ANTIMICROBIAL RESISTANCE PATTERN OF GRAM NEGATIVE BACTERIA ISOLATED FROM HOUSEFLIES IN JIMMA UNIVERSITY MEDICAL CENTER, SOUTHWEST ETHIOPIA

BY: WONDWOSSEN TADESSE (BSc)

A RESEARCH PAPER TO BE SUBMITTED TO JIMMA UNIVERSITY, INSTITUTE OF HEALTH, FACULTY OF HEALTH SCIENCE, SCHOOL OF MEDICAL LABORATORY SCIENCES FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENT OF MASTER OF SCIENCE DEGREE IN MEDICAL MICROBIOLOGY

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By: WONDWOSSEN TADESSE (BSc)

Advisors:

1. **Mr. MULATU GASHAW (MSc in Medical Microbiology,PhD Fellow)**
2. **Mrs. RAHEL TAMRAT (MSc in Medical Microbiology)**
3. **Prof. ESAYAS KEBEDE (MD, DTM and H, PhD)**

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Abstract

Introduction: *The spread of drug resistant bacteria arises by different factors in the hospital settings including environment, specifically sanitation and hygiene, inturn such places could be visited by different arthropod vectors like houseflies there by microorganisms mechanically picked by the houseflies. Having knowledge and understanding of such elements is essencial to identify any modifiable interaction to decrease or intrupt the transmission of resistance from the environment in to the hospital settings. Hence, this study aimed to determine antimicrobial resistance pattern of gram negative bacteria isolated from houseflies in Jimma Medical Center.*

Materials and Methods: *A cross-sectional study was conducted from June 2021 to October 2021G.C. One hundred ninety-two houseflies were collected using a homemade sweep net. Houseflies were picked by sterile forceps individually then kept in a sterile glass test tube containing 1ml sterile normal saline then brought to Core research laboratory microbiology unit, within 20 minutes. In the laboratory each tubes were well shaken to dislodge the bacteria in to the the tubes. The concentrate was inoculated on to Mackonkey agar using sterile cotton swab and gram-negative bacteria were further identified using biochemical tests. Antibiotic susceptibility testing was performed by using the Kirby Bauer disk diffusion method and results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline . Data were analyzed using SPSS version 25.*

Results: *A total of 186-gram-negative bacterial isolates were recovered from 192 houseflies collected in Jimma Medical center at different sites categorized into three: solid waste area, liquid sewage, and garbage bins, and 64 houseflies were collected from each site. Among the most isolated bacteria; Providencia species, Proteus species, and E.coli contributed 23.1% (43/186), 19.4% (36/186), and 16.7% (31/186) respectively.The predominant 45.7% (85/186) of the isolates were recovered from houseflies collected in solid waste areas and the least isolated was from garbage bins, 18.3% (34/186). The percentage of isolates that were resistant to ampicillin was 97.5% followed by cefuroxime to which 71.6% of the isolates were resistant. There were 65.9% resistances to amoxicillin+clavulac acid, 62.9%, 61.3%, and 60.2% to piperacillin, Trimethoprim+sulfamethoxazole, and cefotaxime respectively. The overall rate of MDR (resistant to at least one agent in three or more antimicrobial class categories) among isolated gram-negative bacteria was 86%. ESBL production was observed in: E.coli, Proteus*

species, E.cloacae, and K.pneumoniae were confirmed to produce ESBL, contributing 58.1% (18/31), 38.9% (14/36), 40% (4/10), and 27.3% (3/11) respectively.

Conclusions: *Houseflies caught within the hospital environment were carrying antibiotic-resistant bacteria. These flies also carry multidrug resistant bacteria and extended spectrum beta lactamase producing bacteria. Antibiotic resistance to amikacin and meropenem was low in the isolated bacteria. A high frequency of resistance was seen in ampicillin, Cefuroxime, and amoxicillin+clavulanic acid.These bacteria are causes of nosocomial infections and are opportunists. Therefore, control strategies for houseflies should be considered in the hospital environment.*

Keywords: *Houseflies, Antibiotics resistant pattern, Gram-negative bacteria, Multidrug-resistant*

Abbreviation

AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ESBL	Extended spectrum beta lactamase
GC	Gregorian calendar
H2S	dihydrogen sulfide
JMC	Jimma Medical Center
JU	Jimma University
KIA	Klinger Iron Agra
MDR	Multidrug resistant
MAC	MacConkey agar
MHA	Muller-Hinton agar
MLS	Medical Laboratory Science
QC	Quality control
SPSS	Statistical Package for Social Sciences
SOP	Standard operating procedure
SIM	Simmons Indole Motility
UK	United Kingdom

CHAPTER ONE: INTRODUCTION

1.1 Background

Multidrug-resistant (MDR) bacterial microorganisms are primary treatment challenges around the world. Such bacteria are carried and transmitted to different environments through various avenues; identification of major avenues involved in disseminating antibiotics resistant bacteria is crucial for a better understanding of antibiotics resistance dissemination and vital for the devising of targeted control strategies for resistance. Flies were one such mechanical vector implicated in carrying MDR pathogens, which are particularly dangerous in hospital settings (1). MDR gram-negative bacteria which are carried by houseflies pose poor treatment outcomes in hospitalized patients. These bacteria are gram-negative non-spore-forming rods which are most frequently found in decaying matter and on/in mechanical vectors (i.e houseflies) (2). *E.coli, K.pneumoniae, Proteus mirabilis, Proteus vulgaris, Citrobacter freundi, Providencia spp, Enterobacter species, and Morganella morganii* are among the gram-negative bacteria most frequently isolated bacteria from flies (1,2).

Gram-negative bacterial isolates were readily identified from hospital area collected houseflies (15) these pathogens are mostly recovered from body surfaces of the houseflies, predominantly bacteria (16). Houseflies found in hospital environments pose a major threat to human health since flies come in contact with hospital wastes constituted of human flesh and organic tissues (17). Therefore, these flies may play a significant role in nosocomial infections. Nosocomial infections impose a huge impact on the treatment outcome of hospitalized patients in terms of extending hospital stay, mortality of hospitalized patients, and treatment costs around the globe both in resource-limited and developed countries (18).

In attempting to control nosocomial infections, it is crucial to determine whether houseflies are mechanical vectors of pathogenic gram-negative bacteria that are producers of extended-spectrum beta-lactamases and resistant to carbapenems. The potential of MDR transmission is increased in gram-negative bacteria since the isolations of MDR gram-negative bacteria are most frequent than MDR gram-positive bacteria from houseflies (1). The gram-negative bacteria carried by houseflies, *Musca domestica*, are potentially incriminated in transmitting pathogens or opportunistic pathogens to hospital settings (3). The development of antibiotic resistance among

clinical bacterial isolates and commensal bacteria of people and animals, as well as bacteria in other habitats, raises a concern that flies may be vector competent not only for specific pathogens but also for nonpathogenic bacteria carrying antibiotic resistance genes (4).

The house fly belongs to superorder endopterygota as its wings develop internally during the pupa stage and exhibits holometabolous metamorphosis by passing through all stages of insect development like egg, larva, pupa, and adult. Houseflies mostly require an ample amount of food and a suitable temperature for their breeding and survival. Therefore, they feed on organic wastes, simultaneously picking up different pathogenic organisms by their appendages and body parts then dislodge these microorganisms to numerous clean areas (5,6).

Houseflies have been associated with the maintenance and dissemination of cephalosporin- and colistin-resistant gram-negative bacteria (7) and are the potential vector of multiple-antibiotic-resistant, pathogenic bacteria, including methicillin-resistant *S.aureus*, in the hospital environment. Given their mobility, it seems likely that houseflies carry such pathogens from hospitals to surrounding communities, and the reverse (8). They carry several multidrug-resistant Gram-negative bacteria in their body surface played a role in the transmission of serious diseases to humans (9).

Houseflies' close relationship with waste, garbage, and people's habitation has led researchers to give due attention to houseflies' role in the transmission of infectious diseases. Therefore, these vectors have been incriminated for the spread of bacterial infections (10) representing a huge public health threat at times when they have access to hospitals and land on human food (11,12). Houseflies, *Musca domestica* can mechanically carry different types of microorganisms including bacteria, viruses, fungi, and parasites. Careless and indiscriminate disposal of waste has increased the houseflies population (13). Flies are capable vectors for the dispersion of antibiotic-resistant bacteria to various environments (14).

The housefly, *Musca domestica*, transmits various pathogenic bacteria via their external body surfaces- body hairs, appendages, mouthparts, and vomitus. The behavior of houseflies creates a conducive environment for the spread of bacteria. Such behavior of the housefly is not surprising when considering their habit of being attracted to filthy environments. Since they move far from their resting places to their destinations houseflies are not only a nuisance but also are

mechanical vectors of potentially dangerous microorganisms that pose threat to human health (19).

1.2 Statement of the problem

The world is facing an unbearable problem related to antibiotics resistance that is responsible for the mortality of 700,000 people per year around the globe and it is estimated that the mortality will increase up to 10 million people per year by 2050 unless efforts are made to decrease the resistance or new antibiotics are available (22). This problem is huge in the case of Gram-negative rods since there are limited antibiotics in the pipeline than Gram-positive cocci as some potent and novel antibiotics are available (23). In the modern health system, medically significant bacteria are not well known for their resistance to single antibiotic resistance but also for their multiple antibiotics resistance (24).

Gram-negative bacteria, such as *E.coli* and *K.pneumoniae* are pathogens for humans and are becoming resistant dramatically to the available antibiotics including carbapenems which are reserved for severe infections as a last-resort treatment option. Mostly beta-lactamase genes found on mobile genetic elements are at first hand in the resistance process. The causes of antibiotic resistance are many, but overuse of antibiotics in humans and animals is vital, and these bacteria circulate in both the hospitals and the community, through food staff, unhygienic hands, and between animals and humans (23).

