ANTIBIOTIC SUSCEPTIBILITY PATTERNS AND PLASMID PROFILE OF MICROBIAL ISOLATES FROM VEGETABLES COLLECTED IN SELECTED LOCAL MARKET OF JIMMA TOWN, SOUTHWEST ETHIOPIA

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Antibiotic susceptibility patterns and plasmid profile of microbial isolates from vegetables collected in selected local market at Jimma town, southwest Ethiopia.

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Abstract

***Background**: Vegetables can be contaminated with a range of microbial contaminants and pathogens and have long been known to serve as vehicles for transmission of infectious microorganisms* Thus, *this study was aimed to assess antibiotic susceptibility patterns and plasmid profile of microbial isolates from fresh vegetables collected in selected local market at Jimma town, southwest Ethiopia from Feb to April, 2015.*

***Methods**: A cross sectional study design was employed. A total of 150 fresh vegetable samples were purchased in different days from different outlets in Jimma town, from open market and groceries between Feb-April 2015. Equal numbers of vegetable types comprising lettuce, cabbage, carrot, tomato, green pepper, was purchased from farmers and four purposively selected local markets. For microbiological analyses, 25g of sample was aseptically weighed and washed in 225ml of sterile 0.1% (w/v) bacteriological peptone water (Oxoid) for 3 minutes. Appropriate serial dilutions of the suspension were plated on nutrient agar medium. Total plate count and pathogens isolation was done. Antimicrobial susceptibilities test and plasmid profile was performed.*

***Results**: More than 80% of vegetable samples had total viable counts of greater than10^6CFU/g with ranges of 10^5-10^7CFU/g. A total of 102 bacterial isolates of eight genera were identified. Enterobacter spp. (21.60%) was the most dominant followed by Citrobacter spp. (20.6%), Klebsiella spp. (18.6%), Salmonella spp. (11.8%), E. coli (10.8%), Proteus spp. (9.8%), Staphylococcus spp. (4.9%), and Pseudomonas aeruginosa (2%). Ampicillin and amoxicillin were highly resisted by more than 89% of microbial isolates. All the five S. aureus isolates were sensitive to oxacillin and vancomycin but two isolates were intermediately sensitive to erythromycin. Ciprofloxacillin was the least resisted drugs only 3.9 % whereas oxytetracycline was resisted by 31.4 % of the isolates. Resistance to nitrofurantoin, nalidic acid, streptomycin, chloramphenicol, cotrimoxazole, ceftriaxone, kanamycin and Gentamycin were 30.4%, 26.5%, 18.6%, 12.7%, 10.8%, 10.8%, 7.8%, 4.9% respectively. Plasmid was detected in 20 out of 91resistant isolate to at least one antibiotic.*

***Conclusion and Recommendation**: - Occurrence of antimicrobial resistance and plasmid carriage in bacterial populations in fresh vegetables at retails may constitute threats to consumers, possibly via resistance transfer. Thus, these results suggest the necessity to follow the hygienic practices in handling the vegetables.*

Key words: vegetable, microbial isolate, antibiotic resistance pattern, plasmid profile, Jimma

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Table of Contents Page no

Abstract I
ACKNOWLEDGEMENT II
Table of Contents III
LIST OF TABLES V
List of Abbreviations VI
CHAPTER ONE: INTRODUCTION 1
1.1 Background 1
1.2 Statement of the problem 3
CHAPTER TWO: LITERATURE REVIEW 8
2.1 Microbial quality 8
2.2. Microbial isolates 9
2.3 Antibiotic resistance 10
2.3 Screening for plasmids 12
2.4 Significance of the study 13
CHAPTER THREE: OBJECTIVE 14
3.1. General objective 14
3.2. Specific Objectives 14
CHAPTER FOUR: METHODS AND MATERIAL 15
4.1 Study area. 15
4.2 study design and period 15
4.3. Sample Collection and Analysis 15
4.4 Data collection instrument 16
4.5 Data collection process 16
4.5.1 Microbiological analysiss 16
4.5.2 Drug susceptibility testing: 17
4.5.3 Screening for plasmid 17
4.7 Measurement 17
4.7.1 Study variables 17

4.8 Data quality assurance **18**

4.9 Administration monitoring **18**

4.10 Statistical Methods **19**

4.11 Ethical Consideration **19**

4.12 Operational definitions **19**

4.13 Data dissemination plan **20**

CHAPTER FIVE: RESULTS 21

CHAPTER SIX: DISCUSSION 28

Strength and limitation of the study 32

CHAPTER SEVEN: CONCLUSION AND RECOMMENDATIONS 33

7.1 Conclusions **33**

7.2 Recommendations **33**

REFERENCES 35

Annex I:Information Sheet (English Version) 40

Annex-II: Questionnaire 46

Annex VIII: Modified Kirby-Bauer susceptibility testing technique 51

ANNEX: IV Procedure for plasmid isolation 55

LIST OF TABLES

Table 1: Socio-demographic characteristics of vender of vegetables of selected local markets of Jimma town Southwest Ethiopia, 2015 23

Table 2: knowledge and practice related to vegetable farming for farmer respondents of vegetable venders Jimma town south west Ethiopia, 2015. 24

Table 3: Mean total aerobic mesophilic bacterial counts (CFU/g) of fresh vegetables by type of product purchased from selected local markets Jimma town southwest, Ethiopia 2015. 25

Table 4: Frequency distribution of bacterial isolates from fresh vegetables purchased in selected local market of Jimma town southwest Ethiopia, 2015. 25

Table 5: Antibiotic resistance patterns of gram negative rods isolated from fresh vegetables sold in selected local market of Jimma town southwest Ethiopia, 2015..... 26

Table 6: Antibiotic resistance patterns of *S.aureus* isolated from fresh vegetables sold in selected local market of Jimma town southwest Ethiopia, 2015 27

List of Abbreviations

CFU	Colony forming units
FAO	Food and agricultural organization
NA	Nutrient agar
WHO	World health organization

CHAPTER ONE: INTRODUCTION

1.1 Background

Vegetables eaten raw can be contaminated with a range of microbial contaminants and pathogens from soil, animal and human sources at any point during growing, harvesting, sorting, packaging, and storage and have long been known to serve as vehicles for transmission of infectious microorganisms both in developing and developed countries over the past decade. Moreover food borne illness associated with vegetables includes infections caused by some of the more serious pathogens associated for foods of animal origin such as *Salmonella*, *Shigella*, *E. coli O157:H7*, *Listeria monocytogenes* and *Campylobacter*. The rapid growth of international trade in fresh product has also resulted in outbreaks due to imported food(1).

Consumption of microbial contaminated fresh vegetables is food safety concern, as these produce may represent a potential risk for the consumer's health, particularly in debilitated or immune compromised individuals(2). Since bacteria serving as a reservoir for resistance determinants may have great influence on resistance gene transfer in natural habitats, such as the human colon, fruit and vegetable surface the presence of antibiotic-resistant bacteria in fresh vegetables may constitute an additional food safety concern(3,4).

The use of good agricultural practices during growing, harvesting, sorting, packaging, and storage operations for fresh vegetables is the key to preventing pathogen contaminations. Prevention of food from primary sources of contamination is the most efficient way to ensure food safety and prevent foodborne illness. Factors thought to influence the occurrence and epidemiology of these diseases include the quality of irrigation water, fertilization practices, harvesting/sorting, the equipment used, packing, washing, storage, and cooling. The general level of hygiene in handling fruit and vegetables is also a major problem contributing to cross-contamination from animal products as well as direct contamination from the food handler(2).

According to FAO/WHO joint report, leafy green vegetable were given highest priority particularly lettuce, cabbage and spinach in combination with pathogenic organism for their

strong involvement in outbreaks on a worldwide level. The safety of leafy green vegetables has thus become an important issue worldwide. WHO-based evaluation by experts from multiple countries supports the prioritization of leafy greens(5).

