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Drug susceptibility pattern of gram negative bacterial isolates among hematological malignancy patients undergoing chemotherapy TikurAnbesa Specialized Hospital, Addis Ababa, Ethiopia

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This thesis prepared by Manie Asres which is entitled with "**Drug susceptibility pattern of gram negative bacterial isolates among hematological malignancy patients** undergoing **chemotherapy at TASH"** is my original work and submitted for the partial fulfillment of the requirements for the degree of Master of Clinical Laboratory Sciences (Public health and diagnostic microbiology Specialty) complies with the regulations of the University and meets the accepted standards with respect to originality and quality. All sources of materials used for the thesis have been duly acknowledged.

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List of Abbreviations

AAU	Addis Ababa University
ACT	Acinetobacter
AG	Augmentine
ALL	Acute Lymphoblastic Leukemia
AM	Amikacin
AM	Ampicillin
ANC	Absolute Neutrophil Count
BAP	Blood Agar Plate
BM	Bone marrow
CBC	Complete blood count
CML	Chronic Myeloblatice Leukemia
CRE	Carbapenem-resistant Enterobacteriaceae
CRO	Ceftriaxone
CTX	Cefotaxime
DCH	Duration of Chemotherapy
DN	Duration of Neutropenia
ESBL	Extended spectrum beta-lactamase
GM	Gentamicin
GNB	Gram Negative Bacteria
GNBI	Gram negative Bacterial Infection
HM	Hematological malignancy
LOS	Length of hospital stays
MAC	MacConkey Agar Plate

MDR	Multidrug Resistance
MEROK	Meropenem
NHL	Non-Hodgom Lymphoma
PS	Pseudomonas
SN	Sever Neutropenia
SXT	Trimetophin+Sulfaemethazol
TASH	Tikur Anbessa Specialized Hospital

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Abstract

Background: Gram negative bacterial infections are a major cause of morbidity and mortality in patients with hematological malignancies (HM) undergoing chemotherapy. The choice of empiric antimicrobial treatment is based on susceptibility pattern of locally isolated gram negative bacteria.

Objective; to determine susceptibility pattern of gram negative bacterial (GNB) isolates and potential risk factors among hematological malignancy patients who undergo chemotherapy in Tikur Anbessa Specialized Hospital(TASH).

Methods: A case control study was carried out for determining drug susceptibility pattern and associated risk factors for gram negative infections in patients with hematological malignancies in TASH Between March to August, 2016. A convenient sampling technique was used to select the study participants. Controls were patients admitted in the same ward during the same period but without gram negative infections. Equal number of cases and controls were included. Data was analyzed by using SPSS for windows version 21.0 and EPIINFO version 7.0. Differences between group proportions were assessed by chi-square X^2 or McNemar's test. Potential risk factors were analyzed by using univariate and multivariate analysis. For risk factor analysis p value and likelihood ratio were calculated. P<0.05 was considered significant.

Results: Forty seven patients with gram negative infection (GNBI) and forty seven matched patients without gram negative infection (GNBI) were identified from TASH microbiology laboratory from March2016 to August 2016. Multivariate logistic regression analysis showed that four independent risk factors were significantly associated with GNBI in patients with HM. They were neutropenia (OR 12.2; 95% CI, 4.44-33.49; p<0.001), Durations of Neutropenia (OR, 28.42; 95% CI, 7.62-105.94 P=0.004), Length of Hospital Stay (OR 83; 95% CI, 5.11-1349.24; p<0.001), and Sever Neutropenia (OR 7.96; 95% CI, 2.46-52.82p=0.0004). The predominate isolate was maximally sensitive to carbapenems (92%) followed by aminoglycosides 80%. The multi-drug resistance rate of all GNB isolates in this study, *K.pneumonia* (37%) was predominating followed by *E.coli* 3(26%).Except cefepem all third generations' cephalosporin drugs had low sensitivity.

Conclusions: Our findings suggest that gram negative infections in patients with hematological malignancies strictly correlate with severity of neutropenia and extended hospital stays. The results of this study initiate the hematology unit for the development of effective interventions to minimize the impact of GNBI in TASH. Continuous surveillance of the bacterial etiologies and its antimicrobial susceptibility pattern is crucial.

Key words: Gram negative bacteria; chemotherapy, Risk factors

1. Introduction

1.1 Background

Infectious complications are a serious cause of morbidity and mortality in Patients with underlying hematological malignancies. Infections related death is approximately 60 % in patients with HM (1).However the malignancy itself and intensive chemotherapeutic treatment associated with greater risk factors for infections complications (2). Hematological malignancies constitute group of cancers that arise from malignant transformation of peripheral blood, lymphatic system and bone marrow-derived cells (3).According to WHO report, cancer has emerged as a major public health problem in developing countries, matching its effect in industrialized nations. The mortality rate of leukemia in developed and Less developed areas were 2.8% and 2.6% of total cancer cases in 2012 respectively (4) . In sub-Saharan Africa, Hematologic malignancies (HM) have emerged as a major cause of morbidity and mortality (5).

The patients received myelosuppressive chemotherapy with immune deficiencies generate a potential risk factors .These risk factors increased the susceptibility to infections and associated with substantial morbidity, mortality and costs (6). In most patients With HM who undergoing chemotherapeutic treatment, 80% of neutropenia will be occurred within the following 2-3weeks (7).Bacterial infection in these patients with underlining neutropenia is high due to the immunosuppressive effects of chemotherapy. They are also affected by higher burden of extended chemotherapeutics treatments and prolonged hospital stay (8).

Endogenous flora ,which include *Enterobacteriaceae* in the gastrointestinal tract, passing through the damaged mucosal barriers which breakdown by chemotherapy treatments (9).A Qualitative or quantitative phagocyte defects co-exist with Gram-negative bacterial infections associate with the most frequently encountered complications by releasing endo-toxin in the bloodstream resulting in hypotension, renal failure, and shock in patients with hematological malignancies (10).

These patients exposed to endogenous flora of bacterial infections are estimated to be 80%.*Pseudomonas aeruginosa, Escherichia coli and Klebsiella* are the most common endogenous gram negative bacteria which cause infections through gastrointestinal tract. These bacterial isolates account 40-60% of all GNBI in hematological malignant patients (11).

Prophylactic antibiotics are administered in attempt to protect neutropenic patients from their own normal flora, exposed to multidrug resistant (MDR) Gram-negative organisms which colonize the GI tract. They are the only microorganisms left after treatment with multiple broad spectrum antibiotics and they result in serious infections with increased mortality rates (12).This burden of MDR leaves those clinicians caring for the majority of HM patients, without option of drug in TASH. In favor of quality health care delivery in patients with HM, specific tests, Cultures and antibiotic susceptibilities should be performed during the initial assessment .Culture specimens from sites of suspected infection, sensitivity of antibiotics that are set for empirical treatment (13).

The spectrums of GNB isolates and their antibiotic susceptibilities differ by time and by hospitals as a result of therapeutic and medical manipulations (14).The microbiology laboratory in TASH should find solutions to provide an institutional GNB pattern, to determine antibiotic susceptibilities of the isolate for deciding empirical treatment and to promote early and comprehensive critical care for standard and quality health managements in time.

In order to get immediate health care and appropriate antibiotic therapy, assessing risk factors for GNBI and determining drug susceptible pattern in the institutional level is very important. Therefore the purpose of the study is to enable for health worker to give priority for the potential risk factors for GNBI and to get a preliminary reports from primary samples gram stain results for adjusting the empirical treatments. In order to take immediate actions for GNB isolates that are susceptible to the antibiotic that has been given empirically if resistance can be identified in time. The primary objective of this study was to assess main risk factors which predispose for GNBI, to isolate GNB and to assess drug susceptibility pattern in patients with hematological malignancies who underwent chemotherapy in TASH.

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1.2. Statement of the problem

The latest world cancer statistic showed that Global burden rises to 14.1 million new cases and 8.2 million cancer related deaths (15). The annual new cancer cases report in USA indicated that HM accounted for an estimated 111,310 (9%) and 53,920 death in 2006(1).The developing world, including Africa, shares 53% of the burden which already has taxed its limited resources. The incidence of hematologic malignancies accounted 10% of the overall cancer burden in sub-Saharan Africa (16).

Patients with HM undergoing chemotherapy for hematologic malignant treatments are vulnerable to infections (17). These patients with undergoing intensive chemotherapy generate major risk factors which include, sever and longer duration of neutropenia, MDR and extended hospital stay (18). Bacterial infection continues to be the most common complication in patients with chemotherapy-induced neutropenia (19).

Especially infections due to Gram-negative rods used to be significant threats to hospital-acquired bacteremia .On the occasion of extended duration of neutropenia, gram negative bacterial infection (GNBI) in patient with HM is the main cause of infection-related mortality (20). The risk to infections in these patients highly increased when absolute neutrophil counts (ANC) falls below 100 and approaches to zero (21). According to infectious disease society of America, high-risk patients with severe and prolonged duration of neutropenia after chemotherapy, the mortality rate increased by more than three times after >7 days of duration of Neutropenia (22). This risk factor was predominate predisposed factors for developing GNBI (23).

These patients with underline burden of prolonged duration of Neutropenia also require extended LOS .A patient stay more than 6 days in the hospital may exposed to a broad-spectrum beta lactams drug used for empirical treatment of fibril neutropenia which is resistance to gram negative bacteria. Therefore the exposure to the risk of multi-drug-resistant (GNB-MDR) infection is increased in prolonged length of stay in hospital which causes extra usage of health care resource and costs (24).

Although high-risk patients with significant medical co-morbid conditions initially admitted to the hospital for empirical antibiotic treatments (25),the patients with hematology malignancy

infected by GNB-MDR strains become a challenging problems for the physicians to get drug of choice (26).

Majority of the Gram-negative isolates were multidrug resistant with high prevalence of ESBL producing *E. coli* and *Klebsiella* strains. Regional differences in the ESBL proportion and distribution, therapeutic decisions should be based on local guidelines derived from local bacterial spectrum and drug susceptibility pattern (27). Although most patients are treated empirically by using the institutional profiles of GNB isolates and drug susceptibility pattern, approximately 35% patients required change in empirical line of antibiotic treatment depending on culture sensitivity report (28).It is also important for the change in the spectrum bacterial isolates and antibiotic susceptibility pattern for the reemergence of Gram-negative bacteria in patients with HM (27, 28).

The patients with HM coming from all over the country are managed in our institution. The susceptibility pattern to antibiotics, the choice of empiric therapy has become problematic and must be evaluated on the basis of local patterns of GNBI. Therefore to improve this problem, the result of the study was important for the institution to develop guidelines and to aid in the choice of the most effective empirical treatment based on the profile of local GNB isolates and the local drug resistance pattern in TASH.

