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STUDY OF BACTERIOLOGY AND ANTIMICROBIAL SENSITIVITY PATTERNS IN PATIENTS WITH OPEN FRACTURES AT A TERTIARY CARE CENTER OF RURAL INDIA

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ABSTRACT

Aims: To isolate and identify the bacteria in open fractures and determine the antimicrobial susceptibility pattern of isolates.

Methods: A prospective study was carried out in a tertiary care hospital of rural India. The study was carried out on 100 patients of all age groups presenting with open fractures at Maharishi Markandeshwar Institute of Medical Sciences and Research, Mullana between March 2021 and September 2022. Two wound swabs, (one for culture and other for microscopy) and tissue specimens were collected to isolate and identify the bacteria causing infection in open fractures. All the bacterial isolates were then subjected to antibiotic sensitivity.

Results: The results were statistically analyzed using SPSS version 17.0. Significant growth was reported in 46% of the debridement samples. *Staphylococcus aureus* was the most common organism isolated in 30% of cultures, followed by *Pseudomonas aeruginosa* in 22% of the cultures. In the post operative follow up period, 40 % of the patients reported significant growth. *Pseudomonas aeruginosa* was the most commonorganism isolated in 28% of cultures in follow up period. Among gram positive organisms, *Staphylococcus aureus* was 77% susceptible to Amikacin & 68% susceptible to Erythromycin. Gram negative organisms like Enterobacteriaceae were 100% susceptible to Imipenem, 71% susceptible to Amikacin and 71% to ciprofloxacin.

Conclusion: Initial contamination in open fractures is by both gram positive and gram-negative organisms with *Staphylococcus aureus* being commonest. Broad spectrum antibiotics like ciprofloxacin along with amikacin form cornerstone therapy for prevention of infection.

Keywords: open fracture, bacterial isolates, gram positive, gram negative, antibiotic susceptibility.

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1. INTRODUCTION

Open fractures are fractures which communicate to the external environment through a wound, exposing the bone. They are also referred to as compound fractures [1].
Infection is one of the most common and disastrous complications of an open fracture, with reported incidence of 2-25% [2]. All open wounds are usually considered to be contaminated with microorganisms. If the wound bio-burden is not effectively handled by the immune system, it will continue to increase and greatly enhance the risks of clinical infection, unless intervened with aggressive management such as debridement and antimicrobial prophylaxis.
The source of pathogenic organism in open fracture may be

1. Environmental, contamination of wounds during time of injury;
2. Endogenous, from patients own flora and
3. Exogenous, from another patient or hospital staff [3].

Wound management practices become more difficult once there is an established infection. It results in delayed wound healing and treatment cost also rises. The factors which influence the nature and frequency of infection are low resistance of patients, contaminated environmental sites, contact with infectious persons, or drug resistance of pathogenic bacteria [4]. Further contamination occurs in the course of management at the hospital. The primary goal in the management of open fractures is to prevent the infection of bone and soft tissue. The treatment protocol includes surgical debridement, wound irrigation, broad spectrum antibiotics, stabilization of fracture and early soft tissue coverage [5]. Thorough wound debridement as soon as possible after the injury is recommended as the standard of care for all compound fracture wounds [6]. Swab or tissue samples will be collected to isolate and identify the bacteria from open fractures in long bones. It will be subjected to culture, staining techniques, identification by standard bacteriological tests followed by antibiotic susceptibility testing.
Tissue specimen**:** Piece of deep tissue collected after cleansing all the superficial debris is considered the most valuable method for determining the bio burden in the wound [15]. The tissue is biopsied from the leading edge of the wound where the colonizing organisms are less and pathogens are likely to occur. Tissue is collected aseptically and cultured on appropriate media for determining growth.
Swab**:** Wound swabbing can be done using a cotton tipped swab to sample the wound fluid and tissue debris. Swab method enables growth detection by qualitative and semi quantitative methods [18]. Wound swabbing can also be done with an alginate tipped swab which will dissolve completely in the diluent and hence quantitative analysis of the wound is possible [16]. The procedure is easy to perform, and less traumatic to the patient, though the efficacy of swab samples is questioned [17]. If a wound swab is collected without prior cleansing of the wound and removal of superficial debris, the culture may represent only surface contamination and provide with misleading information. The administration and route of antimicrobial therapy should be taken into account before swab sampling a wound, because microbial isolation from swabs may be considerably reduced in the presence of topical antibiotic therapy, but the microorganisms in the deeper tissues is more influenced by systemic therapy [19-23]. Semi quantitative analysis of the swab will provide an indication about the microbial burden and the qualitative analysis of the microorganisms present.
This problem is overcome when tissue is sampled as it is considered the most appropriate standard procedure. But for routine patient management, semi quantitative sampling of the

