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Original Research Article

Acinetobacter Baumannii Complex and Its Beta-Lactamase Production: Are We Moving Towards Pre Antibiotic Era?

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ABSTRACT

Introduction: *Acinetobacter* species has been increasingly reported as one of the common cause of nosocomial infections and poses a serious threat to the health care system due to development of its multidrug resistance in debilitated and hospitalized patients. Carbapenem resistance in *Acinetobacter sp.* is an emerging problem and is a cause of concern as many nosocomial infections with *Acinetobacter sp.* are resistant to most other antibiotics. The present study was conducted over a period of 6 months in a tertiary care rural hospital to study *Acinetobacter baumannii* complex strains isolated from clinical samples and their antibiogram.
Materials and Methods: 60 strains of *Acinetobacter baumannii* complex isolated from various clinical samples like sputum, endotracheal tube secretions, pus, blood etc. were studied. The antimicrobial susceptibility of these isolates was performed by Kirby-Bauer disk diffusion method and Extended Spectrum Beta-lactamase (ESBL), AmpC, Metallo beta lactamase (MBL) and Klebsiella pneumoniae carbapenemase (KPC) production was detected phenotypically.
Results: The maximum sensitivity was seen for Colistin 60 (100%) and least for Ceftazidime 13 (21.6%). MBL production was detected in13.3% Acinetobacter strains. 10% strains produced all 4 types of β-lactamases i.e., ESBL plus AmpC plus MBL plus KPC in combinations.
Conclusion: Proper infection control measures and judicial use of antibiotics is necessary to combat the problem of development of drug resistance. Phenotypic detection of newer β-lactamases should be done routinely in Clinical Microbiology laboratory to prevent development and spread of these β-lactam producing strains in Health care setup.
Keywords: *Acinetobacter baumannii* complex, Antibiotic resistance, Newer β-lactamases,

INTODUCTION

The control of hospital-acquired infection caused by multidrug resistant Gram-negative bacilli has proved to be a major global problem over the last two decades. [(1)] During 1970s, an increasing incidence of resistant members of the family *Enterobacteriaceae* involved in noscomial infections, has led to the therapeutic introduction of newer broad-spectrum antibiotics in hospitals and a subsequent increase in strictly aerobic Gram-negative bacilli, including *Pseudomonas aeruginosa, Stenotrophomonas maltophilia, and Acinetobacter spp. etc.* causing noscomial infections. Presently Antimicrobial resistance (AMR) is a major threat to patient care in any healthcare setup worldwide. [(2)] The emergence and rapid spread of multidrug-resistant isolates causing noscomial infections are of great concern worldwide. Although methicillin-resistant Staphylococcus aureus (MRSA), Vancomycin-resistant Enterococci (VRE),

and Extended spectrum β-lactamase (ESBL) and AmpC -producing Enterobacteriaceae have been the subject of much attention, multidrug resistance among some non-Enterobacteriaceae organisms such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* have also emerged nowadays. [(3)]

Considering all the achievements of mankind in Medical Sciences, actually the pace of development of drug resistance of bacteria is much higher than the rate of development of newer antimicrobials. [(4)] Of these "newer" pathogens, it is now recognised that *Acinetobacter spp.* play a significant role in the colonization and infection of patients admitted to hospitals. *Bergey's Manual of Systemic Bacteriology* [(5)] classified the genus *Acinetobacter* in the family *Neisseriaceae* with one species, *A. calcoaceticus*. The genus Acinetobacter currently comprises 30 named species and nine genomic species. Four of the 30 species, i.e., *A. calcoaceticus*, *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13TU, are very closely related and difficult to distinguish from each other by phenotypic properties. So, alternative genotypic methods, including sequencing of the 16S rRNA (rrs) gene, and several housekeeping gene such as gyrb and rpoB, constitute rapid helpful tool for the precise identification of uncommon Acinetobacter species in clinical Microbiology. [(6)] It has therefore been proposed to refer to these species as the *A. calcoaceticus-A. baumannii* complex. [(7, 8)]

