Isolation and Speciation of Acinetobacter Species with Special Reference to Antibiotic Resistance in Tertiary Care Hospital

Manasi Vikas Yadav1, Dr. G. S. Karande1*, Dr. S.R. Patil1, Dr. R.V. Shinde1, Dr. S.K. Pawar1, Dr. H.V. Patil1, Dr. P. M. Mane1 and Shanu Sharma1.

1Department of Microbiology, Krishna Institute of Medical Sciences, (Deemed to Be University), Karad, Malkapur, Karad - 415539 (District Satara), Maharashtra, India.
1*, Professor, Department of Microbiology, Krishna Institute of Medical Sciences, Karad.

Abstract: Acinetobacter can survive longer in the environment and is also known for developing resistance against agents like disinfectants and nutritional deprivation. Very restricted information about Acinetobacter is available because of their confused taxonomic status. The isolation and identification of resistance pattern help in the selection and search for new antibiotics, reducing the morbidity and mortality of patients. The present study was conducted to find the different types of resistant mechanisms in Acinetobacter species and their antimicrobial susceptibility pattern from various clinical samples. Gram’s stain and biochemical reactions identified Acinetobacter isolated from various clinical samples. Antibiotic susceptibility testing was done, and their resistant pattern was observed. Phenotypic methods for drug resistance were carried out - Detection of Extended Spectrum Beta Lactamase (ESBL) by Double disc synergy test (DDST), detection of Metallo Beta Lactamase (MBL) by Imipenem with EDTA, and detection of Carbapenemase production by Modified Hodge test (MHT). The results showed that out of 150 isolates of Acinetobacter species, Acinetobacter baumannii 138 (92%) was the most common species isolated, followed by Acinetobacter lwoffii 10 (7%) and Acinetobacter hemolytic 2 (1%). Of these, 22 (15%) isolates showed ESBL production by Double disc synergy test, 80 (53%) isolates were MBL producers, and 11 (7%) were Carbapenemase producers by Modified Hodge test. The study observed that ESBL production in Acinetobacter was 22(15%) and MBL 80(53%). It demonstrated that most of the Acinetobacter isolated were found to be Multi-Drug Resistant (95%). It brings out the need for active surveillance combined to eradicate the curtail of this organism in hospital settings.

Keywords: Acinetobacter, ESBL, MBL, AST, MHT, MDR.

*Corresponding Author
Dr. G. S. Karande , Professor, Department of Microbiology, Krishna Institute of Medical Sciences, Karad.