Drug resistance presents an ever increasing global public health threat that involves all major microbial pathogens and antimicrobial drugs. The presence of antibiotic resistances both in normal flora and pathogenic microorganisms in fresh vegetables may contribute to horizontal spreading of resistances between different isolates, species and genera. Widespread use of large amounts of antibiotics in plant agriculture, animal husbandry are prime areas of antibiotic resistance development which could lead to a direct selection or co-selection of resistances bacteria; applying manure from animal farming to agricultural fields or the use of contaminated water for irrigation could also spread resistant bacteria to vegetables. Transfer of antimicrobial resistance genes from live bacteria to other bacteria in the foodstuff or in the intestines after ingestion by humans may occur by means of conjugation (6)(7)(8)(9).

Every year, billions of people experience one or more episodes of foodborne disease, without ever knowing that it was caused by food. The most common symptoms of foodborne disease are: Stomach pains, Vomiting, Diarrhea. The symptoms depend on the cause of the disease. Symptoms may occur very quickly after eating the food, or may take days or even weeks to appear. For most foodborne diseases, symptoms occur 24 -72 hours after the food has been eaten. It is estimated that 3% of foodborne disease cases can lead to long-term health problems. Very severe diseases, including arthritis and neurological disorders can be caused by contaminated food. Some foodborne diseases can be transferred from person to person. Caregivers can become sick from family members with a foodborne disease. For infants, the sick people, pregnant women and the elderly, the consequences of foodborne disease are usually more severe and more often fatal. Eating vegetables contaminated with dangerous microorganisms is a source of foodborne disease. (10).

1.2 Statement of the problem

The emergence of outbreaks of foodborne illness associated with fresh vegetables has revived interest among public health agencies and sparked a new wave of research on food safety issues related to microbial contamination of fresh product. The epidemiology of foodborne disease has changed rapidly over the past two decades. Shortly after some major human pathogens were recognized as being spread from animal reservoirs, fresh vegetables emerged as new vehicles for the transmission of these zoonotic diseases(11).

The role of fresh vegetables in nutrition and healthy diets is well recognized and in recent years many countries have undertaken various initiatives to encourage consumers to eat more of these products. This, together with increasing consumer demands for variety and availability, and the changing structure of global trade, and has led to an increase in international trade in these products. For many countries, particularly developing countries, such products have become valuable, making a substantial contribution to the economy as well as to the health of a country's population. However, recent food safety problems linked to these products threaten this. For nutritional, health and economic reasons, it is important that the consumption of fresh product continues to increase(5).

Fresh vegetables normally carry natural non-pathogenic epiphytic micro flora, but during growth, harvest, transportation and further handling the produce can be contaminated with pathogens from animal and human sources. As most of these products are eaten without further processing, their microbial content may represent a risk factor for the consumer's health and becoming a food safety problem. The consumption of fresh vegetables has been increasing in recent years, and since the early 1990s the reported outbreaks associated with consumption of fresh vegetables have grown steadily. Most of the reported outbreaks of gastrointestinal disease linked to the fresh product have been associated with bacterial contamination, particularly with members of the *Enterobacteriaceae* family(12).

Globally, foodborne illnesses are accountable for significant morbidity and mortality. According to, S.F. Altekruse, Centers for Disease Control and Prevention, Atlanta, Georgia, USA comprehensive review on some selected and important foodborne pathogens and associated

illnesses. Thus, factors contributing to the emergence of foodborne diseases are changes in human demographics and behavior, technology and industry, and international travel and commerce; microbial adaptation; economic development and land use; and the breakdown of public health measures(13).

Fresh products have been implicated in a number of documented outbreaks of foodborne illness particularly in Europe, Japan, United States, and Canada. Outbreaks of illness caused by bacteria, viruses and parasites have been linked epidemiologically to the consumption of a wide range of vegetables. Surveillance of vegetables has indicated that these foods can be contaminated with various bacterial pathogens, including *Salmonella*, *Shigella, E. coli O157:H7*, *Listeria monocytogenes* and *Campylobacter*. However, the prevalence of foodborne pathogens on vegetables and their involvement in outbreaks are not well documented in developing countries(1).

During 1998–2008, a total of 13,352 foodborne disease outbreaks, causing 271,974 illnesses, were reported in the United States. 37% of the outbreaks and 47% of the illnesses with a single etiology were implicated to food vehicle(14).

Studies conducted in the United States to estimating the overall number of foodborne illnesses showed that 31 known agents of foodborne illness isolated and caused 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths each year. Non-typhoidal *Salmonella* spp., norovirus, *Campylobacter* spp., and *T. gondii* caused the most hospitalizations and non-typhoidal *Salmonella* spp., *T. gondii*, *L. monocytogenes*, and norovirus caused the most deaths(15).

In developing countries, food borne illnesses caused by contaminated vegetables are frequent and in some areas they cause a large proportion of illness. However, due to lack of food borne disease investigation and surveillance in most of these countries, most outbreaks go undetected(16).

Food borne diseases are common in developing countries including Ethiopia because of the prevailing poor food handling and sanitation practices, inadequate food safety laws, weak regulatory systems, lack of financial resources to invest in safer equipment's, and lack of

education for food-handlers. National Hygiene and Sanitation Strategy program reported that about 60% of the disease burden was related to poor hygiene and sanitation in Ethiopia(17). Unsafe sources, contaminated raw food items, improper food storage, poor personal hygiene during food preparation, inadequate cooling and reheating of food items and a prolonged time lapse between preparing and consuming food items were mentioned as contributing factors for outbreak of food borne diseases (18).

.There is no effective antimicrobial treatment at any step from planting to consumption for vegetables which means that pathogens introduced at any point may be present on the final food product. Washing and rinsing some types of vegetables prolong shelf-life by reducing the number of microorganisms on the surfaces. However, only a portion of pathogenic microorganisms may be removed with this simple treatment. Use of a disinfectant can enhance efficiency of removal up to 100 fold, but chemical treatments administered to whole and cut product typically will not reduce populations of pathogens by more than 2 to 3 log10 CFU/g(19).

The number of documented outbreaks of human infections associated with the consumption of raw fruits, vegetables, and unpasteurized fruit juices has increased in recent years. Outbreaks with identified etiology were predominantly of bacterial origin, primarily *Salmonella* and *E. coli* O154:H7. More recently, salmonellosis has been linked to tomatoes, seed sprouts, cantaloupe, mamey, apple juice, and orange juice(20). However, study done on microbiological safety of fruit juices served in cafes/restaurant, Jimma town, southwest Ethiopia by Tsige K. et al(21) did not isolate *Salmonella* from avocado, papaya and pine-apple juices.

Numerous pathogenic microorganisms have been isolated from a wide variety of vegetables, sometimes at relatively high frequencies(22).Previous studies conducted in Ethiopia indicated the occurrence of pathogens including *Salmonella* and *Shigella* in different food such as fresh fruit(23) and vegetables(24). In addition, outbreaks of infections somehow related with poor hygiene and consumption of contaminated food were reported in Ethiopia and some were caused by *Salmonella* and *Shigella*.

The emergence of antimicrobial resistance in disease-causing bacteria is a public health concern that poses unique communication challenges. Antimicrobials are essential for treating infectious

disease in both humans and animals. However, their improper use may lead to the emergence of new strains of bacteria that cannot be treated with commonly used antimicrobials. Sometimes, pathogens emerge that are resistant to multiple antimicrobials, making treatment extremely difficult. A further complication is the fact that antimicrobials are commonly used in food animals, such as cattle, swine, and poultry, which are a common source of exposure to human pathogens linked to food(25).

The prevalence of antimicrobial resistance among foodborne pathogens is reported to have increased, probably as a result of selection pressure created by the use of antimicrobials in agriculture and animal health. Recent study on antimicrobial susceptibility of *Listeria ivanovii* and *Enterobacter cloacae* isolates from food samples signified an alarming multi-drug resistance of at least four or more of the test antibiotics (26).

