Addis Ababa University

College of Health Science

School of Allied Health Sciences

Department of Medical Laboratory Sciences

Antibiotic resistance patterns of gram negative fermentative and non-fermentative bacilli isolated from patients referred to Arsho Advanced Medical laboratory, Addis Ababa, Ethiopia.

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A thesis submitted to the Department of Medical Laboratory Science, College of Health Science, School of Allied health science, Addis Ababa University in partial fulfillment of the requirements for the degree of masters in clinical laboratory science (diagnostic and public health microbiology specialty track).

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List of abbreviations

AAML	Arsho Advanced Medical laboratory
AMR	Antimicrobial Resistance
AST	Antibacterial Susceptibility Testing
EGNR	Enteric Gram Negative Rods
EQA	External Quality Assessment
ESBL	Extended Spectrum Beta-Lactamase
GNB	Gram -Negative Bacteria
McF	McFarland
MDR	Multidrug Resistance
MIC	Minimum Inhibitory Concentration
NF	Non Fermentative
SOP	Standard Operating Procedure
V2C	Vitek 2 Compact.

Abstract

Background: Gram negative bacilli are the most frequently isolated bacteria which recovered from various clinical samples. Conventionally, these microorganisms subdivided into two major groups' fermentative and non-fermentative gram-negative bacteria. The aim of this study is to determine antibiotic resistance patterns of fermentative and non-fermentative gram negative bacilli from various clinical samples in study area.

Method: A laboratory based cross-sectional study conducted at Arsho Advanced Medical laboratory, Addis Ababa, Ethiopia from January to April 2017; Study participants were required Urine , CSF, wound, Nasal fluid ,Ear ,Blood, Body fluid, Semen were collected and cultured on Blood agar, MacConkey agar and chocolate agar and incubated according to standard conditions. Then Species identification and antimicrobial susceptibility testing of gram negative bacteria were determined by automated Vitek 2 compact system using GN and AST GN72 card.

Result: From the total of 824 various specimens, 284(34.5%) were culture positive , of which (90.8%, 258/284) of the isolates were fermentative and (9.2%), 26/284) were non fermentative Gram-negative bacteria from wide range of clinical specimens such as in urine 197(69.4%), in wound and abscess 34(11.9%), in Ear 20(7.0%), in CSF 11(3.87%), in Nasal 11(3.9%), in Blood 4 (1.4%), in Bodyfluid4 (1.4%) and in semen 3(1.1%) .The most common Gram- negative bacterial isolates being *Escherichia coli* 152(53.5%), followed by *Klebsiella pneumonia* 40(14.1%), *Pseudomonas aeruginosa* 13(4.6%), *Proteus mirabilis* 13(4.6%), *Acinetobacter baumannii* 8(2.8%) were the most dominant isolate respectively. Among gram negative bacteria, *K.pneumoniae* (82.5%), *E.coli* (82.9%), *P.aeruginosa* (100%), and *A. baumanii* (100%) were multi drug resistance.

Concussion: High antimicrobial resistance and multi drug resistance was demonstrated over the study period, which may be due to associated with the improper antibiotic consumption or lack of a proper guideline for empirical therapy.

Keywords: Antibiogram, *Enterobacteriaceae,* fermentative and non-fermenting gram-negative bacilli, antimicrobial susceptibility test and antimicrobial resistant.

1. **Introduction**

1.1 **Background**

Gram negative bacilli are the most frequently encountered bacterial isolates recovered from different clinical specimens [1, 2]. Conventionally, these microorganisms are subdivided into fermentative gram negative bacteria (fermenters) and non-fermentative gram-negative bacteria (non- fermenters).The fermenter by large belong to the family *Enterobacteriaceae.* The non-fermentative gram-negative bacilli are a group of aerobic, non-spore forming, gram-negative bacilli that obtain energy by degrade carbohydrates through metabolic pathways other than fermentation[2].The non-fermentative gram-negative bacilli are known to account for nearly 12-16% all bacterial isolates from a clinical microbiology laboratory. They can be recovered from hospital environment, commonly cause device related infections, are often resistant to disinfectants and have the potential to spread from patient-to-patient via vomits or the hands of medical personnel [3, 4].

Antibiotic resistance is a public health problem of increasing magnitude, and a search for effective solutions to address this problem is an active field of research. Gram-positive and Gram-negative bacteria are both affected by the emergence and rise of antimicrobial resistance. However, Gram-negative bacteria are typically more resistant to antimicrobials than are Gram-positive bacteria, and this has long been explained by the presence, in the former, of the outer-membrane permeability barrier, which limits access of the antimicrobial agents to their targets in the bacterial cell [5].Furthermore several intrinsic factors such as point mutation, gene amplification and extrinsic factors like horizontal transfer of resistant gene between bacteria within and across species by transposons, integrins or plasmids that facilitate drug resistance are more common in gram negative bacteria than gram positive[6]. In addition to these, increased use of over-the-counter antibacterial drugs inappropriately and incomplete course of therapy have been suggested as a possible explanation for more frequent isolation of drug resistant bacteria [7].

Prevalence and antimicrobial susceptibility pattern of bacterial pathogen from different clinical samples have been conducted in Ethiopia [8, 9]. However, identification and their drug susceptibility pattern were determined by using convection laboratory procedures with a number of limitations. As the result, bacterial distribution and drug susceptibility pattern of the isolates

vary from one study to another making to arrive at an accurate conclusion and recommendation difficult. Geographical location, local antimicrobial prescription practice, and temporal changes have been identified to play a role in changing the spectrum of bacterial pathogens and their drug susceptibility pattern and such conditions are never stable and may change rapidly especially in places where misuse of antibiotics are common particularly in developing countries[10].

Early provision of microorganism identification and susceptibility data permits efficient management of patients with infectious diseases and is associated with significant clinical and financial benefits, via the reduction of mortality rates and overall hospitalization costs [11]. In view of this assumption, identification and antimicrobial susceptibility testing of clinical isolates are mainly achieved by means of fully automated systems. Apart from shortened turnaround times, improved specimen handling, enhanced quality control, reproducibility, accuracy, and the ability to track results are further advantages prompting routine laboratories to adopt automated technologies for bacterial processing [12].With regard to identification, VITEK 2 compact system utilizes 64 biochemical and substrates to cover a total of 115 Gram-positive and 135 Gram-negative taxa in an approximate turnaround time of 10 hours[13].Speciation of an isolate provides essential information on its pathogenic potential and is of utmost importance for the correct interpretation of antibiotic susceptibility testing. Against these backgrounds, the aim of this study was to identify and determine drug susceptibility pattern of gram-negative bacteria recovered from different clinical samples from patients referred to Arsho Advanced Medical Laboratory by employing the automated VITEK 2 compact system.

1.2 Statement of the problem

Antibiotics are indispensable in virtually all modern medicine; for example major surgery, organ transplantation, treatment of preterm babies and cancer chemotherapy would not be possible without effective treatment and prevention of bacterial infections .However, the extensive use and misuse of antibiotics have resulted in selection and worldwide spread of antibiotic resistant bacteria[14]. Antimicrobial resistance is an increasingly emerging problem worldwide. The development of antibiotic resistance limits the choice of antibiotics to be used. Several of the clinically important non fermenters are multiresistant organisms and treatments for infections caused by nonfermenters are somewhat different from those for infections caused by fermenters. It is generally recognized that narrow- and expanded-spectrum cephalosporins are minimally active against nonfermenters. However, Aminoglycoside or quinolone-resistant strains of*Enterobacteriaceae* are relatively rareof the broad-spectrum cephalosporins (e.g., ceftazidime and ceftriaxone) are effective for the clinicallyimportantnonfermenters. Other antibiotics useful for nonfermenters are monobactams, quinolones, imipenem, and piperacillin [15, 16].However, this is only a general rule, and some resistant strains of *Enterobacteriaceae* may be encountered.

Therefore, many previous studies suggested that only restricted drugs can be recommended as empirical antibiotics against suspected infections caused by Gram-negative bacteria based on their effectiveness.

In our country, Ethiopia, it is wide practice that antibiotics can be purchased without prescription and without real etiological agent identification [17]. This leads to misuse of antibiotics by the public thus contributing to increase spread of antimicrobial resistance of gram negative bacilli.

So this study could provide:

- The appropriate information about the alternative of drug of choice.
- An insight into the present situation regarding the etiology and antimicrobial susceptibility of major negative bacteria isolated from different anatomical sites or clinical samples.

1.3 significance of the study

The antimicrobial susceptibility patterns of common pathogenic bacteria are essential to guide empirical and pathogen-specific therapy; therefore, empiric antibiotic treatment is not effective in elimination of these pathogens much time in clinical practice.

So identifying the various bacteria belonging fermentative and non-fermenting gram negative bacilli and also studying their antibiotic susceptibility patterns by using Vitake 2 compact analyzer in our study area could be:

- One indicator or base line in the appropriate treatment of patients.
- It may help physicians to be specific for the choice of appropriate antibiotics for empiric treatment.
- As a base line for further study.

2. Literature review

A study from Belgium by Shilpa. K. From a total of isolated 130 Non fermentative gram negative bacilli [FGNB] clinical samples, were isolated from pus samples (n=76) followed by blood (n=30), urine (n=11), sputum (n=6), pleural fluid (n=3), catheter tip and ear swab [n=2] each. *P.aeruginosa and A.baumanii* were the commonest NFGNB isolated in their study. Their role as health care associated pathogens is well established and they have caused UTI, Septicemia and systemic infection. *P.aeruginosa* showed good sensitivity to meropenem, ciprofloxacin and amikacin. *A.baumanii* showed good sensitivity to meropenem and ciprofloxacin. The different species of NFGNB have shown a varied sensitivity pattern in their study. Therefore, they concluded that identification of NFGNB and monitoring their susceptibility pattern are important for proper management [18].

Another study in Hyderabad southern India carried out on Antibiotic susceptibility and resistance patterns of *Enterobacteriaceaea* in a teaching hospital in a rural area. The result showed that *Enterobacteriaceae* constituted 86% of the gram negative isolates in their study and 14 % belonged to *non Enterobacteriaceae,* of the enterobacteriaceae,45% belonged to 51-70 age group followed by 31-50 yrs age group (38 %). About 58 % of these isolates detected in males and 42 % detected in females. When the source of these isolates was analyzed, 62% of samples were urine samples followed by sputum samples (22 %), exudates (15 %) and blood (1 %). Among the *Enterobacteriaceae*, *E. coli* was the predominant isolate (58 %) followed by *Klebsiella* (32 %), *Proteus* (3 %),*Citrobacter* (6%) and *Enterobacter* (1%). *Enterobacteriaceae* showed maximum susceptibility to Colistin (100 %) and Tigecycline (100 %) followed by Amikacin (76 %) and Carbapenems (62%). Maximum resistance was seen in *Enterobacteriaceae* to Quinolones (98%).The antibiotic sensitivity of ESBL isolates revealed 100 % sensitivity to Colisin and Tigecycline followed by 89 % and 79 % sensitivity to Meropenem and Imipenem. Maximum resistance to quinolones was observed [19].