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1. INTRODUCTION

Acinetobacter species are Gram-negative, strictly aerobic, non-fastidious, non-fermenting, encapsulated coccobacillus that mainly causes nosocomial infections.\(^1\) Acinetobacter is a heterogeneous group of organisms, typically free-living saprophytes found almost everywhere and commonly distributed in the environment. However, different species of the genus are generally associated with various habitats, e.g., soil, water, sewage, human, foods, and animals.\(^2\) They are isolated as commensals from the skin and throat.\(^3\) Acinetobacter has emerged as one of the most troublesome pathogen classes in healthcare-associated infections like hospital-acquired pneumonia, community-acquired pneumonia, bacteremia, trauma, wound infection, urinary tract infection, meningitis and other manifestations of endocarditis, peritonitis, ophthalmitis.\(^4\) Genus Acinetobacter comprises more than 50 validated species, among which are A. baumannii group, A. Iwoffii, A. johnsonii, A. junii, and A. hemolytic. The presence of a polysaccharide capsule formed of L-rhamnose, D-glucose, D-glucuronic acid, and D-mannose,\(^5\) which probably renders the surface of strains more hydrophilic, although hydrophobicity may be higher in Acinetobacter strains isolated from catheters or tracheal devices.\(^6\) The Acinetobacter infection is the most recent of all bloodstream infections occurring during hospitalization. It is the 10th common etiologic agent responsible for nosocomial bloodstream infections in the United States during 1995-2002, accounting for 1.3% of mono-microbial bloodstream infections.\(^7\) Hospital-acquired pneumonia is still the most common infection caused by Acinetobacter. Acinetobacter in the community commonly causes meningitis.\(^7\) Secondary meningitis is predominant due to the Acinetobacter infection, although primary meningitis also has been reported during neurological procedures or head trauma.\(^8\) It is a microorganism, characterized by the rapid development of resistance to most antimicrobials. It has appeared as a pathogen frequently incriminated in lower respiratory tract infections in critically ill patients. The biochemical tests are carried out to speciate the Acinetobacter species. The study of antibiotic resistance is necessary to help treat infections.\(^9\) Due to long-term evolutionary exposure to soil organisms that produce antibiotics, Acinetobacter sp. can develop antibiotic resistance extremely rapidly. It contrasts with other clinical bacteria, which require greater time to acquire resistance, usually in response to therapeutic strategies. Conjugation, plasmids, and transposons (in conjunction with integrons) are important in transferring resistance determinants between different strains.\(^10\) They are innately resistant to many antibiotics and are known to produce extended-spectrum Beta-lactamases and Metallo-Beta-lactamases. The carbapenems are Beta-lactam antimicrobial agents with an exceptionally broad-spectrum activity. A. baumannii infections are often difficult to eradicate due to high-level resistance to many antibiotics due to intrinsic and acquired mechanisms.\(^9\) Acinetobacter baumannii production of Metallo-beta-lactamase (MBL) has become a worldwide therapeutic concern.\(^11\) The present study was done to investigate the prevalence, bacteriological profile, and antimicrobial susceptibility pattern of Acinetobacter species isolated from various clinical samples with an objective to look for different types of resistant mechanisms which will provide valuable information and opportunity to other scientists in order to uncover the precise drug molecule to fight against infections caused due to Acinetobacter and standardize the living conditions. It is generally agreed that A. baumannii is the most medically significant Acinetobacter spp. The clinical impact of Acinetobacter was increasing morbidity or mortality, and their infections are responsible for the increase in patient mortality in critically ill patients.\(^12\) It is considered a low virulence organism except when isolated in critically ill or immunocompromised patients. These organisms are most often associated with nosocomial rather than community-acquired infections.\(^13,14\) The ability of A. baumannii to develop multidrug resistance and to persist in harsh environmental conditions makes infections by Acinetobacter very dangerous, especially in individuals who have recently undergone major surgery, had malignant diseases or burnt or immunosuppressed patients such as the elderly, neonates with low birth weights, and patients with prolonged illnesses.\(^12\) Patients with mechanical ventilation, particularly of prolonged duration, longer hospital or ICU stay, and a greater degree of exposure to infected or colonized patients in the neighboring hospital environment, have an increased risk for the acquisition of multidrug-resistant outbreak strains.\(^15\)

2. METHODOLOGY

"Isolation and Speciation of Acinetobacter Species with Special Reference to Antibiotic Resistance in Tertiary Care Hospital" was conducted in the Microbiology laboratory at Krishna Institute of Medical Sciences and Krishna Hospital and Medical Research Centre, Karad.

2.1. Study Setting

The study was undertaken in the Department of Microbiology, Krishna Institute of Medical Sciences, deemed to Be University, Karad, India.

2.2. Study Design

Observational cross-sectional study

2.3. Study Period

November 2020 to November 2022

2.4. Inclusion criteria

Isolates of Acinetobacter spp. from all clinical samples received in the laboratory were included. In addition, patients of both sexes were included.

2.5. Exclusion criteria

Isolates of Acinetobacter species from the same patients and specimens were excluded from the study to avoid duplication of isolates.

2.6. Statistical analysis

Data were filled in the MS Excel Software. Then, analyzed results were expressed as percentage and p values by Chi-square test using Graph Pad Instant software. If the probability is less than 0.05, the association or difference is said to be significant.