For the first time, antibiotic resistance was observed in the health care settings causing nosocomial infections and then observed in the community. The worrisome trend is that the increasing bacterial pathogens to multiple antibiotics are observed worldwide in recent years and the magnitude of the problem is alarming when coupled with the antibiotics developed and approved (25).

Controlling antimicrobial resistance is a demanding and complex task that requires the integration of various areas of expertise-infectious disease, molecular biology, human-animal-environmental epidemiology, clinical, health, and social policy to grasp the scope of the problem and embark on the solution. Antimicrobial resistance is identified as One Health problem. Resistance can spread within hospitals, communities, farms, and wastewater systems. It is a well-

established scenario that domestic animals and misuse of antibiotics pave the way for the expansion of antimicrobial-resistant microorganisms. But much attention is not given to arthropod populations in the hospital. 20% of mechanical vectors including flies carry carbapenem resistance bacteria and 80% carry extended-spectrum cephalosporin resistance bacteria from samples obtained in the hospital area (26).

Houseflies, *Musca domestica,* originated on the steppes of central Asia, but are now found all over the world where humans inhabit because of their synanthropic behavior. Houseflies' presence in the hospital, restaurants, and food preparations is not tolerated even in small numbers since it feeds on decaying organic matters and garbage it can transmit microorganisms from such places to the above-mentioned settings. The most significant medical damage related to these flies is the potential mechanical transmission of pathogenic organisms, which are picked from garbage, sewage, and other sick sources by their body, mouth, and leg parts (27).

Investigations in Germany and Belgium, Europe, revealed that flies act as vectors for numerous pathogens and reservoirs for antimicrobial-resistant microorganisms and flies carry various clinically relevant pathogens mainly via their external body surfaces (30,31). Studies done in Iran found that all bacteria isolated from houseflies as pathogenic (19) and houseflies sampled from hospital dumps could take part in the dispersal of drug-resistant bacteria and increase the possibility of human exposure to antibiotic-resistant bacteria (32). The carriage rate of *K. pneumonia* from houseflies collected in the human hospital was 9.0% and 43.3%, these suggest how much of this environment is contaminated by these microorganisms (33,34). Another study done in the same country found that houseflies collected from hospital environments carry more antibiotic-resistant bacteria than non-hospital collected houseflies, confirming these flies as mechanical carriers of antibiotic-resistant bacteria (35).

A study done in Iraq revealed the isolation and identification of *E.coli, S.typhi, P.mirabilis, Shigella spp., K. pneumonia, C.freundii, Hafnia, and Ser.marcescens* from houseflies samples. These bacteria showed resistance to different levels of resistance to erythromycin, ceftriaxone, amikacin, tetracycline, Trimethoprim + Sulfamethoxazole, and Chloramphenicol (36). Research conducted in Thailand revealed that bacteria recovered from houseflies showed resistance to multiple antibiotics including cephalothin, ampicillin, amoxicillin, cefotaxime, ciprofloxacin, meropenem, and concluded that *E.coli* dissemination in a hospital environment as high (37).

Another study that strengthen this conclusion was a study done in Japan drawn a similar conclusion as houseflies are threatening the healthcare outcome by taking part in disseminating pathogenic bacteria (38).

Investigations done on houseflies in South and North America concluded that these flies have the ability and means of inserting, taking out, or maintenance of antimicrobial resistance in any environment and are highly recommended for studying strains recovered from external surfaces of these flies since they come in contact with numerous surfaces (39). Of the bacterial isolates recovered from these flies majority of them were human pathogens (40). and are multidrug-resistant strains posing a great risk as far as public health is concerned (41). Among the bacteria isolated from the houseflies, *E.coli* and *K. pneumoniae* were identified to be extended-spectrum beta-lactamase producers (42).

Studies done in Nigeria, Africa, identified bacteria isolates that are multidrug resistance disease-causing organisms from houseflies establishing the fact that houseflies are mechanical vectors to various disease-causing organisms that pose a health risk to children, the elderly, and immune-compromised people (43). The most isolated bacteria were *E. coli, K. pneumonia, S. aureus, and P. aeruginosa.* The study Concluded that houseflies collected from the hospital environment may participate in the dissemination of disease-causing and antibiotics resistance bacterial strains (44).

In Assela, Ethiopia a study was able to show multidrug-resistant bacterial species collected from houseflies in different sites in a teaching hospital, including the Neonatal intensive care unit, orthopedic ward, and waste disposal area. Hence, the extended-spectrum beta-lactamase (ESBL) multidrug resistance production was 82%, 90%, and 57% respectively (33). Therefore, the problem of antibiotic-resistant bacteria carriage by houseflies became a major challenge in the hospital and community settings. However, the resistance rate is higher in the hospital than in community settings (35).

1.3 Significance of the study

Antibiotic resistance has become a global issue for quite sometimes and new strains of bacteria that are highly drug-resistant are being reported from different places of the world. This study has provided more comprehensive data on the isolates of antimicrobial-resistant gram-negative bacteria on houseflies at Jimma Medical Center. Addressing the problem of antibiotics resistance rate of gram-negative bacteria isolated from houseflies in the hospital environment is vital for understanding the magnitude of the problem and helps to look futher for the factors that are contributing the increment of antibiotics resistance. And also this study addressed multidrug-resistant bacteria isolated from the houseflies in the study area which is strong evidence of drug resistance is a major problem. Hence, it is essential to take appropriate measures to control these mechanical vectors by eliminating their resting places. This study provided scientific information to monitor the environment and hospital sanitary conditions. Furthermore, the finding of this study will be used as current information in the area of antibiotic-resistant gram-negative bacteria isolated from houseflies' external surfaces.

CHAPTER TWO: LITERATURE REVIEW

2.1. Antibiotics and their classifications

Antibiotics are products that are capable of completely killing (bactericidal) or inhibiting (bacteriostatic) microorganisms' growth. These products were lifesaving since the 20th century. Antibiotics classification is most commonly classified according to their molecular structures, mode of action, and spectrum of activities. Based on their chemical structures antibiotics are classified as Beta-lactams, Macrolides, Tetracyclines, Quinolones, Aminoglycosides, Sulphonamides, Glycopeptides, and Oxazolidinones (47,48).

2.2. Antibiotics Mode of Action

Antibiotics act on different sites of the bacterial cell or their metabolic processes. Of note, the major ways in which antibiotics exert their potency include cell wall synthesis inhibition, breakdown of membrane structures, nucleic acid synthesis inhibition, inhibition of protein synthesis, and blocking of metabolic pathways (47).

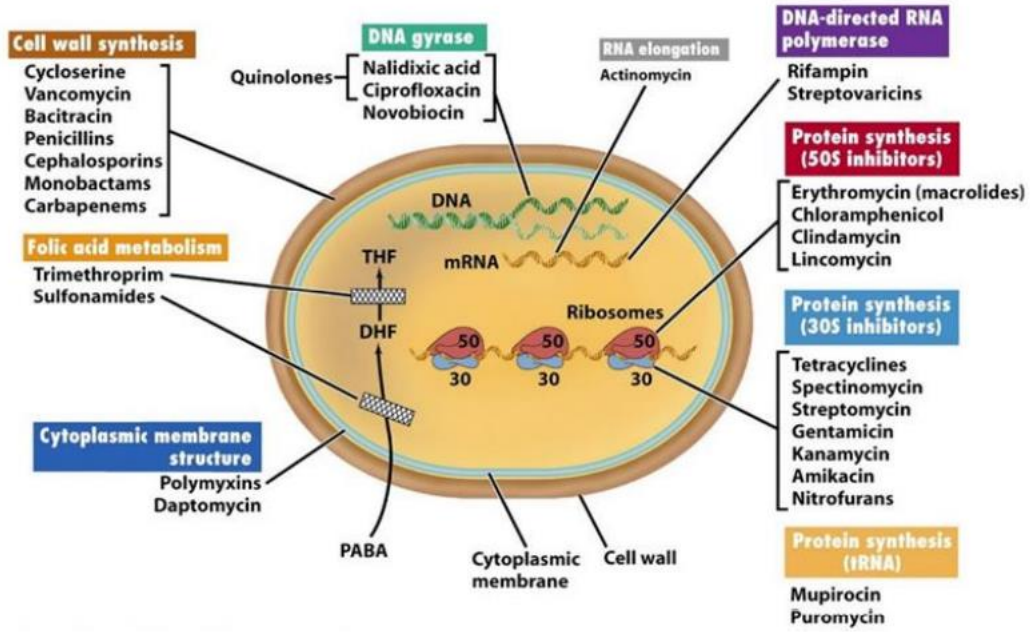

Figure 1. Major target sites of antimicrobial agents (Adopted from Madigan and Martinko 2006)

2.3. Antibiotics resistance

Antibiotics resistance prevails when bacteria alter in some way that decreases or destroys the effectiveness of drugs, chemicals, or agents aimed to cure or prevent infections. Therefore, such resistance including multidrug resistance poses an important public health threat across the world be it in the hospital or community (48,49). Most pathogenic microorganisms can confer resistance to at least some antibiotics. The major resistance mechanisms are limiting drug uptake, target modification, drug inactivation, and active efflux. The origin of resistance could be natural (intrinsic or induced) or acquired. Decreased outer membrane permeability and the natural activity of efflux pumps are common mechanisms of intrinsic resistance. Acquired resistance occurs via the horizontal gene transfer mechanisms (transformation, transposition, and conjugation) and mutation. Generally, Plasmid-mediated transmission of resistance genes is the most common route for the acquisition of outside genetic material (50).

