavulanic acid

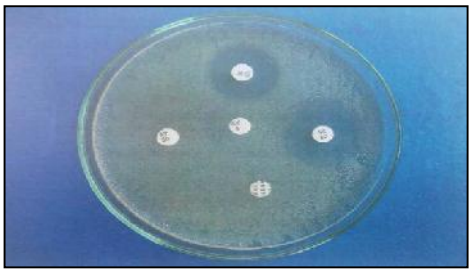
| S.No | Name of the organisms | Ampicillin Medium | Ampicillin with Sulbactam | Ampicillin with Clavulanic acid |
|---|---|---|---|---|
| 1. | *S.aureus* | Resistance | Sensitive | Sensitive |
| 2. | *E.coli* | Resistance | Intermediate | Intermediate |
| 3. | *B.subtilis* | Resistance | Intermediate | Intermediate |
| 4. | *P.aeruginosa* | Resistance | Resistance | Resistance |

**TABLE 6:** Ceftriaxone with Sulbactam Medium and Clavulanic acid

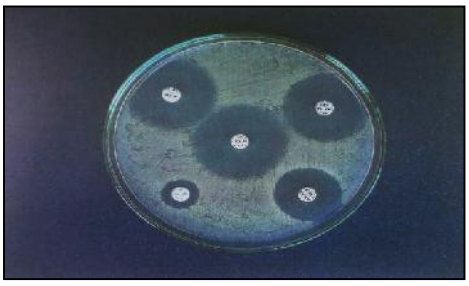
| S.No | Name of the organisms | Ceftriaxon Medium | Ceftriaxon with Sulbactam | Ceftriaxon with Clavulanic acid |
|---|---|---|---|---|
| 1. | *S.aureus* | Resistance | Sensitive | Sensitive |
| 2. | *E.coli* | Resistance | Intermediate | Intermediate |
| 3. | *B.subtilis* | Resistance | Intermediate | Intermediate |
| 4. | *P.aeruginosa* | Resistance | Intermediate | Intermediate |



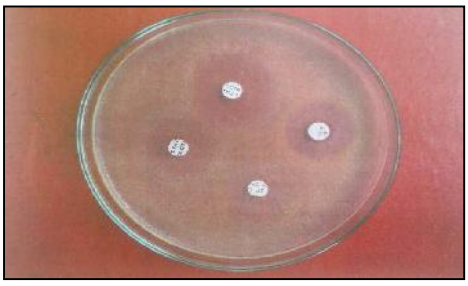
Antibiotic sensitivity of *E. coli*



Antibiotic sensitivity of *S.aureus*



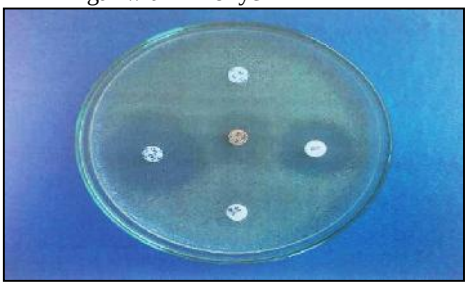
Antibiotic sensitivity of *B. subtilis*



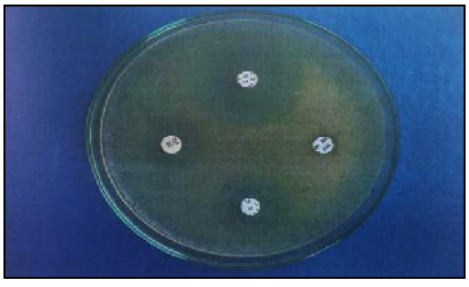
Antibiotic sensitivity of *B. subtilis*

Growth *of S. aureus* in Muller Hinton Agar with Amoxycillin

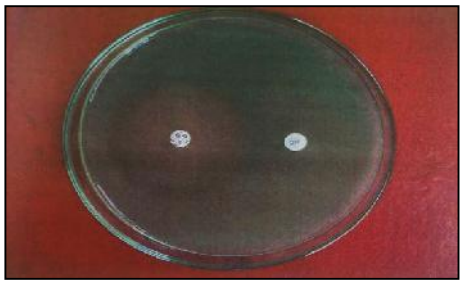
*S. aureus* in Muller Hinton Agar with Amoxycillin combined with sulbactum



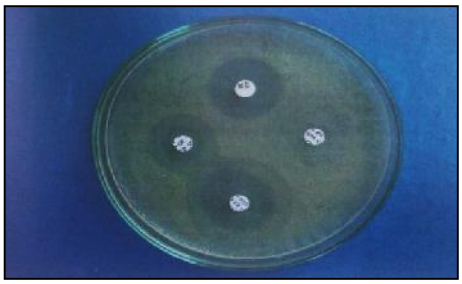
Antibiotic sensitivity of *S. aureus*



Antibiotic sensitivity of *S.aureus* (Resistance Amoxycillin –Right disc)

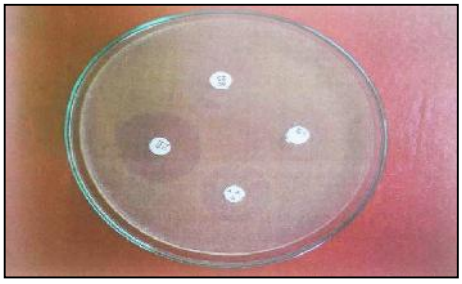


Antibiotic sensitivity of *P. aeruginosa*



Antibiotic sensitivity of *P. aeruginosa*

*S.aureus* Amoxycillin with Clavulanic acid disc



Antibiotic sensitivity of *B. subtilis*

The present study determines sulbactum and clavulanic acid are still the first choice of treatment for serious infections with ESBL-producing organisms *S. aureus, E. coli, B. subtilis* and *P. aerugnosa* with limited susceptibility to antimicrobials in different environment. In order to combat these problems proper antibiotic policies should be formulated. Further, it was observed that all the ESBL-producing isolates were susceptible to combined form of antibiotic with sulbactam and clavulanic acid. This brings due relief as these are the drugs of choice in the treatment of ESBL bacterial infections.

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