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wound is sufficient. Thus, semi quantitative swab culture proves to be an easy and non-invasive method to determine the etiology of wound infection, whereas quantitative tissue culture remains the gold standard for diagnosis of wound infections [24,25].
A Gram staining technique demonstrating the presence of even a single bacterium predicts a tissue load >105 microorganisms. Breidenbrach and Trager [7] have shown that, a critical level of ≥104 bacteria should be achieved to cause infection in the wounds and quantitative cultures are effective in predicting the likelihood of infection than swab cultures. The 10^5 CFU/g of tissue suggests that the microorganism has the potential to cause infection if sustained at or above this level. In an acute wound, infection occurs as the microbial burden increases with the multiplying bacteria, but the virulence and pathogenicity of the bacteria present in the wound bed should also be taken into account without focusing merely on the numbers. The primary agents of skin and soft tissue infection are Staphylococcus aureus, Pseudomonas aeruginosa and members of Enterobacterales. Both Staphylococcus aureus and Pseudomonas aeruginosa are avid biofilm formers and prevent migration of keratinocytes which is significant in wound healing. Microbial synergy increases the pathogenic effect and severity of infection. Oxygen is utilized by the aerobic bacteria which induces hypoxia in the wound. Also, specific nutrients produced by one bacterium encourages growth of other cohabiting bacteria which may be pathogenic to the host [26-28]. Though polymicrobial growth occurs, it is not possible to establish a bacterium as the etiology for delayed healing unless a pure mono-bacterial growth is obtained. The differentiation of pathogenic and non-pathogenic organisms in a mixed culture should be primarily based on clinical signs such as erythema, pain, edema, suppuration and fever.

The selection of antibiotics to treat these infections is still controversial [8]. In most cases cephalosporins, are recommended along with aminoglycosides, which provide protection against both Gram positive and Gram-negative bacteria which may have entered the wound at the time of injury. The aim of this study is to observe the density of common bacterial flora contaminating open fractures and antibiotic sensitivity pattern of various bacterial isolates to implement the most effective antibiotics for open fractures in our population, as common pathogenic organisms and their sensitivity pattern may vary from population to population

2. MATERIALS AND METHODS

The prospective study was carried out on 100 patients of all age groups presenting with open fractures at Maharishi Markandeshwar Institute of Medical Sciences and Research, Mullana between March 2021 and September 2022. Two wound swabs, (one for culture and other for microscopy) and tissue specimens were collected to isolate and identify the bacteria causing infection in open fractures. All the bacterial isolates were then subjected to antibiotic sensitivity.
• SAMPLE COLLECTION Method of Wound swab collection:
After debridement, two swabs were collected under aseptic precautions.
The specimen was collected by rolling the swab over a 1cm2 area on the wound with sufficient pressure to extract the exudate. The swabs were transported to the Microbiology laboratory without delay.
Method of Tissue specimen collection:
Tissue specimen was collected in the operating room after thorough irrigation and debridement of the wound with copious amounts of normal saline.

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2x1cm of tissue was biopsied from the depth of the wound and transferred to a sterile screw capped container.

a) The container with the piece of tissue was weighed on an analytical balance.

b) The tissue was removed aseptically and homogenized by the sterile scalpel and petri dish method.

c) The tissue was then placed in 5 ml sterile 0.85% sodium chloride. This is a 1:5 dilution of tissue

d) The empty specimen container was reweighed again and subtracted to determine the weight of the tissue in grams or milligrams.

e) Homogenize the tissue for 15 to 30s using vortex.

• PROCESSING OF SAMPLE

Direct Gram Stain (for tissue and swab specimens): 0.2 ml of tissue homogenate was applied on a clean glass slide and was spread as a thin smear. It was allowed to air dry for 15 minutes and heat fixed.