A prospective study conducted by RituBhatnagar and his colleagues in India from of 252 isolates of non-fermenters from various clinical samples was done during the two year study period at 2006, the majority of the non-fermenters were isolated from pus 124(49.20%), followed by sputum 50(19.84%), urine 32(12.69%), bronchial aspirate 23(9.12%), Pleural fluid 10(3.96%), Blood culture 3(1.19%), CSF 2(0.79%), the rest of the isolates were from other

clinical specimens as indicated 8(3.17%). The most common isolates were from the genus *Pseudomonas* (210), among them predominant species being *P.aeruginosa* (200), were isolated followed by *P.stutzeri* (08) and *P.putida* (02). From the genus *Acinetobacter* (41), *A.baumanii* (35) and *A.lwoffii* (06) were isolated. Only one isolate was *S.maltophila*.

From the finding majority of the isolates of *P. aeruginosa* were resistant to ceftazidime (44%), ciprofloxacin (41%) and gentamincin (35.5%). The least resistance was observed in the cases of piperacillin (20%), Imipenem (17.5%) and Amikacin (14.5%). They also observed in their study 163 (81%) isolates were found to be resistant to two or more drugs.

Ceftazidime (68.6% 64.8% Vs 35.5%, P=0.05%) and Ciprofloxacin (86.8% Vs 41%, P=0.001). finally they conclude that Of all the non fermenters isolated, *P. aeruginosa* was most common followed by *A.baumanii, P.stutzari, A.lwoffii, P. putida, S.smaltophila* .Irrational use of powerful antibiotics for prolonged periods added to the compromised host conditions might be responsible for multi-drug resistance (MDR).They suggested that only Imipenem, Piperacillin and Amikacin can be recommended as empirical antibiotics against suspected *P.aeruginosa* infections[20].

Jaya S. k, and Soumya K conducted a study from total of 967 clinical samples in TUMKUR [Karnataka state of India] teaching hospital, in microbiology laboratory. Result showed that Gram negative isolates were predominant isolates; 84.8% of the Gram negative isolates belonged to *Enterobacteriacea*. The most predominate isolates of *E.coli 150[55.3%]* were found to be the most common organisms followed by *K. pneumonia* 45[16.6%], *P. aeruginosa* 28 (10.32%), *P.vulgaris*12 (4.42%), *E. aerogenes 7*(2.58%). Their study was also noted high amount of resistance to ampicillin, amoxyclav, and cephalosporins and70 to 75% resistance was noted to cephalosporins, Fluoroquinolones were sensitive in 60% of *E.coli*and 68% of *Klebsiellaspp*, but only 35% sensitivity among *Pseudomaonas spp*. High sensitivity was noted to amikacin and gentamycin among *Enterobacteriacea* observed in their finding. So they concluded that the microbial pathogens, as well as their antibiotic sensitivity patterns may change from time to time and place to place it is necessary to have antibiogram for hospitals to know the common organisms and their susceptibility patterns [21, 22].

From Pakistan a study conducted by Salma G.N and his colleagues, from 2091 Enteric Gram Negative Rods (EGNR) isolated over a period of two years. Gram-negative bacteria showed a high rate of resistance to commonly prescribed antimicrobials like ampicillin, amoxicillin, amoxicillin/clavulanic acid. Carbapenems, β lactamase inhibitor combinations proved to be most effective agents against these organisms. Resistance against Meropenem (MRP) ranges from (0-17.5 %), imipenem (2-20%), piperacilin, tazobactam(4.0-30%), Amikacin (23-37.5%),Ceftazidime (49.7-76.5%), Cefotaxime (33.3-75.6%), Aztreonam (34.3-73.7%), Ciprofloxacin (32.8-56.7%), Gentamicin (22.2-62%), Ofloxacin (60%), Norfloxacin (50-62.3%),Pipemidic acid (62.-66%), Cefuroxime (65.5%), Coamoxiclav (60-86%), Co-trimoxazole (62-81.4%)Cephradine (84-94%), Amoxicillin (86.9%), Doxycycline(87-70%), Ceftriaxone (90.0%), Ampicillin (91-94%) [23].

A study was conducted in Yaoundé Central Hospital; Cameronand their findingsuggested high rates of antimicrobial resistance among Gram-negative bacilli [24].

The presence of *E.coli* and *Klebsiella* spp. Isolates resistant to third generation cephalosporin suggested the importance of monitoring this phenotype. Particularly alarming were the appearance of low level imipenem resistance among different species of Gram negative bacilli studied. According to their result there is an indication that more resources shouldbe allocated to encourage good antibiotics utilization and practice. In addition, to provide information for choosingeither empirical or direct therapy to physicians, surveillance to antimicrobial resistance is necessary [24].

A cross sectional study was conducted at Jimma University specialized hospital ,South West, Ethiopia by Mama M, *et al* and 145 bacterial isolates were recovered from 150 specimens which showed that the isolation rate of 87.3%. The predominant bacteria isolated from the infected wounds were *S. aureus* 47 (32.4%) followed by *E.coli* 29 (20%), Proteus species 23 (16%), Coagulase negative Staphylococci 21 (14.5%), *K. pneumoniae* 14 (10%) and Pseudomonas aeruginosa 11 (8%). All isolates showed high frequency of resistance to ampicillin, penicillin, cephalothin and tetracycline. The overall multiple drug resistance patterns were found to be 85%. They conclude that on in vitro sensitivity testing, ampicillin, penicillin, cephalothin and tetracycline were the least effective. Gentamicin, norfloxacin, ciprofloxacin, vancomycin and amikacin were the most effective antibiotics [25].

A thesis work conducted in Ethiopia by Sosina A. revealed that from Out of 300 samples, 205(68.3%) samples were culture positive while 95 (31.7%) of wound swab cultures. 102(42.9%) were Gram positive while the rest 136 (57.1%) were Gram where as*P.aeroginesa* 53(22.3%) was the most frequently isolated Gram negative bacteria. *P.aeroginesa* showed the highest resistance to Amoxillin (83%), Ampcillin(84.9%) and Chloramphenicol (62.5%) while norfoxacilin and Gentamaycine were the most effective antibiotics with resistance rates of 5.7% and 11.3%. respectively. Out of 24 isolates of *Proteusspp*, (95.8%), (87.5%), and (62.5%) were resistant to Ampicillin, Amoxicillin, and tetracycline respectively. *Klebsiella SPP.* demonstrated high level of resistance to Amoxillin (90.5%)and ampicillin 85.7% Furthermore, *E.coli*showed 78.9% resistance to Ampcillin and 84.2% resistance to amoxillin,whereas *E.coli* were sensitive to Gentamaycine, ceftraxone and norfoxacilin with 5.3% resistance rates for each them[26].

Her study revealed that most of Gram negative bacterial isolates showed low level of resistance to all antibiotics tested except for ampicillin and amoxicillin. She suggested that this might be due to these antibiotics are the most commonly used antibiotics and resistant pattern were reported from many studies.

Another thesis work was conducted at Tikur Anbessa Specialized Hospital by Frehiwot T/H at 2014 from total of 384 different body fluid patients' samples. All age groups were included in her study, bacteria were 54 (14.1%) isolated from 384 body fluids. Among these, 32(59.3%) were gram-negative bacteria, while the reaming 22(40.7%) were gram-positive bacteria. Her result showed that greater than 60% of gram-negative bacteria isolates from body fluids were resistance to Gentamycin65%,Ampcillin 62%, Ciprofloxacin 53.1%, and Ceftriaxone (50%) and Tobramycin (50%),whereas sensitive to Amikacin 68 %, and Chloramphenicol (62%).*K. pneumoniae*9/32(28.1%) were the predominant isolated among gram-negative bacteria showed that more than 90 %(n= 8 /9) isolated had multi drug resistance. Finally she recommended that aregular monitoring of antimicrobial resistance patterns of infected body fluid could be essential to prevent further emergency and the spread of antimicrobial resistance [27].

Mesfn.W and Muluken. B. conducted a study on Bacterial isolate of ear infection in 2014. From the total of 117 ears swab cultures, 61 (52.1%) were bacterial cultures positive**.** Then they have

been identified from the ear swabs were: *Staphylococcus aureus 24 (20.5%), Pseudomonas aeroginosa 17 (14.5%), Klebsiella species 10 (8.5%), Proteus species 7 (6.0%), Entrobacter species 4 (3.4%), Escherchia coli 3 (2.6%), Citrobacter species 2 (1.7%),* and *Providentia species* 2 (1.7%).*P.aeruginosa,* and *Enterobacteriacea* were commonly identified etiology of ear infection both in children and adult. Most of these isolate were resistant to commonly prescribed drug in the study area. However, some of the drug like Amikacin and Gentamycin were highly active against to the isolated organism, whereas Ciprofloxacilin was moderately active [28].Therefore, their suggestion was culture and susceptibility test is vital for appropriate management of ear infection.

3. Objectives

3.1 General objective:

- To assess the Antibiotic resistance patterns of gram negative fermentative and non fermentative bacilli isolated from patients referred to Arsho Advanced Medical laboratory, Addis Ababa, Ethiopia.

3.2 Specific objectives:

- To determine the susceptibility pattern of fermentative and non fermentative gram negative bacteria to the commonly used antibacterial drugs.
- To analyze the distribution of gram negative bacteria on different age group and sex.
- To indicate the prevalence of the most and least frequent bacterial isolate from various clinical sample in the study area.

3.3 Hypothesis

- The Antibiotic resistance patterns of gram negative fermentative and non fermentative bacilli isolated in our laboratory could be similar with previous studies conducted in Ethiopia.

4. MATERIALS AND METHODS

4.1. Study site

The study was conducted from January to April, 2017 in patients referred to Arsho Advanced Medical laboratory (AAML) from different sites (hospitals, health centers, clinics) and from different Arsho branch laboratories. Arsho Advanced Medical Laboratory is a private diagnostic advanced medical laboratory in Addis Ababa Ethiopia that delivers diagnostic testing in the country. Many clinical specimens are referred from all over the country to this advanced medical laboratory. Currently the microbiology laboratory runs around 25-30 microbiological samples on average per day most of them are referral specimens from different sites as indicated above.