2.7. Specimen collection

Clinical samples including pus, sputum, urine, ETT secretions,
blood, body fluids, wound swab, CSF, catheters, and various prosthetic devices from both gender and all groups of patients included. Samples were collected aseptically in sterile and appropriate containers storage and transportation in cold conditions till processing, except blood culture bottles.

2.8. Sample Processing

For primary identification of the *Acinetobacter* based on their microscopic observation, firstly, a clean, grease-free slide was taken. Upon that, a drop of normal saline was taken under aseptic condition. A small colony of bacteria was picked, and a thin, uniform smear was prepared. The smear was heat fixed. The heat-fixed smear was stained by the Gram stain technique and examined under the oil immersion objective of the light microscope. In Gram stain, Gram-negative bacilli were observed. The isolates were identified based on colony morphology on agar and Gram stain of the smear made from the colonies. Oxidase, catalase, and biochemical reactions were performed to identify colonies. All collected specimens were inoculated on Nutrient, MacConkey, blood, and chocolate agar and incubated at 37°C for 24 hours. All clinical specimens received in the laboratory were inoculated on Nutrient agar, Cysteine lactose electrolyte deficient (CLED) agar, MacConkey agar, Blood agar, and Chocolate agar. The biochemical tests were performed according to the standard operating procedure mentioned in Mackie & McCartney Practical Medical Microbiology 14th edition.16

2.9. Antibiogram

Antimicrobial susceptibility testing of isolates was performed on Mueller Hinton agar using the Kirby-Bauer disc diffusion method. Four to five colonies of the same morphology were selected from an agar culture plate. With a sterile bacteriological loop, the growth was inoculated into a broth medium which was incubated for 3 to 5 hours to achieve a turbid suspension. It was compared with 0.5 McFarland standards. A swab was submerged in bacterial suspension and was inoculated on a Mueller Hinton agar plate. The surface of the plate was swabbed in three directions so that there was an even and complete distribution of the inoculum. Within 15 minutes of inoculation, antibiotic discs were applied using sterile forceps. The plates were incubated at 37°C for 24 hrs., after which the zone of inhibition was measured by using a zone measuring scale, and interpretation was made as per the CLSI guidelines.17,18

2.10. Detection of resistance mechanism

- **Detection of Extended Spectrum of Beta-Lactamase (ESBL) production by Double Disc Synergy test (DDST)**

Gram-negative bacilli isolated was suspected to be an ESBL producer if it was resistant to Aztreonam (30 µg), Cefotaxime (30 µg), Cefodoxime (10 µg), Cefazidime (30 µg), and Ceftriaxone (30 µg). The inoculum of test and control organisms was prepared and matched with turbidity 0.5 McFarland standard. ESBL production was tested by the Double Disc Synergy Test (DDST), using a disk of cefazidime + clavulanic acid and cefazidime (cephalosporin). The bacterial strains were cultured on Mueller Hinton agar plates per CLSI guidelines. A disc that contained cefazidime + clavulanic acid (30µg+10µg) was placed on the plate at a distance of 25mm from that of cefazidime (30µg) and allowed to diffuse at room temperature (for 1hr). The plate was incubated for 18-24 hr (at 35°C). An increase greater or equal to 5 mm in the inhibition diameter of the cefazidime disc was applied after the pre-diffusion of cefazidime + clavulanic acid compared with the cefazidime disc considered as positive for ESBL production.19

- **Detection of Metallo Beta Lactamase (MBL) by Imipenem-EDTA combined disc diffusion test**

If the increase in inhibition zone with Imipenem - EDTA disc is greater than or equal to 7 mm than the Imipenem (10 µg) alone, it is interpreted as an MBL producer.10

- **Detection of Carbapenemase production by Modified Hodge test**

A distorted zone of inhibition or clever leaf-like indentation, at the intersection of the test organism and the E. coli ATCC 25922, within the zone of Meropenem susceptibility disc, was interpreted as positive for Carbapenemase by Modified Hodge Test.20