Acinetobacter baumannii is ubiquitous in nature since they are found frequently in soil, water and dry environments. However, this group of organisms comprises not only the three most clinically relevant species that have been implicated in the vast majority of both community-acquired and nosocomial infections mostly in ICU patients. Their contribution to noscomial infections has increased over the past three decades, and many outbreaks involving these microorganisms have been reported worldwide. Various risk factors predispose to severe infections with Acinetobacter spp. The susceptible patients include elderly patients those who have undergone major surgeries, or those with severe underlying diseases (e.g., malignancy, burns or immunosuppression etc). Although Acinetobacter spp. are considered to be a relative low-grade pathogen, its certain characteristic may enhance the virulence of the strain. These include i) the presence of a polysaccharide capsule, which renders the surface of strain more hydrophilic (ii) the property of adhesion to human epithelial cells in presence of fimbriae and/ capsular polysaccharide (iii) the production of enzymes which may damage tissue lipids and (iv) the potentially toxic role of the lipopolysaccharide component of the cell wall and the presence of lipid A. [(9)] They particularly represent an important cause of ventilator-associated pneumonia and catheter-associated bacteremia. They are regularly recovered from urinary tract and wound infections, some sporadic cases of peritonitis following peritoneal dialysis, endocarditis, meningitis, osteomyelitis, arthritis, pancreatic and liver abscesses, and eye infections. [(6)] Such infections are often extremely difficult for the clinician to treat because of the widespread resistance of these bacteria to the commonly used antibiotics. [(10)] Acinetobacter species cause infections that are difficult to control due to multi-drug resistance. Acinetobacter species are known for their intrinsic resistance to antibiotics and for their ability to acquire genes encoding resistance determinants. Among the mechanisms of antibiotic resistance in bacterial strains the production of beta lactamases and aminoglycoside-modifying enzymes, are also imported. [(11)]

The noscomial epidemiology of this organism is a bit complex. Villegas and Hartstein reviewed Acinetobacter outbreaks occurring from 1977 to 2000 and hypothesized that endemicity, increasing rate of new resistance to antimicrobial drugs in a collection of isolates suggest easy transmission. They further suggested that transmission should be confirmed by using a

discriminatory genotyping test. [12] From 1992 to 1996, annual susceptibility summaries showed that all A. baumannii epidemic or endemic isolates were resistant to two or more antibiotic groups, which uniformly included β-lactams and gentamicin, and were susceptible only to carbapenems, sulbactam, and colistin. Currently the spread of hospital populations of resistant microorganisms is of great concern, thereby raising the idea that we may be approaching the preantimicrobial era. [3] During the last few decades, noscomial infections caused by multidrug resistant A. baumannii have been reported. Initial concern about Carbapenem resistant A. baumannii (CRAB) began when the first noscomial outbreak occurred in the United States in 1991. [3] In May 1998, the first isolate of CRAB – which was also resistant to almost all commercially available antibiotics, including all cephalosporins, aztreonam, aminoglycosides and ciprofloxacin (pandrug-resistant A. baumannii, PDRAB) was recovered from a leukaemia patient with bacteremia in an oncology ward. [3] Since then emergence of multidrug resistant *A. baumannii* in hospitalized patients have become the cause of concern.

AIMS AND OBJECTIVES

Hence, the present study was undertaken with the aim to study *Acinetobacter baumannii* complex, isolated from different clinical samples and their antibiotic susceptibility profile.

MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology and was approved by Institutional Ethics Committee (IEC). It was a short term cross sectional study.60 clinical isolates of *Acinetobacter baumannii* were studied.

Selection criteria- A total number of consecutive 60 *Acinetobacter baumannii* complex strains isolated in the department of Microbiology from different clinical specimens like urine, pus, blood, body fluids, endotracheal aspirate etc. were studied. The strains of *Acinetobacter baumannii* complex were detected by conventional methods e.g. Gram staining, motility, biochemical tests especially catalase and oxidase test etc. [11]

Antibiotic susceptibility profile of *Acinetobacter baumannii* complex strains was studied by Kirby-Bauer disc diffusion method [13] as per Clinical Laboratory Standard Institute (CLSI) guidelines. [14] Lawn culture was done on Mueller Hinton (MH) agar plate with overnight broth culture of Acinetobacter baumannii strains (turbidity adjusted to 0.5 Mc Farland standard). With all aseptic precaution, the antibiotic discs like Ceftazidime (30 µg), Ciprofloxacin (10µg), Amikacin (30µg), Imipenem (10µg), etc. were put on inoculated Mueller Hinton [MH] agar plate. After overnight incubation at 37^0C, susceptibility profile of *Acinetobacter baumannii* complex strains was recorded.