4.2. Study Design and period

A single descriptive cross-sectional prospective study was conducted to assess the Antibiotic resistance pattern of gram negative fermentative and non fermentative bacilli isolated from patients referred to AAML from January to April, 2017Addis Ababa, Ethiopia.

4.3. Population

4.3.1 Source population

All patients referred to Arsho Advanced medical laboratory for microbiology investigation from January, 2017to April, 2017Addis Ababa, Ethiopia.

4.3.2 Study population

All patients referred to AAML for culture microbiology laboratory during the study period were source of Population.

4.4 Inclusion and Exclusion Criteria:

4.4.1 Inclusion criteria

All age groups referred to microbiology culture test.

All patients who given samples and confirmed by consent and ascent to participate on the study.

4.4.2 Exclusion criteria

All microbiology test finding other than gram negative bacteria from various clinical samples were excluded.

4.5. Study variables:

4.5.1 Dependent variables

- All isolated gram negative bacteria from various clinical samples.
- Antibiotic resistances patterns of gram negative bacilli.

4.5.2 Independent variables

Age group, sex, specimen types.

4.6 Sample size and sampling technique

4.6.1 Sample size

Unfortunately, Limited published data is available specifically on FN and NFGNB in Ethiopia by using this method, 50% of population proportion was used to determine sample size based on single population proportion. The proportion is assumed to be 50% and expected margin of error (d) 5%and with 95% confidence level (Zα/2), and to minimize errors arising from the likelihood of

Noncompliance, ten percent of the sample size will be added to the normal sample. Accordingly, the required sample size will be 422.

$n= (Z\alpha/2)^2 P (1-P) /d2$ Where:

n = required sample size

P = prevalence

d = expected margin of error

Absolute precision $Z1-\alpha/2= 1.96$

But in our study area, more than 20 microbiology test per day have done, therefore; we collect and processed 824 various clinical sample sizes what we obtained during the study period.

4.6.2 Sampling technique.

Convenient sampling technique procedure was used.

4.7 Data collection

4.7.1 Socio-demographic data

Socio-demographic status and history of the participants were taken from the consent form and the information sheet.

4.7.2 Sample Collection and laboratory procedure.

Sample collection and inoculation of primary isolation culture media

Sample collection

Different clinical samples were collected and processed following standard procedures. Specimens collected from each patient and processed were inoculated onto primary isolation culture media such as Blood Agar base (Oxoid, Basingstoke, Hampaire, UK) to which 10% sheep blood is incorporated, Cysteine Lactose Electrolyte Deficient medium (Oxoid, Basingstoke, Hampaire, UK], MacConkey agar (Oxoid, Basingstoke, Hampaire, UK) , Thayer martin agar (Oxoid, Basingstoke, Hampaire, UK), Chocolate agar (Oxoid, Basingstoke, Hampaire, UK), SS agar (Oxoid, Basingstoke, Hampaire, UK) by using inoculating loop. Clean-catch midstream urine collected frompatients was inoculated onto Blood Agar and Cysteine Lactose Electrolyte Deficient medium by using a calibrated loop with a capacity of 1µl. Colony counts yielding bacterial growth of $>10^5$/ml of urine (≥100,000 colonies) were regarded as significant for bacteriuria. Urine samples yielded three and more bacterial species were not considered for further study. Blood was transferred to blood culture bottles containing brain heart infusion broth. All inoculated plates were incubated at 37 ^{0}C for 18-24 hours aerobically, but Blood agar, blood culture bottles, and Chocolate agar were incubated in 5 % CO_2 incubator. Pure isolates of bacterial pathogen were preliminary characterized by colony morphology, gram-stain, and oxidase test before inoculating them into AST-GN72.

AST-GN72 cards consist of an array of biochemical method and substrates for identification and array antibiotics for drug susceptibility testing. Substrates and biochemical tests used for identification of gram negative bacteria were Ala-Phe-Pro-Arylamidase, Adonitol,

L-Pyrrolydonyl-Arylamidase, L-Arabitol, D-Cellobiose, Beta-Galactosidase, H_2S production, Beta-N-Acetyl-Glucosaminidase, GlutamylArylamidasepNA, D-Glucose, Gamma-Glutamyl-Transferase, Fermentation/Glucose, Beta-Glucosidase, D-Maltose, D-Mannitol, D-Mannose, Beta-Xylosidase, Beta-Alanine arylamidasepNA, L-ProlineArylamidase, Lipase, Palatinose, Tyrosine Arylamidase, Urease, D-Sorbitol, Saccharose/Sucrose, D-Tagatose, D-Trehalose, Citrate(Sodium), Malonate, 5-Keto-D-Gluconate, L-Lactate alkalinisation, alpha-Glucosidase, Succinate alkalinisatio Beta-N-Acetyl-Galactosaminidase, Alpha-Galactosidase, Phosphatase, Glycine Arylamidase, Ornithine Decarboxylase Base, Lysine Decarboxylase, Decarboxylase Base, L-Histidine assimilation, Coumarate, Beta- Glucoronidase, O/129 Resistance (comp.vibrio.), Glu-Gly-Arg-Arylamidase, L-Malate assimilation, Ellman, L-Lactate assimilation. Antibiotics with their different concentration used for determination of drug susceptibility profile of gram-negative bacteria in this investigation were: Ampicillin (4,8,32), Amoxicilin/Clavulanic Acid (4/2,16/8,32/16), Cefalotin (2,8,32), Cefazolin (4, 16, 64), Cefepime (2,8,16,32), Cefoxitin (8,16,32), Cefpodoxime (0.5, 1, 4), Ceftazidime (1,2,8,32), Ceftriaxone (1,2,8,32), Cefuroxime (2,8,32), Ciprofloxacin (0.5,2,4), Gentamicin (4,16,32), Levofloxacin (0.25,0.5,2,8), Nitrofurantoin (16,32,64), Piperacillin/Tazobactam (2/4,8/4,24/4,32/4,32/8), Tetracycline (2,4,8), Tobramycin (8,16,64), Trimethoprim/sulfamethoxazole (1/19,4/76,16/304).

Inoculation of primary isolation culture media.

Quality control bacteria and pure cultures of bacterial isolates were suspended in 3 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 6.8-7.2) in a 12 x 75 mm clear plastic (polystyrene or glass) test tube to achieve a turbidity equivalent to that of a McFarland 0.50 standard (range, 0.50 to 0.63), as measured by the DensiChek (bioMe´rieux) turbidity meter. These suspensions will be 145 micro liter transferred in to AST-GN72. [21].

Inoculation

Identification cards were inoculated or filled with microorganism suspensions using an integrated vacuum apparatus. A test tube containing the microorganism suspension was placed into a special rack (cassette) and the identification card were placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The cassette can accommodate up to 10 tests (VITEK 2 Compact). The filled cassette were placed manually (VITEK 2 compact) into a vacuum chamber station. After the vacuum applied and air was re-introduced into the

station, the organism suspension was forced through the transfer tube into micro-channels that fill all the test wells [21].

Card sealing, loading and incubation

Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. The carousel incubator can accommodate up to 30 cards. All card types were incubated on-line at 35.5 $\pm$ 1.0ºC. Each card removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next read time. Then data collected at 15-minute intervals during the entire incubation period [21].

4.7.3 Culture and identification

Reagent Cards

The reagent cards have 64 wells that can each contain an individual test substrate. Substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances. An optically clear film present on both sides of the card allowed for the appropriate level of oxygen transmission while maintaining a sealed vessel that prevents contact with the organism-substrate admixtures. Each card has a pre-inserted transfer tube used for inoculation. Cards have bar codes that contain information on product type, lot number, expiration date, and a unique identifier that can be linked to the sample either before or after loading the card onto the system [21].

Identification Techniques

Test data from an unknown organism were compared to the respective database to determine a quantitative value for proximity to each of the database taxa. Each of the composite values compared to the others to determine if the data were sufficiently unique or close to one or more of the other database taxa. If a unique identification pattern was not recognized, a list of possible organisms was given, or the strain determined as to be outside the scope of the database [21].

4.7.4 Antibacterial susceptibility testing

Antimicrobial Susceptibility testing with the VITEK-2 compact system was performed using an AST GN 72 card according to the Manufacturer's instructions. The GN 72 card were used for the automated identification of 135 taxa of the most significant fermenting and non-fermenting

Gram-negative bacilli. The GN card is based on established biochemical methods and newly developed substrates measuring carbon source utilization, enzymatic activities, and resistance. The VITEK-2 AST N72 susceptibility card is intended for use with the VITEK-2 systems in clinical laboratories as an *in-vitro* test to determine the susceptibility of clinically significant aerobic gram negative bacilli to antimicrobial agents. Antibiotics tested in AST GN72 card included 19 antibiotics [21, 29, and 30].

The cards were filled with inoculums in filling chambers .The VITEK-2 System automatically processes the antimicrobial susceptibility cards until MIC's were obtained. The VITEK-2 compact system subsequently corrects, where necessary for MIC's or clinical category in accordance with the internal database of possible phenotypes for microorganism antimicrobial agent combinations [8]. Preparation of inoculums were done by transferring 145µL of culture suspension from the 0.5 McFarland culture suspension used for filling the identification cards into a fresh 3mL(0.45% of) sterile saline solution obtaining a final turbidity of 8x106 cfu/mL) in the filling chamber according to the instruction of the insert kit of The AST card[29].

4.8 Quality control

For quality control of susceptibility tests *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 were used, and Also the VITEK-2 compact automated microbiology analyzer found at AAML is participated external quality assessment scheme (Proficiency testing) from one world accuracy and from National health Laboratory service on bacterial identification & antibiotics susceptibility study. Standard Operating Procedures (SOP) were strictly followed.

4.9 Data analysis

Data entry and analysis was done using SPSS (Statistical Package for social sciences statistical software) version 20. The descriptive statistics analysis was used to see the relation between dependent variable and independent variables. During analysis frequencies of the different variables were determined, odd ratio and test was used to compare frequencies. The association was assessed by using chi-square test. In all cases P-value less than 0.05 was considered as statistically significant. Finally, the results were presented on words, charts, and tables.

4.10 Ethical consideration

Ethical clearance was obtained from "Departmental Ethics and Research committee" of the Department of Medical Laboratory Sciences, Collage Health Science, and School of Allied Health Science of Addis Ababa University. Permission was also obtained from Arsho Advanced medical laboratory. Study proceeds after patients become informed about the objectives and use of the study and after they give informed consent. All the laboratory result and information obtained from the patients with in the study period was kept as confidential.