3. OBSERVATION AND RESULTS

**Table 1: Age and gender-wise distribution of Acinetobacter**

<table>
<thead>
<tr>
<th>Age group</th>
<th>Male (n)%</th>
<th>Female (n)%</th>
<th>Total (n)%</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-20</td>
<td>8 (5)</td>
<td>6 (4)</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>21-40</td>
<td>28 (19)</td>
<td>18 (12)</td>
<td>46</td>
<td>31</td>
</tr>
<tr>
<td>41-60</td>
<td>42 (28)</td>
<td>15 (10)</td>
<td>57</td>
<td>38</td>
</tr>
<tr>
<td>&gt; 60</td>
<td>22 (15)</td>
<td>11 (7)</td>
<td>33</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>100 (67)</td>
<td>50 (33)</td>
<td>150</td>
<td>100</td>
</tr>
</tbody>
</table>

$X^2 = 0.5993$, $p$-value = 0.8966, Not significant

Age and gender-wise distribution of Acinetobacter isolated. The isolates in the age group of 0-20 years were 14(9%), followed by the age group 21-40 years 46(31%), 41-60 years 57(38%), >60 years 33(22%) respectively. (Table No.1)

**Table 2: Distribution of the Acinetobacter species isolated from various clinical specimens**

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Total</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pus</td>
<td>38</td>
<td>25</td>
</tr>
<tr>
<td>Tracheal aspirate</td>
<td>36</td>
<td>24</td>
</tr>
</tbody>
</table>
Sample-wise distribution of *Acinetobacter* species. The majority of the isolates were from pus 38 (25%), followed by tracheal aspirate 36 (24%), urine 30 (20%), sputum 16 (11%), blood 16 (11%), body fluids 12 (8%), CSF 2 (1%). (Table No.2)

<table>
<thead>
<tr>
<th>Location</th>
<th>Isolation</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indoor</td>
<td>139</td>
<td>93</td>
</tr>
<tr>
<td>Outdoor</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>100</td>
</tr>
</tbody>
</table>

Location-wise isolation of *Acinetobacter* species from patient’s samples. In the present study, a maximum number of isolates were from Indoor patients, 139 (93%), then Outdoor 11 (7%). (Table No.3)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>43</td>
<td>107</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>32</td>
<td>118</td>
</tr>
<tr>
<td>Cefepime</td>
<td>26</td>
<td>124</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>32</td>
<td>118</td>
</tr>
<tr>
<td>Imipenem</td>
<td>44</td>
<td>106</td>
</tr>
<tr>
<td>Meropenem</td>
<td>38</td>
<td>112</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>38</td>
<td>112</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>42</td>
<td>108</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>106</td>
<td>44</td>
</tr>
<tr>
<td>Colistin</td>
<td>113</td>
<td>37</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>46</td>
<td>104</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>22</td>
<td>128</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>20</td>
<td>130</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>24</td>
<td>126</td>
</tr>
</tbody>
</table>

The different resistance pattern of bacterial isolates was observed against antimicrobial agents. Maximum sensitivity to Colistin 113(75.3%) was showed by *Acinetobacter* species, followed by Tigecycline 106(71%), whereas, maximum resistance was to Ampicillin 130(87%), followed by Nalidixic acid 128(85.3%), Ceftazidime 126(84%). (Table No.4)

<table>
<thead>
<tr>
<th>Test</th>
<th>Number (n)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHT Positive</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>MHT Negative</td>
<td>139</td>
<td>93</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>100</td>
</tr>
</tbody>
</table>

Out of 150 isolates, 11 (7%) were Modified Hodge test positive, and 139 (93%) were negative. (Table No.5)
Fig 1: Distribution of the *Acinetobacter* from various clinical specimens

Fig 2: Speciation of *Acinetobacter* isolates
Fig 3: Antibiotic susceptibility pattern of Acinetobacter species isolated from various clinical sample

Fig 4: Comparative study of antibiotic susceptibility pattern in both ESBL producer and ESBL nonproducer

Fig 5: Comparative study of antibiotic susceptibility pattern in both MBL producer and MBL nonproducer
Fig 6: Multi-Drug Resistance Pattern

Fig 7: Gram-negative coccobacilli

Fig 8: CLED agar
Fig 9: MacConkey agar

Fig 10: Biochemical Tests from left to right – TSI, Indole, MR, Nitrate reduction, Citrate, Urease, VP.