1.3. Significance of study

As far as our knowledge is concerned, in Ethiopia the potential risk factors for GNBI in patients with HM have not been well known. Our study was intended to assess the potential risk factors for GNBI in HM patients. This is a one step forward to improve the health care quality for those patients. Besides it came up with updates on the local spectrum of GNB isolates and their antimicrobial susceptibility profile which help clinicians to choose appropriate empiric treatment for their patients.

Although culture test is much more efficient and reliable in terms of giving the exact species of gram negative bacterial infections , the whole processes need extended length of time to get the final results .In order to adjust the empirical treatments, in addition to the updated documents on the local spectrum of GNB isolates and their antimicrobial susceptibility profile ,an immediate preliminary report of gram stain results from primary samples is very important to increase the survival of high risk patients. Therefore this study can be used as initial documents for the expertise who develop diagnostic protocols and standard operational procedure in the hospital.

In our hospital settings, for patients with hematological malignancies that may be exposed to potential risk factors of the reemerged gram negative strains and MDR ,the culture and susceptibility test is mandatory before empirical treatments .But it requires an extended length of time to get the final results. Therefore this study is used as base line information for laboratory managers and hospital administrative staff to see options like molecular diagnostics tests which can get results within one day .

The profiles of GNB isolates and their antimicrobial susceptibilities pattern will inform all decision makers for change in antibiotic resistance and update empirical treatments options used before infection complications have been occurred in patients with HM. Finally caring out this research is very important to encourage a more selective management strategy for health professionals effectively in resource-limited setting.

2. Literature Review

Patients with underlying hematological malignancies are at risk for bacterial infectious diseases. The treatment-associated is one of predisposing factors in patients with underlying malignancies to an increased risk of infection. Chemotherapeutic agents are treatment-associated factors that predispose to infection in a variety of ways (29).

A febrile neutropenic patient with HM is associated with a significant risk of GNB infections complications Therefore to administrate the parenteral and broad-spectrum antibiotic empirical therapies for patients with HM , immediate standard management is required (30).

2.1. Treatment-Associated Factors

2.1.1 Chemotherapy

Chemotherapy-induced febrile neutropenia (FN) predisposes patients to life-threatening infections and typically requires hospitalization .Gram-negative bacilli such as *E. coli, Klebsiella spp., and P. aeruginosa* cause the earliest infections in neutropenic patients. These usually occur within the first 2–3 weeks after the initiation of chemotherapy and are due to the rapid decrease in the neutrophil count (31).

It is only commonsensical that pretreated patients by chemotherapy develop more infections than untreated cancer patients (32).A study was conducted to determine risk factors for Bacteremia by GNB in patients with hematological malignancies .The patients with neutropenia for more than six days (OR 3.0 (95% CI: 1.7-9.5)) showed an increased risk for bacteremia that strictly correlates with the intensity and length of neutropenia (33).

A study was conducted by collecting Prospective data from the patients who received chemotherapy for HM treatment at National Institute of Blood Disease Center in Pakistan. Parameters analyzed included age, sex, presenting complaints, duration of neutropenia, duration of hospital stay, isolation of microorganism, and their culture susceptibility pattern. Median duration of neutropenia was 6 days while median hospital stay was 7.41 days (34).

A study was conducted to associate factors with LOS among Cancer Patients with febrile neutropenia in Brazil. They found that Hematologic neoplasm, who took a high-dose chemotherapy regimens, duration of neutropenia and MDR involving Gram-negative MDR

bacteria .A patient exposed to these risk factors were statistically significantly associated with LOS(35).

A retrospective study was conducted on hematological malignancy Patients with profound neutropenia and had neutropenia duration of average 16±2 days, in order to show patients have increased risk of septicemia associated with significant morbidity. Thirty eight (67%) isolates were from hem cultures, 12 (20.8%) were from urine cultures and 7 (12.2%) were from catheter, abscess and wound. Twenty six (45.6%) isolates were gram negative bacteria (36).

A study was conducted on the patients with hematological malignancies by relating the high mortality of bacterial infection to the degree and duration of neutropenia. The 7-day mortality rate for patients with bacteremia was 17% (95% CI, 13–21), the 30-day mortality rate was 32% (95% CI, 27–37). Gram-negative organisms caused 50% of the bacteremia (37).

A study done in Israel indicated that among adults and children with underline HM disease the spectrum of pathogens changed with time in hospital. The prevalence of *E. coli* decreased significantly from 23.9% within 48 hours of admission to 7.6% after 14 days in hospital while the prevalence of other Gram-negative bacteria, mainly *Pseudomonas aeruginosa and Klebsiella pneumonia ,*increased throughout the hospital stay (38, 35).

A Study was conducted by M. Paul et al showed that Pathogen distribution changed with the length of hospital stay before bacteremia. The prevalence of *E. coli* decreased significantly from 23.9% within 48 hours of admission to 7.6% after 14 days in hospital ($P < 0.001$ for trend), while the prevalence of other Gram-negative bacteria, mainly *Pseudomonas aeruginosa and Klebsiella pneumonia ,*increased throughout the hospital stay (41).

2.1.2 Antimicrobial Use

A study was conducted on risk factors of Gram-negative bacilli causing blood stream infection in patients with malignancy in Saudi, the researchers found that there was an emergence of drug-resistant GNB, such as ESBL producing GNB. The emergence of carbapenemase-producing *K. Pneumonia* (KPC-Kp) blood stream infection among patients with hematologic malignancies has contributed to 26 (18%) of all gram negative infections (40).

A study was conducted by Andria et al. on Carbapenem-resistant *Enterobacteriaceae* (CRE) to find significant problem of CRE in hospitals in several locations worldwide. Their study indicate

that Patients with the Carbapenem-resistant Gram-negative bacteria (CRGNB) was associated higher rates of inappropriate empirical antibiotic treatment (68/103, 66%) than among patients with carbapenem-susceptible Gram-negative. Bacteremia(CSGNB) (99/320,30.9%), the 14 day mortality rate was 45.6% (47/103) for patients with CRGNB versus 15% (48/320) following CSGNB (P,0.001).These patients also have longer admission period prior to the bacteremia episode (42).

A Case-Control Study was conducted to identify potential risk factors for *P. aeruginosa* bloodstream infection among patients admitted to the hematology unit .Among patients who have a bloodstream infections due to Gram-negative bacteria other than *P.aeruginosa* (GN Controls) and patients who never developed bloodstream infection due to Gram-negative bacteria (Non-GN Controls).The study results showed that multi drug resistance for *P. aeruginosa* infection was associated with hospitalization longer than 60 days (OR= 60, 95% CI 4.73–2879) and imipenem(OR = 7.77, 95% CI 1.2–57.8) were significantly associated with MRPA-BSI(42,43)

A matched case-control study was conducted by to compare the differences in risk factors of patients with multidrug-resistant *Acinetobacter baumanni* (MDRAB) and non- multidrug-resistant *Acinetobacter baumannii* (non-MDRA bacteremia. The result showed that Cases were significantly more likely to receive antimicrobial therapy than controls (92.1% vs. 69.8%, p=0.012).And also Cases were significantly more likely than controls to receive more antibiotic agents within 4 weeks before bacteremia (2.6 vs. 1.7, p=0.008)(43).Dove press conducted study on patients older than 14 years with HM undergone chemotherapy and they developed febrile neutropenia .As the study indicated that 66 (23%) had bacteremia from 126 febrile patients. Gram-negative bacteria caused 74% (n=49) of all bacteremic episodes. Carbapenem-resistant Gram-negative bacteria (n=6) caused 12% and 9% of Gram-negative bacteremia episodes and all bacteremia episodes, respectively. Carbapenem-resistant Gram-negative bacteria included *Acinetobacter baumannii* (n=4), *Pseudomonas aeruginosa* (n=1) 45,(42,43)

The study was conducted at Erciyes University Hospital in Turkey to assess the outcome of nosocomial GNB bacteremia in patients with hematological malignancy. A total of 154 patients with GNB bacteremia were identified and Blood cultures revealed *Enterobacteriaceae* in 120 patients and glucose non-fermenting GNB in 34 patients. Forty (33.3%) out of 120

Enterobacteriaceae were extended spectrum beta-lactamase (ESBL) producers and 18 (52.9%) out of 34 glucose non-fermenting GNB were multidrug resistant (44).

2.2. Commonly Encountered Gram negative isolates in patient with hematological malignancy

The study done at departments of Pediatric Hematology and Oncology showed that Gram-negative infections accounted for 74% of all positive bacterial isolates which include with *E. coli* and *Klebsiella* being the most frequently identified organisms followed by Pseudomonas ,*Acinetobacter, Enterobacter and Proteus* . Probability of isolating a Gram-negative organism from blood, urine, and other sites constituted 6.5%, 16.6%, and 21.6%, respectively ($P < 0.001$) (45).

A case-control study was conducted in India on 71 clinically suspected cases of sepsis patients with HM from deferent types of samples ,47(69%) of the isolates were Gram-negative bacilli. *Klebsiella* pneumonia was the predominant isolate followed by E. Coli 48, (46) .

A study was conducted to assess risk factors of GNB infections among HM patients .From this study the most predominant pathogen was E. coli 29.5% (18/61) followed by *A. baumannii*. 18.0% (11/61), Pseudomonas spp. 16.3% (10/61), K. pneumonia 13.1% (8/61) Salmonella spp. (6.5%), and *Enterobacter spp*. (3.24%)(47).

A study was conducted upon hospital admission in patients with HM in U.S A indicated that the sample collected at admission before 48hr,Escherichia coli was the most common isolate 51%; followed by *Klebsiella* species 16%; *P. aeruginosa*6.8%Enterobacter species 4.9%; and Proteus species 5% (48).

A study was conducted on a neutropenic patient with underline disease of HM showed that among 80 cultures, there were 68 (85%) Gram negative organisms while Gram positive were only 12 (15%).Gram negative cultures included 27 Escherichia coli (40%),12*Klebsiella pneumoniae*(17.6%), 8 *Klebsiella* species. (12%), 10*Pseudomonas aeruginosa*(14.7%), and 8 Pseudomonas spp.(12%).The bacterial isolates from this study ,blood stream infection were 43.75%)and from other sites was reported as throat 12 (15%), urine 11 (13.75%), central line 7(8.75%), and others 9 (11.25%) 50,(49).

A study conducted in Turkey on patients with hematological malignancies and had neutropenia duration of average 16±2 days. Thirty eight (67%) isolates were from hem cultures, 12 (20.8%) were from urine cultures and 7 (12.2%) were from, abscess and wound. Twenty six (45.6%) isolates were gram negative bacteria, whereas 23 (42.1%) isolates were gram positive bacteria (50).

Empirical first-line antibiotics used were piperacillin- tazobactam and amikacin. Fever was responsive to first-line antibiotics in 184 patients (85.9%). The most frequent hematological disease documented was ALL, 91 cases (40.3%), followed by AML, 45 cases (19.9%) (51).