4.11 Operational definitions

Fermentative – they are belongs to the family *Entrobacteriacea,* they obtain energy through carbohydrate fermentation.

Non fermentative- are a group of aerobic, non-spore forming, gram-negative bacilli that obtain energy by degrade carbohydrates through metabolic pathways other than fermentation.

Multidrug resistance – Bacteria those are resistant to antimicrobials for a minimal of three or more of classes of antibiotics tested.

4.12 Dissemination of results.

The findings of this study will be forwarded to the Department of Medical Laboratory Sciences, School of Allied Health Sciences, and Addis Ababa University. And an attempt will be made to present the findings in different conferences and also will be sent for publication.

5. Results

5.1 Socio-demographic characteristics

Out of a total of 824 various clinical samples processed during the study period, 526(63.8%) were collected from female and 298 (36.2%) from male patients. Among the total clinical samples processed, 284 (34.5%) yielded gram negative bacterial pathogens of which 191 (67.3%) were obtained from female and 93(32.7%) from male patients. The isolated organism found dominantly from a wide range of clinical specimens such as in urine 197(69.37%), in wound and abscess 34(11.97%), in Ear 20(7.04%), in CSF 11(3.87%), in Nasal 11(3.87%), in Blood 4 (1.41), in Bodyfluid4 (1.41%) and in semen 3(1.069%) were culture positive for gram negative bacteria.

The association of bacterial pathogen from clinical samples with gender was not statistically significant (p = 0.14). Highest cases of 33.5% (95/284) isolated gram-negative bacteria were recorded among young and middle-age patients with an age group of 25-44 years followed by age groups of 45-64(24.6%, 70/284). Pediatric (0-14 years) and elderly (≥ 65 years) patients accounted for 12.3 %(35/284) and 19% (54/284) of the total number of gram-negative bacteria isolates, respectively (Table 2).In this study result, patients in age group of 1-14,15-24 and 25-44 were significantly affected by gram- negative bacteria [p= 0.019 , p = 0.036 and p=0.001], respectively.

Table 1: Association of socio-demographic variables with various clinical samples culture results at Arsho Advanced Medical Laboratory from January 2017 to April 2017.

			Culture result from various clinical samples			
Variable		total number of Processed samples	Culture positive for Gram negative bacteria		COR [CI; 95%]	P- Value
			Yes	No		
Age	<1	7(0.8%)	02(0.7%)	05(0.9%)	0.467[0.0870-2.5031]	0.378
	1-14	107(13.0%)	33(11.6%)	74(13.7%)	0.523[0.3007-0.9001]	0.019
	15-24	94(11.4%)	30(10.6%)	64(11.9%)	0.547[0.3106-0.9630]	0.037
	25-44	335(40.7%)	95(33.5%)	240(44.4%)	0.462[0.2991-0.7129]	0.001
	45-64	164(19.9%)	70(24.6%)	94(17.4%)	0.869[0.5390-1.4003]	0.564
	>65	117(14.2%)	54(19.0%)	63(11.7%)		
	Total	824(100%)	284(100%)	540(100%)		
Gender	Female	526(63.8%)	191(67.3%)	335(62.1%)	1.257[0.9285-1.701]	0.139
	Male	298((36.2%)	93(32.7%)	205(37.9%)		
	Total	824(100%)	284(100%)	540(100%)		

WHO age classification for health [31].

5.2 Prevalence of bacterial isolates from the processed clinical samples.

A total 284 bacterial isolates belonging to 27 species were recovered, of which 258(90.8%, 258/284) of the isolates were fermentative Gram-negative and 26 (9.2%), 26/284) were non fermentative Gram-negative bacteria. Among from Gram-negative bacteria was the most common one for 53.5% of the total isolates. *E.coli* followed by *K.pneumonea* 40(14.1%), *P.aeruginosa* 13(4.6%), *P.mirabilis* 13(4.6%), *E.cloacae* 11(3.9%), *S.enterica* 10(3.5%), *A.baumanii* 8(2.8%) of the total isolates. These were the seven predominant accounted Gram-negative bacteria (Table 2 and 3).

Table 2: Frequency distribution of fermentative gram –negative bacterial pathogen isolated from different clinical specimens (n=258).

Specimen	*A.caviae(1)*	*C. freundii(5)*	*C. sedlakii(3)*	*C.koseri(3)*	*E. cloacae(11)*	*E.coli(152)*	*K. pneumonea(40)*	*K.oxytoca(5)*	*L.adecarboxylata(1)*	*M.morgani(2)*	*P.mirabilis(13)*	*P.shigelloides (1)*	*P.stuarti(2)*	*P.vulgaris (3)*	*R.gilardii(1)*	*R.planticola (1)*	*S.enterica(10)*	*S.fonticola(1)*	*S.groupA(1)*	*S.marcescens(1)*	*S.paratyphi(1)*	*Total[n=258]*
Urine (n=197)	1 (0.5%)	2 (1%)	2 (1%)	1 (0.5%)	6 (3.1%)	138 (70.5%)	24 (12.8%)	1 (0.5%)	0	2 (1%)	2 (1%)	1 (0.5 %)	1 (0.5 %)	0	1 (0.5 %)	0	8 (4.1 %)	0	0	1 (0.5 %0	0	190 (73.6 %)
Wound(n=34)	0	2 (5.8%)	1 (2.9 4%)	1 (2.9%)	1 (2.9%)	7 (20.6%)	5 (14.7%)	1 (2.9%)	0	0	1 (2.9 %)	0	1 (2.9 %)	2 [5.9 %)]	0	1 (2.9 %)	1 (2.9 %)	0	0	0	1 (2.9 %)	26(10 %)
Ear (n=20)	0	0	0	1 (5.1%)	2 (10.2 %)	1 (5.1%)	1 (5.1%)	2 (10.2 %)	0	0	7 (35%)	0	0	0	0	0	0	0	0	0	0	14(5.3 %)
CSF (n=11)	0	0	0	0	1 (9.1%)	5 (45.5%)	2 (18.2%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8(3.1 %)
Nasal(n=11)	0	0	0	0	0	0	5 (45.5%)	1 (9.1%)	1 (9. 1 %)	0	2 (18.2 %)	0	0	1 (9.1 %)	0	0	0	0	0	0	0	10(3.8 %)
Blood(n=4)	0	0	0	0	1 (33.3 %)	1 (33.3%)	1 (33.3%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4(1.6 %)
Body fluid(n=4)	0	1 (25%)	0	0	0	0	1 (25%)	0	0	0	0	0	0	0	0		1 (25%)	1 (25 %)	0	0	0	4(1.6 %)
Semen (n=3)	0	0	0	0	0	1 (33.3%)	1 (33.3%)	0	0	0	1 (33.3 %)	0	0	0	0	0	0	0	0	0	0	3(1.1 %)

Table 3: Frequency distribution of non-fermentative gram –negative bacterial pathogen isolated from different clinical specimens (n=26).

Specimen	*p.aeruginosa(13)*	*A.baumannii(8)*	*S.maltophilia(1)*	*P.fulrescens(2)*	*C. pauclus(1)*	*A.calcoacticus(1)*	*Total (n=26)*
Urine(n=197)	4(2.03%)	3(1.52%)	0	0	0		7(27 %)
Wound(n=34)	4(11.74%)	2(5.88%)	1(2.94%)	1(2.94%)	0	1(2.94%)	8(34.6%)
CSF(n=11)	1(9.09%)	2(18.18%)	0	0	0	0	3 (11.5%)
EAR(n=20)	3(15%)	1(18.8%)	0	1(5%)	1(5%)	0	6(23.1%)
Nasal	1(9.1%)	0	0	0	0	0	1(3.8%)

5.3 Antibacterial susceptibility pattern of gram-negative bacteria isolates.

The overall drug susceptibility profile of Gram negative bacteria against the nineteen anti-bacterial drugs tested is summarized in Table 4 and 5. Ampicillin had the highest overall resistance rate (82.33%) against Gram negative bacteria, followed by cefatoline (65.33%), and tetracycline (63.7%). Gram-negative bacteria showed better sensitivity towards tazobactum (70.15%), followed by gentamycine, cefoxitine, and tobramycine with the overall sensitivity rates 64.13%, 63.5%, and 63.37%, respectively.

As far as species specific antimicrobial resistance rates are concerned, *E. coli*, the most frequently isolated bacterium, showed resistance rates of 77.0% for ampicillin, 65.8% for both trimethoprime/sulfamethoxazole and tetracycline. The second most predominant isolate was *K.pneumoniea* was also exhibited high resistance for Ampicilline(97.5%), (cefatoline and cefazolin)70% each, 67.5% (cefuroxime and cefatzidime),the least resistance observed in cefoxitin(67.5%), peperaciline/tazobactum(62.5%),levofloxine(60%), the third commonly isolated gram negative bacteria were *P.aeruginosa and P.mirabilis* respectively. *P.aeruginosa* showed highly resistance for many drugs as compared to others it was resistances for more than ten drugs (100%). The least resistance was observed in the case of tobramicin (7.7%), levofloxine (23.1%), and peperaciline (38.5%). And *p.mirabilis* had a resistance range from 15.4 %(ciprofloxaciline, levofloxine) to 92.3 %(nitrfurantoin).

The fourth and fifth bacteria were *E.cloacae* and *A.baumani. A.baumanii* which showed that high resistance for 87.5% for (ampcillin, amoxicillin/Clavulinicacid, cefuroxime, cefoxitin, nitrofurantoine) next 75% for (cefatoline, cefazolin, cefpodoxime, cefteriaxone, cefepime) and the sensitivity range were 12.5% to 37.5 %(trimethoprime/sulfamethoxazole).

In this study the most frequent isolates were E*. coli, Klebsiella* spp. *P.aeruginosa, P.mirabilis* and *E.cloacae* isolates resistant to third generation cephalosporin suggests the importance of monitoring this phenotype. And also in the result high resistance rate of non fermentative gram negative bacteria was observed than fermentative gram negative bacteria.