Fig 11: ESBL production
4. DISCUSSION

A total of 450 samples were collected during the study period. Out of the 450 samples, 150 samples were positive for Acinetobacter species. A total of 150 isolates of Acinetobacter species were processed. There were 150 patients included in the study, of which, males were 100 (67%) and 50 (33%) females. A maximum number of male cases, 42 (28%), were in the age group 41-60. Maximum isolates were from females aged 21-40, 18 (12%). 42 (28%) Acinetobacter species isolates were from the 41-60 age group with male predominance. In our study, a total of 150 isolates, in the majority of infections, were in the age group of 41-60 years (38%). Similar results were reported by Swarnatrisha Saha et al. showing a majority of isolates in the age group 41-60 years (35%). In our study, most of the Acinetobacter isolates obtained were from pus sample 38 (25%), followed by tracheal aspirates 36 (24%), urine 30 (20%), sputum 16 (11%), blood 16 (11%), body fluids 12 (8%), CSF 2 (1%). This finding can be correlated with the study conducted by B Apoorva et al. in which they reported a maximum number of isolates from pus 22.5%, followed by blood 17.5%, endotracheal aspirate 17.5%, urine 15%, sputum 12.5%, BAL (Bronchoalveolar lavage) 5%, swab (gluteal abscess) 2.5%, throat swab 2.5%, CVP tip 5%. Similarly, Sana Islahi et al. reported that most isolates were from pus samples 36.95%. Shivaranjani V et al. also reported maximum isolation from pus sample 38.5%. Acinetobacter was most commonly isolated from ICU 52 (35%) mainly Surgical 41 (27.3%), Neurology 24 (16%), OBGY 6 (4%), Pediatric 6 (4%), Orthopedic 6 (4%), Oncology 4 (3%), Radiology 3 (2%), Casualty 3 (2%), Medicine 2 (1%), Cath lab 2 (1%), CVTS 1 (0.7%). Acinetobacter subspecies Acinetobacter baumannii 138 (92%) was the most common species isolated, followed by Acinetobacter Iwoffii 10 (7%) and Acinetobacter hemolytic 2 (1.3%). The gender wise observation showed Acinetobacter baumannii in males 91 (61%), females 45 (30%), followed by Acinetobacter Iwoffii in males 5 (3.3%), females 5 (3.3%) and Acinetobacter hemolyticus in males were 1 (0.7), females 1 (0.7%). The above results elucidated that maximum resistance was observed to Ampicillin 130 (87%), Nalidixic acid 128 (85.3%), Ceftazidime 126 (84%), and Cefepime 124 (83%). Maximum sensitivity was seen to Colistin 113 (75.3%) and Tigecycline 106 (71%). In the present study, Acinetobacter species were found to be resistant to the most commonly used antibiotics. In our study, Acinetobacter showed an extremely high degree of resistance to Ampicillin (87%), Nalidixic acid (85.3%), Ceftazidime (84%), Cefepime (83%), Ciprofloxacin (79%), Piperacillin (79%), Gentamicin (75%), Amikacin (73.3%), Meropenem (75%), Imipenem (71%) which correlates with
the studies by Guckan R et al. 23, Peymani et al.24 found that the resistance to Piperacillin +Tazobactam was 89%, Ticarcillin +Clavulanic acid 83%, Ceftazidime 92%, Cefepime 88%, Ceftriaxone 94%, Meropenem 56%, Imipenem 54%, Gentamicin 86%, Amikacin 81% and Ciprofloxacin 86%. These findings were similar to the results of our study. It shows that the extensive use of carbapenems has created a selective antibiotics pressure, increasing the prevalence of carbapenem-resistant Acinetobacter (CRA). The findings of our study showed 15% ESBL production, comparable to the study by Owlia P et al. 27 documented 21% ESBL production, and MBL production was 53%, similar to the findings by Amrita Talukdar et al. 9, documenting 53% MBL production. Most of the Acinetobacter species were Multi-Drug Resistant and also Carbapenem group resistant. The Acinetobacter are the ones showing the highest resistance to Carbapenems. The Modified Hodge test observed by K Lee et al. 28 was 73%, Amjed A et al. 29 169%, A.V Kumar et al. 30 71%, Muneez Anwar et al. 31 83.3%, Mojtaba Moosavian et al. 32 53%, J. Thiriveni et al. 33 45% and 14.8% in S John et al. 34 The present study revealed a 7% positive result for MHT. Compared to the further studies, the present study exhibited less positive percentage for MHT. In the present study, out of 150 Acinetobacter species isolated, 95% were found to be Multi-drug resistant (MDR). In a study conducted by Mostofi S.et.al35, 54% of isolates were multidrug-resistant (MDR). Other studies conducted by Rekha S et al. 36 noted 74% MDR, A. S. Mathai et al. 37 observed 70% MDR, and Dash M et al. 38 studied 55% MDR. Bhattacharyya S et al. 39 and I. D. Khan et al. 40 reported 29% and 88.20% MDR isolates, respectively. Thus, Drug formulation needs to be changed according to the MDR pattern to treat more serious problems like ventilator-associated pneumonia, a nosocomial infection among critically ill patients. Acinetobacter baumannii is one of the most prevalent VAP-causing pathogens. Ventilator-associated pneumonia is one of the most common ICU-acquired infections, associated with a prolonged duration of microbial treatment, length of hospital stays (LOS), mechanical ventilation (MV), and high mortality rate.