A study done in west Africa, Ghana showed that54.8 % of blood stream infection occurred in patients with hematological malignancies and 52.6 % of them had Gram-negative bacteria which included *Enterobacteriaceae, Pseudomonas aeruginosa*, and *Acinetobacter species* (52).

A study conducted in TASH, Ethiopia on high mortality due drug resistance of blood stream infections indicated that the predominate bacteria was *K.pneumonia (55)*

3. Objective of the study

3.1 General objective

To assess the potential risk factors for gram negative bacterial infections (GNBI) and to determine susceptibility profiles of gram negative bacterial isolates among hematological malignancy patients who undergo chemotherapy in Tikur Anbessa Specialized Hospital

3.2 Specific Objective

- To assess the profile of GNB isolates from different samples in patients with HM
- To determine drug susceptibility pattern of GNB isolates
- To assess the potential risks factors and determine their level of significance in patients with undergoing chemotherapy.

3.3 Hypothesis

Ho: There is no association between developing GNB infections and undergoing chemotherapy in patients with hematological malignancy

Ha: There is an association between developing GNB infections and undergoing chemotherapy in patients with hematological malignancy.

4. Materials and Methods

4.1 Study design and study period

This study was undertaken in the microbiology unit of laboratory department and hematology wards at Tikur Anbessa Specialized Hospital .All cases of hematological malignancies admitted from March 2016to August 2016 were included in the study. A hospital based case-control study was conducted among 94 patients of both sexes greater than two years of age. All patients diagnosed with hematological malignancies and receiving induction chemotherapy were eligible for the study .

4.2 Study Area

Tikur Anbessa Specialized Hospital (TASH) is located in the nation's capital Addis Ababa. The faculty is the oldest and the largest among the health training institutions in the country, staffed with the most senior specialists. It is administered by Addis Ababa University, providing teaching for about 300 medical students and 350 Residents every year. The hospital has 800 beds, with 130 specialists, 50 non-teaching doctors. In the hematology wards, around 400 patients are admitted a year. This study was conducted at TASH which have the largest number of patients with HM undergone chemotherapy.

The hospital has BACTEC machine to detect bacteria in blood with a capacity of 50samples at one time. Approximately from 40 up to 60 samples per day come to micro laboratory for culture and susceptibility testing (25).

4.3 Study population

All types samples that come to Tikur Anbessa Specialized Hospital microbiology laboratory for culture investigations who were admitted in hematology ward for undergoing chemotherapy in all patients with hematological malignancies less than two years of age is not important to recruit in this study. The selection of Patient with HM takes place at the time of or after incidence of the GNBI and data was collected to look back toward exposure to risk factors of the GNBI. In Patients with HM were selected based on those with the GNBI undergoing chemotherapy as case subjects and those without GNBI undergoing chemotherapy as control subjects.

4.4 Study Variables

Dependent variable-gram negative isolates, drug susceptibility pattern.

Independent variables- Age, sex, durations of chemotherapy, empirical treatments .MDR, LOS and neutrophil counts.

4.5 Sample size estimation and Sampling technique

The sample size was assessed with the intention to identify patients with hematological malignancies predisposed to GNB infections with and without exposure to risk factors by using convenient Sampling Technique. The number of case with infections and the number control with non-infections were included in the study. The numbers of cases with HM and control with HM were included in this study

4.6 Source population

All samples that come to Tikur Anbessa Specialized Hospital laboratory from March to August, 2016 were the source population for this study.

4.7 Eligibility criteria

4.7.1 Inclusion criteria

All hematological malignant patients admitted to pediatrics and adult wards during the study period.

4.7.2 Exclusion criteria

Patient with evidence of infections prior to chemotherapy

Patients less than two years of age

4.8 Data collections procedure

4.8.1 Sample collection

For patients with suspected of infections, blood cultures were aseptically collected from each adult patient 8-10 ml of blood sample and for pediatric3-5 ml of blood sample was collected by physician /nurses and by microbiologist inoculated into a BACTEC vial for aerobic culture . The samples were collected in to BACTEC bottles for blood cultures, cups for urine and sputum cultures and aspirate samples for different fluids from admitted patients for undergoing chemotherapy by their attending physician and laboratory professionals at TASH. The samples

were transported to the TASH micro laboratory Within 20 Minutes. No additional samples were collected for our study; we used left over samples.

4.8.2 Sample processing's

The collected blood samples were incubated in to BacT/TECH machine immediately. Others collected samples were inoculated directly in to media. For aspirate fluids, sputum and BACTEC 9050 with a positive flag, we made a gram stain immediately for preliminary reports.

Blood; Samples of the contents of all instrument-positive vials were gram stained and subculture. Gram stain Findings from gram stain determines media for inoculation. Isolates were identified by means of microscopic examination as well as biochemical and serological tests. Findings in gram stain from positive bottles were always reported by phone.

Urine; the samples are inoculated onto BAP and MAC with a quantitative loop, 1 µl on each plate. Blood agar plate: Non-selective MAC: Selective for gram negative bacteria and finally Plates are incubated for 24 hrs in 35^oC**.**The amount of bacteria is counted from the blood agar plate (BAP) and is important to determine further processing of the sample. One colony on BAP equals 1000 cfu/ml urine = 10^3cfu/ml.

CSF and other fluids; the samples were inoculated onto three plates with loop; blood agar plat and chocolate agar Non-selective MAC: Selective for gram negative bacteria and nutrient broth for enrichments. Before inoculate into plates we Centrifuged >1 ml for 15 min at 3600 rpm (2000g). If the collected specimens were <1ml ,we inoculated directly without centrifugation. BAP and CAP were incubated in Atmospheric conditions of 5% CO_2 at 35^oC for 4 days for CSF and 2 days for other fluids. Findings in gram stain from primary samples are always reported by phone to prescribing physician (53).

4.8.3 Isolation Procedures

Isolating Procedures for the Maximal recovery of GNB from fluids specimens is obtained by using an enrichment broth, although isolation from acutely ill persons is usually possible by direct plating of specimens.

4.8.4 Principles of BACTEC machine

Detection of bacterial growth was instrument-assisted and predicated on infrared spectroscopic analysis of air in the head space of the blood culture bottles for the presence of evolved CO2.BACTEC 9050 is a fully automated system, which monitors increase in CO_2 concentration produced by growing microorganisms by means of a fluorescent sensor present at the bottom of

the bottle. Being a fully automated system for detecting growth, it also has the advantage of earlier detection time (54).

Many differential plating media that are available for isolation of GNB from deferent specimens are selectivity include MAC, chocolate and blood agar which are helpful for the detection of lactose-fermenting and lactose fermenting GNB strains. Suspect colonies may be inoculated onto a screening medium such as KIA or TSI, Trypton, citrate urea, Manitol, malonat and motility Drug susceptibility Patterns.

Gram stains; is a rapid and sensitive method for presumptive diagnosis of gram negative bacteria and gram positive bacteria. Sufrenine aqueous basic was used as the counter stain for smears of pure cultures .it may be detected by direct gram stain examination from samples. The sensitivity ranges from 66% to 94% and specificity above 95%. During GNB Infection, white cells have been reported in 25% to 80% of culture proven Cases (53).

4.8.5 Drug susceptibility Patterns

Antibiotic susceptibility test was carried out by disc diffusion technique and after 16-18hours of incubation at 37 ºC. The zone of inhibition was measured and interpreted as recommended by the Clinical and Laboratory Standards Institute (CLSI), 2016. Pure colonies of isolated organisms was picked into nutrient broth and emulsified for GNB. Standard inoculums adjusted to 0.5 McFarland was swabbed onto Muller -Hinton agar (dispensed on 150mm plate). Incubate at 37ºC in aerobic condition. Standard antibiotic discs were then placed on the plate and this is incubated at 37°C for 24 hours. Antibiotics were selected based on CLSI, 2016 in the recent guideline and included; Ceftazidime (10ug, Oxoid), Ceftriaxone (30ug, BD), TMP-SXT (1.25ug, BD), Cefoxitime (30ug, Oxoid), Tetracycline (30ug, BD) meropenem and Ciprofloxacin (5ug, BD). Diameters of growth inhibition around the discs was be measured and interpreted as sensitive, intermediate or resistant as per the standard protocol (CLS) (54).

4.8.4 Quality Control

Standard Operating Procedures (SOP) were strictly followed.

Pre-analytical Phase; Steps starting in chronological order, from the clinician's request and including the examination requisition, preparation of the patient, collection of the primary sample, and transportation to and within the laboratory, and ending when the analytical examination procedure begins. The pre-analytical phase is known to be error-prone, only

recently have data been collected to demonstrate that the errors occurring are mainly related to procedures performed outside the laboratory walls, by healthcare personnel not under the direct control of the clinical laboratory. We checked all medias were not expired and passed the quality control parameter recommended by CLSI, 2016. Visual inspections of cracks in media or plastic Petri-dishes, unequal fill, hemolysis, and evidence of freezing, bubbles, and contamination were performed.

Analytical phase

Quality control (QC) was performed to check the quality of medium. Each new lots were tested against quality controller strain before use include; the *E.* coli ATCC25922,*K.pneumonia* ATCC 700603 and *P. aeruginosa* ATCC 27853 strains. In addition Site assessment and pre-test were done prior to data collection (54).

Post-analytical phase

Post analytical activities review and evaluate effectiveness of the corrective actions , procedures and policies to prevent re occurrence , accuracy and completeness of result and report , disposition of un acceptable samples, corrected reports, procedures for notification of test results with statistic ,and assurance of confidentiality of patient information(54).

4.9 Statistical analysis and interpretation

Statistical analyses were performed with the SPSS statistical software package (version 21).Differences in group proportions were assessed with the chi-square test or, McNemar's test. Potential risk factors and prognostic indicators for bacteremia were analyzed by univariate methods in order to determine possible inclusion in multivariate models. To facilitate the statistical analysis of the outcome, the fatalities directly or indirectly related to GNBI Were included in a single category when they occurred before the episode of GNBI was considered resolved. Multivariate analysis was performed with logistic regression models, and 95% test-based confidence intervals (CI) were used to determine the statistical significance of the odds ratio (OR). Two tailed tests of significance at the $p < 0.05$ level were used to determine statistical significance. Finally, the study findings were explained in words, tables, charts, graphs.

Operational definition

Hematological malignancy; constitute group of cancers that arise from malignant transformation of peripheral blood, lymphatic system and bone marrow-derived cells

Gram negative Infection; the presence of a pathogenic GNB in site (such as blood, Urine Sputum Cerebrospinal Fluid, Or As Cites) Or Clinically Suspected Infection That Needed administration of antibiotics.