The Multidrug resistant (MDR), Extensively drug resistant (XDR) and Pandrug resistant (PDR) bacterial strains will be detected as per definition given by European Society for Disease Control (ECDC) and Centre for Disease Control (CDC), Atlanta.: [15]

a. Multidrug resistant (MDR): MDR is defined as acquired resistance to at least one agent in three or more antimicrobial agents.
b. Extensively drug resistant (XDR): *XDR is defined as nonsusceptibility* to atleast one agent in all but two or fewer classes of antimicrobials.
c. Pandrug resistant (PDR)*:* PDR is defined as nonsusceptibility to all agents in all antimicrobials categories.

Detection of newer β-lactamases such as ESBL, AmpC, MBL, KPC production:

As different types of β-lactamases can be produced in combination by single strains of *Acinetobacter baumannii complex*, all strains were tested for all types of newer β-lactamases and also for detecting both ESBL and AmpC β-lactamase producing strains and both MBL and KPC producing strains.

1) Detection of Extended spectrum β-lactamases (ESBLs):

The screening test and phenotypic confirmatory tests were done for all 60 *Acinetobacter baumannii complex* strains according to CLSI guidelines described for Enterobacteriaceae as the principle is same.

Screening Tests: Reduced susceptibility to Ceftazidime was used as screening test. [(16)]

Confirmatory test: It was done by combined disc method and E-Test.

1. **Combined Disc Method:** [(17)]

Lawn culture of test strains (turbidity adjusted to McFarland 0.5 standard) was made on Mueller Hinton (MH) agar plates with a sterile swab. The disc containing Ceftazidime (CAZ-30µg) and Ceftazidime plus clavulanic acid (CAC-30/10 µg) respectively were used in this method. An increase in diameter of ≥5 mm with Ceftazidime plus clavulanic acid (CAC) disc as compared to Ceftazidime (CAZ) disc alone was considered positive for ESBL production. The discs of Piperacillin (100 µg), Piperacillin-Tazobactam (100 /10 µg) was also used for Combined Disc method.

2. **E test ESBL strip** [(18)]

ESBL producing strains detected by combined disc method were also confirmed by E-test ESBL strip (bioMerieux). The E-test strip has concentration gradients of Ceftazidime (TZ) 0.5 to 32 µg/ml on one half and Ceftazidime 0.064 to 4 µg/ml plus 4 µg/ml Clavulanic acid (TZL) on another half. The ESBL E-test was performed and interpreted using test strains and Quality Control strains according to the manufacturer's instruction. MIC ratio of Ceftazidime/ Ceftazidime plus clavulanic acid (TZ/TZL) ≥ 8 or deformation of ellipse or phantom zone present was considered as positive for ESBL production.

Detection of AmpC β- Lactamases:

For detection of AmpC β-lactamases, no satisfactory technique has been established till date as per CLSI guideline for *Acinetobacter baumannii complex*.

Screening Test: Acinetobacter baumannii lacks an AmpR gene so that its AmpC β-lactamase is noninducible. [(12)] All 60 *Acinetobacter baumannii complex* strains were also screened for AmpC β-lactamase production with CAC ≥ 2mm less than CAZ and PIT ≥ 2mm less than PI. [(19)]

Disk potentiation test [(20)] for Ampc β-lactamases production:

Confirmatory Test: It was done by using Ceftazidime/Ceftazidime plus 3-aminophenyboronic acid (CAZ/CAZ+ 3-APB). [(20)]

All 60 *Acinetobacter baumannii complex* strains were tested by disc potentiation test for AmpC β-lactamases production. An overnight broth culture of test strain (turbidity adjusted to 0.5 Mc Farland Standard) was inoculated on Mueller Hinton agar plate with sterile swab. Two Ceftazidime (30µg) disc with center to center distance of 30mm were placed on that inoculated plate. 3-amino phenyboronic acid (3-APB) was dissolved in Dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml. 10µl of this 3-APB solution was added to one of the Ceftazidime disc. After overnight incubation at 37^0C, an increase in zone size of ≥5mm around the Ceftazidime plus 3-APB disc compared to Ceftazidime alone was considered as positive for AmpC β-lactamase production.