5. CONCLUSION

The study showed that ESBL production in Acinetobacter was 22(15%) and MBL 80(53%) and is on the rise across the globe, thus making these infections difficult to treat. The detection of ESBL and MBL production would be important for the reduction of mortality rate and spread of multidrug-resistant organisms. In the present study, Acinetobacter species isolates showed higher resistance to carbapenems such as Imipenem and meropenem. Most Acinetobacter isolates were found to be Multi-Drug Resistant (MDR), i.e., resistant to more than or equal to three antibiotics. MDR Acinetobacter is widely increasing due to the inappropriate use of antibiotics in healthcare hospitals. MDR Acinetobacter isolates were susceptible to Colistin and Tigecycline, which can be used as a treatment for patients. The modified Hodge test is an easy and simple test that can be performed to detect carbapenemase-producing bacteria. Active surveillance combined with education of the health care worker on hand hygiene, environmental cleaning, contact precautions, antimicrobial stewardship, and handwashing practice will help curtail this organism in hospital settings.

6. ETHICAL APPROVAL STATEMENT

Ethical and research clearance was procured from the Institutional Ethics Committee, Krishna Institute of Medical Sciences, Deemed University Karad, for conducting the present research, with protocol number (054/2021-22). All the participants were informed about the research and the study, conducted to provide the required data and informed consent before participating.

7. DATA AVAILABILITY

The article contains the appropriate and proper data obtained during the experiment, supporting the research article's result, discussion, and conclusion.

8. AUTHOR'S CONTRIBUTION STATEMENT

Miss Manasi Vikas Yadav: Conceptualization, Designing the study Methodology, Dr. Geeta Satish Karande: Supervision, Writing-Reviewing, Software, Data curation, Editing, and provided valuable input towards designing the manuscript. Dr. S.R. Patil: Writing-Reviewing, Data curation, and Editing, Dr. R.V. Shinde, Dr. S.K. Pawar, Dr. H.V. Patil, Dr. P. M. Mane: Editing, Visualization, investigation, Conceptualization. Shana Sharma: Writing- Original draft preparation, Editing, Data curation, submission, and looking for the manuscript's progress.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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36. S R, B N G, Pm B, Sr P Multidrug-resistant Acinetobacter Isolates from patients Admitted at...
Microbiology