Table 4: percentage of antibacterial susceptibility pattern of fermentative gram-negative bacteria (n=258)

Species	P	AMP	AMC	TZP	CFA	CFZ	CFU	CFXA	FOX	CPD	CAZ	CRO	CFP	GM	TBM	CIP	LEV	TEC	NFT	SXT
E.coli(n=152)	I	1.3	22.3	8.6	16.4	0.7	3.9	9.2	7.9	1.3	0	0	0	0.7	4.6	0.7	0.7	0	13.8	0.7
	R	77.0	35.5	12.5	52.9	45.4	46.1	46.1	10.5	40.8	42.1	42.8	41.4	32.9	30.9	46.1	45.4	65.8	4.6	65.8
	S	21.7	42.1	77.0	28.3	52.6	48.0	42.8	80.3	56.6	55.9	56.6	56.6	65.8	63.2	53.3	52.6	32.2	81.6	31.6
K.pneumonea (n=40)	I	2.5	15	10	5	o	5	7.5	0	0	0	0	0	0	22.5	12.5	10	5	25	0
	R	97.5	52.5	27.5	70	70	67.5	65.0	32.5	67.5	65.0	62.5	62.5	45	37.5	37.5	30	42.5	40	32.5
	S	0	32.5	62.5	25	30	27.5	27.5	67.5	32.5	35.0	37.5	37.5	55	50.0	50.0	60	52.5	35	47.5
S.enterica(n=10)	I	0	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0
	R	70	20	20	70	80	80	80	60	30	30	40	30	70	80	40	40	70	0	60
	S	30	50	80	30	20	20	20	40	70	70	60	70	30	20	60	60	30	90	40
P.mirabilis (n=13)	I	0	0	7.7	7.7	15.4	15.4	0	15.4	7.7	0	0	0	15.4	7.7	0	0	0	7.7	0
	R	76.9	15.4	61.5	69.2	61.5	61.5	61.5	53.8	61.5	61.5	61.5	61.5	30.8	15.4	15.4	15.4	84.6	92.3	76.9
	S	23.1	84.6	30.8	23.1	23.1	23.1	38.5	30.8	30.8	38.5	38.5	38.5	53.8	84.6	84.6	84.6	15.6	0	23.1
C.ferundii(n=5)	I	0	0	40	0	0	0	20	0	0	0	0	0	0	0	0	0	0	20	0
	R	80	80	20	100	80	40	60	100	80	40	40	0	0	0	20	20	40	0	60
	S	20	20	40	0	20	60	20	0	20	60	60	100	100	100	80	80	60	80	40
K.oxytoca(n=5)	I	0	20	20	20	20	0	0	0	0	0	0	0	0	0	0	0	0	40	0
	R	100	40	0	20	60	60	40	20	20	20	20	20	20	20	0	0	0	0	20
	S	0	40	80	60	20	40	60	80	80	80	80	80	80	80	100	100	100	60	80
P.vulgaris(n=3)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	100	0	66.7	100	100	100	100	33.3	33.3	33.3	33.3	33.3	0	0	0	0	100	100	33.3
	S	0	100	33.3	0	0	0	0	66.7	66.7	66.7	66.7	66.7	100	100	100	100	0	0	66.7
C.koseri(n=3)	I	66.7	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	66.7	0
	R	33.3	0	0	0	0	0	0	0	0	0	0	0	33.3	0	0	0	33.3	0	33.3
	S	0	100	100	100	100	100	0	100	100	100	100	100	66.7	100	100	100	66.7	33.3	66.7

C.sedlakii(n=3)	I	O	0	0	0	0	33.3	33.3	33.3	66.7	33.3	33.3	66.7	33.3	66.7	0	33.3	66.7	33.3	0
	R	100	66.7	66.7	100	100	66.7	66.7	66.7	33.3	33.3	33.3	33.3	33.3	33.3	66.7	33.3	33.3	0	66.7
	S	0	33.3	33.3	0	0	0	0	0	0	33.3	33.3	0	33.3	0	33.3	33.3	0	66.7	33.7
M.morgani(n=2)	I	O	0	50	0	0	0	0	0	0	0	50	50	0	0	0	0	0	0	0
	R	100	100	50	100	100	100	100	100	0	100	50	50	0	100	100	100	100	100	50
	S	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	50
P.stuartii(n=2)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	50	50	50	50	50	50	50	50	50	50	50	0	50	50	0	50	50	50	50
	S	50	50	50	50	50	50	50	50	50	50	50	100	50	50	100	50	50	50	50
E.cloacea(n=11)	I	O	0	0	18.2	0	36.4	18.2	9.1	0	9.1	0	0	0	9.1	9.1	0	18.2	54.5	0
	R	81.8	81.8	18.2	81.8	81.8	63.6	81.8	18.2	18.2	18.2	27.3	18.2	27.3	18.2	18.2	18.2	36.4	27.3	9.1
	S	18.2	18.2	81.8	0	18.2	0	0	72.7	81.8	72.7	72.7	81.8	72.7	72.7	72.7	81.8	45.5	18.2	90.9
C.sedlakii(n=3)	I	O	0	0	0	0	33.3	33.3	33.3	66.7	33.3	33.3	66.7	33.3	66.7	0	33.3	66.7	33.3	0
	R	100	66.7	66.7	100	100	66.7	66.7	66.7	33.3	33.3	33.3	33.3	33.3	33.3	66.7	33.3	33.3	0	66.7
	S	0	33.3	33.3	0	0	0	0	0	0	33.3	33.3	0	33.3	0	33.3	33.3	0	66.7	33.7
S.marcescens(n=1)	I	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0
	R	0	100	0	100	100	100	100	0	0	0	0	0	0	0	0	0	100	100	0
	S	0	0	100	0	0	0	0	0	0	100	100	100	100	100	100	100	0	0	100
A.cavaie(n=1)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	100	0	100	100	100	0	0	0	0	0	0	0	0	0	0	0	100	0	100
	S	0	100	0	0	0	100	100	100	100	100	100	100	100	100	100	100	0	100	0
R.planticola(n=1)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	100	0	0	100	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	S	0	100	100	0	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
S.paratyphi(n=1)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	0	0	0	100	100	100	100	100	0	0	0	0	100	100	0	0	0	0	0
	S	100	100	100	0	0	0	0	0	100	100	100	100	0	0	100	100	100	100	100
S.fanticola(n=1)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	100	100	100	100	100	100	100	0	0	100	100	100	100	100	100	100	100	0	0
	S	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	100	100
S.groupA(n=1)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	100	100	0	0	100	100	0	0	0	0	0	100	100	0	0	0	0	0	0
	S	0	0	0	0	0	0	0	0	0	0	100	0	0	100	0	0	0	0	0

L.adecarboxylata(n=1)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	S	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
P.shigelloides(n=1)	I	0	100	0	0	0	100	0	0	0	0	0	0	100	0	0	0	0	0	0
	R	100	0	100	100	0	0	0	0	0	0	0	100	0	100	100	100	100	0	0
	S	0	0	0	0	100	0	100	100	100	100	100	0	0	0	0	0	0	100	100
R.gilardii(n=1)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	100	0	0	100	100	0	0	0	0	100	100	0	0	0	100	100		100	100
	S	0	100	0	0	0	0	0	0	0	0	0	0	100		0	0	0	0	0

Ampcillin=AMP, Amox/clavulic acid =AMC , Peperaciline /tazobactum=TZP, Cefatolin=CFA , Cefazoline =CFZ, Cefruxime =CFU CefruximeAxetile= CFXA, Cefoxitin = FOX, Cefpodoxime =CPD, Ceftazidime =CAZ, Ceftriaxone =CRO, Cefepime =CFP, Gentamicin = GM, Tobramicine =TBM, Ciprofloxacilin =CIP, Levofloxacin =LEV, Teteracycline= TEC ,Nitrofuranatoin=NFT, Trimethoprime/sulfamethoxazole =SXT

P =pattern I= intermediate R= resistance S=sensitive.

Table 6:Percentage of antibacterial susceptibility pattern of non-fermentative gram-negative bacteria (n=26).

Species	P	AMP	AMC	TZP	CFA	CFZ	CFU	CFXA	FOX	CPD	CAZ	CRO	CFP	GM	TBM	CIP	LEV	TEC	NFT	SXT
P.aeruginosa(n=13)	I	0	0	0	0	0	0	0	7.7	0	0	0	7.7	15.4	0	15.4	7.7	0	0	0
	R	100	100	38.5	100	100	100	100	92.3	100	53.8	100	38.5	0	7.7	15.4	23.1	100	100	92.3
	S	0	0	61.5	0	0	0	0	0	0	46.2	0	61.5	84.6	92.3	69.2	69.2	0	0	7.7
P.fulorescens (n=2)	I	0	0	0	0	50	0	50	0	0	0	0	0	0	0	0	0	0	0	0
	R	0	0	0	50	0	0	0	50	0	0	0	0	0	0	0	0	0	50	0
	S	100	100	100	50	50	100	50	50	100	100	100	100	100	100	100	100	100	50	100
A.calcoacticus(n=1)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C.pauclus (n=1)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	0	0	0	100	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	S	100	100	0	0	0	100	100	100	100	100	100	0	0	0	100	100	0	100	0
A.baumanii (n=8)	I	12.5	0	25	0	0	12.5	12.5	12.5	0	12.5	0	0	25	37.5	0	37.5	12.5	12.5	0
	R	87.5	87.5	62.5	75	75	87.5	87.5	87.5	75	62.5	75	75	62.5	37.5	75	37.5	75	87.5	62.5
	S	0	12.5	12.5	25	25	0	0	0	25	25	25	25	12.5	25	25	25	12.5	0	37.5
S.maltophilia(n=1)	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	100

Percentage of antibacterial susceptibility pattern of all fermentative and non fermentative gram-negative bacteria (n=284).																			
P	**AMP**	**AMC**	**TZP**	**CFA**	**CFZ**	**CFU**	**CFXA**	**FOX**	**CPD**	**CAZ**	**CRO**	**CFP**	**GM**	**TBM**	**CIP**	**LEV**	**TEC**	**NFT**	**SXT**
I	1.1	16.2	7.8	10.6	1.1	4.1	8	5.8	0.8	1.5	0	0.7	1.8	8	2.8	3.3	1.1	15.8	0.4
R	82.3	42.8	22	65.3	60.2	57.6	58.5	30.7	50.2	44.4	47.5	42.8	34.1	28.6	39.2	37.1	63.7	24.4	59.7
S	16.6	41	70.2	24.1	38.7	36.3	33.5	63.5	49	54.1	52.5	56.5	64.1	63.4	58	59.6	35.2	59.8	39.9

5.4 Multidrug resistance patterns for the isolated gram negative bacteria.

Based on the finding of the present study result as shown that in table 6 below, the overall prevalence of multidrug resistance gram negative bacteria for three or more antibiotics rate was 58 %. However, 3.9 % resistance only for one antibiotic, 11.3% bacterial isolates were sensitive for all antibiotics tested this raveled that wide range of antibiotics becomes resistance for gram negative bacteria.