This means that the problem of antibiotics resistance requires the involvement of an array of disciplines including microbiology, molecular genetics, agriculture, and environmental sciences since transmission of resistant pathogens and antimicrobial resistance determinants across different components of the ecosystem transforms antibiotic resistance into a topic that extends beyond the scope of clinical medicine. Knowledge of the complexity of antimicrobial resistance will enable limiting the emergence and distribution of resistant strains and safe utilization of antibiotics (25).

2.4. Housefly's life cycles

Housefly develops its wings internally during the pupal stage and has complete metamorphosis passing through all the stages of insect development: egg, larva, pupa, and adult. Suitable temperature and ample amount of food are requirements for its development. Therefore, they must fly, land, and feed in clean and ill environments since they are indiscriminate feeders. It produces a high number of populations due to high egg production. A female housefly may lay 4-6 hatches and each hatch consists of 75-150 eggs. The life span of an adult housefly is about 15 to 30 days. Just on the day of their emergence, males are ready to mate but mating occurs when a female is three days old. Finally, the pupa changes into an adult house fly within 5 days. In warm climatic conditions, the house fly completes its life cycle from 2-3 weeks. In a year, it may produce 10-12 generations in temperate regions. But in contrast, they may produce 4-6 generations in cold regions where their breeding is limited to warmer months(6,27).

2.5. Prevalence of bacteria from houseflies

A review conducted by Onwugamba et al. pinpointed that flies easily pick antibiotic-resistant bacterial pathogens. The bacteria are resistant to different antibiotic types including extended-spectrum beta-lactamases, carbapenemase-producing, and colistin-resistant (28). Graczyk et al. concluded in their review that Synanthropic flies as the main epidemiological factors for transmission of acute gastroenteritis and trachoma especially in developing regions and houseflies' involvement in the mechanical transmission of nosocomial infection with multiple antibiotic-resistant bacteria in the health care settings (29).

Flies pick up various microorganisms due to their structural fitness, thus adding to their pathogen transmission potential(51). Houseflies were implicated in the carriage of both gram-positive and gram-negative bacteria. The bacteria species which are responsible for causing nosocomial infections that have been isolated from these flies are *E.coli, Pseudomonas aeroginosa, K. pneumoniae*, and *S. aureus* (18,42). Even though, the transmission of these microbes relays on the capability of the flies' specific parts in picking up, retaining, maintaining infectivity of microbes during travel and dislodging the infectious microorganisms on host or surfaces, the exterior and all exposed surfaces of houseflies are potential carriage sites for microorganisms (21).

Geographical origin influences the external bacterial microbiota of houseflies. It's pointing out that house flies carriage of specific bacterial signatures when the sampling area has an ample amount of source bacteria. Therefore, the external body surfaces of houseflies may play a significant role in vectoring a wide variety of environmental and potentially pathogenic microbes (31). Bacterial contamination of houseflies is different based on their collection sites. This may be the reason for the prevalence of bacteria to be higher in flies collected from hospital environments than in non-hospital environments (35).

2.6. Antimicrobial resistance in Houseflies

Arthropods are rarely well-controlled in healthcare facilities in low-income countries. Arthropods have a role in the dissemination of extremely drug-resistant Enterobacterales that have clonal links with clinical samples (26). Different investigations have revealed flies' ability in carrying antimicrobial-resistant bacteria. Multidrug resistance including phenotypes of clinical interest, extended-spectrum beta-lactamase (ESBL), and carbapenemase-producing gram-

negative bacteria have been spread by flies in different parts of the world. Of note, resistance genes for different antibiotics can be transmitted via mobile genetic elements and these mobile genetic elements can be carried by houseflies between and/or among various environments considering the strong flying ability of these flies(28).

Bacteria isolated from flies showed different degrees of resistance to the antibiotics tested. A study, of the isolated *Pseudomonas aeruginosa, Salmonella spp, and Proteus mirabilis* showed multiple resistance to different classes of antibiotics including streptomycin, cotrimoxazole, augmentin, and amoxicillin (44). Houseflies also harbor ESBL and carbapenem (i.e. the last resort antibiotics) resistance bacteria (9,42). Isolates recovered from houseflies collected in the hospital environment are more resistant to different antibiotics than non-hospital (34,35).

2.7. The medical importance of antimicrobial resistance in houseflies

Houseflies, *Musca domestica*, prevention, and control are very important given human health concerns. The most medical significance is that these flies play their part as a vector of potentially pathogenic microorganisms transmission including bacteria, viruses, and fungi. These flies pick pathogenic organisms from different unhygienic places such as garbage and sewage then transfer them to human food via their contaminated external surfaces. Such contaminated houseflies pose a serious health risk if there are nearby outdoor food markets, hospitals, and slaughterhouses. Of note, potential medical pathogens like *Salmonella* spp, *Shigella* spp, *Campylobacter* spp, *Enterococcus* spp, and *Chlamidia* spp are carried by houseflies. These flies are implicated in the outbreaks of diarrhea, shigellosis, food poisoning, typhoid fever, dysentery, tuberculosis, anthrax, and ophthalmia (27,28).

The most pathogenic and nosocomial infection-causing bacteria like *P. aeruginosa*, *E. coli,* and *S. aureus* are also carried by houseflies (18). Of note, the flies collected from hospitals may play in the distribution of pathogenic and drug-resistant bacteria (34). Therefore, increases the burden of drug-resistant bacteria in the hospital environment.

2.8. Antibiotics resistance in the hospital environment

Many low- and middle-income countries (LMICs) are particularly vulnerable to the antibiotic resistance crisis. This is because of, for example, limited surveillance and diagnostic opportunities, less-controlled use of antibiotics in both humans and animals, overcrowding in hospitals, insufficient hygiene control, The environmental dimensions can also be more

important in these regions, for example, as a consequence of inferior infrastructure for managing waste, leading to greater environmental contamination by resistant bacteria. Resolving the resistance crisis is needed. Strategies to improve water quality, sanitation and hygiene also needed (59).

The overuse and abuse of antibiotics have contributed to the global epidemic of antibiotic resistance. Current evidence suggests that widespread dependency on antibiotics and complex interactions between human health, animal husbandry and veterinary medicine, have contributed to the propagation and spread of resistant organisms. The lack of information on pathogens of major public health importance, limited surveillance, and paucity of standards for a harmonised and coordinated approach, further complicates the issue. Despite the widespread nature of antimicrobial resistance, limited focus has been placed on the role of environmental factors in propagating resistance. There are limited studies that examine the role of the environment, specifically water, sanitation and hygiene that contribute to the development of resistant pathogens. Understanding these elements is necessary to identify any modifiable interactions to reduce or interrupt the spread of resistance from the environment into clinical settings (58).

The damage caused by nosocomial infection because of multidrug-resistant is serious in terms of poor treatment outcomes and increased hospital stay. Most of the studies done on multidrug-resistant organisms originate from clinical samples; the laboratory should have the ability to perform environmental surveillance, as cultures from the environment may be necessary to fully define the flora of a particular hospital area. The first step in the process of controlling resistance is to have a clear understanding of the resistance patterns of the different hospital ecosystems (21). Therefore, in Ethiopia, specifically in the study area there is a paucity of data about houseflies' carriage of antimicrobial resistant (AMR) gram-negative bacteria more comprehensively from hospital area. Hence, investigation of determining antimicrobial resistance pattern of gram-negative bacteria isolated from houseflies collected in Jimma medical center, Southwest Ethiopia was undertaken.

CHAPTER THREE: OBJECTIVS

3.1. General objective

To determine profile and antimicrobial resistance pattern of gram-negative bacteria isolated from houseflies in Jimma Medical Center, southwest Ethiopia.

3.2. Specific objectives

To determine the bacterial profile of gram-negative bacteria isolated from houseflies in Jimma Medical Center

To assess the magnitude of multidrug-resistant gram-negative bacteria isolated from houseflies in Jimma Medical Center.

To determine the prevalence of potential extended-spectrum beta-lactamase and carbapenemase-producing gram-negative bacteria isolated from houseflies in Jimma Medical Center.

CHAPTER FOUR: METHOD AND MATERIALS

4.1. Study area

Jimma Medical Center (JMC) is one of the oldest public hospitals in the country. It was established in 1930 E.C by Italian invaders for the service of their soldiers. Geographically, it is located in Jimma town 352km southwest of Addis Ababa the capital of Ethiopia. After the withdrawal of the colonial occupants, it has been governed under the Ethiopian government by the name of Ras Desta damtew hospital and later Jimma hospital during dergue regime after the downfall of the dergue until 2009 E.C the name was Jimma University specialized teaching hospital and currently its name is changed to Jimma university medical center (52).

JMC has a catchment population of about 15 million people that is expected to serve. The center has an annual out-patient caseload of 160, 000 and 45, 000 in-patients. It provides services to a diverse population from three regional states; namely, Oromia, Southern Nations, Nationalities and Peoples, and Gambella.

It has a total of 1846 workers of which 827 are technical staff (health professionals), 766 are administrative staff and the remaining 253 are temporary staff. JUMC is categorized into different departments (units) which is suitable for service provisions like the medical ward, surgical ward, pediatrics ward, maternity ward, gynecology ward, stroke unit, major operation room (OR), minor OR, neonatology ward, oncology ward, dialysis unit, psychiatry ward, maxillofacial ward, orthopedic OR, maternity OR, ophthalmology ward, ART Unit, chronic illness/derma ward, physiotherapy unit, gynecology OPD, pediatrics OPD, EOPD, cold OPD, CSR unit, Recovery unit, orthopedics ward, dental unit, ICU unit, Endoscopy unit and sexual violence clinic(52)

The geographical coordinates of the town are approximately 7◦41’N latitudes and 36◦ 50’E longitude. The town is located at an average altitude of 1,780 meters above sea level. The town is generally characterized by a warm climate with a mean annual maximum temperature of 30 ◦C and a mean annual minimum temperature of 14◦C. Annual rainfall ranges from 1138 to 1690 mm (53).