The specimen was stained with crystal violet for 1 min. Gram iodine was poured over the slide for 1 min.

The smear was washed with iodine and decolorized with acetone for 2-4 seconds. Washed again with water and finally counterstained with safranin for 10-30seconds. The smear was observed under a microscope at 100x (oil immersion) to detect gram positive or gram-negative organism.

Swabs were also subjected to Gram stain for the presence of pus cells, epithelial cells and bacteria, if any were noted.

Culture: Tissue samples, wound swabs and aspirates were cultured on routine laboratory media such as MacConkey Agar and Sheep Blood Agar. The whole process was performed under aseptic conditions using standard laboratory protocols.

• IDENTIFICATION

All the bacteria isolated from the samples were identified by the standard bacteriological tests.

Gram positive cocci were identified using, cultural characteristics on MacConkey and Sheep blood agar, morphology on Gram stain, catalase, slide coagulase and tube coagulase tests, Methyl red, Voges Proskauer, Nitrate reduction tests, mannitol fermentation and urease reactions.

Gram negative bacilli of Enterobacteriaceae family were identified by the following reactions: Growth characteristics on MacConkey agar, Gram stain, motility by hanging drop method, catalase and oxidase tests, Nitrate reduction, Indole, Methyl red, Voges Proskauer and Citrate (IMViC) tests, urease production, phenylalanine deamination, reaction on Triple sugar iron agar fermentation of 1% sugars and amino acid decarboxylation tests.

Identification of Non-Fermenter Gram Negative Bacilli was done as follows: Growth characteristics on MacConkey agar, Gram stain, pigment production on Nutrient agar, motility by hanging drop method, catalase andoxidase tests, reaction on Hugh and Leifson's oxidation fermentation (OF) medium, nitrate reduction, Methyl red and Voges Proskauer tests, reaction on triple sugar iron agar, and amino acid decarboxylation tests.

Non pigmented Pseudomonas was speciated by growth at 42ºC, gelatin liquefaction, sensitivity to polymyxin B 300 units, reactions on Hugh and Leifson's OF glucose medium and amino acid decarboxylation.

Acinetobacter species identification was done by inoculation onto 1% OF glucose and lactose and 10% OF lactose, coccobacillary appearance on Gram stain and growth at 44ºC.

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The isolates that were difficult to identify by manual method were identified by using VITEK 2 system.

• ANTIBIOTIC SUSCEPTIBILITY TESTING

All the isolates were then subjected to antibiotic sensitivity by two methods

1) Conventional method using Kirby-Bauer Disc Diffusion method

2) Automated method: VITEK 2 COMPACT (BIOMERIEUX INDIA)

ANTIMICROBIAL SUSCEPTIBILITY PATTERN TESTING BY KIRBYBAUER DISC DIFFUSION METHOD [9]

Inoculum Preparation and procedure: 3-5 similar colonies from 24-hour culture were transferred to a sterile test tube containing 3 ml of peptone water with the help of a sterile bacteriological loop.

The colonies were emulsified and turbidity matched with 0.5 McFarland standards.

1. Using a sterile cotton swab, the suspension was evenly streaked over Mueller Hilton agar in three directions approximately at 60° to evenly distribute the inoculum.

2. Antibiotic disks (HiMedia) were placed on the agar plate using a sterile forceps after allowing the plates to dry for 3-5 minutes.

The petri dishes were incubated overnight at37°C aerobically for 24 hours. The diameter of zone of inhibition were read with the ruled template. Interpretation was done according to the CLSI guidelines [9].

QUALITY CONTROL: The quality control for antimicrobial susceptibility testing was done with the following standard strains; Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853.