Among gram negative bacteria, *K.pneumoniae* (82.5%),*E.coli* (82.9%)**,** *P.aeruginosa* (100%), and *A. baumanii* **(**100%) were MDR. High level of resistance to different antibiotic particularly to ampicillin in almost all gram negative bacterial isolate was observed **(**in Table 6**)**.

P.aeruginosa 13(100%)), *K.pneumoniae27* (67.5%), *A.baumanii*6 (75%), *E.coli72* (43.7%) and *E.cloacae*7 (63.6%) *S.enterica* 07(70%) were resistance for more than five antibiotics tested.

Table 6: Multiple antibiotics resistance pattern of bacterial isolates from various clinical patient samples at Arsho Advanced medical laboratory.

Isolated gram negative bacteria	**Resistant antibiotics (%)**						
	Ro	R1	R2	R3	R4	R5	R>5
A.baumanii(n=8)	0	0	0	01(12.5%)	0	01(12.5%)	06(75%)
E.coli(n=152)	22(14.5%)	04(2.6%)	12(7.9%)	25(16.4%)	08(5.3%)	09(5.9%)	72(43.7%)
P.aeruginosa(n=13)	0	0	0	0	0	0	13(100%)
K.pneumonea(n=40)	2(5%)	05(12.5%)	03 (7.5%)	01(2.5%)	01(2.5%)	01(2.5%)	27(67.5%)
P.mirabilis(n=13)	01(7.7%)	01(7.7%)	0	02(15.4%)	0	01(7.7%)	08 (61.5%)
S.enterica(n=10)	02(20%)	01(10%)	0	0	0	0	07(70%)
E.cloacae(n=11)	0	0	02(18.2%)	0	0	02(18.2%)	07(63.6%)
C.freundii(n=5)	0	0	0	0	0	01(20%)	04 (80%)
C.sedlakii(n=3)	0	0	0	0	01(33.3%)	01(33.3%)	01(33.3%)
M.morganii(n=2)	0	0	0	0	0	0	02(100%)
P.shigelloides(n=1)	0	0	0	0	0	0	01(100%)
R.gilardii(n=1)	0	0	0	0	0	0	01(100%)
S.marcescens(n=1)	0	0	0	0	0	0	01(00%)
K.oxytoca(n=5)	0	0	02(40%)	02(40%)	0	0	01(20%)
C.koseri(n=3)	02(66.7%)	0	0	0	0	0	01(33.3%)
A.calcoacticus(n=1)	0	0	0	0	0	0	01(100%)
S.paratyphi(n=1)	0	0	0	0	0	0	01(100%)
P.fulorescens(n=2)	01(50%)	0	0	0	0	0	01(50%)
P.stuartii(n=2)	01(50%)	0	0	0	0	0	01(50%)
C.pauclus(n=1)	0	0	01(100%)	0	0	0	0
A.cavaie(n=1)	0	0	0	0	0	0	01(100%)
L.adecarboxylta(n=1)	01(100%)	0	0	0	0	0	0
S.fanticola(n=1)	0	0	0	0	0	0	01(100%)
S.groupA(n=1)	0	0	0	0	0	0	01(100%)
R.planticola(n=1)	0	0	0	01(100%)	0	0	0
Total (n=284)	32(11.3%)	11(3.9%)	20(7.04%)	32(11.3%)	10(3.5%)	16(5.6%)	159(55.9%)

Ro- no resistant for any antibiotic **,R1**-resistant for 1 class of antibiotic, **R2**- resistant for 2 different class of antibiotics, **R3**- resistant for 3 classes of antibiotics, **R4**-resistant for 4 different class of antibiotics, **R5**- resistant for 5 different classes of antibiotics,**>R5**- resistant for more than 5 different class of antibiotics .

NB: Class of antibiotics made based on CLSI 2014 category.

6. Discussion

Antimicrobial resistance among gram negative bacteria is showing a global rise. Empirical antimicrobial therapy plays a very important role in the successful management of infections in patients where as inappropriate therapy affects both patients mortality rates and patients time spent in the hospital [32].

Out of 824 various clinical samples collected from patients referred to our laboratory with different cases of infections, among these 284 patients showed significant growth of gram negative bacteria giving a prevalence rate of gram negative bacteria were 34.5%(284/824), Our study agrees with a similar study carried out by Kibret M, *et al* [33] discovered on prevalence of gram negative bacteria. But, our study result was lower than that a study done in Southern Ethiopia by Anteneh A [34] which showed 67.8%.

In the present study, the automated analyzer identified among 284 bacterial isolates, 258 (90.8%) fermentative and 26(9.2%) were non fermentative Gram-negative.Our study is similarprevalence rate to what has been reportedbyDipakBhargavin Pakistan [35].But also the prevalence of non fermentor was lower than a study conducted byMinhas Akbar et al in Peru[36]such variation may be due to the Geographical variations and drug resistance properties.

The isolation of predominant gram negative bacteria from various clinical samples was much similar with many local [34, 37 and 38] and international studies demonstrated by Rudrajit Paulin India [50] .

The overall drug resistance of Gram-negative bacteria isolates for three or more bacteria drug resistance rates ranged from 58%, 3.9% for only one antibiotic, and 11.3% were sensitive for all bacteria tested[39]. High level of drug resistance to ampicillin and amoxicillin was exhibited in this study. Similar studies conducted locally by Mulugeta KA [40] and internationally in Nigeria and India [41, 42] respectively.

A significant observation showed that the majority of Gram-negative bacteria isolates were more sensitive towards Nitofurantoin [81.6%], cefoxitine [80.3%], Piperacillin/tazobctum combinations, and toberamycine [77%], were the most active agent against *E. coli;*which was the most frequently isolated Gram-negative bacterium. Our finding was similar to earlier study carried out against *E. coli* isolated from urine sample by Tamirat M.[37] and Lue et al [43].

Contrarily study conducted in Mekelle concludes that most bacterial isolates were sensitive to Gentamicin, Ciprofloxacin and Amoxicillin clavulanic acid [44].

Among the gram negative bacteria *P.aeruginosa* (100% each), *A. baumanii* (100%) *E.coli (82.9%),* and *K.pneumoniae* (82.5 %), was MDR which is in line with Zenebe T [45].

High level of resistance to different antibiotic particularly to ampicillin in almost all gram negative bacterial isolate was observed. *P.aeruginosa* **13**(100% each), *K.pneumoniae**27*** (67.5%), *A.baumanii**6*** (75%), **E.coli 72** *(43.7%)* and ***E.cloacae*7** (63.6%) *S.enterica* 07(70%) were resistance for more than five antibiotics tested agree with study conducted by DipakBhargav and Zenebe T [35, 45], respectively.

Multidrug resistance is a major problem with non fermenting gram negative bacilli and so the infections caused by them are very difficult to be treated [46]. In this study from the isolated non fermentative gram negative bacteria *P.aeruginosa*(50%) was the most predominant isolates followed by *A.baumanii*(30.7%] similar study were conducted in Nepal and Saudi [36, 49] respectively.

P.aeruginosa showed highly resistance for many drugs it also exhibited resistance for more than ten drugs (100%),but 100 % sensitivity showed only for Genatamicine agree with the study conducted by [46,48]. Similarly to *P.aeruginosa* and *A.baumanii* also showed that high resistance for 87.5% for (ampcillin, amoxicillin/Clavulinicacid**,** cefuroxime, cefoxitin, and nitrofurantoine similar with [32, 48, and 49]. A study conducted by F. Daxboeck in Viena which completely in contrast with our finding [50].

The most important thing to be considered in this study finding is that identification of spectrum bacterial species implicated in causing for various infections or diseases has been changing as the result of geographical variations and drug resistance properties depending on the improper use of antibiotics by the physician or poor local anti-microbial prescription practices. Isolation of twenty-seven bacterial at the species level in the present study in contrast to earlier studies in Ethiopia was in line with the above fact.

Hence, accurate identification of bacteria to a species level by employing accurate method is important because; (a) different species have different antibiotic susceptibilities, (b) serious bacterial infections caused by true "pathogens" have decreased in recent years in

proportion to those caused by opportunistic bacteria that once were considered to be of low virulence (i.e. the incidence of opportunist infections is increasing),and (c) such infections cannot be traced epidemiologically or documented without identification of bacteria to a species level.

7. Strength of the study.

- A standardized dilution method was used for testing isolates, and antibiotic susceptibilities were interpreted using a common standard.
- Accurate identification of bacteria to a species level by employing accurate method.

8. Limitation of the study

- Lack of patient's clinical history in their request paper which could be a good variable for our study.
- Being the study as a single laboratory based it may decrease or increase the detection rate.

9. Conclusion.

In this study high antimicrobial resistance and multi drug resistance was demonstrated over the study period, such as ampicillin followed by trimethoprim/sulfamethoxazolewas the least active agent against gram negative bacteria while piperacillin/tazobactam and tobramycinme were exhibited the most susceptible drug. Therefore, this may be due to with the associated improper antimicrobial consumption of antibiotic, lack of a tool for a proper guideline for clinical antibiotic therapy, and prescribing antibiotic without identification of the etiological agent by laboratory.

10. Recommendations

- Establishing health laboratories with modern methods such as VITEK 2 compact system for accurate identification of bacteria pathogens to the species level and determining drug susceptibility pattern of the etiologic agents for efficient management of bacterial infections should be considered for routine laboratory diagnosis.
- A periodically surveillance of antimicrobial resistance pattern record and report is recommended.
- Establish antibiogram based on the susceptibility pattern for empiric therapy at national level.

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Annex I: participants information sheet [English version].

Principal Investigator: EsitifanosTsige

Addis Ababa University College of Health Sciences, department of medical laboratory science.

Purpose: The purpose of this study is to determine theantibiotic resistance pattern of gram negative fermentative and non fermentative bacilli isolated from patients in our laboratory.

Procedures to be carried on: you are invited to participate in the study after giving your consent and by giving the requested sample for investigation.

Risks associated with the study: There is no risk and serious invasive procedure at the beginning as well as at the end of the study and there is no additional time required from you tostay during study.

Benefits of the study: There will be no financial benefit to you. But the result of the study will be used as a base lineto developantibiogramand it helps patients avoiding empirical treatment.

Confidentiality of your information: The results of the laboratory findings will be kept confidential and could only be accessed by the researcher and the responsible physician. There will be no personal information to be attached to your data.

Termination of the study: We will respect your decision if you later on change your mind and you can refuse to participate or withdraw from the study at any time. Refusal to participate will not result in loss of medical care provided or any other benefits. You can get your results of the analysis.

Annex II. Informed consent [English version]

I, the undersigned individual, am oriented about the objective of the study. I have informed that all of my information will be kept confidential and used only for this study.