4.2. Study Design and period

A cross-sectional study was conducted at Jimma Medical Center. The study period was from June 2021 G.C up to Oct. 2021 G.C.

4.3. Study Population

The study populations were houseflies, *Musca domestica*, collected in Jimma Medical Center area and around the hospital.

4.4. Sample size and sampling technique

One hundred ninety-two houseflies were collected by convenience sampling technique.

4.5. Data collection method

The houseflies were captured using a homemade sweeping net and kept in 1ml of sterile normal saline in separate sterile glass test tubes and immediately shipped to the Core research laboratory microbiology unit, located at Jimma University.

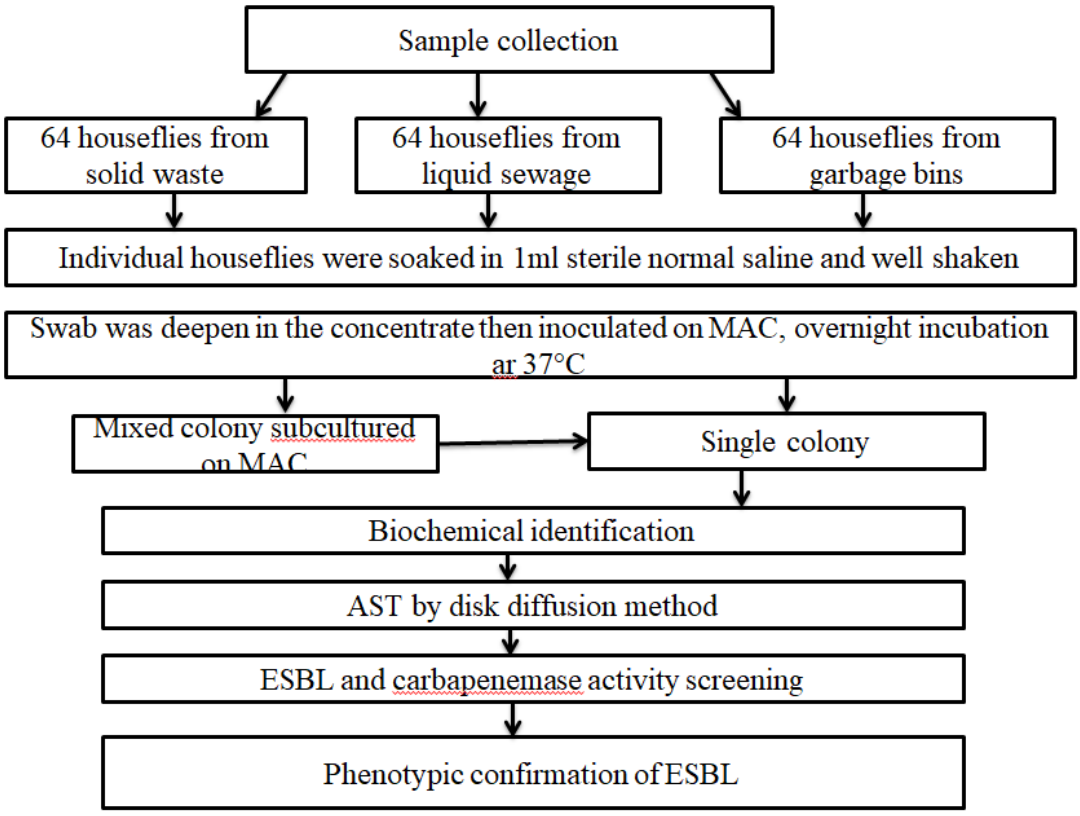

Figure 2. Research workflow

4.6. Houseflies collection by sweeping net

The study sites were categorized into three; two solid waste area, sewage, and garbage bins located in three locations. During sample collection we observed open disposal, unsegregated disposal of solid waste constituted of gloves, food leftovers, catheter bags, syringes, and saline bags and there was water near the disposal site which makes suited for resting and continuing their developmental stages. In the sewage, there was a mixing of solid waste and food leftovers. While we were collecting houseflies from garbage bins we observed open and unclean garbage bins in which houseflies hover and rest on them. A total of 192 houseflies were collected using a homemade sweeping net from 9:00 am to 1:00 pm (32). Sixty-four houseflies were collected from each of the above-mentioned sites in Jimma Medical Center, then houseflies were individually placed in a sterile test tube containing 1.0ml sterile normal saline by using a sterile forceps and brought to the Core research Microbiology Laboratory for further Bacteriological analysis according to protocol done by Ibrahim AW (13).

4.7. Bacteria isolation

In the Core research Microbiology laboratory, the houseflies collected in a tube containing 1.0ml normal saline were washed by shaking the tube for 30 seconds to obtain microbial flora on the external parts of the houseflies into the normal saline then a drop of the mix from each tube was inoculated onto MacConkey (Oxoid UK) agar plates using a sterilized cotton swab and streaked by a sterilized wire loop, and incubated aerobically at 37°C for 16-18 hours. After 16-18 hours of incubation separate colonies were selected and sub-cultured again on MacConkey agar (Oxoid, UK) to get pure cultures. Then MacConkey agar plates were incubated aerobically at 37°C for 16-18 hours. For the maintenance of isolates for further identification of the specific bacteria 16-18 hours of the pure colony were transferred to vials containing aseptically prepared mixtures of skim milk, glucose, glycerol, distilled water, and Tryptic Soy broth and stored at -81 °C (13).

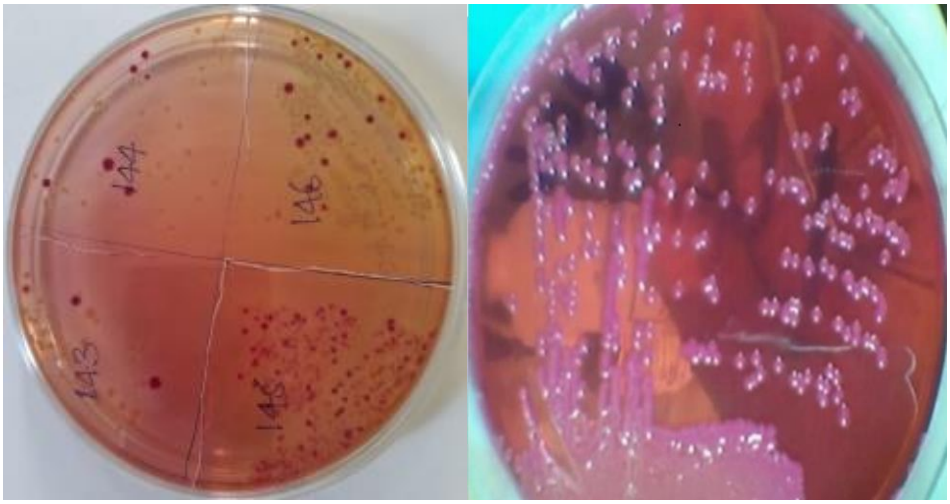

Figure 3. Figure showing gram negative bacteria isolated from houseflies on MacConkey agar in JMC, 2021.

4.8. Bacterial identification

All positive cultures were characterized by colony characteristics, Gram stain, and standard biochemical tests using the WHO guideline for the identification of gram-negative bacilli (54). The isolated bacteria were grouped into a lactose fermenter and a non-lactose fermenter for proper identification by using different biochemical tests. The two groups were characterized to species levels using indole production, Oxidase, H_2S production, gas production, hydrolysis of urea, citrate utilization, lysine decarboxylation, motility test, methyl red, and Voges-Proskauer biochemical tests.

4.9. Antibiotics Sensitivity Test

The Antibiotics sensitivity testing was performed using the Kirby–Bauer disc diffusion method following the recommendation of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (55). The inoculum suspension was prepared from pure colonies which were 16-18 hours old on a MacConkey agar and 3-to 5 selected colonies of bacteria were taken and transferred to a tube containing sterile normal saline to make homogenous suspension and the turbidity of the suspension was adjusted comparably to 0.5 McFarland standard.

The suspension density was measured by Densi check which has been calibrated with a McFarland standard based on the manufacturer's instructions. A sterile cotton swab was used to inoculate the plates and the excess suspension was removed by gentle pressing and rotation of the swab against the inside wall surface of the tube. Then the swab was used to distribute the

bacteria evenly over the entire surface of Mueller Hinton agar (MHA) (Oxoid, UK). The inoculated plates were left at room temperature to dry for 3-5 minutes.

The isolated bacteria was tested against the following antibiotics namely Ampicillin (10 µg), Amoxicillin-clavulanic acid (30 µg), Amikacin (30 µg), Ceftazidime (30 µg), Ciprofloxacin (5 µg), Cefotaxime (30 µg), Cefuroxime (30 µg), Cefepime (30 µg), Cefoxitin (30 µg), Gentamicin (10 µg), Meropenem (10 µg), Moxifloxacin (5 µg), Piperacillin (100 µg), Trimethoprim+sulfamethoxazole (1.25+23.75 µg), Tobramycin (10 µg), Ceftraxone (30 µg), and Piperacillin+tazobzctam (100+10 µg) were firmly placed. Within 15 minutes of antibiotics discs application, then plates were incubated in an inverted position at 37 °C aerobically for 16-18 hours. After incubation, zones of inhibition were read where there was no obvious growth. The inhibition zone diameters were measured to the nearest millimeter by the investigator (55).

Interpretation of the results was based on the EUCAST clinical breakpoint table as resistant, and sensitive. Intermediate results were also considered resistant. (55). Extracted data were recorded in an excel sheet, and bacterial isolates were considered MDR when they showed resistance to at least one antibiotic in three or more antibiotics classes.