Neutropenia; an absolute neutrophil count of less than and equal to _0.5×109/L of white blood cells counts. Neutropenia is characterized by an abnormally low number of neutrophil granulocytes.

Case control study; A study with retrospectively selected diseased cases and matched non-disease controls

Odds ratio calculation; considers only the discordant pairs. It can be explained intuitively: pairs where both case and control were exposed or where both were unexposed give no information about the relationship of the exposure to disease

Multidrug resistance (MDR) : gram-negative bacteria resistance to three or more of the antimicrobials in different groups.

High-risk patients; to be those with anticipated prolonged and profound neutropenia following cytotoxic chemotherapy and/or significant medical co-morbid conditions: Admitted patients with HM who are High-risk and admitted to the hospital for empirical therapy.

4.10 Ethical consideration

The study was conducted after ethical clearance was obtained from the research ethical committee of Department of Medical Laboratory sciences .An informed consent was obtained before collection of specimens preliminary and final results were provided for the management of patients. For hematological malignant patients who were admitted in hematology wards and also had willing to participate in the study and able to give sample during the study period were informed about the purpose of the study and their written signed consent was obtained for the study. Any information related with the patient and clinical history was kept confidential.

4.11 Dissemination of results

After conducting the research, the result of the study will be submitted to the Department of Medical Laboratory Sciences, School of Allied Health Sciences, College of Health Sciences, and Addis Ababa University, Tikur Anbesa Specialized Hospital and other concerned bodies. The result of the thesis will be submitted to the international or national peer reviewed journal for publication.

5. Results

5.1 Characteristics of study participants

In total, 94 patients with hematological malignancy were identified, among them, we identified 47 patients with GNBI as case and 47 patients without GNBI were chosen as the controls. The two groups did not differ in baseline characteristics such as, age gender and underline hematological malignancy. A total of 94 episodes of GNBI occurred in 47 patients (60 males and 34 females with a mean age of 25±3.2 years), there is no statistically significant relationship between the GNBI and gender (X^2 0.002; p = 1.00).

Table 1. Demographic and Clinical characteristics of hematology patients, March to August, 2016

	Number(%)	**Estimated risk of GNBI (95% CI)**
Types of hematological malignancy		
Non-Hodgkin's lymphoma	9(56.3)	1.4(0.4-4.9)
Acute lymphocytic leukemia	15(42.6)	0.6(0.3-1.5)
Acute myeloid leukemia	12(49.2)	1.1(0.4-2.9)
chronic myeloid leukemia	11(64.7)	2.1(0.7-6.2)
Age (years)		
2-18	17(46.0)	0.8(0.3-1.8)
19-30	15(65.2)	2.3(0.9-6.1)
31-49	9(40.9)	0.6(0.2-1.6)
≥50	6(50)	0.9(0.3-3.4)
Gender		
Male	30(50)	1.0(0.4-2.3)
Female	17(50)	1.0(0.4-2.3)

5.2 Gram negative bacteria profile among types of hematological malignancy

By organism type, the distribution of the 47 incident gram negative bacterial isolates comprising in the case series was: *K. pneumoniae,*12 (26%) was the predominant isolate followed by *E. coli, 11 (24%); P. aeruginosa,* 1 (2%);); and polymicrobial13(28%),*Acinetobacter* species 6(12%),others 4(8%).Incidence of GNBI was maximum in patients With CML 64.7%,11/17 and NHL 56.3%, 9/16) followed by AML 52.2%(12/23) and ALL 42.9%(15/35). A chi-square test was performed, there was no association between type of HM and incidence of GNB isolates.

A total of 112 specimens which included 74 blood cultures, 17 Urine, 7sputum, 6 stool and 8 other were processed. Sixty isolates were obtained from 47 culture positive hematological malignancy patients. Within the cases, more number of GNB isolates were from blood (51%) followed by urine (28%) samples (fig 1).

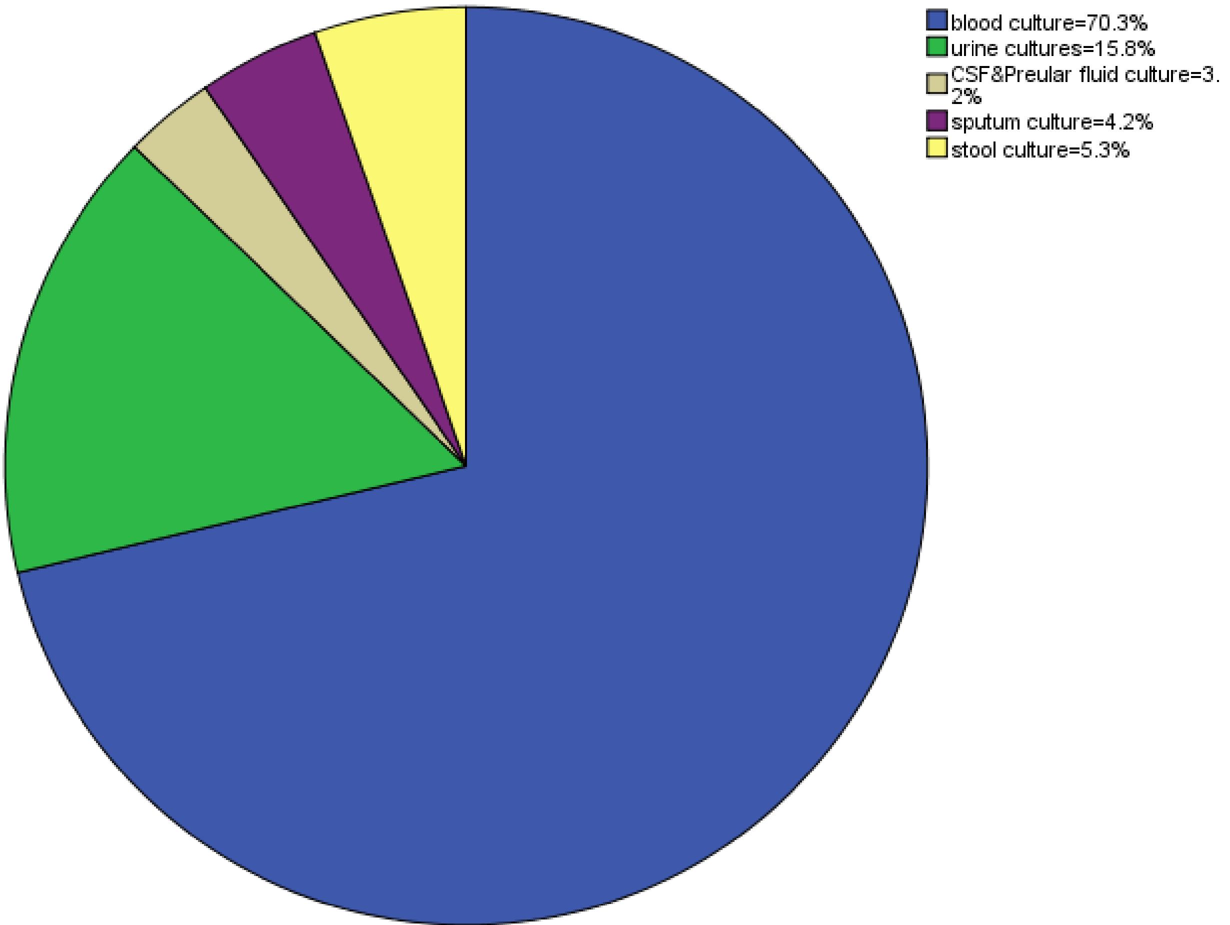

Figure 1 Types of specimen and culture positive among hematological malignancy in TASH

Table -2 Gram negative bacterial isolates in patients with hematological malignancies in TASH,2016

Organism isolated	blood	urine	sputum	CSF &pleural fluids	total
Gram-positive isolates	12	8	4	1	25(34.7%)
Gram-negative isolates	25	14	4	3	47(65.3%)
Klebsiella spp.	12	4	2		18(38.3%)
Escherichia coli	9	5			14(29.8%)
Acinetobacter spp.	2	2	2		7(14.9%)
Pseudomonas aeruginosa	0	2	1		3(6.4%)
Enterobacter pp.	2	3			5(10.6%)
Total					72(100%)

5.3 Induction of chemotherapy is the main predisposing risk factors for GNBI in patient with hematological malignancy in TASH

All 94 patients with hematological malignancy were given induction chemotherapy per protocol. Among the 47patients with GNB, 39 patients for more than six days were neutropenic, while 8 patients for less than six days were not neutropenia. All 32 patients with neutropenia had evidence of GNB infections. 15 patients who did not have neutropenia also developed infections. By using univariate analysis we identified five risk factors which were significantly associated with GNBI (table-2) .

In patients with HM undergone Chemotherapy had a statistically significant association to duration of neutropenia of more than 7days (P <0.0001), sever neutropenia (P<0.0001) and a mean duration of hospitalization of 9.49 days (P <0.0001), which were important risk factors for developing GNBI. AS the test of Mantel-Haenszel statistic showed that Caring or treating for patients with HM had a significant difference between undergoing chemotherapy more than 2weeks and less than 2weeks with GNB infections rates , $\chi 2$ (1, N = 94) =30.46, p <0.001(table-2) .

Table 3 Risk factors for developing gram negative bacterial infections in TASH ,2016.

Risk factor	Case (n=47) *No. (%)*	Control (n = 47) No. (%)	Odd ratio	P value	Likelihood ratio	95% confidence interval
Duration of chemotherapy ≥14days <14days	27(94.4) 20(30.3)	1(3.6) 46(69.7)	62.1	*P<0.001	$1.3x10^{-15}$	7.89-489.10
Absolute neutrophil count(ANC) ≤500 >500	32(82.1) 15(27.3)	7(17.95) 40(72.73)	12.2	*P<0.001	0.000001	4.44-33.49
Durations of neutropenia ≥7days <7days	31(92.2) 16(26.7)	3(8.8) 44(73.3)	28.42	*P<0.001	<0.0001	7.62-105.94
Sever neutropenia ≤100 >100	20(83.3) 27(38.6)	4(16.7) 43(61.4)	7.96	*P=0.00004	$1.6x10^{-4}$	2.46-52.82
Length of hospital stay ≥6days <6days	45(98.3) 2(4.3)	3(6.3) 44(95.7)	83.0	*P<0.001	0.0001	5.11-1349.24
Gender Male Female	30(50.0) 17(50.0)	30(50.0) 17(50.0)	1.00	P=1.00	1.00	0.43-2.32
Median of male with GNBI	29.40±6.8	29.21±8	2.27	P=0.15	0.09	0.86-6.07

*p<0.05

DCH=durations of chemotherapy, ANC=absolute neutrophil counts, DN=durations of neutropenia, LOS=Length of hospital stay, SN=sever neutropenia

Risk factor analysis (Table-2) revealed neutropenia as the major risk factor for development of GNBI (P <0.001). The odds of having the GNBI were 12.0 times greater for subjects in the exposed group than for subjects not exposed to the neutropenia (OR =12.20, 95% CI =4.44,

33.59). Thus, the odds ratio is significantly greater than 1, suggesting that the true odds of having the gram negative bacterial infections were greater for the neutropenic patients.