Most dangerous microorganisms do not change the appearance of the food, so you usually can't tell that the food is contaminated with dangerous microorganisms by just looking, smelling or tasting it. Several smaller surveys have demonstrated the presence of pathogenic enteric bacteria on produce and in unpasteurized fruit or vegetable juices and vegetable sampled during production or at retail markets in different parts of the world among them some survey were done in Addis Ababa, Gondar, Hawassa, Bahirdar, Bangladesh, Pakistan, South Africa (27),27,32, (29), (30), (31), (26).

Thus, adequate information should be gathered to develop an effective strategy to reduce the outbreak of food borne illnesses related to fresh vegetables and resistance burden in the community. *Salmonella* and other enteropathogens have easy access to the food chain in areas where wastewater/sewage is used for irrigation. In Ethiopia, where wastewater flows without proper treatment into irrigation channels or rivers, it is important to know the microbiological quality of vegetables. In fact, very little is known about the microbial quality of fresh vegetables in Ethiopia.

Efforts to resolve food safety problems linked to fresh products are essential and timely(5). So far there is one recent publication(32) on parasitic contamination of fruits and vegetable and the other publication(21) is on microbiological safety of fruit juices served in cafes/restaurant's

which did not determine antibiotic resistance pattern another recent study published in the study area determined resistance pattern of *Salmonella* spp and *S. aureus* only and did not perform resistance pattern for other isolates. However, there is no publication available regarding information on plasmid profile among antibiotic resistance isolate from fresh vegetables in the study area. Thus, this study is aimed at investigating microbial load, plasmid profile, prevalence and antibiotic susceptibility pattern of microbial isolate from selected fresh vegetable in Jimma town, Southwest Ethiopia.

CHAPTER TWO: LITERATURE REVIEW

The importance of vegetables in nutritious and healthy diets is well recognized, and in recent years consumers have been encouraged to eat more of these products. For many countries, particularly developing countries, these products have become a valuable commodity. At the same time, food safety problems linked to the consumption of fresh vegetables contaminated with microorganisms are increasing. Recent foodborne outbreaks linked to the consumption of leafy greens, tomatoes, sprouts and green peppers clearly demonstrate that the consumption of contaminated vegetables represents an important source of foodborne disease. Efforts to minimize the microbial contamination of fresh vegetables are essential and timely(10).

2.1 Microbial quality

Preventing microbial contamination is the best way to prevent disease and improve our health and that of our family and community. Researchers have investigated the microbiological quality of street vended foods in different countries; high bacterial counts and a high incidence of foodborne pathogens in such foods have been reported. In Ghana, Thermotolerant coliforms on lettuce varied from 2.3×10^3 to 9.3×10^8 on farm, 6.0×10^1 to 2.3×10^8 on market and 2.3×10^6 to 2.4×10^9 at street food vendor sites. Indicator bacterial numbers on farm lettuce were higher compared to the irrigation water (1.5×10^3 to 4.3×10^6) used on the farms. Thermotolerant coliform numbers in market refreshing water (9.0×103 to 4.3×10^{10}) were higher compared to that on the market lettuce and *Salmonella* spp were also detected and documented in the street foods of Kumasi (33). Similar study in Accra(34) reported total bacterial count of 6.3 ± 0.78 log10 CFU g−1 in vegetable salad and 2.5 ± 2.32 log10 CFU g−1 in tomato. Another study in South Africa reported High levels of total aerobic count in vegetables, 6.8 ± 0.07 log10 CFU g−1 (26).

Fresh vegetables could be contaminated at any point from farm to tables. Different survey demonstrated different contamination rate. Study conducted in Jordan (35)Philippines (36) showed 61/150 (40.6%) and 50/300 (16.7%) contamination rate respectively with *E. coli*.

Gbonjubola et al reported high prevalence of bacterial contamination, high bacterial load (6.0 x 10^4 cfu/g to 2.0 x 10^6cfu/g) which varied between the different locations moreover he reported non-availability of water in good quantity for washing of ready-to-eat vegetable salad(37).

Guchi and Ashenafi reported over 90% of the vegetable samples had aerobic mesophilic counts of ≥ log 6 cfu/g(38). More over the majority of lettuce and green pepper samples had high microbial load and multiple drug resistant pathogens were also isolated from some samples.

Akter et al reported total bacterial load in tomato sample between 4.98 to 5.10 log CFU/g in 4 different local markets in Dhaka city. However, the bacterial loads in the carrot samples were 5.50 to 5.70 log CFU/g(39).

2.2. *Microbial isolates*

Different researchers reported different result for varieties of microbial isolate from fresh product. Halablab M. et al reported the presence of significant number of *E. coli* in lettuce samples (42.30%) than in parsley samples (13.8%) and *S. aureus* was significantly more often detected in lettuce samples (51.5%) than in parsley samples (38%). Moreover, his study demonstrated that lettuce and parsley which are usually consumed raw may contain pathogenic microorganisms and represent a risk for human health(40). In contrary study conducted in Jordan reported highest contamination rate with *E. coli* in parsley and lowest in Lettuce(35). According to study conducted in Spain to determine presence and antibiotic susceptibilities of coliform bacteria in fresh vegetables identified isolates mainly species belonging to *Klebsiella, Enterobacter*, and other genera, as well as four identified as *Escherichia coli*(41). In a survey from South Africa Organisms isolated include: *Listeria* spp. *252*(22%), *Enterobacter* spp. (18%), *Aeromonas hydrophila* (12%), *Klebsiella oxytoca* (8%), *Proteus mirabilis* (6.3%), *Staphylococcus aureus* (3.2%) and *Pseudomonas luteola* (2.4%). Interestingly, *Salmonella* spp. and *Escherichia coli were* not isolated in any of the vegetable samples(26).

.In a study of Gbonjubola et al a total of 32 bacteria species were isolated from 25 vegetable salad samples, 12 (37.5%) were pathogens. Three of the total pathogenic bacteria isolated were *Staph. aureus* (25.0%), three *Salmonella spp.* (25.0%), four *E. coli* (33.3%) and two *Ps.*

aeruginosa (16.%). The pre-dominant bacteria isolated from the samples were *Staphylococcus aureus*, *Salmonella* spp, *Escherichia coli* and *Pseudomonas aeruginosa*(37).

Guchi and Ashenafi reported isolation of *Salmonella* and *Shigella* from eight (10%) and 24 (30%) vegetable samples, respectively(38). In addition other species including *Pseudomonas* and *staphylococci* were also isolated from lettuce and green pepper. Over 80% of the green pepper and lettuce samples harbored *staphylococci*.

Akter et al reported presence of *Escherichia coli*, *Shigella dysenteriae, Klebsilla pneumonia, Salmonella Typhimurium, Proteus Vulgaris* and Gram-positive bacteria including, *Staphyloccus aureus* in the fresh vegetable samples(39). Moreover their study demonstrated that the fresh vegetable samples collected from local markets were heavily contaminated with resistant bacteria and is of special concern for human consumption.

2.3 Antibiotic resistance

Antibacterial resistance is a worldwide threat, and concerns have arisen about the involvement of commensal and pathogenic bacteria in the maintenance and spread of resistance genes(42). A study conducted in Jodan showed a total of 17 (27.8%) *E. coli* isolates were found to be resistant to three or more antimicrobial agents. More over this study demonstrated the absence of common diarrheagenic *E. coli*, but indicates widespread of antimicrobial resistance in *E. coli* contaminating fresh green product which may increase the reservoir of antimicrobial resistance in the intestinal tract(35).

Vegetables may act as a reservoir and vector for antimicrobial resistance and antimicrobial resistance in *Escherichia coli* is a useful indicator of resistance levels expected in pathogenic bacteria (4). Holvoet K, et al reported a total of 54 (11.4%) of resistant *E. coli* isolated from lettuce to one or more antimicrobials. The highest resistance rate was observed for ampicillin (7%), followed by cephalothin, amoxicillin/clavulanic acid, tetracycline, trimethoprim and streptomycin with resistance rates between 4.4 % and 3.6 %. No resistance was observed for amikacin, ciprofloxacin, gentamicin and kanamycin. Among the multi-resistant isolates (n = 37), ampicillin and cephalothin showed the highest resistance rate, respectively 76 % and 52 %. The

E. coli isolated from the lettuce showed higher resistance rates compared to *E. coli* isolates obtained from the soil or irrigation water samples (4).