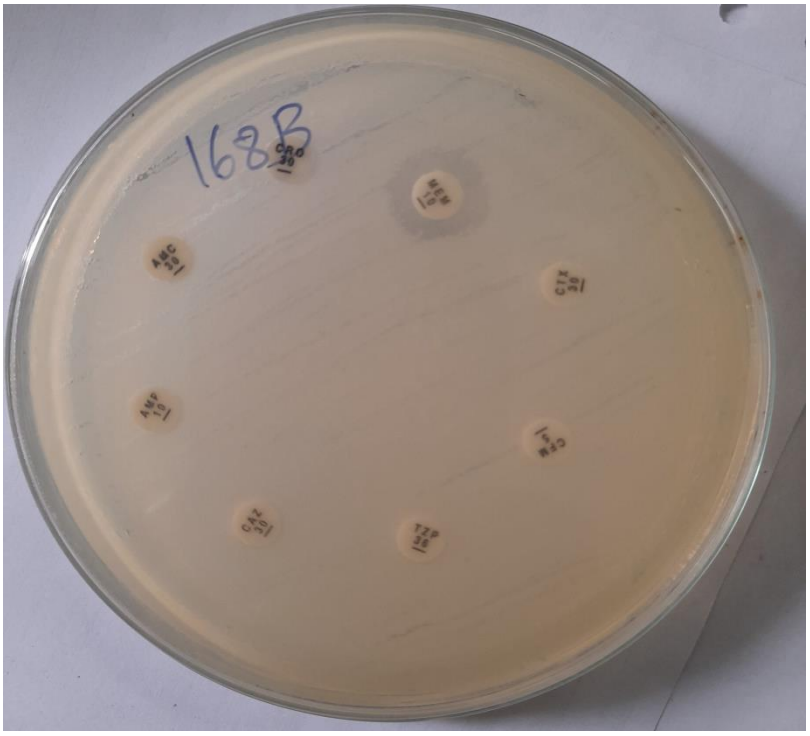

Figure 4. Image showing antimicrobial sensitivity of bacteria isolated from houseflies in JMC, 2021.

4.10. Screening of Extended spectrum beta lactamase producing bacteria

Isolates which showed decreased susceptibility and/or resistance to ceftriaxone, cefotaxime, and ceftazidime by Kirby-Bauer disk diffusion method were considered for ESBL screening. If the zone of inhibition is less than or equal to 22 mm for ceftazidime, less than or equal to 27 for cefotaxime and less or equal to 25 mm for ceftriaxone they were considered as potential ESBL isolates and selected for further phenotypic confirmatory test as described below.

4.11. Phenotypic confirmation of Extended spectrum beta lactamase production

The presence of an ESBL and/or AmpC was determined with Cefpodoxime (10 µg), Cefotaxime (30 µg), Cefepime (30 µg) and Ceftazidime (30 µg) containing antibiotic discs (Mast Group ltd, UK) by disc diffusion confirmation test. For those isolates that were resistant to Ceftiaxone, Ceftazidime and/or Cefepime: disc A (Cefpodoxime), discB (Cefpodoxime +ESBL inhibitor), disc C (Cefpodoxime + Ampc inhibitor), discD (Cefpodoxime + ESBL inhibitor + AmpC inhibitor) were used to determine presence of an ESBL and/or AmpC. After inoculating plates with direct suspension of colony equivalent to turbidity of 0.5 McFarland the four discs (A, B, C and D) were placed at a distance of 24mm apart to each other from center to

center, and then they were incubated at 37 °C for 18–24 hrs aerobically. Finally, zones of inhibition were read and recorded on excel sheet. The data from the excel sheet was transported to Mast group ESBL/AmpC and CARBA plus calculator spreadsheet (Mast group, UK) and reported as negative or positive for ESBL or/and AmpC and finally the results were recorded.

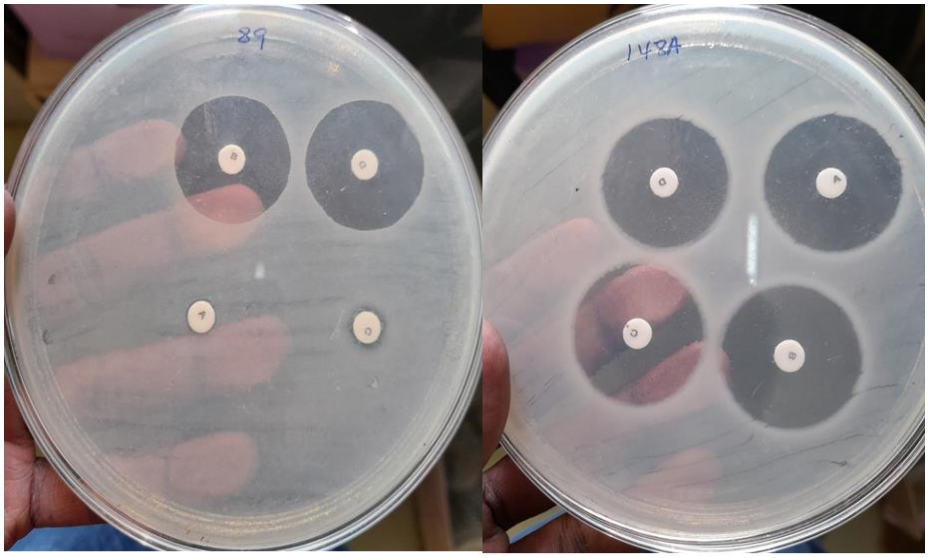

Figure 5. image showing Phenotypic confirmation of ESBL and AmpC production from houseflies collected in JMC, 2021.

4.11. Data Entry, Processing, and Analyzing

The collected and laboratory-generated data were entered into Epi-Data software v4.6.0.6, processed, and analyzed using the SPSS version 25.0 computer software. Types and resistance patterns of the bacterial isolates with the respective antibiotics were calculated by SPSS, and graphs were generated by excel. Data was presented in descriptive measures such as tables, figures, and percentages.

4.12. Data Quality Assurance

The reliability of the study findings was guaranteed by implementing Quality control (QC) measures throughout the whole process of the laboratory work. All materials and equipment were sterilized before the procedure. Standard operating procedures (SOPs) were applied during the three steps of laboratory work (pre, analytical and post-analytical) to evaluate and monitor the quality of the test result and to maintain a high standard of accuracy of the result. The manufacturer's instructions were followed for different media preparation strictly. From the prepared media, 5% were incubated at 37°C for overnight to see if there is any contamination or

not. *K.pneumoniae* ATCC 700603 (positive control) and *E. coli* ATCC 25922 (negative control) were used for ESBL detection. Data was double entered to ensure the quality of output information.

4.13. Ethical Consideration

Ethical clearance was obtained from the Ethical Review Board of Jimma University, Institute of Health. A letter of support was obtained from Jimma University School of Medical Laboratory.

4.14. Result Dissemination Plan

- Results will be presented and discussed with Jimma University (JU), Institute of Health, School of Medical Laboratory Science (MLS) staff, and students.
- The final result will be submitted to JU, Institute of Health, School of Medical Laboratory Science (MLS), and Jimma Medical Center management offices.
- Finally, the manuscript will be prepared and published in a peer-reviewed reputable journal

CHAPTER FIVE: RESULTS

5.1. Distribution of Gram-Negative bacterial isolates

A total of 186-gram-negative bacterial isolates were recovered from 192 houseflies collected in Jimma Medical center at different sites categorized into three: solid waste area, liquid sewage, and garbage bins, and 64 houseflies were collected from each site. The genera of *Providencia, Proteus, Escherichia, Klebsiella, Morganella, and Enterobacter* presence were confirmed. Among the most isolated bacteria; *Providencia* species, *Proteus* species, and *E.coli* contributed 23.1% (43/186), 19.4% (36/186), and 16.7% (31/186) respectively. Furthermore, non-lactose fermenting bacteria, *Acinetobacter* spp, and *Pseudomonas* spp were isolated (Figure 5, Table 1).

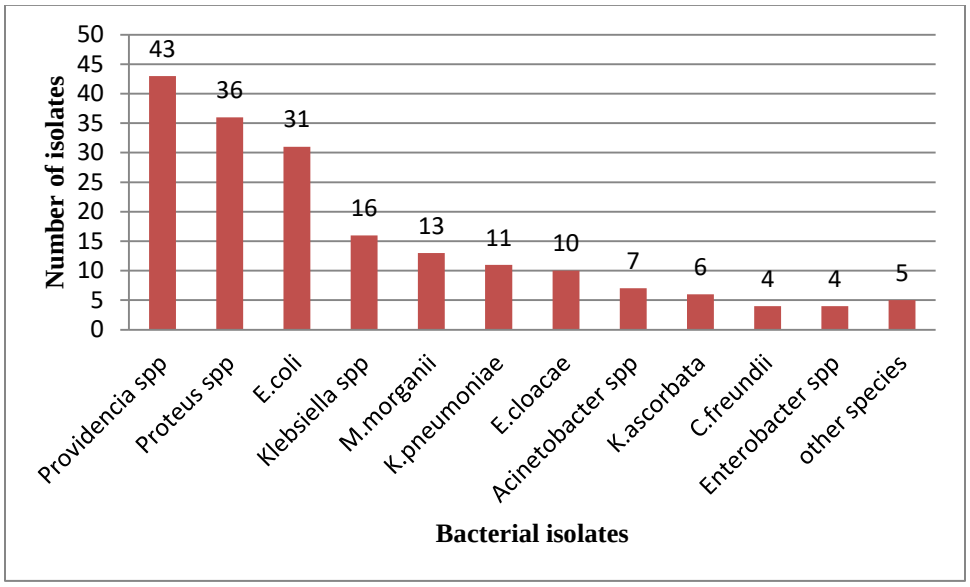

Figure 6. Frequency distribution of gram-negative bacteria isolated from houseflies collected in JMC, 2021. Note: other species include isolates of *Pseudomonas spp (n=2), S.maltophilia(n=1),* and two isolates of *Escherichia spp*.