Detection of both ESBL and AmpC β-lactamase producing strains:

As ESBL and AmpC β-lactamase can be produced by single strain and ESBL production is suppressed if the same strain also produces AmpC β-lactamases.. Caudron et al. recommended the use of boronic acid in combination with Clavulanic acid, for detection of ESBL among AmpC β-lactamase producing strains. [(21)] The plate was then incubated at 37^0C overnight. The zone diameter of ≥5mm around CAC disc with 3-APB compared to CAZ only was recorded as ESBL and AmpC β-lactamase positive.

Detection of Carbapenem resistant *Acinetobacter baumannii complex:*

All 60 *Acinetobacter baumannii complex* strains were screened for Carbapenemase activity by Classical Hodge Test [(22)] and were tested for Metallo beta lactamases (MBL) production by disc potentiation test [(23)] and MBL E-test strip (bioMerieux). The MBL E-test is considered as a standard Reference method for MBL detection. [(24)] Detection of *Klebsiella pneumoniae* carbapenemases (KPC): producing strains was done by combined disc method.

Classical Hodge Test: [(22)]

Classical Hodge test was done to detect Carbapenemase activity. The indicator organism, *Escherichia coli* ATCC 25922 (turbidity adjusted to 0.5 Mc Farland Standard) was used to inoculate the Mueller Hinton agar plate as lawn culture and the test strain was heavily streaked from the center of the plate to the periphery. Then the plate was allowed to stand for 15 minutes at room temperature and a 10 µg Imipenem disc was placed at the center. The plate was incubated overnight at 37^0C. The presence of distorted inhibition zone was interpreted as a positive result for Carbapenem hydrolysis screening.

Imipenem-EDTA disc potentiation test for detection of MBL: [(23)]

The Imipenem-EDTA combined disc test was performed for detection of Metallo-beta-lactamases (MBL) producing strains. Test strains (turbidity adjusted to 0.5 McFarland standards) were inoculated on to Mueller Hinton agar plate. Two Imipenem disc (10 µg) were placed on the plate wide apart and 10 µl of 50 mM zinc sulphate solution was added to one Imipenem disc. Then 10 µl of 0.5 M EDTA solution was added to other IPM disc. The inhibition zone of the Imipenem disc and Imipenem plus EDTA disc were compared after 16-18 hours of incubation at 35^0C. If the inhibition zone with the Imipenem plus EDTA disc was ≥7mm than the Imipenem disc alone, it was considered as MBL positive.

MBL E-Test: [(24)]

The MBL E- Test strip (bioMerieux), containing Imipenem i.e IP (4-256 µg/ml) on one side of strip and IPI (1 to 64 µg/ml) i.e. Imipenem in combination with fixed concentration of EDTA was used for MBL detection. The MBL E test was done and interpreted according to manufacturer's instructions. Overnight broth culture of test strain (turbidity adjusted to 0.5 Mc Farland standards) was used to inoculate Mueller Hinton (MH) agar plate. The MBL E-Test strip was put on that inoculated MH plate with a sterile forceps. MIC ratio of Imipenem/Imipenem- EDTA (IP/IPI) of ≥ 8 or deformation of ellipse or phantom zone indicated MBL production.

Detection of *Klebsiella pneumoniae* carbapenemases (KPC): [(25)]

Detection of KPC producing *Acinetobacter baumannii complex* was done by combined disc method. Lawn culture of test strain (turbidity adjusted to 0.5 McFarland) was put on Mueller Hinton (MH) agar plate and 2 Imipenem (10 µg) discs were put widely apart. To one Imipenem disc 10 µl Phenyl boronic acid (PBA) solution (400 µg/ disc) was put. The PBA solution was made with Dimethyl sulfoxide. Then the MH agar plates were incubated at 37^0C overnight. After incubation, the test should be considered positive when growth inhibitory zone around the disc containing Imipenem plus Phenyl boronic acid was ≥5 mm compared to zone diameter of Imipenem alone.

Detection of Metallo beta lactamase (MBL) and *Klebsiella pneumoniae* Carbapenemase (KPC): [(23)]

If the strain produces both MBL and KPC, the KPC production may be masked by MBL. In this test, four discs of Imipenem (10 µg) alone, Imipenem plus EDTA, Imipenem plus Phenyl boronic acid and Imipenem plus EDTA plus Phenyl boronic acid were used.