DRUGS USED FOR SUSCEPTIBILITY TESTING OF GRAM-POSITIVE COCCI [9]

1. Penicillin 10U 2. Cefoxitin 30µg 3. Amikacin 30µg 4. Erthromycin 15µg 5. Ciprofloxacin 5µg 6. Trimethoprim-Sulfamethoxazole 1.25/23.75µg 7. Vancomycin by MIC for MRSA isolates

DRUGS USED FOR SUSCEPTIBILITY TESTING OF ENTEROBACTERALES [9]

1. Cefotaxime30µg 2. Amikacin30µg 3. Ciprofloxacin5µg 4. Cotrimoxazole 1.25/23.75µg 5. Imipenem 10µg

DRUGS USED FOR SUSCEPTIBILITY TESTING OF NONFERMENTERS [9]

1. Piperacillin-Tazobactam 100/10µg 2. Ceftazidime 30µg 3. Ciprofloxacin 5µg 4. Amikacin 30µg 5. Imipenem 10µg 6. Cotrimoxazole 1.25/23.75µg

VITEK-2 Compact system:

It is an automated microbiology system used for organism identification and sensitivity by utilizing growth-based technology. This system accommodates the colorimetric reagent cards that are incubated and interpreted automatically on basis of advanced caloriemetric technology and sensitivity cards utilizes turbidometry principle.

Antimicrobial susceptibility testing with the VITEK-2 Compact system was performed using an AST P628 card for Gram positive cocci, AST N280 card for oxidase negative, Gram-negative bacilli (except Acinetobacter) AST N281 card for oxidase positive and Acinetobacter species, according to the Manufacturer's instructions.

Antibiotics tested in AST P628 card include: Benzylpenicillin, Cefoxitin screen, Ciprofloxacin, Clindamycin, Daptomycin, Erythromycin (for CLSI only), inducible Clindamycin resistance, Levofloxacin, Linezolid, Oxacillin, Rifampicin, Teicoplanin, Tetracyclin, Trimethorprim/ Sulphamethoxazole, Vancomycin.

AST N280 card includes: Amikacin, Amoxillin/Clav. acid (CLSI), Ampicillin, Cefepime, Cefoperazone/Sulbactam, Ceftriaxone, Cefuroxime, Ciprofloxacin, Colistin, Ertapenem,

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Gentamicin, Imipenem, Meropenem, Tigecycline, Trimethoprim/Sulfamethoxazole.
AST N281 card include: Levofloxacin, Gentamicin, Cefepime, Meropenem, Imipenem, Doripenem, Ceftazidime, Cefoperzone/Sulbactam, Amikacin, Ciprofloxacin, Minocycline, Tigecycline, Colistin, Trimethoprim/Sulfamethoxazole, Ticarcillin/ Clavulanic acid, Piperacillin/ Tazobactam, Aztreonam.
The card was filled with inoculum (prepared by transferring 200ul of culture suspension from the 0.5 McFarland). The VITEK-2 system will automatically processes the antimicrobial susceptibility card until MIC's are obtained.

3. RESULTS

This study was conducted on 100 patients with open fractures of long bones and the results were analyzed statistically using SPSS version 17.0. Age distribution was almost equal, but 95% of the study population were males and 5% were females. 91% of the fractures were of the lower limb among which fractures of both bones of the leg were the commonest. Significant growth reported in 46% of the debridement samples. Direct Gram Stain was used as a screening procedure to predict microbial burden in tissue samples taken at the time of debridement with SENSITIVITY = 78%, SPECIFICITY = 100%.
Staphylococcus aureus was the most common organism isolated in 30% of cultures, followed by Pseudomonas aeruginosa in 22% of the cultures (Table 1). 40 % of the patients reported significant growth in the post operative follow up period among which Pseudomonas aeruginosa was the most common organism isolated in 28% of cultures (Table 2). Sensitivity of debridement cultures in predicting infection in follow up period was found to be 69.23% while specificity was 77%, positive predictive value was 66%, negative predictive value=80% and kappa value=0.458.
Antimicrobial susceptibility pattern of Gram-positive bacteria in post debridement cultures showed susceptibility to amikacin in 77%, to Ciprofloxacin in 61%, to penicillin in 58%, to cotrimoxazole in 16% and cefoxitin in 58%. 23 Staphylococcus aureus were isolated from all the cultures i.e debridement and follow up cultures, out of which 42% were Methicillin resistant on Cefoxitin disc diffusion screening test. All Methicillin resistant isolates were sensitive to Vancomycin.
Antimicrobial susceptibility pattern of Gram-negative bacteria-ENTEROBACTERIACEAE in post debridement cultures showed 100% susceptibility to Imipenem, 71% susceptibility to Amikacin and ciprofloxacin, and 86% susceptibility to cefotaxime clavulinate in case of E. coli and 100% susceptibility to Imipenem and cefotaxime clavulinate, and 80% susceptibility to Amikacin and ciprofloxacin in case of K. Pneumoniae (Table 3). Non-fermentative Gram-negative bacilli such as Pseudomonas aeruginosa were 100% susceptible to Imipenem and 96% susceptible to ceftazidime and Piperacillin-Tazobactam (Table 4).