5.2. The recovery rate of gram-negative bacterial isolates from different sampling sites

Of the total 186 gram-negative bacteria isolated the predominant 45.7% (85/186) of the isolates were recovered from houseflies collected in solid waste areas and the least isolated was from garbage bins, 18.3% (34/186). Of which, the most frequent isolated bacteria was *E.coli* 12.4% (23/186) isolated from houseflies collected from the solid waste area, and 3.2% (6/186) isolated

from garbage bins. While that of *Providencia* species 9.7% (18/186), and *Proteus* species 8.1% (15/186) were isolated from liquid sewage area collected houseflies (Table 1).

Table 1. Gram-negative bacteria isolated from houseflies collected at different sampling sites in JMC, 2021.

Isolates	Solid waste (n/%)	Liquid sewage (n/%)	Garbage bins (n/%)	Total (n/%)
Providencia species	17 (9.1)	18 (9.7)	8 (4.3)	43 (23.1)
Proteus species	12 (6.5)	15 (8.1)	9 (4.8)	36 (19.4)
E.coli	23 (12.4)	2 (1.1)	6 (3.2)	31 (16.7)
Klebsiella species	9 (4.8)	4 (2.2)	3 (1.6)	16 (8.6)
M.morganii	7 (3.8)	4 (2.2)	2 (1.1)	13 (6.9)
K.pneumoniae	6 (3.2)	2 (1.1)	3 (1.6)	11 (5.9)
E.cloacae	2 (1.1)	7 (3.8)	1 (0.54)	10 (5.4)
Acinetobacter species	1 (0.54)	5 (2.7)	1 (0.54)	7 (3.8)
K.ascorbata	1 (0.54)	5 (2.7)	0 (0.0)	6 (3.2)
C.freundii	4 (2.2)	0 (0.0)	0 (0.0)	4 (2.2)
Enterobacter species	2 (1.1)	2 (1.1)	0 (0.0)	4 (2.2)
Other species	1 (0.54)	3 (1.6)	1 (0.54)	5 (2.7)
Total	85 (45.7)	67 (36.0)	34 (18.3)	186(100)

5.3. Antimicrobial-resistant pattern of Gram-negative bacterial isolates

Antibiotic resistance patterns of the isolated bacteria are presented in table 2. The percentage of isolates that were resistant to ampicillin was 97.5% followed by cefuroxime to which 71.6% of the isolates were resistant. There were 65.9% resistances to amoxicillin+clavulac acid, 62.9%,61.3%, and 60.2% to piperacillin, Trimethoprim+sulfamethoxazole, and cefotaxime respectively. Few isolates showed low resistance to amikacin (4.3%), and meropenem (9.6%). Resistance to meropenem was observed in 32.3% of *E.coli*, 25% of *C.freundii*, 10% of *E.cloacae*, and 7.7% of *M.morganii* respectively (table 2).

Table 2. Percentage resistance of gram-negative bacteria isolated from houseflies collected from hospital sampling sites in JMC, 2021.

Isolates	**AM**	**AMC**	**AN**	**CAZ**	**CIP**	**CTX**	**CXM**	**FEP**	**FOX**	**GM**	**MEM**	**MXF**	**PIP**	**SXT**	**TM**	**TZP**
Providencia species(n=43)	93	100	0	58	37.2	65.1	N	39.5	16.3	37.2	0	60.5	60.5	60.5	30.2	11.6
Proteus species(n=36)	100	19.4	5.6	30.6	69.4	69.4	97.2	66.7	0	69.4	0	80.6	61.1	75	66.7	0
E.coli (n=31)	100	80.6	6.5	83.4	70.9	77.4	100	77.4	45.2	35.5	32.3	77.4	93.5	77.4	54.8	51.6
Klebsiella species(n=16)	100	37.5	0	37.5	37.5	43.8	100	31.3	6.3	31.3	0	50	93.8	50	31.3	12.5
M.morganii (n=13)	100	100	0	30.8	38.5	30.8	N	15.4	61.5	15.4	7.7	53.8	15.4	30.8	15.4	0
K.pneumoniae (n=11)	100	72.7	0	63.7	54.5	72.7	100	63.7	18.2	45.5	0	54.5	90.9	72.7	45.5	36
E.cloacae (n=10)	100	100	20	70	40	90	N	100	100	80	10	60	90	80	70	30
Acinetobacter species(n=7)	N	57.1	0	0	85.7	42.9	85.7	0	14.3	28.6	0	14.3	0	42.9	28.6	0
K.ascorbata (n=6)	100	33.3	0	33.3	16.7	50	0	50	0	50	0	33.3	50	66.7	33.3	16.7
C.freundii (n=4)	100	75	0	75	50	50	50	50	75	25	25	50	75	75	50	50
Enterobacter species(n=4)	100	75	0	50	50	50	N	25	100	75	0	75	25	25	25	0
Other species(n=5)	80	40	20	60	40	80	40	60	20	40	40	20	100	80	60	40

Total (n=186)	97.5	65.9	4.3	49.4	49.2	60.2	71.6	48.3	38	44.4	9.6	52.5	62.9	61.3	42.6	20.7
Note: AM;Ampicillin, AMC;Amoxicillin-clavulanic acid, AN;Amikacin, CAZ;Ceftazidime, CIP;Ciprofloxacin, CTX;Cefotaxime,CXM;Cefuroxime,FEP;Cefepime,FOX;Cefoxitin,GM;Gentamicin,MEM;Meropenem,MXF;Moxifloxacin,PIP;Pepracillin, SXT;Trimethoprim+sulfamethoxazole, TM;Tobramicin, TZP;Piperacillin-tazobactam, N: not done, other species include; , Pseudomonas spp (n=2), Escherichia spp (n=2), & S.maltophilia(n=1).																

5.4. Multidrug-resistant Gram-negative bacteria isolates

The multidrug resistance pattern of isolated bacteria from houseflies collected in the Medical Center is shown in table 3. The overall rate of MDR (resistant to at least one agent in three or more antimicrobial class categories) among isolated gram-negative bacteria was 86%. Out of the total 10 *E.cloacae* isolates tested, all isolates were MDR. While only 3.1% of the isolates were resistant to at least a single antibiotic in only one class of antibiotics.

Table 3. Multidrug resistance level of gram-negative bacteria isolates to different classes of antibiotics in JMC, 2021.

Isolates	Level of resistance (n/%)									
	R1	R2	R3	R4	R5	R6	R7	R8	R9	Total MDR(>=R3)
Providencia species(n=43)	3 (6.9)	3 (6.9)	3 (6.9)	11 (25.6)	8 (18.6)	9 (20.9)	5 (11.6)	1 (2.3)	0 (0.0)	37 (86.0)
Proteus species (n=36)	1 (2.8)	5 (13.9)	4 (11.1)	5 (13.9)	15 (41.7)	6 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	30 (83.3)
E.coli (n=31)	0(0.0)	2(6.5)	3(9.7)	4(12.9)	2(6.5)	5(16.1)	3(9.7)	4(12.9)	8(25.8)	29(93.5)
Klebsiella species(n=16)	0 (0.0)	4 (25.4)	3 (18.8)	3 (18.8)	4 (25)	1 (6.3)	1 (6.3)	0 (0.0)	0 (0.0)	12 (75.0)
M.morganii (n=13)	0(0.0)	1(7.7)	5(38.5)	3(23.0)	1(7.7)	2(15.4)	1(7.7)	0(0.0)	0(0.0)	12(92.3)
K.pneumoniae (n=11)	0(0.0)	2(18.2)	1(9.0)	1(9.0)	3(27.3)	1(9.0)	3(27.3)	0(0.0)	0(0.0)	9(81.8)
E.cloacae (n=10)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	3(30)	4(40)	0(0.0)	2(20)	1(10)	10(100)
Acinetobacter	0(0.0)	1(14.3)	2(28.	4(51.4)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0	6(85.7)

species (n=7)			6)						)	
K.ascorbata (n=6)	1(16.7)	2(33.3)	0(0.0)	2(33.3)	0(0.0)	0(0.0)	1(16.7)	0(0.0)	0(0.0)	3(50)
C.freundii (n=4)	0(0.0)	1(25)	0(0.0)	1(25)	0(0.0)	0(0.0)	0(0.0)	2(50)	0(0.0)	3(75)
Enterobacter species(n=4)	0(0.0)	0(0.0)	1(25)	0(0.0)	2(50)	1(25)	0(0.0)	0)0.0)	0(0.0)	4(100)
Other species (n=5)	0(0.0)	0(0.0)	1(20)	0(0.0)	3(60)	0(0.0)	1(20)	0(0.0)	0(0.0)	5(100)
Total (186)	5 (3.1)	21 (13.1)	23 (14.4)	34 (21.3)	41 (25.6)	29 (18.1)	15 (9.4)	9 (5.6)	9 (5.6)	160 (86.0)

R1= resistant to one class of antibiotics, R2=resistant to two classes of antibiotics, R3=resistant to three classes of antibiotics, R4=resistant to four classes of antibiotics, R5= resistant to five classes of antibiotics, R6=resistant to six classes of antibiotics, R7= resistant to seven classes of antibiotics, R8= resistant to eight classes of antibiotics, R9= resistant to nine classes of antibiotics.