The presence of antibiotic resistances both in epiphytic and pathogenic microorganisms in fresh vegetables may contribute to horizontal spreading of resistances among bacterial populations. Study conducted in Valencia city (Spain) to determine presence and antibiotic susceptibilities of coliform bacteria in fresh vegetables, as an indicator of their microbiological quality and their potential as a risk factor for consumer's health on a sample collected directly from cultivated lands and supermarkets isolated coliforms and other enterobacterial species in significant proportion of individual vegetable samples (average >50%) Susceptibility of isolates to eleven common chemotherapeutic agents was tested and reported most isolates to be resistant to ampicillin, and to amoxicillin/clavulanic acid; some isolates showed multiresistance to 3-5 agents.(41).

In a study of Gbonjubola et al the mean diameter zones of inhibition showed by the used antibiotics against the bacteria isolates ranged between 0.0 mm – 38.0 mm. The highest zone of inhibition of 38 mm was produced by Ofloxacin against *Salmonella sp.* while the lowest zone of inhibition of 0.0 mm was produced by Nalidixic acid and Nitrofurantoin against *Staph. sp.* and *E. coli* respectively(37).

Escherichia coli are an important gastrointestinal flora which has been known to be capable of accepting and transferring plasmids and these plasmids can be transferred readily under stress to other species. Therefore, this attribute has made *E. coli* to be considered as an important reservoir of transferable antibiotic. Gbonjubola et al reported Multiple Antibiotic Resistance *Salmonella* and *E. coli* isolates from vegetable salads(37).

According to Guchi and Ashenafi all of the *Salmonella* and 97% of *Shigella* isolates form vegetable samples showed resistance to penicillin. Ampicillin resistance was observed in 42% of *Salmonella* and 79% of *Shigella* isolates. Multiple drug resistance was seen in 8 and 24 isolates of *Salmonella* and *Shigella* isolates, respectively(38).

According to Akter et al amoxicillin resistant bacteria were 42%, ciprofloxacin resistant bacteria were 18% and multi drug resistant bacteria were 3% .The percentage of amoxicillin resistant bacteria to all resistant bacteria was found to be at the highest frequency. Moreover an antibiogram study of 22 isolates showed that the isolates were also resistant to streptomycin, ampicillin, oxytetracyclin, cephalosporin and chloramphenicol. Most of isolates were resistant to ampicillin (18), followed by oxytetracyclin (14), and very low resistance to ciprofloxacin (4) and cephalosporin (4). These experimental results also suggested that multidrug resistance to environmental bacteria was increasing and almost every bacterium was resistant to more than one antibiotic(39). The most common resistant bacteria were found to be *E. coli*.

2.3 Screening for plasmids

Study done in Australia to check the involvement of plasmids in the resistance to antibiotics observed in some of the isolates, plasmid DNA was extracted from all 86 isolates that were resistant to at least one antibiotic. Plasmids of varying numbers and sizes were found in 74.4% of resistant isolates, while 25.6% did not possess any plasmids(43). Another study conducted to determine bacterial Load of Fresh Vegetables and Their Resistance to currently used antibiotics in Saudi Arabia revealed presence of plasmid DNA in all a preselected multidrug-resistant isolates tested(44). Moreover study conducted by Akter et al reported presence of plasmid in 22 isolate tested for plasmid profile(39).

The above review mainly focus on the finding of different researchers from different parts of the world. Those original articles which investigate microbial load, microbial isolate and resistance patterns of microbial isolate from salad vegetables were included in the review. According to the above review there was high rate of microbial contamination; high microbial load and resistance to antibiotics were reported.

2.4 Significance of the study

Diets high in fruits and vegetables are widely recommended for their health-promoting properties. However, Food safety problems linked to the consumption of fresh vegetables contaminated with microorganisms are increasing. Efforts to minimize the microbial contamination of fresh vegetables are essential and timely since there is no effective method to remove microbial contaminant once contamination occurred. Thus, the present study determined microbial load, microbial profile and resistance patterns of microbial isolates from lettuce, cabbage, tomatoes, carrot and green pepper collected in selected local market at Jimma town, southwest Ethiopia from Feb to April, 2015.

Food safety is more importantly a public health issue as it plays a noteworthy role in health development and consequently national economic development. Thus, great endeavors should be made to improve it at all levels of the food chain. In Ethiopia, where wastewater flows without proper treatment into irrigation channels or rivers, it is important to know the microbiological quality of vegetables. In fact, very little is known about the microbial quality of fresh vegetables in Ethiopia. So far there is no published data available regarding information on microbial safety of fresh vegetables in the study area. Thus, this study was aimed at investigating microbial load, prevalence and antibiotic susceptibility pattern of microbial isolate from fresh vegetable in Jimma town, Southwest Ethiopia. Therefore the present study;

- Determined the current bacterial profile, prevalence, antibiotic resistance patterns and plasmid profile
    - Provide relevant information for government bodies, non-governmental organizations, policy makers and health planners, for future planning and interventions on microbial safety of salad vegetable.
    - Provide base line information for further studies.
    - Data on the prevalence and types of antibiotic resistance in microorganisms isolated from fresh produce may help explain the role of foods in the transmission of antibiotic-resistant strains to human populations

CHAPTER THREE: OBJECTIVE

3.1. General objective

To assess prevalence, plasmid profile and antibiotic susceptibility patterns of microbial isolates from lettuce, cabbage, tomatoes, carrot and green pepper collected in selected local market at Jimma town, southwest Ethiopia from Feb to April, 2015

3.2. Specific Objectives

1. To determine microbial load and microbial profile of lettuce, cabbage, tomatoes, carrot and green pepper

2. To determine number of pathogenic and indicator organisms isolated from lettuce, cabbage, tomatoes, carrot and green pepper

3. To characterize the antibiotic resistance profiles of microbial isolates from lettuce, cabbage, tomatoes, carrot and green pepper

4. To screen for the presence of plasmid among drug resistant isolate

CHAPTER FOUR: METHODS AND MATERIAL

4.1 Study area. The study was conducted in Jimma Town, which is located at south west of Ethiopia, about 352 km from Addis Ababa, the capital of Ethiopia. Based on the 2007 Census conducted by the CSA, Jimma Zone has a total population of 2,486,155 of whom 1,250,527 are men and 1,235,628 women; with an area of 15,568.58 square kilometers, Jimma has a population density of 159.69. While 137,668 or 11.31% are urban inhabitants, a further 858 or 0.03% are pastoralists. A total of 521,506 households were counted in this Zone, which results in an average of 4.77 persons to a household(45).

4.2 study design and period. A cross-sectional study was conducted to determine microbial load, microbial profile and antibiotic susceptibility patterns of microbial isolates from lettuce, cabbage, tomatoes, carrot and green pepper collected from selected local market of Jimma town, southwest Ethiopia from Feb to April, 2015.

4.3. Sample Collection and Analysis. All vegetables including lettuce, cabbage, carrot, tomato, green pepper brought to Jimma town for sale during the study period was the target sample for this study. Purposive sampling techniques was employed to purchase a total of 150 vegetable samples.50 vegetables from farmers and 100 vegetables from merchant were included in the study. each five types fresh vegetables from a single market including lettuce, cabbage, carrot, tomato, green pepper from one selected market at different sampling days from different outlets in Jimma town, Ethiopia from conveniently selected open local markets, namely, “Bishishe,” “Hirmata Merkato,” “Kochi,” and “Agip” found in Jimma Town between Feb -April 2015. The sample size for each market was 25 and 25/5 vegetable type = 5. All the samples was purchased and put in sterile plastic containers, properly labeled and immediately transported to the Microbiology laboratory at medical laboratory and pathology department, Jimma University for microbiological analysis. Microbiological analysis was conducted within three hours of sample collection.