4. DISCUSSION

In this study, most of the open fractures occur in males in between ages 15-30 years. This is in concordance with the research done by Ahmad et al that showed the incidence of open tibial shaft fractures most common in this age group [10]. This is explained by the fact that most of the transportation and industrial works are associated with males of this age group. Males of this group are also usually involved in driving activities and hence vehicular accidents which results in open fractures.
Road traffic accidents (RTA) are the main cause of open fractures with male predominance

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[10].

In this study, the debridement culture was done by collecting a tissue bit, performing Gram's stain and culturing by quantitative method. A quantitative count of ≥104 cfu/gm was taken as the significant microbial burden which must be reached to cause infection in the post-operative period as proposed by Breidenbach and Trager [7]. A colony count of ≥104 cfu/gm was observed in 46% of the debridement cultures among which 12% reported polymicrobial growth. A comparison was drawn between the presence of bacteria on Gram's stain and the quantitative bacterial load. 35 out of 46 debridement samples with bacteria observed on Gram's stain were culture positive and 11/46 tissue samples which did not demonstrate the presence of a bacterium turned out to be positive on culture. This study showed the sensitivity of Gram staining in predicting culture positivity to be 78% and specificity, 100%. This was in line with the findings by Bowler et al [11], that a rapid Gram stain technique predicted a reliable microbial load of>10^5 organisms even if a single bacterium was observable on the slide preparation. In a study by Kaftandzieva et al, on "Bacteriology of Wound - Clinical Utility of Gram Stain Microscopy and the Correlation with Culture", Gram stain reported a low sensitivity of 38% and a fair specificity of 90% when compared with culture. The author however concluded that, Gram stain should not be a substitute for culture. The information obtained on Gram stain will not be sufficient to guide the possible choice of antimicrobial chemotherapy [12].

Staphylococcus aureus was isolated in 30% of the cultures and Pseudomonas aeruginosa in 22%. Members of Enterobacterales were isolated in 46% of debridement cultures, among which Escherichia coli was the commonest, accounting for 15% of the debridement cultures. This may be because Escherichia coli is an intestinal commensal and many orthopaedic patients are bedridden, so the contamination of wounds, dressings, linen and even hands during perineal hygiene plays an important role in transmission of the organism [3]. Other members of Enterobacteriaceae isolated at the time of debridement were, Klebsiella pneumoniae (11%), Klebsiella oxytoca (9%), Proteus mirabilis (2%), Proteus vulgaris (2%) and Enterobacter aerogenes (2%). This trend was in concordance with the study by Gupta et al [1] which reported that Gram negative bacteria dominated the debridement cultures.

In this study, polymicrobial growth with a quantitative count of ≥104 was reported in 12% of debridement cultures and all the patients developed postoperative infection on follow up. Ikem et al [13] has proposed the same fact in his study that polymicrobial growth of organisms are more virulent than the growth of a single bacterium.

Post operative follow up was done for 6 weeks. 46% of patients showed clinical signs of infection. 40% of patients turned out to be culture positive confirming the presence of post-operative infection. Polymicrobial growth was reported in 12% of cultures.

Gram negative bacteria contributed to 77.5% and the Gram-positive cocci were isolated in 22.5% of the patients. The Gram-negative bacteria belonging to Enterobacteriaceae family were the predominant pathogens, accounting for 48% of the cultures. Overall Pseudomonas aeruginosa was the commonest bacteria isolated in 28% of the cultures in the post operative period. The higher rate of isolation of Pseudomonas aeruginosa in the post operative period may be due to the production of several virulence factors and also the property to form biofilms adhering to the wound, progressing from colonization to infection.