5.5. Screening of the isolates for production of extended spectrum beta lactamase and carbapenemase

Among the total of 186 isolates 116 (62.4%) were screening positive for ESBL production, and 13 (6.9%) were positive for carbapenemase production. The predominant isolates for ESBL production screening positive were *E.coli* 77.4% (24/31), *K.pneumoniae* 72.7% (8/11), while for carbapenemase screening positive were *E.coli* 32.3% (10/31). (Table 4).

Table 4. Gram negative bacterial isolates screening positive for ESBL and carbapenemase production in JMC, 2021.

Isolates	ESBL screening positive	Carbapenemase screening positive
Providencia species(n=43)	28 (65.1)	0 (0.0)
Proteus species (n=36)	23 (63.9)	0 (0.0)
E.coli (n=31)	24 (77.4)	10 (32.3))
Klebsiella species(n=16)	7 (43.8)	0 (0.0)
M.morganii (n=13)	4 (30.8)	0 (0.0)
K.pneumoniae	8 (72.7)	0 (0.0)

(n=11)		
E.cloacae (n=10)	9 (90)	1 (10.0)
Acinetobacter species (n=7)	3 (42.9)	0 (0.0)
K.ascorbata (n=6)	3 (50.0)	0 (0.0)
C.freundii (n=4)	2 (50.0)	0 (0.0)
Enterobacter species(n=4)	1 (25.0)	0 (0.0)
Other species(n=5)	4 (80.0)	2 (40.0)
Total (n=186)	116 (62.4)	13(6.9)

5.6. Phenotypic confirmation of ESBL production

Out of the total screened positive for ESBL 62.4 (116/186); 33.6% (39/116) of isolates were found to be ESBL producers as confirmed using combination disk test method. Of the 62.4% isolates screened positive for ESBL production; *E.coli*, *Proteus species*, *E.cloacae*, and *K.pneumoniae* were confirmed to produce ESBL, contributing 58.1% (18/31), 38.9% (14/36), 40% (4/10), and 27.3% (3/11) respectively.

CHAPTER SIX:DISCUSSION

The high burden of antibiotic-resistant microorganisms, encountered in recent times, in addition to treatment difficulties, make it essential to limit the dissemination of these potential pathogens through houseflies, most importantly in hospital environments (15). Most hospitalized patients carry pathogenic microorganisms. These pathogenic bacteria cost a great deal when they infect people. Thus, assessing houseflies for bacteria carriage and their antibiotic resistance is a significant perspective for public health. The current study analyzes houseflies from a hospital environment as mechanical vectors of antimicrobial-resistant gram-negative bacteria.

Our results confirm the presence of pathogenic gram-negative bacteria on the external surface of houseflies, *Musca domestica*. Therefore, these flies gaining access to garbage, open wound, and contaminated medical equipment can meddle between hygienic and non-hygienic areas in the hospital environment. We identified the presence of many bacterial species on the external surface of houseflies, *Musca domestica*. High frequency of isolated bacteria were *Providencia* species, followed by *Proteus* species, and *E.coli* . Of note, these bacteria are pathogenic and very dangerous for hospitalized patients. In our study, we isolated bacteria genera of the following from houseflies: *Klebsiella, Escherichia, Morganella, Providencia, Kluvyera, Acinetobacter, Enterobacter, Proteus, and Citrobacter.* This observation was comparable with the study reported from Poland and Czechoslovakia (1,45,57) which also isolated genera of *Klebsiella, Escherichia, Providencia, Enterobacter, Proteus,* and *Citrobacter* on the external surface of flies.

In the current study, all isolated gram-negative bacteria can cause human diseases including catheter-related bacteremia, pneumonia, haemorrhagic colitis, wound infection, and resistant neonatal bacteremia (15). In the present study, eleven isolates of *K. pneumoniae and sixteen Klebsiella species* were isolated. Studies reported from India (12) and Iran (33) confirmed the presence of *Klebsiella* species isolated from the hospital environments. The similarity in identifying *Klebsiella* species could be due to sample collection sites. *K. pneumoniae* and *E. coli*, are in the family of Enterobacteriaceae, in addition to *Acinetobacter* species and *Pseudomonas* species they are the common etiological microorganisms causing nosocomial infections (1).

In the present study, the carriage rate was 5.9% *K. pneumoniae* 16.7% *E.coli*, 19.4% Proteus species, 8.6% *Klebsiella* species 2.2% *Enterobacter* species were identified from houseflies

collected in the hospital environment. A report of a study done in Iran found the carriage rate of *K. pneumoniae* to be 9.0% (33) and a study from India reported *E. coli* (33.8%); *Klebsiella* spp. (33.8%); *Enterobacter* spp. (13.8%), and *Proteus* spp. (9.2%) isolation from houseflies collected from hospital surgical wards (10). This difference in the carriage rate could be explained by the methodological differences used for the identification of isolate and sample collection site variation. A study done in Libya was able to isolate the serotype of enteropathogenic *E.coli* and enterotoxigenic *E.coli* (8). In our study, we identified thirty-one *E.coli* even though; we have not done serotyping of the species. Studies done in the United Kingdom (15), Iran (35), and Poland (1) reported the presence of *E.coli* isolated from flies.

Antibiotic resistance is a major problem in the world. Nowadays, bacteria are characterized not only by single antibiotic resistance rather than by multiple antibiotic resistance (24). Medically important antibiotic-resistant bacteria are carried and transmitted mechanically by flies (28). In our study, we assessed antibiotic resistance of gram-negative bacteria; *K.pneumoniae, E.coli, M.morganii, Providencia species, Enterobacter species, Acinetobacter species, Proteus species, Kluyvera ascorbata, C.freundii,* and *Klebsiella species.*

In our study, 86% of the isolates were MDR. A study done in the USA reported that 9.0% of bacteria isolated from houseflies were multidrug-resistant (50). In our study, more than ninty percent of *E.coli* isolates showed MDR resistance to more than three classes of antibiotics and were called multidrug-resistant isolates. Resistance to different antibiotic classes was found in most of the isolated *bacteria*.

High percent of *K.pneumoniae* isolates were also Multidrug-resistant except for Amikacin and Meropenem in which all isolates were sensitive to both antibiotics. Of note, these antimicrobial agents are prescribed in clinical settings, thus, the observation of such bacteria being resistant to these agents is very challenging for the proper treatment outcome in cases of houseflies transmitting the bacteria. Houseflies carrying bacteria that are resistant to last-resort antibiotics like that of Meropenem call due attention to the strategy of controlling these flies. In Thailand, a study reported that *E.coli* is resistant to classes of Penicillin, Cephalosporins, folate pathway inhibitors, and carbapenems (37). This similarity of resistance pattern for different classes of antibiotics could be explained by similarity in study sites and the common utilization of such antibiotics in hospitals.

Gram-negative nosocomial pathogens are a major concern of the health care system since some of these microorganisms are showing resistance to all currently available antimicrobial agents. The increased presence of such multidrug-resistant (MDR) bacteria is associated with high morbidity and mortality(57). In our study, we found 100% (10/10) of *E.cloacae*, 93.5% of *E.coli*, and 81.8% of *K.pneumoniae* isolates resistant to multiple antibiotics and are MDR. A study done in the United States of America reported that 35.7% of *E. coli* and 10.8% *of K. pneumoniae* isolates were resistant to one or more antibiotics (42). Such variation in the resistant pattern could be explained by the sites where the flies were collected.

In our study, all isolated gram-negative bacteria were resistant to all antimicrobial agents tested, to a varying degree. *K.pneumoniae* isolates were 18-45% resistant to cefoxitin, gentamicin, tobramycin, and piperacillin+tazobactam, 54-90% resistant to amoxicillin-clavulanic acid, ceftazime, ciprofloxacin, cefotaxime, cefepime, moxifloxacin, trimethoprim+sulamethoxazole, and piperacillin and 100% resistant to ampicillin and cefuroxime. On the other hand, *K.pneumoniae* isolates were 100% sensitive to amikacin and meropenem. We also found that the resistance of *E.coli* for ciprofloxacin was 70.9% and that of *Enterobacte*r species resistance was 10% for meropenem. A study from Bangladesh reported that *E.coli* 66-77% resistance to ciprofloxacin and *Enterobacter* species' resistance to imipenem was 66% (9). Such a slight difference could be explained by methodological differences.

In our study, we found 15.5% of *E.coli* was ESBL producer followed by 12% *Proteus species*, 3.5% *E.cloacae* , and 2.6% of *K.pneumoniae.* A study done in Ethiopia reported that 50% (5/10) of *E.coli* and 44% (8/18) of *Klebsiella* species were confirmed as ESBL producers (45). This difference in the production of ESBL could be explained by sample size, methodlogy and sample collection site variation. Hence, clinicians and nurses may need training on up-to-dated antimicrobial stewardship and the Microbiology laboratory unit should take its part by performing antimicrobial sensitivity testing.

6.1. Limitation of the study

- We are unable to determine the level of co-expression of ESBL and AmpC and carbapenemase production due to unavailability of confirmatory tests for AmpC and carbapenemase.

CHAPTER SEVEN: CONCLUSION AND RECOMMENDATION

7.1. Conclusion

Houseflies caught within the hospital environment were colonized with antibiotic-resistant bacteria. These flies may have the potential in disseminating multidrug-resistant bacteria in hospitals. The most isolated gram-negative bacteria from external surfaces of the houseflies were *Providencia* species followed by *Proteus* species, and *E.coli*. A high frequency of resistance was seen in ampicillin, Cefuroxime, and amoxicillin+clavulanic acid. Almost all isolates were sensitive to amikacin and meropenem. Eighty-six percent of the isolates showed multidrug resistance. These vectors were also carring extended spectrum beta lactamase producing bacteria. Therefore, houseflies control should be considered in the fight against antibiotics resistance.