McNemar's chi-square statistic showed that there is a statistically significant difference in the proportion of patients with HM underline GNBI in the DN>7days group and the proportion of patients with HM underline GNBI in the DN<7days group . According to the results, GNBI was significantly associated with both the duration of neutropenia (P=0.004) and the severe neutropenia (P=0.001) . A higher proportion of patients exposed to the DN >7days (91.2% or 31 of 34) showed a GNBI than did the non-exposed group (26.7% or 16 of 60), x2 (1, N =94) =36.15, p =0.001. A higher proportion of the exposed group (83.3% or 20 of 24) showed a GNBI to the patients with severe neutropenia than did the non-exposed severe neutropenic group (75% or 12of 16), X2(1, N =94) =25.33, p =0.0001(table-4).

Using McNemar's test, significant tendency was found for subjects who had more than 6 days of LOS and also likely to have GNBI in patients with HM after undergone chemotherapy (*p* <0.001). A significant associations was found for subjects who had a long hospital stay their durations of neutropenia to be more likely to have longer DN of in patients with HM after GNBI (*p<0.0001*) (table-4).

The odds of having the DN >7days were 57.85 times greater for chemotherapy more than 2 weeks than for DN<7dys to chemotherapy less than 2 weeks (OR=57.85, 95% CI=7.36, 454.89.0),p<0.0001.

A higher proportion of the patients who had a LOS more than 6days (96% or 45/48) showed a GNBI to the patients who had LOS less than 6 days group (31.8%,or 21/66), x2(1, *N* =94) =32.84, *p* <0.0001(table-4).

Table 4.Association between infections risk factors and the most common GNB isolates among Admitted patients with hematological malignancies in TASH ,2016.

GNB isolates	**DCH,≥14days n=27**			**Neutropenia n=32 ANC≤0.5x10^{-9}**			**Duration of neutropenia ,n=34 ≥7days**			**SN, n=16 ANC≤0.1X10^{-9}**			**LOS,≥6days n=48**		
	n	*P	OR	n	*p	OR	n	*p	OR	n	*p	OR	n	*p	OR
Klebsiella species	6	*0.1	2.7	10	*0.002	9.14	10	*<0.001	12.1	5	*0.04	4.6	11	*0.003	13.8
E. coli	6	*0.06	3.3	7	*0.113	2.79	7	*0.04	3.6	1	*0.8	0.5	10	*0.005	11.8
Acinetobacterspecies	5	*0.003	14.1	3	*0.66	1.6	3	*0.66	1.8	2	*0.6	2.6	6	*0.013	
Poly-GNB	7	*0.041	3.3	9	*0.03	3.8	9	*0.008	5.0	5	*0.8	1.6	13	*0.003	15.8
Pseudomonas	3	*0.04	7.8	3	*0.16	-	2	*0.56	4.5	2	*0.39		4	*0.045	

The antimicrobial susceptibility pattern revealed a high level of resistance to routinely used antimicrobials including third generation cephalosporin and quinolones. The *Enterobacteriaceae* were maximally sensitive to carbapenems (92%) followed by Amikacin77% . The sensitivity of *Acinetobacter spp.* and *Pseudomonas aeruginosa* to carbapenems was 88%and100%, respectively. Amongst the aminoglycosides, tobramycin and amikacin had a better sensitivity as compared to gentamicin.

More than 75 % of the predominating isolate were expressed susceptibility towards amikacin and tobramycin. Multi-drug resistance rate among the predominate gram negative bacteria was high(table-4)

The multi-drug resistance rate of all GNB isolates in this study, *K.pneumoniae* 6(37%) was the predominate followed by *E.coli* 3(26%).Except cefepem all third generations cephalosporin drugs had low sensitivity.

Table 5 Antimicrobial susceptibility pattern of Gram-negative isolates (n = 43), % sensitive TASH,2016

ORGANISM	AM	AG	GM	SXT	CTX	CFP	CRO	CAZ	CIP	MERO	AMK	TORB	MDR
Klebsiella pneumoniae(n = 15)	0	47	47	0	40	80	47	16.7	33	92	76.9	80	37%
Escherichia coli (n = 12)	0	67%	58	0	17	74	42	33.3	67	100	80	92	26.%
Acinetobacter spp. (n = 7)	0	71	85	14	71	85	75	25	-	75	75	76	15.%
Pseudomonas SPP(n =2)	0	0	100	0	100	100	100	16.7	-	100	100	100	0
Enterobacter cloace=3	0	33.3 %	0	-	67	100	0	-	-	75	67	67	5.6%
Entro .divers n=1	0	0	0	-	100	100	0	-	-	100	-	67	10.5 %
k.oxytica n=3	0	0	33			100	33	0	-	100	-	67	5.3%

AM=Amoxicillin AG=Augmentine, GM=Gentamicin,SXT= Sulfamethoxazole + Trimethoprim=SXT,CTX=Cefotaxime,CRO=Ceftriaxone,ceftazidine=CAZ=,MEROPENEM (MEM)=,AMK=,Amikacin

6. Discussions

This case control study, which covered a period of seven months, identified 72 bacterial isolates in patients with hematological malignancies. The results of this study indicated that from all bacterial isolates during this period, gram negative bacteria were predominant in patients with HM. The antimicrobial susceptibility pattern to *Klebsiella species* revealed a high level of resistance to routinely used antimicrobials third generation cephalosporin and also for cefepem. Our study also revealed that *Klebsiella* species followed closely by *E.coli* were frequently isolated pathogens. This result was similar to previous studies, where *Klebsiella spps*.followed by *E.coli* was the most prevalent organism (49). In contrast to this finding revealed that *E.coli* was the most common cause, followed by *Klebsiella spps(47).* The spectrum of GNB isolates and its drug susceptibility pattern is influenced by different factors in every institution.

At univariate analysis we observed that longer duration of neutropenia, sever Neutropenia and extended length of hospital stays were significantly associated with an increased risk of gram negative infections in hematological malignancy patients. In particular, longer durations of neutropenia and extended LOS significantly correlated with the development of a *Klebsiella* species and poly gram negative bacterial infections ($p<0.001$). The prevalence of other Gram-negative bacteria, mainly hospital acquires infections, increased throughout the hospital stay (42).

Our findings indicate through univariate and multivariate analysis that LOS more than 6 days and durations of neutropenia more than 7 days are important source of GNBI in significant percentage of hematological patients. Patients in the Carbapenem-resistant Gram-negative bacteria (CRGNB) was associated higher rates of inappropriate empirical antibiotic treatment than among patients with Carbapenem-susceptible Gram-negative. These patients also have longer admission period prior to the GNBI episode (41)

The spectrum of GNB isolates of this study results had a different findings compared to previous studies. In our study the spectrum of GNB isolates and drugs susceptibility pattern in patients with HM underwent changes and were influenced by different factors. The spectrum of organisms associated with infections in neutropenic patients is showing a change with higher rates of resistant pathogens. the choice of empiric therapy has become problematic and must be

evaluated on the basis of local patterns of infectious agents and local and regional resistance patterns .A Similar spectrum of GNB isolates study was conducted in patients with HM ,the *Klebsiella spps* was the predominates followed by *Escherichia coli* (36).Another findings also showed that the spectrum was the same as our study but different prevalence of Gram-negative bacteria(32).In contrast to this finding revealed that a neutropenic patient with underline disease of HM showed that among gram negative isolates *Escherichia coli* was the predominates followed by *Klebsiella pneumonia*(42) and in other study the most predominant pathogen was *E. coli* followed by *A. baumannii*(47).In our study the spectrum of GNB and susceptibility pattern were different from the previous studies .This might be due to an emergence of drug-resistant GNB, such as ESBL producing GNB.The emergence of carbapenemase-producing K. pneumonia blood stream infection among patients with hematologic malignancies and a high rate of cefepem resistance or ESBL production in *E. coli* and *K. pneumonia* are the major concern (49).

A Statistical Analysis, a data obtained from registrations logs, showed a significant result was found for patients with hematological malignancy who underwent chemotherapy to be more likely to have GNBI of these patients after two weeks ($p <0.001$). The overall GNBI rates for HM patient underwent chemotherapy more than 2weeks was 96 .4% (27/28), while for less than 2 weeks, it was only 30.3% (20/66). Gram-negative bacilli such as *E. coli, Klebsiella spp., and P. aeruginosa* cause the earliest infections in neutropenic patients. These usually occur within the first 2–3 weeks after the initiation of chemotherapy and are due to the rapid decrease in the neutrophil count (45).

In this prospective series of patients receiving chemotherapy for more than 2 weeks, we had observed a higher incidence of episodes of neutropenia78.6%(22/28), extended LOS 96.4%(27/28), longer duration of neutropenia 96.4(22/28) and sever neutropenia 25%(7/28) than in a well-matched control group of patients who underwent chemotherapy less than 2weeks at the hospital (p<0.001). Immune deficiencies seen in patients with HM, associated with the immunosuppressive effects of chemotherapy, generate a major risk for infections (4)

The epidemiological pattern of bacterial infection in patients with HM undergoes periodic changes and is influenced by the severity and duration of neutropenia, empirical antibiotic treatments and extended hospital stay. Bacterial infection continues to be the most common

complication in patients with chemotherapy-induced neutropenia (18).Microbiological tested infections were statistically associated with higher morbidity in patients with hematological malignancies. Gram negative bacterial infections are complications frequently encountered in these patients who undergone chemotherapy. At univariate analysis we observed that the patients with neutropenia, extended hospital stay and durations of neutropenia more than 7 days were significantly associated with an increased risk of GNBI in hematological malignant patients($p<0.001$). Based on the results of multivariate analysis we can affirm that neutropenia and length of hospital stay more than 6 days were the influential independent risk factors for the development of GNBI. Similar findings in the case control study done up on admitted HM patients ($p<0.001$)(47).But in that study the spectrum of GNB isolate was different from our study.

7. Strength and limitations

7.1. Strength

- In hematological wards ,the study has tried to show the drug sensitivity pattern of Carbapenem and 4th generations of cephalosporin groups of drug for better care and management of the HM patients with GNBI.
- The importance of preliminary laboratory reports is well emphasized through this study to start or change empirical treatment for improving patients output
- Initial attempt to identify causal or preventive risk factors for GNBI in patients with HM in TASH

7.2. Limitation

Some gram negative bacteria were missed in cultures or not detected due to lack of co2 incubator and ATCC strain used for anaerobic and microaeophilic bacteria isolation.