The production of both KPC and MBL were considered when the growth inhibitory zone diameter seen around Imipenem disc with both PBA plus EDTA had increased to ≥5mm, as compared to the

growth inhibitory zone diameter seen around the Imipenem disc alone.

Several workers have reported that class D enzymes i.e. OXA-48 types are the most difficult Carbapenemase producers to be identified phenotypically. [(26)] Hence, we did not include detection of class D Carbapenemase in our study.

OBSERVATION AND RESULTS

A total number of 60 *Acinetobacter baumannii* complex strains were isolated from different clinical samples e.g. pus and wound swab, urine, blood, body fluids etc.

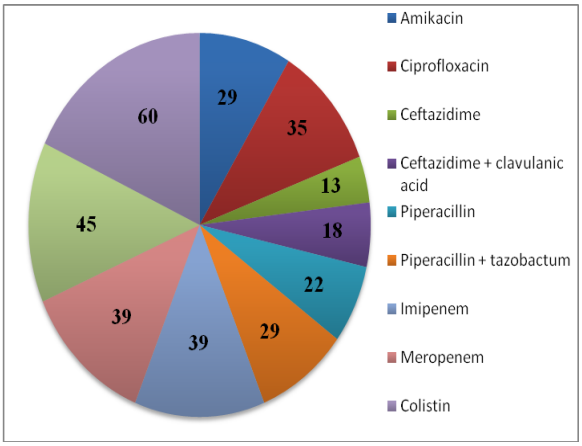


Figure 1: Antibiotic susceptibility profile of *Acinetobacter baumannii* complex strains (n=60)

Figure 1 shows the antibiotic susceptibility profile of Acinetobacter baumannii complex strains. All 60 (100%) strains were sensitive to Colistin followed by Aztreonam 45 (75%), Imipenem and Meropenem 39 (65%). The lowest sensitivity was observed with Ceftazidime 13 (21.7%).

Table 1. Isolation of *Acinetobacter baumannii* complex strains from different clinical samples (n=60)

SPECIMEN	NO. (%)
Pus and wound swab	23(38.3)
Blood	8(13.3)
Urine	9 (15)
Body fluids	3(5)
Medical devices	12 (20)
Others*	5(8.3)

*Others include sputum (3), tracheal secretion (2).

Table 1 shows the isolation of *Acinetobacter baumannii* complex strains from different clinical samples. 23 (38.3%) Acinetobacter baumannii complex strains were isolated from pus and wound swab followed by 12 (20%), 9(15%) and 8(13.3%) from medical devices, urine and blood respectively. Out of 12 medical devices 5 were IV catheter tips, 4 were Foley's catheter tips and 3 were endotracheal tube tips.

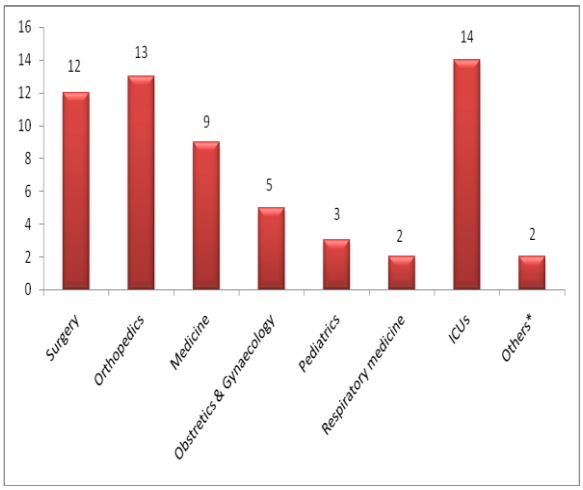


Figure 2: Isolation of *Acinetobacter baumannii* complex strains from different clinical specialties (IPD) (n=60)

Figure 2 shows the isolation of *Acinetobacter baumannii* strains from different clinical specialties of Indoor Patient Departments (IPD). Maximum 14 (23.3%) *Acinetobacter baumannii* strains were isolated from ICUs followed by 13 (21.7%) from Orthopedics ward and 12 (20%) from Surgery ward respectively.