Your signature below indicates that you have read /or listened, and understand the information provided for you about the study. Before you sign,please understand purpose of the study, procedure,risks and benefits of participation, right to refuse or withdraw, confidentiality and privacy, and who to contact if you have any question.

I have read /or listened to the description of the study and I understand what procedures are and what will happen to me in the study.

Based on the above information I agree to participate in the research

Signature: _____ Date: _____

Name of Data collector _____ Signature _____

If you have any question you can ask the principal investigator

Principal investigator EstifanosTsige [Msc candidate]

Mobile 0912121618

e-mail.tsigeestifanos@gmail.com

Annex III: participant's information sheet [Amharic version]

ጥናቱ ን የሚያጠናው ፤እስጢፋኖስ ፅጌ

በአዲስ አበባ ዩኒቨርሲቲ ጤና ሳይንስ ኮሌጅ የህክም ና ላቦራቶሪ ሳይንስ ዲፓርትመንት፡፡

የጥናቱ አላማ

መድሃኒት የተለማመዱ ፈርመንታቲቭ እና ፈርመንታቲቭ ያልሆኑ ግራም ኔጌቲቭ ባክቴሪያዎችን በአርሾ አድቫንስድ ሚዲካል ላቦራቶሪ ከተለያየ ለማይክሮባዮሎጂ ምርመራ ከተላኩ ናሙናዎች ውስጥ በመለየት አሁን ያሉበትን ደረጃ ማሳየት፡፡

ጥናቱ ቀጣይነት የሚኖረው

በጥናቱ ውስጥ እረሰዎ ተሳታፊ ለመሆን ፈቃደኛ መሆንዎን በስምምነት ሲያረጋግጡ እና ናሙናውን ለምርመራ መስጠት ሲችሉ ብቻ ይሆናል፡፡

በጥናቱ ተሳታፊዎች ላይ ያለው ጉዳት

በጥናቱ መጀመሪያም ይሁን መጨረሻ በዚህ ጥናት ላይ በመሳተፍዎ ሊደርስብዎ የሚችል አንድም ጉዳት አይኖርም፡፡በጥናቱ ምክንያት የሚያባክኑት ተጨማሪ ጊዜም አይኖርም፡፡

ለ ጥናቱ ተሳታፊዎች ያለው ልዩ ጥቅም

በጥናቱ ተሳታፊ በመሆንዎ ምንም አይነት የገንዘብ ክፍያ አያኙም ነገር ግን ከጥናቱ የሚገኘው ውጤት ወደ ፊት ለተለያዩ ህመሞች መድሃኒት ማዘጋጀ የሚረዳ መመሪያ ለማዘጋጀት ግብዓት ይሆናል፡፡

የመረጃ ሚስጥራዊ አጠባበቅ

የሚሰጡት መረጃ በጥናቱ ወቅትም ሆነ ከዚያ በኋላ ባሉት ጊዜያት ሙሉ በሙሉ ሚስጥራዊነቱ የሚጠበቅና መረጃውም የሚያዘዉ በስም ሳይሆን በመለያ ቁጥር ይሆናል፡፡

በጥናቱ ላይ ያለመሳተፍ መብት አለዎት፡፡

በጥናቱ ለመሳተፍ ከወሰኑ በኋላም ሃሳቦዎትን መቀየር ቢፈልጉ እንኳዎን ውሳኔዎትን በማክበር በማንኛው ሰዓት ማቋረጥ ይችላሉ፡፡ ይህ ደግሞ ተሳትፎን ቢያቋርጡም የምርመራ ውጤትዎን ይሁን ከምርመራ በኋላ ማግኘት የሚገባዎትን የህክምና ግልጋሎት ሁሉ ማግኘት ይችላሉ፡፡

Annex IV. Informed consent [Amharic version]

ከእኔ የሚወሰደዉ ናሙና ለጥናቱ አላማ ብቻ እንደሚዉል ተረድቻለሁ። ሁሉም መረጃዎች እና የናሙና ዉጤቱ ምስጢራዊ መሆኑን ተገንዝቤአለሁ። በጥናቱ ላይ በመሳተፌ ምንም የገንዘብ ክፍያ እንደማላገኝ ተረድቻለሁ። በጥናቱ ያለመሳተፍ እንዲሁም በማንኛዉም ጊዜ የማቋረጥ መብት እንዳለኝ አዉቄአለሁ።

ስለዚህ ጥናት ማንኛውም ጥያቄ ካለዎት በማንኛውም ጊዜ ከዚህ በታች በተጠቀሱት አድራሻዎች መጠየቅ ይችላሉ።

እኔም የጥናቱ ተሳታፊ ይህንን በመገንዘብ ጥናቱ ላይ ለመሳተፍ ተስማምቼያለሁ።

ፊርማ ----------ቀን----------

መረጃውን የሰበሰበው ግለሰብ ስም----------

ፊርማ ----------

የዋና ተመራማሪው አድራሻ

እስጢፋኖስ ፅጌ

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ስልክ፣ +2510912-121618

Annex V. SOPs for Bacteriology Laboratory Procedures

SOP of Blood agar plate (BAP)

AIM of Blood agar plate

A non-selective medium for the isolation and cultivation of many pathogenic and non-pathogenic microorganisms. The medium is often used to investigate the forms of haemolysis from pathogenic microorganisms from clinical specimen.

Blood Agar Base formulation has been used as a base for preparation of blood agar and to support good growth of a wide variety of fastidious microorganisms.

Because it is a highly nutritious medium it can also be used as a general purpose growth media without adding blood. For example it was used for studyingirradiated*Escherichia coli*,

Meat extract and Peptone provide nitrogenous compounds, vitamins, carbon, sulphur and amino acids in Blood Agar Base.

Blood Agar Base is suitable to isolate and cultivate a wide range of microorganisms with difficult growth.

Procedure Preparation

To make about 30-35agar plates

Measure 500ml of distilled water using a measuring cylinder.

Transfer the distilled water into a 1litre capacityconical flask.

Weigh 20g of Blood Agar Base IIpowder using a weighing balance.

And then add into the 500ml of distilled water and mix thoroughly.

Boil until completely dissolved

Autoclave at 121°C for 15 minutes.

Allow to cool to 45-50°C in a water bath.

Once the medium has been melted and cooled to 45-50 ºC you can add 5-10% of defibrinated sterile sheep blood, in this case you can recuperate Haemophylus. Be careful to avoid bubble formation when adding the blood to the cooled medium and rotate the flask or bottle slowly to create a homogeneous solution.

Aseptically add 25 ml of sterile defibrinated sheep blood with constant shaking. When mixing, avoiding froth formation.

Arrange the petri-dishes onto the clean safety hood.
Gently pour 15-20 ml of the ready media on to the plates by using dispenser and allow to set. If air bubbles occurred, using a Bunsen burner gently invert and pass the flame over the poured blood agar in the plate such that the air bubbles are removed.

Leave standing for thirty minutes to solidify.

Label on the bottom top of the blood agar plates the batch number & date prepared.

Store the culture media plates upside down at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.

Shelf life: up to *sixteen weeks* provided there is no change in the appearance of the medium to suggest contamination, haemolysis, or deterioration.

Sop of Chocolate (Heated Blood) Agar

AIM of Chocolate (Heated Blood) Agar

Chocolate agar; a non selective media supplies the factors X and V required for the proper growth of *H. influenza*. It is also used to culture nutritionally demanding pathogens such as *N.meningitidis* and *S.pneumoniae*.

When Blood agar is heated, the red cells are lysed and the medium becomes brown in colour; it is referred to as chocolate agar. It is appropriate for isolating pathogenic bacteria in sputum, throat swabs, eye swabs, ear swabs, urogenital swabs, cerebrospinal fluid.

Procedure for preparation:

Prepare as described for Blood agar except after adding blood, heat the medium in a 70^0C water bath until it becomes brown in color. This takes about 10-15 minutes during which time the medium should be mixed gently several times.

Allow the medium to cool to about 45^0C, remix and dispense in sterile petri dishes using a dispenser as described for blood agar.

Leave standing for thirty minutes to solidify.

Perform sterility testing as described for blood agar plate.

Label the bottom of each plate with date of preparation and batch number.

Store the culture media plates upside down at 2-8^0C sealed in plastic bags to reduce chances of contamination.

Important: Care must be taken not to overheat or prolong the heating of the medium because this will cause it to become granular and unfit for use.

Up to sixteen weeks provided there is no change in the appearance of the medium to suggest contamination or deterioration.

Sop of Mac Conkey Agar

AIM of Mac Conkey Agar

It is preferable for the isolation and differentiation of clinically important gram negative rods by inhibiting gram positive cocci.

Principle

MacConkey agar is selective and differential medium to distinguish gram negative *Entrobactericae* and lactose fermenting bacteria from non lactose fermentors.

MacConkey Agar is a selective and differential medium. It is only slightly selective since the concentration of bile salts, which inhibit gram-positive microorganisms, is low in comparison with other enteric plating media. Crystal violet also is included in the medium to inhibit the growth of gram-positive bacteria, especially *Enterococci* and staphylococci. Differentiation of enteric microorganisms is achieved by the combination of lactose and the neutral red indicator.

Colorless or pink to red colonies are produced depending upon the ability of the isolate to ferment the carbohydrate.

Procedure for preparation:

Prepare as instructed by the manufacturer.

Suspend 51.1g of powder in 1 liter of distilled or deionized water.

Heat and boil until completely dissolved with frequent agitation.

Sterilize in autoclave at 121^0C for 15 minutes.

Cool to 45-50 ^{0}C.

Mix well and dispense by dispenser (15-20 ml) aseptically into sterile petri dishes.

Leave standing for thirty minutes to solidify.

Perform sterility testing as described before.

Label the bottom of each plate with date of preparation and batch number.

Store the culture media plates upside down at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.

Test Samples for performance, using stable, typical control cultures.

Sop of XYLOSE LYSINE DESOXYCHOLATE AGAR [XLD]

AIM of XYLOSE LYSINE DESOXYCHOLATE AGAR [XLD

It is a selective differential medium suitable for isolation of *Shigella* and *Salmonella* from stool specimens and other clinical specimens.

Differentiation of these two species from non-pathogenic bacteria is accomplished by xylose and lactose fermentation, lysine decarboxylation, and hydrogen sulfide production.

I.e. Xylose is incorporated in the medium as carbohydrate source to provide a differential mechanism for *shigellae*. Enteric generally ferment xylose rapidly but *shigellae* and *providencias* do so slowly or not at all.