4.4 Data collection instrument

A semi-structured questionnaire was used that contained questions concerning the pre- harvest and post-harvest vegetable contamination. The questionnaire was adapted from reviewing similar studies and prepared first in English language and was then translated into Amharic; and then pretested for its appropriateness. The questionnaire was further modified after pretesting. Laboratory format was used to record the laboratory test results.

4.5 Data collection process

For data collection process standard operating procedures that are attached under annex was followed

4.5.1 Microbiological analysiss: For microbiological analyses, 25g of sample was aseptically removed from each sample using a sterile spatula and vigorously shaken in 225ml of sterile 0.1% (w/v) bacteriological peptone water (Oxoid) for 3 minutes. Serial dilutions of 10^{-2}, $_{10}{}^{-3}$ were made and then 0.1ml of the suspension from each dilutions were plated in duplicate on a pre-dried surfaces of nutrient agar and average count were recorded after multiplying by reciprocal of dilution factor and reported as colony forming unit per gram.

For isolation of *Salmonella* spp and *Shigella* spp. vegetable samples (25 g) was added to 225 ml bacteriological peptone water, vigorously shaken and the suspension incubated at 37^{o}C for 24 hours for the metabolic recovery and proliferation of cells. From this, 1ml of culture was transferred into tubes containing 10 ml of Selenite F Broth. Selenite F broth was incubated at 37^{o}C for 24 hours. After secondary enrichment, culture from selenite F broth was separately streaked on plates of MacConkey Agar, Xylose Lysine Desoxycholate (XLD) medium (all from Oxoid). Characteristic colonies from each selective medium was picked, purified and tested biochemically on Kligler‟s Iron Agar (Oxoid), Lysine Iron Agar (LIA) (Oxoid), Urea Agar (Oxoid), oxidase, indole, Simmons Citrate Agar (Oxoid) and SIM Medium (Oxoid). For isolation and identification of *S. aureus* a loop full of sample from the homogenate was inoculated on Manitol salt agar (MSA) and yellow colonies on MSA which was catalase positive and coagulase positive isolates was identified as *S. aureus*.

4.5.2 Drug susceptibility testing: The criteria used to select the antimicrobial agents tested was based on the availability and frequency of prescription for the management of bacterial infections in Ethiopia and WHO Recommended antimicrobials for surveillance of *Salmonella* and *E. coli* as they are the major food borne pathogen around the globe(25).

Antimicrobial susceptibility testing for microbial isolate was performed using the disk diffusion method and results was interpreted using the criteria of the National Committee for Clinical Laboratory Standards(CLSI 2012). The antibiotics used was Ampicillin (A-10µg), Amoxicillin/clavulanic acid (AM-10µg), Chloramphenicol (C-30µg), Streptomycin (S-10µg), OxyTetracycline (T-30µg), Ciprofloxacin (Cf-5µg), Nalidixic acid (Na-30µg), Gentamicin (G-10µg), Kanamycin (K-30µg) and Co-trimoxazole (SXT-25µg). Antibiotic susceptibility testing for *S. aureus* was determined for Ampicillin (A-10 µg), Amoxicillin/clavulanic acid (Am-10 µg), Erythromycin (E-15 µg), Oxacillin (Ox -1 µg), Streptomycin(S-10 µg), Co-trimoxazole (SXT-25 µg) and Vancomycin (Va-30 µg). A standard reference strains *E. coli* (ATCC 25922) and S.aureus (ATCC 25923) with known sensitivity were used in this study. Interpretation of readings as sensitive, intermediate or resistant was made according to a chart

4.5.3 Screening for plasmid

Sample preparation was performed by culturing isolates on Nutrient agar. Colonies grown on NA were transferred to and grown in 12 ml of tryptic soya Broth (Oxoid) containing 50µg/ml ampicillin and incubated at 37°C for 24 hours. The plasmid DNA was extracted by using cold alkaline lysis method protocol described by (C. Rohde & B. Henze) attached under (annex IV) using sodium acetate as neutralizing agent. Agarose gel electrophoresis (1%) was run for 1.30 hrs at 100 V using horizontal gel electrophoresis apparatus. The plasmids were visualized with ultraviolet light.

4.7 Measurement

4.7.1 Study variables

- Microbial load
- Types of microbial isolate

- Antibiotic resistance
- Kind of vegetable
- Market place
- Means of display
- Washing before display
- Sources of water for washing

4.8 Data quality assurance

The following measures was undertaken so as to control the quality of the data and laboratory investigation. Properly designed and pre-tested data collection instrument was used. Every day the collected data was cross checked for completeness, consistency and on site corrective action was made.

A standard operational procedure tools was strictly used for sample collection, transportation, processing and storage. Special emphasis was given during coding each culture media as well as the collected vegetable samples. Before use all disks, reagents and culture media was checked being at appropriate temperature and within specified shelf life. Antibiotic sensitivity test was performed according to clinical laboratory standard institute guide. *E. coli* strain ATCC 25922 and *S. aureus* strain ATCC 25923 was included. For plasmid isolation optimization test were performed using some of the isolate for gel concentration, running time and volt.

All media, even those that have been sterility tested at the time of preparation, was checked visually before being inoculated for any change in appearance that could indicate contamination or deterioration. Most biochemical testing media was controlled when they were used. The medium was inoculated with bacterial species of known positive and negative reactions.

4.9 Administration monitoring

Validity and completeness of the overall study was supervised by advisors.

4.10 *Statistical Methods*

Data were organized and summarized in simple descriptive statistics methods. Moreover, all components of the data entered and analyzed using SPSS 20.0 computer software. Chi-square test (X^2) results were used and a *p*-value of less than 0.05 was considered statistically significant.

4.11 Ethical *Consideration*

Ethical clearance was obtained from Jimma University research and ethical review committee. The purpose and procedures of the study was explained to the respondents (vendors of vegetables) a verbal consent was obtained from all study participants. Privacy and confidentiality of the study participants response and laboratory test result was maintained.

4.12 Operational definitions

Indicator organisms: are defined as large group of bacteria including certain pathogenic bacteria, which are relatively easy to measure as a group and the presence of this in food, is likely to indicate the presence of pathogenic bacteria. An indicator organism by itself is considered to cause no diseases in man or animals, but its presence usually indicates the presence of pathogenic or disease-causing organisms.

Pathogenic bacteria: bacteria that are known to cause infectious diseases in human

Index organism: an index organism is one whose presence implies the possible occurrence of a similar but pathogenic organism. *E. coli* is used an index organism and its presence indicates possible presence of pathogenic enterobacteriacea e.g. *Salmonella* sp.

Manure: is a mixture of animal faeces, urine and vegetable waste

***Resistant*:** A pathogen reported as 'resistant' implies that the infection it has caused will not respond to treatment with the drug to which it is resistant irrespective of dose or site of infection.

***Intermediate*:** A pathogen reported as intermediately susceptible suggests that the infection it has caused is likely to respond to treatment when the drug is used in larger doses than normal or

when the drug is concentrated at the site of infection, e.g. in the urinary tract. Consideration should be given to using other drugs that may provide more optimal therapy.

Susceptible: A pathogen reported as susceptible suggests that the infection it has caused is likely to respond to treatment when the drug to which it is susceptible is used in normal recommended doses and administered by an appropriate route.