7.2. Recommendation

Depending on our result the following recommendations are made:

- Responsible body should work towards devising control mechanisms for these vectors to manage the spread of antibiotics resistance.
- Characterization and identification of genes responsible for the resistance should be considered in the future.
- Characterization and genomic sequencing of bacteria isolated from clinical samples and houseflies should be considered to make sure if these flies transmit pathogenic bacteria in the hospital.

References

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Appendices

Appendix I*:* Study protocol

Sampling collection observational checklist

1. What do you observe during houseflies collection from the solid waste sampling site?
    a. Open waste disposal system
    b. Segregation of waste during disposal
    c. Unsegregation of waste during disposal
    d. Water source
2. What do you observe during houseflies collection from the liquid sewage sampling site?
    a. Sewage was mixed with solid waste
    b. Blocked sewage flow
    c. Unblocked sewage flow
3. What do you observe during houseflies collection from garbage bins located in different places of the Medical Center?
    a. Open garbage bins
    b. Closed garbage bins
    c. Unclean garbage bins
    d. Unclean garbage bins

Houseflies' collection procedures

1. The net was held with the hoop.
2. And swung rapidly to capture the specimen.
3. Followed through to force the flies into the very bottom of the net.

4. The wrist was twisted as followed through the bottom of the net hanging over the rim; this entrapped the specimen.
5. Then with caution individual housefly was taken from the net by a pair of sterile forceps and kept in the sterile test tube.
6. Then transported to the microbiological laboratory immediately. The number of collected houseflies was recorded at every collection time and sites

MacConkey Agar media preparation procedure

Principle

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.

Materials

Supplies

1. MAC agar powder (oxoid)

2. Weighing paper

3. Distilled water

4. Spatula

Equipment

1. Balance
2. Autoclave
3. Hot plate
4. Bunsen burner

5. distiller
6. dispenser
7. graduated cylinder
8. flask
9. test tube
10. Ph meter

Procedures

1. Weigh and Suspend 51.5grams of powder in1liter of distilled water
2. Mix thoroughly and heat to boil to dissolve the medium completely with frequent agitation
3. When cool adjust the ph to 7.1$\pm$0.2
4. Autoclave at 15 lbs pressure at (121^{o}c)for 15 minute
5. Cool the medium at 50-55^{o}c
6. Dispense about 20ml of the solution into a sterile Petri dish
7. Allow the medium to solidify label with date and store at room temperature

Nutrient Agar media preparation

Principle

Nutrient Agar is a general-purpose, nutrient medium used for the cultivation of microbes supporting the growth of a wide range of non-fastidious organisms.

Procedure

1. Suspend 28g of nutrient agar powder in 1L of distilled water
2. Heat this mixture while stirring to fully dissolve all components
3. Autoclave the dissolved mix at 121 degree Celsius for 15 minutes
4. Pour nutrient agar into each plate and leave plates on the sterile surface until the agar has solidified
5. Replace the lid of each petri dish and store the plates in the refrigerator

Storage media Preparation

Principle

Storage media is prepared for prolonging the viability of microorganisms for further processing of Microorganisms.

Procedure

1. Measure 75 ml distilled water and 25ml of glycerol
2. Weigh 3g TSB, 0.5 g glucose and 2g skim milk
3. Mix and heat all the above-mentioned ingredients for proper mixing
4. Pour into storage media vials and sterilize at 121 degree Celsius for 15 minutes and store all the storage media in a 2-8 degree Celsius refrigerator by labeling

Muller-Hinton Agar media preparation

Principle

Beef Extract and Acid Hydrolysate of Casein provide nitrogen, vitamins, carbon, and amino acids in Mueller Hinton Agar. Starch is added to absorb any toxic metabolites produced. Agar is the solidifying agent. A suitable medium is essential for testing the susceptibility of microorganisms to sulfonamides and trimethoprim. Antagonism to sulfonamide activity is demonstrated by para-aminobenzoic acid (PABA) and its analogs. Reduced activity of trimethoprim, resulting in smaller growth inhibition zones and inner zonal growth, is demonstrated on medium possessing high levels of thymide. The PABA and thymine/thymidine content of Mueller Hinton Agar are reduced to a minimum, reducing the inactivation of sulfonamides and trimethoprim.

Materials

Supplies

- MHA powder (Oxoid, UK)
- Distilled water
- Flask
- Petri dish
- Graduated cylinder

Equipment

- Balance

- Distiller
- Bunsen burner
- Autoclave
- Hot plate
- PH meter

Procedures

1. Suspend 38 gm of MHA powder & transfer into a flask containing 1000 ml of distilled water.
2. Boil until the powder is completely dissolved.
3. Autoclave at 121^0c for 15 minutes.
4. Mix well and dispense aseptically into a sterile Petri dish.
5. Final PH at 25oc is 7.3 +/- 0.2.

Biochemical tests

- **Urease Test**

Material and equipment

- Urea agar base
- Sterile 40% urea solution
- Distilled water
- Flask
- Sterile graduated cylinder
- Sterile separating funnel
- Screw caped test tube
- Balance
- Distiller
- Bunsen burner
- Autoclave
- Hot plate
- PH meter

Procedure

1. Suspend 24 gm of urea agar base powder & transfer into a flask containing 950 ml of distilled water.
2. Boil until the powder is completely dissolved. Autoclave at 121^0c for 20 minute.
3. Cool to 50oc and aseptically add 50 ml of sterile 40% urea solution and mix well.
4. Dispense into the sterile test tube and allow to set in the slanting position.
5. Keep it in RT to cool.

- **Simmons citrate agar**

Material and equipment

- SCA powder
- Distilled water
- Erlenmeyer flask (pyrex)
- Sterile graduated cylinder
- Screw caped test tube
- Balance
- Distiller
- Bunsen burner
- Agar dispenser
- Autoclave
- Hot plate
- PH meter

Procedure

1. Suspend 24 gm of the SCA powder & transfer into a flask containing 950 ml of distilled water.
2. Mix thoroughly, heat with frequent agitation, and boil to dissolve the medium completely.
3. When cool adjust the media PH to 6.9
4. Dispense 4ml each into the sterile test tube
5. Autoclave at 121^0c for 15 minute.

6. Allow the media to solidify in the slanting position.

C. Lysine decarboxylase test

Materials and equipment

1. LIA powder

2. weighing paper

3. distilled water

4. spatula

5. flask

6. test tube

7. graduated cylinder

8. Balance

9. Autoclave

10. Hot plate

11. Bunsen burner

12. distiller

13. water bath

14. dispenser

Procedure

1. Suspend 34.56grams of LIA powder in1 a liter of distilled water
2. Heat to boiling to dissolve the medium completely
3. Dispense 4-5 ml of the solution into test tubes
4. Autoclave at 15 lbs pressure at (121^{o}c)for 15 minute
5. Cool the tubes in a slanted position to form slant and deep butts

6. Close capes tightly to prevent evaporation

- KIA

Material and equipment

- KIA media powder
- Distilled water
- Erlenmeyer flask (pyrex)
- Sterile graduated cylinder
- Screw caped test tube
- Balance
- Autoclave
- Distiller
- Bunsen burner
- Agar dispenser
- Hot plate
- PH meter
- **Indole production**

  Material and equipment

- Pasteur pipette
- Rubber tit
- Wire loop
- Incubator 37ºC
- Bunsen burner

Procedures

1. Take nutrient Broth tube
2. Take pure colony on MacConkey Agar Plate near Bunsen burner
3. Suspend in Nutrient Broth
4. Incubate at 37ºC incubator overnight
5. Add drops of kovacs reagent
6. Observe the production of red Ring or not

Oxidase test

Material and equipment

- Commercially prepared oxidase strip
- Slides
- Sterile stick or glass loop disposable pipettes
- cotton swabtest tubes

Procedure

1. Soak a piece of filter paper in the oxidase reagent solution
2. Scrap some fresh growth from the plate with a disposable loop or stick & rub on onto the filter
3. Examine for blue color within 10 seconds

Methyl red Test procedure

1. Allow the medium to room temperature
2. Inoculate with pure colony
3. Incubate at 37 degree c
4. Add 2 to 3 drops of methyl red indicator to aliquot
5. Observe for red color immediately

Voges-Proskauer

Procedure

1. Inoculate the medium with pure culture
2. Incubate aerobically at 37 degree c
3. Add 6 drops of 5% alpha-naphthol and mix
4. Add 2 drops of 40% potassium hydroxide mix
5. Observe for pink-red color at the surface within 30 min.

Appendix II: Information sheet

Title of the Research: Antimicrobial resistance pattern of gram-negative bacteria isolated from houseflies in Jimma Medical center.

Name of Principal Investigator: Wondwossen Tadesse

Organization/University: Jimma University

Name of Sponsoring Organization: Wachamo University

Purpose of the study: The purpose of the study is to determine the resistance pattern of gram-negative bacteria isolated from houseflies.

Whom to Contact: Wondwossen Tadesse, +251913784908

Mr. Mulatu Gashaw, +251913629953

Mrs. Rahel Tamirat, +251912128654

Research approval sheet

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

Name of the Student	**Signature**	**Date**
WONDWOSSEN TADESSE (MSc candidate)	_____	_____

Approved by

Name of advisor 1 Signature _____ Date_____

Mr. MULATU GASHAW (MSc, Assistant Professor, PhD Fellow) _____ _____

Name of Advisor 2 Signature Date

Mrs. RAHEL TAMRAT (MSc) _____ _____

Name of Advisor 3 Signature Date

Prof. ESAYAS KEBEDE (MD, DTM and H, PhD) _____ _____

Signature Date

Name of assessor _____ _____ _____

School Head _____ _____ _____

JUNE, 2022

JIMMA, ETHIOPIA