In this study, 77% of the Staphylococcus aureus isolates were sensitive to Amikacin. This finding was in concurrence with Agarwal et al who has stated that Aminoglycosides are the most sensitive group of drugs in both Gram positive and Gram-negative bacteria [3]. In this study all the MRSA isolates were sensitive to Vancomycin by Minimum inhibitory

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concentration method.
Also 68% of Staphylococcus aureus showed susceptibility to erythromycin and 61% to ciprofloxacin, but only 16% were susceptible to cotrimoxazole. Members of the Enterobacteriaceae family showed 100% susceptibility to imipenem, 71% to amikacin and ciprofloxacin. This is in agreement with the study conducted by Barakat et al that, the most effective drugs against Gram-negative bacteria were gentamicin, ciprofloxacin, imipenem, aztreonam and tazocin [14].

5. CONCLUSION

Open fracture wounds are at a high risk of developing infections and other related complications. The management of open fractures is targeted on effective wound debridement, appropriate antimicrobial therapy and early wound closure.
Gram's stain has been a rapid, sensitive and specific technique to identify the load of organisms present at the wound site.
Role of culture of the wound and determination of the antimicrobial susceptibility pattern of the isolated microorganisms cannot be overlooked in treatment of open fractures, because antibiotic therapy is considered therapeutic and not prophylactic owing to the high degree of contamination involved. Debridement cultures provide guidance regarding the choice of antimicrobial therapy, which when combined with a thorough wound debridement will enable an early wound closure and lesser complications.

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ETHICAL CLEARANCE:
The ethical clearance was taken from ethical committee of the institute before starting the study.
CONFLICT OF INTERESTS:
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Table 1: PATTERN OF BACTERIAL ISOLATES IN DEBRIDEMENT CULTURES (n=46)

Organism	No. of isolates	% age
Staphylococcus aureus	14	30
Klebsiella pneumonia	5	11
Klebsiella oxytoca	4	9
Escherichia coli	7	15
Proteus mirabilis	1	2
Proteus vulgaris	1	2
Pseudomonas aeruginosa	10	22
Enterobacter aerogenes	1	2
Acinetobacter baumannii	3	7

Table 2: PATTERN OF BACTERIAL ISOLATES IN PATIENTS WITH CLINICAL SIGNS OF INFECTION IN THE POST OPERATIVE PERIOD (n=40)

Organism	No. of isolates	% age
Staphylococcus aureus	9	22.5

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Klebsiella pneumonia	6	15
Klebsiella oxytoca	4	10
Escherichia coli	7	17.5
Proteus mirabilis	2	5
Pseudomonas aeruginosa	11	27.5
Acinetobacter baumannii	1	2.5

Table 3: ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF GRAM-NEGATIVE BACTERIA-ENTEROBACTERIACEAE IN POST DEBRIDEMENT CULTURES

Organism	CTX%	CEC%	AK%	CIP%	COTRI%	IMP%
Escherichia coli (n=7)	(n=1) 43%	(n=6) 86%	(n=5) 71%	(n=5) 71%	(n=4) 57%	(n=7) 100%
Klebsiella pneumoniae(n=5)	(n=3) 60%	(n=5) 100%	(n=4) 80%	(n=4) 80%	(n=0) 0%	(n=5) 100%
Klebsiella oxytoca (n=4)	(n=1) 25%	(n=4) 100%	(n=3) 75%	(n=3) 75%	(n=1) 25%	(n=4) 100%
Proteus mirabilis (n=1)	(n=0)0%	(n=1) 100%	(n=0)0%	(n=1) 100%	(n=1) 100%	(n=1) 100%
Proteus vulgaris (n=1)	(n=0) 0%	(n=1) 100%	(n=0) 0%	(n=1) 100%	(n=0) 0%	(n=1) 100%
Enterobacter aerogenes (n=1)	(n=0)0%	(n=1) 100%	(n=1) 100%	(n=1) 100%	(n=1) 100%	(n=1) 100%

Table 4: ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF GRAM NEGATIVEBACTERIA-NON-FERMENTERS

Organism	PT%	CAZ%	AK%	CIP%	COTRI%	IPM%
Pseudomonas aeruginosa (n=10)	(n=10) 100%	(n=10) 100%	(n=7) 70%	(n=3) 30%	NT	(n=10) 100%
Acinetobacter baumannii (n=3)	(n=3) 100%	(n=3) 100%	(n=2) 67%	(n=2) 67%	(n=1) 33%	(n=3) 100%

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