4.13 Data dissemination plan

The final finding of the current study will be submitted to Jimma University collage of health sciences, department of medical laboratory sciences and pathology. Moreover, publication of the study finding in a peer reviewed journals and presentation in scientific meeting will be considered

CHAPTER FIVE: RESULTS

Socio-demographic characteristics

A total of 150 vegetable samples comprising lettuce, cabbage, carrot, tomatoes and green peppers were included in this study (**Table 1**). A total of 30 vegetable venders were interviewed comprising 10 farmers and 20 merchant at different vending market days. The mean age of the respondents was 31.6 with standard deviation of 3.85 the minimum was 25 and maximum was 40. Majority (80%) of the vegetable venders was female and male accounts for only 20% (**table 1**). With respect to educational status majority (43%) had no formal education, 36. 7 % had primary education while the rest 20% had secondary education (**Table 1**). Almost all (9 out of 10) of the farmer participant used decayed manure as fertilizer while only one farmer used organic fertilizer to cultivate vegetables as soil amendment(**Table 2**). Moreover all farmers used surface water for irrigation and washing purpose whereas almost all merchant used pipe water for washing purposes. Almost all the participants had latrine and almost all washed their hands after visiting toilet. All the vending area observed was infested by insects. All vegetable venders participated in the study had direct contact with vegetable with bare hand without prior washing of their hands during vending and harvesting.

Microbial quality

The bacteriological finding of the mean total aerobic mesophilic count was displayed in the **table 3.** More than 80% of vegetable samples had total viable counts of greater than10^6CFU/g with ranges of 10^5-10^7CFU/g for all vegetable types.

Microbial isolates

A total of 102 bacterial isolates of eight genera were identified. *Enterobacter spp. (21.60%)* was the most dominant followed by *Citrobacter spp. (20.6%), Klebsiella spp. (18.6%), Salmonella spp. (11.8%), E. coli (10.8%), Proteus spp. (9.8%), Staphylococcus spp. (4.9%), and Pseudomonas aeruginosa (2%).* The prevalent bacterial species were the genera *Enterobacter*

spp, *Citrobacter spp, Klebsiella spp, Proteus spp, Pseudomonas spp, E.coli, Salmonella spp,* and *S.aureus* as presented in (**table-4**).

Antibiotic resistance

Ampicillin and amoxicillin were highly resisted by more than 89% of microbial isolates. All the five *S. aureus* isolates were sensitive to oxacillin and vancomycin but two isolates were intermediately sensitive to erythromycin. Ciprofloxacillin was the least resisted drugs only 3.9 % whereas oxytetracycline was resisted by 31.4 % of the isolates. Resistance to nitrofurantoin, nalidic acid, streptomycin, chloramphenicol, cotrimoxazole, ceftriaxone, kanamycin and Gentamycin were 30.4%, 26.5%, 18.6%, 12.7%, 10.8%, 10.8%, 7.8%, 4.9% respectively. Multiple drug resistant isolates to more than one class of antibiotics were also detected (**table-5&6).**

Screening for plasmid

All (102) bacterial isolates were screened for the presence of plasmid. Out of 91 isolate resistant to at least one antibiotics plasmid were detected in 20 and plasmid were not detected in sensitive and intermediately sensitive isolate which were 8 and 3 in number respectively.

Table 1: Socio-demographic characteristics of vender of vegetables of selected local markets of Jimma town Southwest Ethiopia, 2015

Variables	Frequency (n)	Percent (%)
Gender		
Male	6	20
Female	24	80
Total	**30**	**100**
Educational status		
No formal education	13	43.3
Primary education	11	36.7
Secondary education	6	20
Total	**30**	**100**
Market place		
Kochi	25	16.7
Bishishe merkato	25	16.7
Hirmata merkato	25	16.7
Agip	25	16.7
From farmer	50	33.3
Total	**150**	**100**
Type of product		
green pepper	30	20
Carrot	30	20
Lettuce	30	20
Cabbage	30	20
Tomato	30	20
Total	**150**	**100**
Occupation		
Merchant	20	66.7
Farmer	10	33.3
Total	**30**	**100**
Place of display		
Open market	21	70
Grocery	9	30
Total	**30**	**100**
Water source for washing		
Pipe water	19	63.3
Well water	1	3.4
Surface water	10	33.3
Total	**30**	**100**

Table 2: knowledge and practice related to vegetable farming for farmer respondents of vegetable venders Jimma town south west Ethiopia, 2015.

	Frequency (n)	Percent (%)
Put animals in fenced area		
Yes	10	100
No	0	
Harvest container used to carry other materials		
Yes	10	100
No	0	
Type of fertilizer used		
Decayed manure	9	90
Inorganic fertilizer	1	10
Water source for irrigation		
Pipe water	0	
Well water	0	
Surface water	10	100
Habit of washing harvest equipment		
Yes	10	100
No	0	
Hand washing before harvesting		
Yes	0	
No	10	100
Keep container off the ground		
Yes	0	
No	10	100

Table 3: Mean total aerobic mesophilic bacterial counts (CFU/g) of fresh vegetables by type of product purchased from selected local markets Jimma town southwest, Ethiopia 2015.

Mean aerobic mesophilic count		type of product					Total
		green pepper	carrot	lettuce	cabbage	tomato	
	10^5	4	4	2	3	3	16
		13.3%	13.3%	6.7%	10.0%	10.0%	10.7%
	10^6	25	25	26	26	26	128
		83.3%	83.3%	86.7%	86.7%	86.7%	85.3%
	10^7	1	1	2	1	1	6
		3.3%	3.3%	6.7%	3.3%	3.3%	4.0%
Total		30	30	30	30	30	150
		100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

Table 4: Frequency distribution of bacterial isolates from fresh vegetables purchased in selected local market of Jimma town southwest Ethiopia, 2015.

Microbial isolates		Frequency(n)	Percent (%)
	E.coli	11	10.8
	Salmonella spp	12	11.8
	Proteus spp	10	9.8
	Klebsiella spp	19	18.6
	Citrobacter spp	21	20.6
	Enterobacter spp	22	21.6
	Pseudomonas spp	2	2.0
	S.aureus	5	4.9
	Total	102	100.0

Table 5: Antibiotic resistance patterns of gram negative rods isolated from fresh vegetables sold in selected local market of Jimma town southwest Ethiopia, 2015

Bacterial isolate		Antimicrobial agents											
	Numb	AM	AMX	C	S	SXT	OXT	CIP	GN	NA	K	CRO	F
E. coli	11	7	7	4	4	0	6	3	0	4	1	4	6
	%	64	64	36	36		54.5	27		36	9	36	54.5
Salmonella spp	12	12	12	2	0	0	8	0	0	2	0	2	2
	%	100	100	16.6			66.7			16.6		16.7	16.7
Proteus spp	10	10	10	0	5	7	8	0	1	6	0	0	7
	%	100	100		50	70	80		10	60			70
Klebsiella spp	19	19	17	2	4	2	1	0	1	6	1	1	7
	%	100	89.4	10.5	21.1	10.5	5.3		5.3	31.6	5.3	5.3	36.8
Citrobacter spp	21	20	20	0	2	1	5	1	0	4	2	2	6
	%	95.2	95.2		9.5	4.8	23.8	4.8		19.1	9.5	9.5	28.5
Enterobacter spp	22	21	21	3	2	1	4	0	3	2	4	2	3
	%	95.5	95.5	13.6	9	4.5	18		13.6	9	18	9	13.6
Pseudomonas spp	2	2	2	2	0	0	0	0	0	2	0	0	0
	%	100	100	100						100			

Key: AM=Ampicillin (10µg), AMX=Amoxicillin (10µg) ,C=Chloramphenicol(30µg) ,S=Streptomycin (10 µg), SXT= Cotrimoxazole (1.25µg) ,OXT=Oxytetracycline (30µg) ,CIP=Ciprofloxacillin (5µg) GN=Gentamycin (10µg) ,NA=Nalidic acid (30µg) ,K=Kanamycin (30µg),CRO=Ceftriaxone (30µg) F=Nitrofurantoin (300µg)

Table 6: Antibiotic resistance patterns of *S.aureus* isolated from fresh vegetables sold in selected local market of Jimma town southwest Ethiopia, 2015

Bacterial isolate		Antimicrobial agents														
	Numb	AM	AMX	C	S	SXT	OXT	CIP	GN	NA	K	CRO	F	OX	VA	E
S.aureus	5	0	3	0	2	0	0	0	0	1	0	0	0	0	0	0
	%		60		40					20						