8. Conclusions and Recommendations

8.1 Conclusion

Gram negative bacterial infections are serious common complication in hematological malignant patients on chemotherapy, as the longer durations of neutropenia and extended hospital stay may result in significant morbidity and mortality. In this study ,*Klebsiella pneumonia*e, *Escherichia coli, Acinetobacter spp*. Were commonly isolated from HM patients. Moreover, their drug resistance pattern is alarming.

8.2 Recommendations

- Patient should give samples for culture and sensitivity test before any antibiotic treatments to shift the empirical treatment in time.
- The annual antimicrobial resistance pattern in TASH should be revised and documented to use the antibiotics for routine empirical therapy.
- Hygienic measures are crucial and this has to be seriously evaluated and monitored in HM wards.
- Further large scale studies are essential with better sample size and follow up studies

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10. Annexes

Annex I. Participant Information Sheet (English version)

Name of the organization: Department of Medical Laboratory Science, School of Allied Health Sciences, Collage of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia.

Title of the Research Project: Drug susceptibility pattern of gram negative bacterial isolates among hematological malignancy patients undergoing chemotherapy in TikurAnbessa Hospital of Addis Ababa Ethiopia .

Name of Investigator: Manie Asres

First of all I would like to thank you in advance for your cooperation and consent in participation in this study. Please take as much time as you need to read or listen the information sheet. If you have any question regarding the study please ask freely.

Background information

Gram negative bacterial infections are a major cause of morbidity and mortality in patients with hematological malignancies (HM) undergoing chemotherapy. The choice of empiric antimicrobial treatment is based on susceptibility pattern of locally isolated gram negative bacteria.

Purpose of the Research Project

We are asking you to take part in this study because we are trying to learn more about *Gram negative bacteria and their*drug susceptibility pattern among hematological malignancy patients who undergoing chemotherapy with associated risk factors in different clinical sample at Tikur Anbessa Specialized Hospital.

Benefits of participation

By participating, you will get no financial benefits. Even though there is no direct benefit due to participation in this study, the findings of the study is useful for better understanding of the problems of ***gram negative infections among hematology malignant patients***. You will also obtain all the results of the analysis for free and communicated to your physician for the appropriate management.

Expected from participants

As a participant of this study, you are expected to give blood. Being asked to give sample does not necessarily mean that you have the disease. When you are found to be positive for the micro-organism, you will be informed by the health worker and receive proper treatment. You need to know that your results might be discussed with other appropriate individual out of this hospital. But your name, address will not be disclosed rather an identification code will be used in such conditions.

Risks and complications

There is no risk to the participants in participating in the study other than benefiting from the research.

Confidentiality

In order to keep the confidentiality of the participants the sample will be labeled with code instead of giving name. No personal information will be disclosed to third party or will not appear in any report from this study. You can choose whether to be a part of this study or not and you have a right to get a laboratory diagnosis result for free.

Assurance of Principal Investigator

I put my signature below to confirm you that I take over the responsibility for the scientific ethical and technical conduct of the research project and for provision of progress reports for all stakeholders of the research project. If you have any question you can contact and ask at any time you want.

Manie Asres: Department of Medical Laboratory Sciences, Collage of health Sciences, Addis Ababa University, Addis Ababa, Ethiopia

Cell phone: +251-9 12172606 E-mail: maniework@gmail.com

Annex II:Amharic version of Participant Information sheet

በአዲስአበባዩኒቨርሲቲ፤የጤናሳይንስኮሌጅ

የህክምናላቦራቶሪት/ክፍል

በአዲስአበባዩኒቨርሲቲ፤የጤናሳይንስኮሌጅየህክምናላቦራቶሪትምህርትክፍልበሁለተኛዲግሪተማሪየመመረቂያጥናትላይእንዲሳተፉተጋብዘዋል፡፡እባክዎበዚህጥናትላይከመሳተፍዎበፊትከዚህቀጥሎየሚገኘዉንምንባብበጥሞናያንብቡ / ይመልሱ፤ግልፅያልሆነነገርካጋጠመዎትይጠይቁ፡፡

መግቢያ

የጥናቱርዕስ

"በጥቁርአንበሳስፔሻላይዝድሆስፒታልበሚገኙትበበሄማቶሎጂታካሚዎችበሆኑትመካከልለገራካምነጊሽባክቴሪያዎችያላቸውየተጋላጭነትእናለመድሃኒትየሚሰጡትምላሽ "

እርስዎበዚህጥናትላይየሚኖሮትተሳትፎሙሉለሙሉበበጎፈቃደኝነትላይየተመሠረተነዉ፡፡በዚህጥናትዉስጥላለመሳተፍከወሰኑበዚህየህክምናቦታዉስጥየሚሰጥዎትአገልግሎትአይቅዋረጥም፡፡በጥናቱለመሳተፍየሚስማሙከሆነየስምምነትቅጹላይበጹሑፍወይምበጣትፊርማዎትንማስቀመጥይጠበቅቦታል፡፡

የጥናቱተሳታፊበመሆኔየሚጠበቅብኝምንድንነዉ?

የጥናቱተሳታፊበመሆንዎምንምዓይነትተጨማሪናሙናእንዲሰጡአይጠየቁም።እርስዎለምርመራበሚሰጡትደምጥናቱየሚካሂድይሆናልእንጂአዲስናሙናእንዲሰጡአይጠየቁም፡፡

በዚህጥናትመሳተፍየሚያስገኛቸውጥቅሞች

በጥናቱውጤትመሰረትየሄማቶሎጂታካሚዎችትክክለኛእናሁሉንምየላብራቶሪምርመራያካተተመረጃለመስጠት፡፡ስለዚህበጥናቱበመሳተፍዎበተዘዋዋሪመንገድለሌሎችህሙማንብሎምለህብረተሰቡይጠቅማሉማለትነው።

በዚህጥናትበመሳተፍየሚከፈልክፊያ

በዚህጥናትስለተሳተፉምንምዓይነትክፍያአይከፈልዎትም፡፡

የተሳታፊዎችሚስጢርስለመጠበቅ

በመጠየቂያውወረቀትላይየተሳታፊዎችስምወይምማንነትኣይገለጽም።በተሳታፊዎችየሚሰጥናሙናለዚህጥናትጥቅምብቻየሚያገለግልይሆናል።

ጥያቄካሎዎት

ይህንጥናትበተመለከተወይምከዚህጋራበተዛመደመልኩስለሚያጋጥሙድንገተኛችግሮችወይምጥያቄካሎትበሚከተለዉአድራሻ ይጠቀሙ።

ማኔአስረስ

የሕክምናላብራቶሪሳይንስት/ክፍል፤የጤናሳይንስኮሌጅ፤አዲስአበባዩኒቨርሲቲ

ሞባይል: +251- 09 12 17 26 06

ኢ-ሚይል: maniework@gmail.com

Annex III. English Version of Informed Consent Form

This page contains an agreement signature to participate in the study entitled **"**Drug susceptibility pattern of gram negative bacterial isolates among hematological malignancy patients attending chemotherapy at Tikur Anbesa Specialized Hospital, Addis Ababa, Ethiopia**."S**o please read the following points and sign your signature at the end in the space provided.

I understand the objective of the study inDrug susceptibility pattern of gram negative bacterial isolates among hematological malignancy patients attending chemotherapy

1. I know that the left over samples that I gave is going to be used for this study only.
2. I understand that, all the information and the results are confidential.
3. I understand that I will not get any money for my participation.
4. All the information is explained by reception and Principal investigator.

Therefore, with full understanding of the situations I agree to give stool for laboratory analysis.

Signature of the participant: __________________

Address of the participant: __________________

Date: _________________

Annex IV. Amharic Version of Informed Consent Form

የስምምነትመጠየቅያቅጽ

የጥናቱተሳታፊመለያቁጥር፡_______________________________

የዚህጥናትርእስ“በጥቁርአንበሳስፔሻላይዝድሆስፒታልበሚገኙትበበሄማቶሎጂ ታካሚዎችበሆኑትመካከልለገራካምነጊ ቭባክቴሪያዎችያላቸውየተጋላጭነትእናለመድሃኒትየሚሰጡትምላሽ “

“ጥናቱየሚካሄደውበጥቁርአንበሳስፔሻላይዝድሆስፒታልይሆናል።እባክዎትንከዚህበታችየተዘረዘሩነጥቦችበጥሞናያንቡቡእናበመጨረሻበተሰጠውክፍትቦታይፈርሙ::

1. በጥቁርአንበሳስፔሻላይዝድሆስፒታልበሚገኙትበበሄማቶሎጂ ታካሚዎችበሆኑትመካከልለገራካምነጊ ቭባክቴሪያዎችያላቸውየተጋላጭነትእናለመድሃኒትየሚሰጡትምላሽ “የሚካየሄደውንጥናትዓላማተረድቻለሁ።
2. የምሰጠውናሙናለዚህጥናትብቻእንደሚዉልአውቂያለሁ።
3. ለጥናቱየምሰጠውናሙናእንዲሁምውጤቱበሚስጥርእንደሚያዝተረድቻለሁ።
4. በጥናቱበመሳተፌየሚከፈለኝክፍያእንደሌለኣውቂያለሁ።
5. ሁሉምየሚያስፈልገውነገርበተመራመረሪውይብራራልኛል።

ስለዚህከላይየተጠቀሱትንነጥቦችበመረዳትናሙና (ደም) ለመስጠትተስማምቻለሁ።

የተሳታፊፊርማ፡____

ቀን፡____

Annex V Data Collection Sheet

Date: _________________
Code No.: _________________
Address: Sub-city_____________Woreda____________Kebele___________Tele:___________

Section I: Socio Demographic Characteristics

1. Sex: M __________ F__________

2. Age: _______ Years

3. Permanent residence place: Rural __________Urban__________

4. Current visiting status: For in patient Ward: __________

5. The highest level of education you have achieved: Illiterate____Writing & reading__________

Primary school____Secondary school _____College_____diploma__________

University 1stdegree____ 2nddegree____ 3rddegree____
7. Occupation: ___________________________

Section II. Risk factors associated with gram negative infection

1.Which hematological ward have you admitted? Ward; ____________

2.Type hematological malignancy __________

3.Date of admission __________

4.Duration of admission to the hospital? for how long?____ <72 hrs, ____7 to 15 days, ____1 Month, ____> 1 Month

5.History of previous repeated hospital admission? Yes____ No____

6. Have you taken any antibiotic in the past 14 days? Yes ____No____

7.History of previous antibiotic treatment: ___________________________

8.If yes how many times? 2 times ____3 times____ 4 time more____

9. History of previous chemotherapy? Yes____ No____

10.If yes, for how long? Days____, week____,months ____

11.Have you ever had underline disease? Yes ____No____

13. If yes;Type of underline disease,____________

Section III: laboratory data

1. Before chemotherapy

WBC____________, Neutrophil counts____________

ANC____________, Hgb ____________

2. Afterchemotherapy

WBC____________, Neutrophil counts____________

ANC____________, Hgb ____________

Section IV: Laboratory results

Date of sample collection: ____________________

Types of sample ____________________

Cultures and Identification:
Type of *gram negative species*____________________

Antimicrobial susceptibility testing:

Ampicillin/AMP_______

Ciprofloxacin/CIP___________

Ceftazidime/CAZ___________

Tobramycin/NN___________

Chloramphenicol/C___________

Augmentin/AG___________

Cefotaxime/CXT___________

Meropenem/MEM___________

Gentamicin/GEN___________

Imipenem/IPM___________

Amikacin/ANN___________

Trim-sulpha/SXT___________

Amoxicilin-Clavulanicacid(AMC)___________

Annex VI. Laboratory procedures

1.BLOOD CULTURE – INOCULATION, IDENTIFICATION AND SUSCEPTIBILITY
Inoculation from positive BACTEC bottles

1. Label two slides with pencil for gram stain.

2. Clean the rubber membrane of the bottle with an alcohol swab, let dry for 30 seconds.

3. Ventilate bottle with a needle and use a syringe to draw blood culture broth from the bottle.

4. Drip 1-2 drops on one slide. Put the other slide on top and pull apart. Stain one slide in the gram stain machine.

5. Assess gram stain microscopically. Document findings. All slides are kept for 1 week.

Code	Gram stain finding
	Gram negative rods
	Gram negative diplococci
	Gram negative cocci

6. Findings from gram stain and type of bottle determines media for inoculation, follow chart.

7.

Incubation	Atmospheric conditions	Temperature	Time
Aerobic growth	5% CO_2	35^oC	24-48 hrs

Report

1. Findings in gram stain from positive bottles are always reported by phone to prescribing physician.

2. A vague identification can be indicated based on tests done directly from bottle.

3. Identification and AST are reported by phone to prescribing physician as soon it is finished.

Inoculation and identification

Gram stain	Media	Tests from bottle	Tests from colony
	Aerobic bottle		
No microbes	BAP CAP	GRAM stain	
Gram negative rods	BAP Mac	Gram stain Before 10.00: Rapid AST with MIC	GN from 1 bottle. Lactose neg ECOL/Shigella ; GN. Check morphology, lactose reaction and indole/oxidase from remaining bottles.
Gram negative small rods	BAP CAP	Gram stain at 35°C	GN from 1 bottle. For non lactose ;Oxidase test

Antibiotic panels

H.influenzae	**Enterobacteriaceae**	**Pseudomonas sp.**	**Acinetobacter sp.**	**Meningococci/genococci**
MHF	MHA	MHA	MHA	MHF
Ampicillin Amoxicillin Cefotaxime Cefuroxime Ciprofloxacin Meropenem	Ampicillin Augmentin Cefotaxime Ceftazidime Cefuroxime Ciprofloxacin Gentamicin Meropenem Tobramycine Trim-sulpha	Ceftazidime Ciprofloxacin Gentamicin Imipenem Meropenem Tobramycin	Ciprofloxacin Gentamicin Meropenem Tobramycin Trim-sulpha	Ampicillin Cefotaxime Chloramphenicol Meropenem Penicillin G

2-ENT – INOCULATION, ASSESSMENT, IDENTIFICATION AND SUSCEPTIBILITY

Ordering analyses

Specimen	Analyses	Remarks
Sputum Traceal secretion, -aspirate, -tube	Aerobic growth	from immunosuppressed patients –Diagnosis cystic fibrosis -Diagnosis aspiration pneumonia - Lung abscess - If requested
Bronchial secretion, -aspirate, BAL	Aerobic growth	-From wards - If requested -Diagnosis aspiration pneumonia - Lung abscess - If requested

Inoculation and incubation

	BAP +OP/ NA aerobic	CAP +BA10 aerobic	MAC aerobic	Comment aerobic
Mouth				
Sputum Tracheal secretion Tube secretion	x	x	x	1) From hematology ward or requested 2) From hematology ward 3) Aspiration pneumonia, lung abscess or requested
Bronchioalveolar lavage (BAL)	x	x		1) From hematology ward or requested 2) From hematology ward 3) Aspiration pneumonia, lung abscess or requested

<u>Incubation</u>

Incubation	Atmospheric conditons	Temperature	Time
Aerobic growth	5% CO_2	35°C	24-48 hrs

Assessment

Ear

Microbe	Identification and susceptibility	Remarks
Pseudomonas sp.	Identification and susceptibility if moderate, rich or massive growth. Smaller amounts if dominating or pure culture.	Comment: Most commonly associated with external otitis.
Enterobacteriaceae	Identification and susceptibility only if pure culture	Comment: Most commonly associated with external otitis.

Nose

Microbe	Identification and susceptibility	Remarks
Enterobacteriaceae	Identification and susceptibility only if diagnosis sinusitis or cystic fibrosis and moderate, rich or massive growth or dominating.	Comment: Uncertain pathogenic significance.

Throat

From BAP/CAP		
Microbe	Identification and susceptibility	Remarks
Enterobacteriaceae	Identification and susceptibility if rich or massive growth and diagnosis cystic fibrosis or immunosuppressed patients	

Sputum, tracheal- and tube secretion

Microbe	Identification and susceptibility	Remarks
Enterobacteriaceae	Identification and susceptibility if rich or massive growth.	Comment: Uncertain pathogenic significance.

Bronchial secretion, -aspirate and –lavage

Microbe	Identification and susceptibility	Remarks
Pseudomonas sp.	Identification and susceptibility regardless of amount	

Keratitis - Every finding in pure culture is considered relevant		
Microbe	Identification and susceptibility	Remarks
	Identification and susceptibility	
Neisseria sp.		
Pseudomonas sp.		
Enterobacteriaceae		

- Every finding in pure culture is considered relevant		
Microbe	Identification and susceptibility	Remarks
Pseudomonas aeruginosa	Identification and susceptibility regardless of amount	

Antibiotic panels

Admitted patients

Acinetobacter sp.	**Enterobacteriaceae**	**Pseudomonas sp.**
MHA	MHA	MHA
Ciprofloxacin Gentamicin Meropenem Tobramycin Trim-sulpha	Ampicillin Cefotaxime Ceftazidime Cefuroxime Ciprofloxacin Gentamicin Meropenem Tobramycine Trim-sulpha	Ceftazidime Ciprofloxacin Gentamicin Meropenem Tobramycin

3.CSF and other fluids – INOCULATION, ASSESSMENT AND SUSCEPTIBILITY
Preparation

Appearance	Note appearance of CSF; turbid, yellow, bloody, clear	
Culture	<0,5 ml	Vortex and inoculate
	≥1 ml	- Centrifuge the rest (≈1 ml) for 15 min at 3600 rpm (2000g). If less than 1 ml, inoculate directly without centrifugation. - Remove supernatant and vortex precipitate for 30 sec. - Inoculate from the precipitate.
Pleural fluid		
Peritoneal fluid		

4. Microscopy

Note if CSF is centrifuged. Assess cells and microbes in gram stain. Type and amount of cells are recorded .Report result of gram stain on request paper.

Inoculation

Sample/diagnosis	BAP	CAP	MAC	serum broth	Comment
CSF for culture	x	x	x	Add 2 drops	Immunodeficiency or if requested. .
PLEULAR FLUID	X	X	X		
PERTIONEAL FLUID	X		X		

- Inoculation of CSF PLEULAR FLUID and PERTIONEAL FLUID are done in the safety cabinet. Use gloves.
- Prepare 2 slides. Use a diamond point marker to make a ring on each slide. Clean slides with alcohols swab. Label slides with pencil.

Drop 1-2 drops of precipitate or un centrifuged CSF PLEULAR FLUID and PERTIONEAL FLUID on each plate, spread with loop. Drop 1 drop on each slide.

Incubation	Atmospheric conditons	Temperature	Time
BAP/CAP	5% CO_2	35^oC	4 days for CSF 2DAYS FOR OTHERS

Assessment

Plates and serum broth is read daily. Primary plates are incubated for 4 days.

Subculture from serum broth is done after 24 hrs on BAP and CAP, incubate in 5% CO_2, 35^oC. Subculture is also done if serum broth looks turbid at later point.

Identification and susceptibility for all findings. Call prescribing physician with preliminary report.

Antibiotic panels

Enterobacteriaceae	**Acinetobacter sp.**	**Pseudomonas sp.**
MIC	MIC	MIC
MHA	MHA	MHA
Ampicillin Cefotaxime Ceftriaxone Chloramphenicol Meropenem *Ceftazidime*	Ciprofloxacin Gentamicin Meropenem Tobramycin Trim-sulpha	 Ceftazidime Ciprofloxacin Gentamicin Meropenem Tobramycin

5. URINE CULTURE

1. Isolate organisms from a simulated urine specimen using the urine streaking technique.
2. Interpret results using the colony count and variety of organisms present.
3. Recognize bacteria as Gram positive or Gram negative based on culture results

PRINCIPLE

Chronic or acute infections of the urinary tract may involve the kidneys, ureters, bladder, or urethra. Such infections may cause high blood pressure, kidney damage or uremia. In some instances the infections are in apparent and go unnoticed for some time. Most infections of this tract enter by way of the urethra; very few originate in the blood. The presence of bacteria in the urine is called bacteriuria. A multitude of organisms can cause urinary infections. The most common cause of such infections in women of child bearing age is Escherichia coli. other related Gram negative rods.

The sequence of steps in performing a complete study of microorganisms in urine includes:

1. aseptic collection (clean catch)
2. culture
3. quantitative evaluation
4. isolation of the pathogen
5. identification
6. antimicrobic sensitivity testing

MATERIALS

1 BAP plate 1 MSA plate

1 MacConkey agar plate (MAC)

4 sterile calibrated inoculating loops (0.001 ml)

PROCEDURE

Day One

1. Label your four plates with your name, seat #, and specimen #.
2. Obtain the urine specimen and gently rotate it so as to mix it thoroughly. Remove lid; place it facing up on the bench top.

3. Aseptically remove a loop from the package. Dip one disposable 0.001 ml calibrated loop vertically into the urine so it goes just below the surface. Withdraw the loop.
4. Examine the loop to be sure that it is full.
5. Touch the loop to the BAP plate at the top edge of the medium and draw the loop down the center of the plate. This deposits the drop of urine on the plate. Streak back and forth as shown in figure 15.1. Dispose of the loop in a biohazard bag.
6. Repeat this procedure with the MAC plates using a NEW loop for each plate.
7. Incubate all plates at 37°C for 24 hours.