Table 2: Isolation of different β-lactamase producing *Acinetobacter baumannii* complex strains (n=60)

Total β-lactamases	No.(%)
ESBL	7 (11.6)
AmpC	14 (23.3)
MBL	23 (38.3)
KPC	14 (23.3)

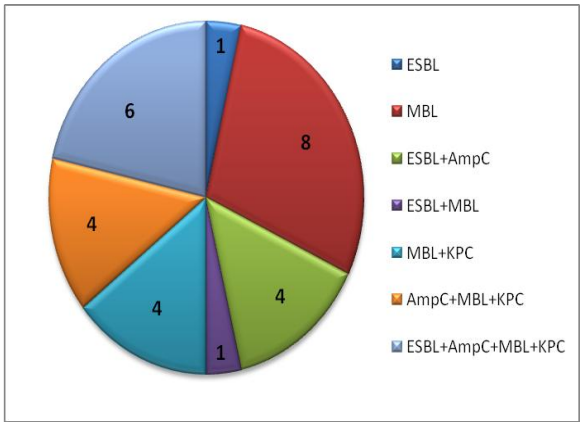
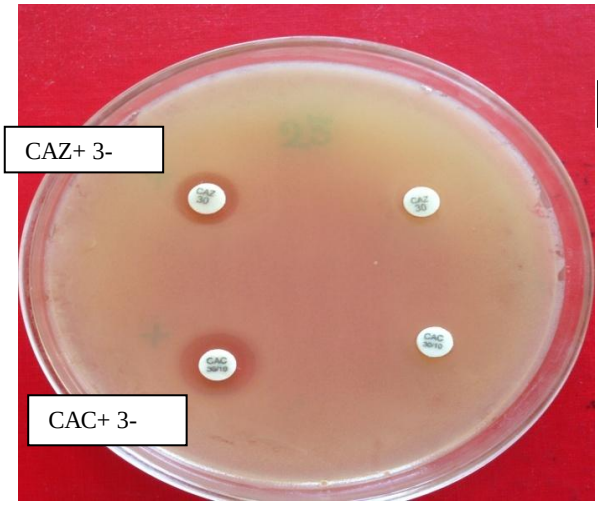


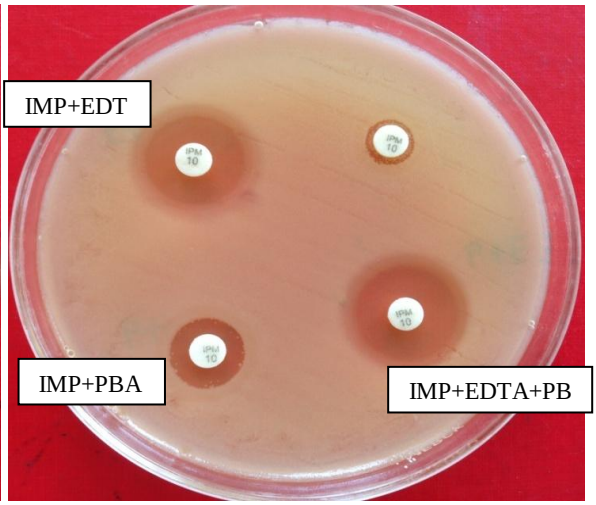
Figure 3. Isolation of *Acinetobacter baumannii* complex strains producing different β-lactamases either singly or in combination. (n=60)

Table 2 shows different β-lactamase producing *Acinetobacter baumannii* complex strains. Maximum 23 (38.3%) strains produced MBL followed by 14 (23.3%) strains produced AmpC β-lactamase and KPC respectively

Out of 60 Acinetobacter baumannii strains 28 (46.6%) strains produced newer β-lactamases either alone or in combination. 8 (28.6%) strains produced only MBL whereas 6 (10%) strains produced all 4 types of β-lactamases in combination i.e, ESBL plus AmpC plus MBL plus KPC.

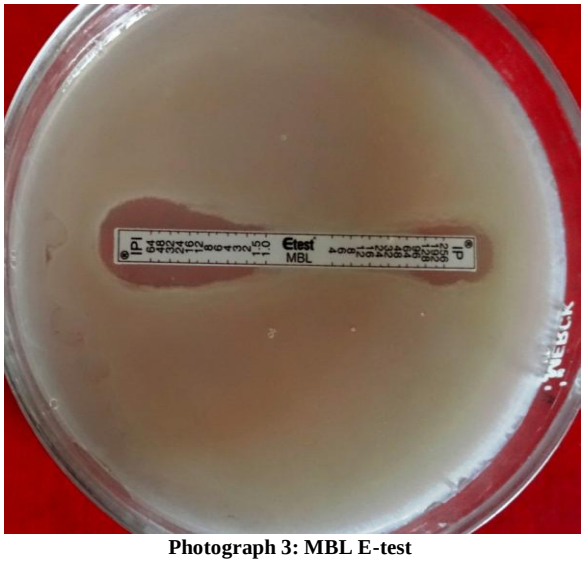


Photgraph 1: ESBL plus AmpC (+ve)