Key: AM=Ampicillin (10µg), AMX=Amoxicillin (10µg) ,C=Chloramphenicol(30µg) ,S=Streptomycin (10 µg), SXT= Cotrimoxazole (1.25µg) ,OXT=Oxytetracycline (30µg) ,CIP=Ciprofloxacillin (5µg) GN=Gentamycin (10µg) ,NA=Nalidic acid (30µg) ,K=Kanamycin (30µg),CRO=Ceftriaxone (30µg) F=Nitrofurantoin (300µg), OX=Oxacillin (1µg) ,VA=Vancomycin (30µg) ,E=Erythromycin (15µg)

CHAPTER SIX: DISCUSSION

In the present study the following points were noted which could have served as the source and cause of microbiological contamination; such as, Bare-handed handling of vegetables, Use of unsafe water by food handlers to wash their hands that had been used over and over again. This water could have been a source of coliform and fecal coliform. The presence of insects around vending area, the presence of waste container without lids, displaying vegetable product on the floor in all market areas. Use of decayed organic manure as soil amendments by most farmers. Transportation of vegetable product with unclean container. Storage of vegetable products at high temperature. Failure to bath and change clothe regularly. The use of surface water for irrigation purpose by all farmers. Keeping vegetable product for display for a long time at elevated temperatures without refrigeration which facilitates bacteria to proliferate.

Microbial quality

The present study demonstrated heavy microbial contamination of fresh vegetables sold in the selected open markets with the ranges of total aerobic mesophilic counts between 10^5-10^7 CFU/g for all vegetable samples. There was no significant variation in microbial load of vegetable samples by market place, educational level, type of venders, type of products and storage condition.

Researchers have investigated the microbiological quality of street vended foods in different countries; high bacterial counts and a high incidence of foodborne pathogens in such foods have been reported. The microbial load of vegetables in the study area is higher compared to study done in Accra(34) for tomato sample and comparable with study done in South Africa (26) and Nigeria(37) Accra(34) but lower compared to study done in Ghana, documented in the street foods of Kumasi (33) and Bangladesh(39). The discrepancy between the present study and previous studies might be as a result of the variations in geographical locations, seasonal, climatic and environmental conditions, the kind of sample and sample size examined, the sampling techniques, methods used for detection of the microbial isolates, and socioeconomic status. So long as these factors differ, consequently the discrepancy of the results would be expected.

More than 85% of vegetable samples had total viable counts of greater than 10^6CFU/g. Similar study conducted in Addis Ababa reported over 90% of the vegetable samples had aerobic mesophilic counts of ≥ log 6 cfu/g(38). The high microbial load of vegetable in this study could be due to fact that in both grocery and open markets vegetables were seen displayed on open stalls in close proximity to waste container without lids where flies are swarming all over the place, mostly close to open gutter, direct hand contact during both harvest and sell. Moreover all farmers used surface water for washing purpose.

Microbial isolates

Consumption of microbial contaminated fresh vegetables is food safety concern, as these product may represent a potential risk for the consumer's health, particularly in debilitated or immune compromised individuals(2). Different researchers reported different result for varieties of microbial isolate from fresh product. In the present study a total of 102 bacterial isolates of eight genera were identified. *Enterobacter* spp. (21.60%) was the most dominant followed by *Citrobacter spp*. (20.6%), *Klebsiella spp.* (18.6%), *Salmonella* spp. (11.8%), *E. coli* (10.8%), *Proteus spp*. (9.8%), *Staphylococcus spp*. (4.9%), and *Pseudomonas aeruginosa* (2%). The prevalence of *E.coli* in the present study was lower compared to study done in Lebanon(40) which reported prevalence of (42.30%) this could be due to sample type, sample size, climatic and seasonal variation. However the present study was comparable with study done in Lebanon in demonstrating the presence of pathogenic microorganism in fresh vegetables consumed which are usually consumed raw and represent a risk for human health(40).

Comparable results was reported from similar study conducted in Nigeria(37),Jordan (35) and Spain (41) However, similar study from South Africa did not isolate *Salmonella* spp. and *Escherichia coli* in any of the vegetable samples(26) which could be due to the difference in hygienic practice.

Guchi and Ashenafi reported isolation of *Salmonella* and *Shigella* from eight (10%) and 24 (30%) vegetable samples, respectively(38). In addition other species including *Pseudomonas and staphylococci* were also isolated from lettuce and green pepper. Over 80% of the green pepper and lettuce samples harbored *staphylococci.*

Akter et al reported presence of *Escherichia coli*, *Shigella* dysenteriae, *Klebsilla pneumonia*, *Salmonella Typhimurium*, *Proteus Vulgaris* and Gram-positive bacteria including, *Staphyloccus aureus* in the fresh vegetable samples(39). Moreover their study demonstrated that the fresh vegetable samples collected from local markets were heavily contaminated with resistant bacteria and is of special concern for human consumption.

Fresh vegetables could be contaminated at any point from farm to tables. Different survey demonstrated different contamination rate. Study conducted in Jordan (35)Philippines (36) showed 61/150 (40.6%) and 50/300 (16.7%) contamination rate respectively with *E. coli*

Antibiotics resistance

The presence of antibiotic-resistant bacteria in fresh vegetables may constitute food safety concern since bacteria serving as a reservoir for resistance determinants may have great influence on resistance gene transfer in natural habitats, such as the human colon, fruit and vegetable surface (3,4). In the present study ampicillin and amoxicillin were highly resisted by more than 89% of microbial isolates. All the five *S. aureus* isolates were sensitive to oxacillin and vancomycin but two isolates were intermediately sensitive to erythromycin. Ciprofloxacillin was the least resisted drugs only 3.9 % whereas oxytetracycline was resisted by 31.4 % of the isolates. Resistance to nitrofurantoin, nalidic acid, streptomycin, chloramphenicol, cotrimoxazole, ceftriaxone, kanamycin and Gentamycin were 30.4%, 26.5%, 18.6%, 12.7%, 10.8%, 10.8%, 7.8%, 4.9% respectively. Multiple drug resistant isolates to more than one class of antibiotics were also detected.

Antibacterial resistance is a worldwide threat, and concerns have arisen about the involvement of commensal and pathogenic bacteria in the maintenance and spread of resistance genes(42). Results of the present study demonstrated multiple antibiotic resistance isolates from fresh vegetable samples which was comparable to a report from Nigeria(37) Addis Ababa(38) , Jodan (35) and Belgium(4)Spain(41) and Bangladesh(39).

Moreover, ampicillin resistance was higher (100%) for *Salmonella* in the present study compared to a report from Addis Ababa. The presence of multidrug resistance isolate was comparable to a

report from Addis Ababa(38). The discrepancy between previous and present study could be due to difference in geographical location, climatic and seasonal variations, and sample size.

In contrary to the present study a report from Belgium indicated no resistance for ciprofloxacin, gentamicin and kanamycin. However, ampicillin resistance was comparable to the present study (4). The discrepancy between previous and present study could be due to difference in geographical location, climatic and seasonal variations.

Screening for plasmid

Plasmids are extra-chromosomal pieces of DNA, which are capable of replicating independently of the genome, and are particularly important in the spread of antibiotic resistance genes(46). In the present study 91 resistant 8 sensitive and 3 intermediately sensitive bacterial isolates were screened for the presence of plasmid. Among which only 20 out of 91 resistant isolates were found to contain plasmid no plasmid was detected in sensitive and intermediately sensitive bacterial isolates. This finding is very low compared to similar study done in other parts of the world for example Study done in Australia to check the involvement of plasmids in the resistance to antibiotics observed in some of the isolates, plasmid DNA was extracted from all 86 isolates that were resistant to at least one antibiotic. Plasmids of varying numbers and sizes were found in 74.4% of resistant isolates, while 25.6% did not possess any plasmids(43). Another study conducted to determine bacterial Load of Fresh Vegetables and Their Resistance to currently used antibiotics in Saudi Arabia revealed presence of plasmid DNA in all a preselected multidrug-resistant isolates tested(44). Moreover study conducted by Akter et al reported presence of plasmid in 22 isolate tested for plasmid profile(39). The low prevalence observed in our study might be due to methodological difference and environmental factors.