Since most salmonellae ferment xylose as readily as do coli forms a second differential mechanism is employed-lysine decarboxylase. If an organism ferments xylose and decarboxylates lysine the specific xylose/lysine ratio causes it to rapidly exhaust the xylose, and with the lysine reaction a reversion to alkaline stimulates the shigella reaction. Lactose and sucrose are added in excess to prevent lysine-positive coli forms from similarly reverting to alkaline condition.

*Shigella*colonies on XLD agar are transparent pink or red smooth colonies 1–2-mm in diameter.

S. dysenteriae1 colonies on XLD agar are frequently very tiny; unlike other *Shigella*spp. Coliforms appear yellow. Salmonella colonies are usually red with black centers but can also be yellow with black centers.

Preparation

Prepare according to manufacturer's instructions. [***Note:*** Several commercial brands of XLD agar are available.

Mix with distilled water thoroughly.

Heat with agitation just until the medium boils. Do not overheat; overheating when boiling XLD or allowing the medium to cool too long may cause the medium to precipitate.

Cool flask under running water until just cool enough to pour; avoid cooling the medium too long.

Pour the XLD into Petri plates, leaving the lids ajar for about 20 minutes so that the surface of the agar will dry.

Plates can be stored at 4°C for up to a week.

Sop of *Salmonella-Shigella* Agar (SS agar)

AIM of *Salmonella-Shigella* Agar (SS agar)

It is highly selective medium for the isolation of *Salmonellae* and *Shigellae* from faeces, foodstuffs and other materials although it should not be used for isolation of *S.dysenteriae*type 1 because some strains are inhibited.

Brilliant green, ox bile and high concentrations of thiosulfate and citrate largely inhibit the accompanying microbial flora. Sulfide production is detected by using thiosulfate and iron ions, the colonies turn black.

S.typhi, which is lactose-negative, produces smooth, colorless, transparent or translucent colonies that may or may not have black centers indicating production of H2S. Lactose-positive colonies are pink surrounded by a zone of bile precipitation.

Procedure for preparation

Suspend 52.0g of powder in 1 liter of distilled or deionized water.

Mix well.

Heat to boiling for 1 minute to completely dissolve the powder.DO NOT AUTOCLAVE and avoid overheating.

Cool to 45-50^{0}C and dispense into Petri dishes.

Pour 15-20 ml of the ready media on to Petri dishes.

Leave standing for thirty minutes to solidify.

Perform sterility testing as described before.

Label the bottom of each plate with date of preparation and batch number.

Sop of Nutrient agar

AIM of NUTRIENT AGAR

Nutrient agar is used for the cultivation of a wide variety of non-fastidious bacteria. It was originally developed in recognition of the need for a standardized medium for use in the examination of water and waste water, dairy products and various foods. Currently it is used as a maintenance medium for *S.aureus, P. mirabilis,* and*E.coli.* Tube slants are used primarily for the cultivation and maintenance of pure cultures.

Procedure for preparation:

Suspend 23 g of the powder in 1 litre of purified water.
Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
Autoclave at 121˚C for 15 minutes.
Cool to 45-50^{0}C.
Pour 15-20 ml of the ready media into sterile 20ml glass universal tubes.
Leave standing for thirty minutes to solidify, resting the tubes leaning at 30˚- 60˚ to produce the slope effects in the tubes.
Perform sterility testing as described in page 13.
Label the side of each tube with date of preparation and batch number.
Store the tubes at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.
Test Samples of the finished product for performance, using stable, typical control cultures.

Shelf life: up to *eighteen months* provided there is no change in the appearance of the medium to suggest contamination or deterioration.
pH of the medium 7.2-7.6 at room temperature.

Sop of Brain Heart Infusion Broth

AIM of Brain Heart Infusion Broth

Brain Heart Infusion is a general-purpose liquid medium used in the cultivation of fastidious and non-fastidious microorganisms, including aerobic and anaerobic bacteria, from a variety of clinical and non-clinical specimens. It serves as a base for supplemented media containing 0.1% agar or 6.5 % sodium chloride. A supplemented pre-reduced formulation in tubes is especially recommended for the cultivation of anaerobes. Described brain-heart infusion broth prepared by adding pieces of brain tissue to meat infusion or beef extract-dextrose broth. It is appropriate for blood culture and culturing specimens likely to contain anaerobes.

Procedure for preparation (BHI Broth):

Suspend 37.0g in 1 liter of distilled water.

Heat if necessary to dissolve the medium completely.

Dispense into bottles or tubes as desired. Sterilize by autoclaving at 15 Ibs (121^0C) for 15 minutes then, Cool to 45-50^0C.

Dispense into 20ml universal tubes.

Perform sterility testing as described in the previous notes.

Label the side of each tube with date of preparation and batch number.

Store the culture medium at 2-8^0C sealed in plastic bags to reduce chances of contamination.

Test Samples of the finished product for performance, using stable, typical control cultures.

Shelf life: Up to *eighteen months* provided there is no change in the appearance of the medium to suggest contamination or deterioration.

PH of the medium 7.2-7.6 at room temperature.

SOP of Vitake 2 compact analyzer

Purpose

To describe the procedures for the preparation and identification of test microorganisms (test microbes and Quality Control Organisms) using the VITEK 2 Compact Instrument.

Procedure and Analysis

Follow the operational instructions below strictly for the proper use and required quality control activities on VITEK 2 Compact anaylazer.

Initiation of the V2C System

A. the V2C Instrument is always "on"; the instrument will say "Ready" or "Not Ready" on the digital screen. Once the computer is initialized, the instrument will say "Ready."The V2C will not run if it is not on ready mode.

B. Select VITEK 2 Compact to initiate the system from the upper left side of the screen. After the system is initiated, log onto the system using the appropriate user name and password.

The system is now initialized and ready for data entry.

Preparation of Organisms

A.QC organisms.

i. If starting from a frozen stock culture, remove the 0.5 mL cryovials from the -80°C freezer. Avoid repeated thawing and freezing of the frozen culture by aseptically removing a small portion (or loopful) of the frozen inoculums, then immediately return cryovials to -80°C freezer.

ii. Streak isolates the inoculums from a frozen stock culture or other source onto agar plate appropriate for the QC organism.

Following this streak isolation, a second streak isolation on the appropriate media is recommended.

B. Non-QC organisms.

A. Use growth on tubes or plates to perform streak isolation on BAP or NA warmed to room temperature. A second streak isolation step is not required unless there is evidence of a mixed culture.

C. For cultures used on BCL and GN cards, incubate cultures for 18-24 h at 36±1°C. For cultures used on GP cards, incubate cultures for 12-48 h at 36±1°C. For cultures used on ANC cards, incubate cultures under anaerobic conditions for 18-24 h (or until sufficient growth is obtained) at 36±1°C. All organisms to be identified must be pure cultures.
D. Perform Gram stain using an isolated colony from a pure culture plate from section 12.4b and document the Gram stain reaction

Preparation of inoculums

a. Select the appropriate card based on the Gram stain reaction and the organism's microscopic appearance. Allow the card(s) to come to room temperature before opening the package liner.

Aseptically transfer at least 3 mL of sterile saline into a clear polystyrene 12×75 mm test tube. Using sterile cotton swabs, prepare a homogenous organism suspension by transferring several isolated colonies from the plates to the saline tube. Adjust the suspension to the McFarland standard required by the ID reagent using a calibrated V2C DensiCHEK plus Meter, see below table.

Required Inoculums Concentrations

Card	McF Range
GN	0.5-0.63
GP	0.5-0.63
YST	2.7-3.3
BCL	1.8-2.2

Place the prepared suspensions in the cassette (see section 15, Instrument User Manual).
d. To use the DensiCHEKPlus Meter to read samples:

i. Ensure the instrument is ON and set to the PLASTIC tube setting.
ii. Blank the DensiCHEK Plus by filling a test tube with sterile saline and inserting the tube into the instrument. Press the "0" key and slowly rotate the test tube. Ensure one full rotation is completed before the reading is displayed. The instrument will display a series of dashes followed by 0.00.

iii. To measure a sample, place a well-mixed organism suspension into the instrument and slowly rotate the test tube. Ensure one full rotation has completed before the reading is displayed.

The instrument will display a series of dashes followed by a reading.

iv. Remove the test tube after completion of a reading. The instrument will automatically shut off when test tubes are not inserted after one minute.
NOTE: If the instrument flashes 0.00 or 4.00, the suspension is either below 0.0 McF or above 4.0 McF and is not within the reading range. Ensure suspensions are within the appropriate reading range to avoid compromised card results. If necessary, re-calibrate the DensiCHEK Plus instrument after processing each cassette.

E. Insert the straw (in the V2C card) into the inoculated suspension tube in the cassette.

NOTE: The age of the suspension must not exceed 30 minutes before inoculating the cards.
f. Proceed to data entry.

Filling the Cards

a. Place the cassette in the Filler box on the left side of the V2C unit and hit Start Fill button on the instrument. Filling the cards takes approximately 70 seconds for a cassette regardless of the number of cards in the cassette holder. The V2C instrument will beep when the filling cycle is complete.

i. Discard individual cards that may have been exposed to multiple fill cycles. NOTE: The cassette must be placed inside the Loader Door within 10 minutes from the end of the filling cycle to avoid the cards being rejected.

b. When the cards are finished filling, the Load Door is automatically unlocked. Place the cassette in the Load Door. The V2C Instrument will verify the scanned barcodes against the Virtual Cassette (the information scanned in by the analyst). Cards are sealed, straws are cut and the cards are loaded automatically into the carousel. The V2C will beep once all cards are loaded into the cassette.

c. When the cards are loaded, remove the cassette and dispose of the tubes and straws in a biohazard container.

d. The V2C automatically processes the cards once all the cards are loaded. NOTE: If the cassette status description in the Navigation Tree is red, the cassette needs more information to completely process the tests cards. Open up the red colored file and make sure all fields are defined.

Results

The VITEK system analyses the data results and determines the identity of the test microbes/QC organism based on colorimetric tests (biochemical reactions).

Results are concurrently printed and the data sent to the Results View folder on the left side of the screen also called the Navigation Tree where the information is archived. A red cassette in the Navigation Tree is indicative of an error.

If an error occurs during processing, refer to the Software User Manual.

Annex VI: Assurance of Principal Investigator

I the undersigned agree to accept all responsibilities for the scientific and ethical conduct of the research project. I was providing timely progress report to my advisor and seek the necessary advice and approval from my primary advisors in the course of the research. I was communicating timely to my advisors all stakeholders involved in the study including any source of funding for this research.

EstifanosTsige [Msc candidate]

Signature---------- Date of submission----------

Advisor: Dr.Adane Bitew [PhD. Associate Professor]

Signature_____ date _____