Day Two

1. Examine all the plates carefully. Identify the number of colony types on each plate; count the number of colonies on each plate. Record this on worksheet
Interpret the results of the bacterial counts (see interpretation chart). Remember that each colony grew from one bacterial cell, therefore each colony that you see represents one bacterial cell in the original urine specimen and is reported as CFU's (Colony Forming Units).
2. Since colony counts are reported as the number of bacterial cells present in one milliliter of urine, the volume of urine that was actually deposited on the plate must be multiplied by a factor that converts it to 1 ml.
The loop was calibrated to deliver 1/1000 milliliter (0.001 ml). To get 1 ml, multiply the amount of fluid in the loop by a factor of 1000.
To determine the number of bacteria in 1 ml of specimen, multiply the number of colonies growing by a factor of 1000.

INTERPRETATION OF GROWTH ON URINE CULTURES

It is important that the number of organisms that are present in urine **at the time that it is Collected** be determined. Because urine itself is a good culture medium, any organisms present in a specimen will multiply unless the specimen is handled properly. Urine specimens should be stored at 2 - 8oC if processing cannot take place within one-half hour of collection.
In the clinical laboratory, the number of organisms in **a clean catch voided** urine

specimen is determined by using loops that deliver an exact volume to streak specimens over the entire surface of plates. When the plates are observed for growth, the number of colony types is noted first, and if **only one**, or at the **most two** are seen, the number of colonies is determined and the following interpretive guideline is used.

Colony Count (1 or 2 types) Interpretation

A. < 10,000 CFU’s/ml. Normal flora, skin contamination

B. 10,000 - 99,000 CFU’s/ml. Specimen stored improperly; or infection just beginning

C. 100,000 = 105 CFU’s/ml. Significant

D. > 100,000 = > 105 CFU’s/ml. Significant

NOTE: These are suggested guidelines only. Some persons may have a significant infection with a colony count of only 10,000 CFU’s/ml. Good judgment and the results of a routine urinalysis are required when evaluating urine culture results. Considerations include the patient’s medical condition, underlying disease, and if the patient took antibiotics before the culture was collected. For our lab exercise we will use these criteria in order to interpret results.

Sterile urine collection such as straight catheterization must be indicated on the lab requisition since any colony count (in pure culture) is considered significant.

6.-Biochemical reactions GNB

Identification of bacterial isolates involves the use of biochemical screening Medias. Indole, Urease, Mannitol, Triple sugar iron (TSI), Citrate, Motility, Lysine Decarboxylase, and Mannolet and Oxidase tests.

Biochemical reactions		Ind o	Urea	Man	TSI H2S	GAS	Ci t	Mot	LDC	OX
Result	**Posetive**									
	negative									
Gram negative rods										

Key: LDC = Lysine Decarboxylase, Man = Mannitol (mannite), Triple sugar iron (TSI), Ox = Oxidase test, Cit = Citrate test, Mot = Motility, Ind = Indole test, Urea = Urease, H2S = Hydrogen sulphide (blackening), R = Red-pink (alkaline reaction), Y = Yellow (acid reaction), d = different strains give different results.

A. **Indole test:** Few colonies of the culture will be inoculated into peptone water and incubated at 37°C for 24 hours. Few drops of indicator (Kovac's reagent) will be added and gently shake to mix well. Color change will be then observed. If the layer of indicator reagent turns to red within 1 minute, it is Indole positive (positive result). If the layer of indicator reagent remains yellow within 1 minute, it is indole negative (negative result).

B. **B. Urease test (Christensen's (modified) urea broth):** Urea agars will be inoculated heavily over the entire surfaces of the slants in bijou bottles. The cap will be loosened

and then incubated at 37 ^{o}C for 3-12 hours. A urease-positive culture produces an alkaline reaction in the medium, evidenced by pinkish red color of the Medium. Urease-negative organisms do not change the color of the medium, which is pale yellow-pink.

C. **Triple Sugar Iron (TSI) Agar Slant:** Using a sterile inoculating needle, stab the butt of the LIA slant twice then streak back and forth along the surface of the agar with the organism. Incubate at 37°c for 18 to 24 h. If acid slant–acid butt (yellow–yellow): glucose and sucrose and/or lactose fermented. If alkaline slant–acid butt (red–yellow): glucose fermented only. If alkaline slant–alkaline butt (red–red): glucose not fermented. The presence of black precipitate (butt) indicates hydrogen sulfide production, and presence of splits or cracks with air bubbles indicates gas production.

D. **D. Citrate utilization test using Simmon's citrate agar:** Simmon's citrate slopes will be prepared in bijou bottles as recommended by the manufacturer (stored at 2-8°C). And the slopes will be then stabbed and incubated at 37°C aerobically for 48 hours. Blue colour indicates a positive reaction and if Simmon's citrate agar slopes remained as green in colour indicate negative reaction.

E. **E. Motility Test (using motility agars):** Motility agar will be prepared and inoculated with a straight inoculating needle making a single stab about 1-2cm down into the medium. The motility will be examined after 35-37^{0}C for 24 hour. Motility will be indicated by the presence of diffuse growth (appearing as coloring of the medium) away from the line of inoculation.

F. Lysine decarboxylase: Decarboxylation of lysine can be detected by culturing bacteria in a medium containing the desired amino acid, glucose, and a pH indicator bromcresol purple. The acids produced by the bacteria from the fermentation of glucose will initially lower the pH of the medium and cause the pH indicator to change from purple to yellow. The acid pH activates theenzyme that causes decarboxylation of lysine to amines and the subsequent neutralization of the medium. This results in another color change from yellow back to purple. Bacteria that decarboxylate lysine turn the medium purple. In addition bacteria that produce H_2S appear as black colonies.

G. Oxidase test: A piece of filter paper is soaked with a few drops of oxidase reagent. A colony of the test organism is then smeared on the filter paper. Alternatively an oxidase reagent strip can be used. When the organism is oxidase-producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour.

3. Antibiotics susceptibility result for Bacteria isolates

Isolated Bacteria	Antibiotics	AML	AMC	C	GM	CTX	NOR	TE	CRO	SXT	FOX	IMI	MEM	
	Susceptibility Patten													S
														I
														R

NOTE: Ampicillin (Amp), Chloramphenicol(C), Ceftriaxone(CRO), Cefotaxime(CTX),), Gentamycin(GM), Amoxicilin-Clavulanic acid (AMC), Norfloxacin (NOR), Imipenem(IMI) and Merepenem(MEM)

Procedure for Performing the Disc Diffusion Test

Inoculums Preparation

At least three to five well-isolated pure colonies of the same morphological type will be selected from CCDA or XLD or SSI agar plate. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of car-Bare or tryptone soy broth.

The turbidity of the broth culture will be adjusted with that of the 0.5 McFarland standards.

Inoculation of Test Plates

Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level.

The dried surface of a Mueller-Hinton agar plate or Mueller-Hinton factor is inoculated by streaking the swab over the entire sterile agar surface.

The lid may be left ajar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

NOTE: Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures or other un standardized inoculum for streaking plates.

Application of Discs to Inoculated Agar Plates

The predetermined battery of antimicrobial discs is dispensed onto the surface of the inoculated agar plate. Each disc must be pressed down to ensure complete contact with the agar surface.

The plates are inverted and placed in an incubator set to 37°C or 42°C in gas pack within 15 minutes after the discs are applied.

Reading Plates and Interpreting Results

After 16 to 24 hours of incubation for NTS or After 23- 25 hours of incubation in the gas pack for TCS, each plate is examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc. Zones are measured to the nearest whole millimeter, using sliding calipers which is held on the back of the inverted plat

ANNEX-VII ANTIMICROBIAL SUSCEPTIBILITY TESTING

Disc diffusion

From colony:

1. Use a fresh culture (not more than 48 hrs). Mixed culture cannot be used due to interference between different strains.
2. It is recommended to make the inoculums from a non-selective plate, but mac, CNA and MSA can also be accepted.
3. Make a 0,5±0,1 McF suspension using a densitometer from a fresh culture (not more than 48 hrs). Touch several colonies with a cotton swab to get a representative bacterial population. Suspension must be homogenous. Suspension should be used within 15 min.
4. With a cotton swab, make a diametric line over the MHF plate. Wetness of the swab is depending on species. Put the plate on a rotator and move swab slowly from rim towards center of the plate.
5. Place plate on a cardboard disc (0,5 mm) and add antibiotic discs within 15 min. Make sure that antibiotic discs are in proper contact with agar. Discs are not to be moved after application.
6. Start incubation within 15 min after application of antibiotic discs. Plates are either placed in low stacks (max. 4 plates) or with an empty plate between every third MHF.

Directly from blood culture bottle:

1. Consider the amount of bacteria from the gram stain. 10-20 drops of blood culture media is added to 5 ml 0,9% saline.
2. Swab directly from tube.

3 Result is only indicative and AST must always be repeated from colonies.

Bacteria	Agar	Cotton swab	Incubation		
			Temperature	Atmosphere	Time
Enterobacteriaceae **Pseudomonas sp.** **Acinetobacter sp.**	MHA	Dry	35±2°C	Normal	18±2 hrs

Dry AST-plates without lid for 15 min at 35°C before inoculation to prevent swarming.

Reading:

1. Consider thickness of AST, there should be confluent growth. To achieve reproducible results it is important that the inoculate is not too thick. Repeat AST if the inoculate is too thick or too thin.
2. Zones are read at full inhibition of growth, this applies to both bacteriostatic and bactericide antibiotics.
3. MHA: Put the plate directly on species specific stencil on marked zones or read from the back with caliper or ruler.
   MHF: Take of lid and read from the front. Caliper or ruler is held close to the agar surface without touching it. Aim perpendicular at edges on both sides of the zone.
4. Measure the diameter, or the radius multiplied by 2. Zones are approximated to the nearest mm.

Considerations:

- Growth inside the zone could be caused by resistant sub-population; take into regard. Exception: Enterobacteriaceae and ampicillin; disregard the inner zone.
- Read outer zone. Mixed culture/contamination: Disregard colonies inside the zone.
  - Swarming: Disregard swarming inside zone.
- Blurred edges: Regard visible growth (no magnifying glass).
- Jagged edges: AST is too thick.
- Growth or hemolysis? Read zone where growth stops, not where hemolysis s

AnnexVIII Declaration

I the undersigned, declare that this is my original work and has not been presented for a degree in this or any other university and all sources of materials used for this thesis have been acknowledged.

Name of the Investigator:_______________

Signature ________________

Date of submission ____________

This thesis has been submitted with our approval as University Advisors

Name: _______________ **(MSc, PhD fellow)**

Signature ______________

This thesis has been submitted for examination with my approval as External Advisor.

Name: _______________ **(MSc, PhD fellow)**

Signature _______________

This thesis has been submitted for examination with my approval as External Advisor.

Name: _______________**(MD, Assistance professor)**

Signature ___________