Photograph 2: MBL plus KPC (+ve)

Photograph 1. Showing Disc potentiation test of *Acinetobacter baumannii* complex strain producing ESBL plus AmPc β-lactamase in combination.
Photograph 2. Showing Disc potentiation test of *Acinetobacter baumannii* complex strain producing MBL plus KPC β-lactamase in combination.



Photograph 3: MBL E-test

Photograph 3 is showing MBL E-test positive where IPI= 1 µg/ml and IP= 48 µg/ml.

In the present study, 39 strains were resistant to Imipenem. Out of 23 strains, MBLs were detected in all 21(91.3%) carbapenem resistant *Acinetobacter baumannii* complex strains and 2 (8.7%) strains were detected from carbapenem sensitive strains. Out of 60 Acinetobacter spp. strains, 10 (16.6%) were Multidrug resistant (MDR) and 27 (45%) were extensively drug resistant (XDR) strains.

DISCUSSION

A.baumannii complex has emerged as an important pathogen that causes hospital acquired infections and leads to treatment failures because of multiple antibiotic resistances. [27,28] Through determination of the clonal relationship between the isolates, the source of the infection, the porter, and the way of spread can be revealed, and proper prevention methods can be chosen. Conjugation plays a significant role in transfer of antibiotic resistance genes for *Acinetobacter* species. There are reports that > 80% of

Acinetobacter sp. carry indigenous plasmids that are difficult to isolate.

The susceptibility rates were 30%, and 10% for amikacin, and meropenem, respectively in a study [(27,28)] and that of ceftazidime, imipenem, meropenem and colistin is 1.8%, 5.2%, 45.3%, and 100% in another study [(29)] respectively. In the present study also 100% strains were sensitive to colistin and maximum 23 (38.3%) *Acinetobacter baumannii* complex strains were isolated from pus and wound swab, followed by urine 9 (15%) and blood 8 (13.3%) respectively. Another study reported maximum 53.5% *Acinetobacter baumannii* complex species were isolated from blood followed by 19.8% from pus and 12.8% from sputum. [(30)]

In our study maximum 14 (23.3%) *Acinetobacter baumannii* complex strains were isolated from ICUs. In 2015 Kamble R reported that maximum 37.2 % strains were isolated from Intensive Care units. [(30)]

23(38.3%) strains produced MBL, compared to very high rate of MBL production as 96.6% [(31)] in another study whereas lower rate of MBL production 7.5% and 14.8% respectively was reported in other studies. [(32,33)]

The major cause of concern is in the present study where all four types of newer β-lactamases i.e., ESBL, AmpC, MBL and KPC were produced by 6 (10%) strains. No other studies have reported all four types of β-lactamases producing A. baumannii complex strains. In our study, about 16.6% strains were Multidrug resistant (MDR) and 45% strains were extensively drug resistant (XDR) whereas in 2015, Kamble R reported 58% strains as extensively drug resistant (XDR) and 14% as multi-drug resistant (MDR) Acinetobacter baumannii complex strains. [(30)]

CONCLUSION

The findings of this study indicate that colistin has the best activity against *A. baumannii* complex strains, whereas Meropenem and Imipenem are good choices for the treatment of non-MDR *A. baumannii* strains. 10 % Acinetobacter baumannii, strains producing all four types of β-lactamases i.e. ESBL plus AmPC plus MBL plus KPC in combination indicate an alarming situation and remind us of preantibiotic era. These 10 % strains were only sensitive to colistin and resistant to all other antibiotics used. Today, while clinicians confront the worst situation of trying to combat even pan drug resistant isolates such as *Acinetobacter baumannii*, Pseudomonas aeruginosa the new antibiotics are not being developed. A concerted effort by industry, Government and academicians is urgently required to improve the situation. Hence to conclude to antimicrobial stewardship, strict infection control measures especially strict implementation of hand hygiene practices are needed to prevent dissemination of antibiotic resistant *Acinetobacter baumannii* complex strains in Health care setup.

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