Strength and limitation of the study

This study sheds light on the microbial load, prevalence, resistance pattern and plasmid profile of microbial isolates from fresh vegetables sold in selected local market of Jimma town. However, due to the expensive cost and budget constraints serotyping of *E.coli* and *Salmonella* spp were not performed which is to be considered as a limitation.

Method used for plasmid isolation was traditional cold alkaline methods and it is not very sensitive to detect low copy number of plasmid.

Distribution of microbial isolate such as *campylobacter spp* and *listeria monocytogenes* were not performed

CHAPTER SEVEN: CONCLUSION AND RECOMMENDATIONS

7.1 Conclusions

The results of the present study indicated that

- The presence of multiple antibiotic resistance isolate from fresh vegetables
- More than 80% of vegetable sample had total viable count greater than 10^6.
- The predominant bacterial isolate was *enterobacter spp* followed by *citrobacter spp*.
- Detection of *Salmonella* spp in 12 vegetable samples.
- Absence significant association of prevalence of microbial isolates by type of vegetable, place of market, type of vender, sources of water used for washing.
- Ampicillin and amoxicillin were resisted by more than 89% of the isolate.
- Heavily contamination of vegetables with a variety of microbial groups and enteric pathogens.
- Plasmid carriage was detected in 20 out of 91 resistant isolates

7.2 Recommendations

- ✓ Considering the high prevalence of microbial contamination revealed by this study it is recommended to create awareness about the microbial contamination of vegetables and food borne infection. Health information should be provided to the food handlers and farmers on the risk factors predisposing to microbial contamination of vegetables from farm to fork, particularly on preventive behavioral and personal hygienic practices such as avoiding direct bare hand contact with vegetables both during harvesting and vending, hand washing after outdoor activities involving soil contact, washing and drying containers used for transportation and harvesting , avoiding use of polluted water for irrigation and washing vegetables.
- ✓ In depth further study on the prevalence of microbial isolate including others such as *listeria monocytogens and campylobacter spp* is recommended as the prevalence of this microbial isolate on fresh vegetable in the study area is not known.

- ✓ This information will enable the policy makers to identify the risks of human exposure to vegetables and guide in decisions of whether vegetables should be included in routine food surveillance for enteric pathogens. The study will also contribute towards the development of national food safety strategies, to protect the consumer from enteric pathogens. It is therefore recommended that concerted efforts should be made towards regular surveillance of vegetables sold for public consumption to ascertain the microbial loads and the antimicrobial resistance profiles. This will guide in formulating prompt and effective control measures.
- ✓ Data on the prevalence and types of antibiotic resistance in microorganisms isolated from fresh produce may help explain the role of foods in the transmission of antibiotic-resistant strains to human populations.

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ANNEXES

Annex I:Information Sheet (English Version)

Title of the project: antibiotic susceptibility patterns of microbial isolates from vegetables collected in selected local market of Jimma town, southwest Ethiopia

Name of Principal Investigator: Eshetu Chilo

Organization: Jimma University College of Public Health and Medical Sciences department of Medical Laboratory Sciences and Pathology

Name of sponsor: Jimma University

This information sheet is prepared for Vegetable vendors who will be involved in project entitled above. We are going to tell you about the whole process that will happen in the study and requesting you to participate voluntarily.

Description and Purpose of the study: Vegetables eaten raw can be contaminated with a range of microbial contaminants and pathogens from soil, animal and human sources at any point during growing, harvesting, sorting, packaging, and storage and have long been known to serve as vehicles for transmission of infectious microorganisms both in developing and developed countries over the past decade. Microbial contaminated vegetables are frequently associated with diarrheal diseases which occur due to irrigation using waste water polluted water and use of untreated manure moreover improper handling and storage of vegetables especially in a developing world like our country, Ethiopia. So this study is designed to determine the bacterial load, profile and antibiotic susceptibility patterns in vegetables at microbiology laboratory of Jimma University.

Procedures: If you are willing to participate in the study, you will be asked to sign a consent form and the following procedures will be done.

- You will provide us 10 minutes interview.

- ❖ We will take/bought yours vending food item.
- ❖ The bought/collected food sample will be processed in Medical Microbiology laboratory of Jimma University.
- ❖ Bacteriological culture studies will be done on food sample.

Risks and discomforts: There is no risk and discomfort in participating in this study. During all food sample collection we will bought food item for sampling and follow Standard operational procedures.

Benefits and Compensation: By participating in this study, there will no financial benefit directly but based on the result that we will recommending on the issue of street food, you will be beneficiary indirectly after implementation by the responsible governmental and/or non-governmental bodies.

Confidentiality: All information that collected from the study subjects and bacterial profile of the street food item will be kept confidential. Any information about the participant that collected for the study will be stored in a file and will not bear a name on it, but only a number assigned to it instead.

Voluntary participation and withdrawal: Your participation in this study is voluntary. You may decide not to participate or may leave the study at any time. Your decision will not result in any penalty or loss of benefits to which you are entitled. Your decision will not put a risk at any time. You should ask the study investigators listed below any questions you may have about this research study. You may ask questions in the future if you do not understand something that is being done.

Use the following address for any question:

Mr. Eshetu Chilo, Phone No +251-932-482041, Email: eshetuchilo@gmail.com

Mss Haimanot Tassew, Phone No +251-917-804249, Email: haimatas@yahoo.com

Mr. Muatu Gashaw, Phone No +251-913629953 , Email: mulatugashaw@yahoo.com

For the success of our study, we will be asking you to give the correct answer for the respective questions. Thank you for your assistance.

የጅማ ዩኒቨርስቲ

የጤና ሳይንስ ት/ቤት

የህክምና ላቦራቶሪ ሳይንስ ትምህርት ክፍል

የአትክልት ብክለት ልየስከትሉ የሚችሉ የሆድ ህመም ተህዋስያን (ባክቴሪያዎች) ለሚሰጣቸው መድሀኒቶች ያላቸው ተጋላጭነትና ለአትክልት ብክለት የሚያጋልጡ ምክንያቶችን ለማረጋገጥ በጅማ ከተማ የተመሬጡ የገበያ ስፈራዎች የተወስደ የአትክልት ናሙና ተወስዶ ለሚሰራዉ ጥናት ላይ ለሚሳተፉ ህመምተገኞች የተዘጋጀ መረጃ::

በጅማ ዩኒቨርስቲ የጤና ሳይንስ ት/ቤት የህክምና ላቦራቶሪ ሳይንስ ትምህርት ክፍል በማስተርስ ድግሪ ተማሪ የመመረቂያ ጥናት ላይ እዲሳተፉ ተጋብዘዋል:: እባክዎ በዚህ ጥናት ለመሳተፍ ከመስማማትዎ በፊት ከዚህ ቀጥሎ የሚገኘዉን ምንባብ በጥሞና ያንብቡና ግልጽ ያልሆነዎትን ማንኛዉም ሃሳብ ይጠይቁ::

ምርምር የማድረጊያ ፈቃድ ለመውሰድ የተዘጋጀ ማብራሪያ

የምርምሩ ርእስ የአትክልት ብክለት ልየስከትሉ የሚችሉ የሆድ ህመም ተህዋስያን (ባክቴሪያዎች) ለሚሰጣቸው መድሀኒቶች ያላቸው ተጋላጭነትና ለአትክልት ብክለት የሚያጋልጡ ምክንያቶችን ለማረጋገጥ በጅማ ከተማ የተመሬጡ የገበያ ስፈራዎች የተወስደ የአትክልት ናሙና ተወስዶ ለሚሰራዉ ጥናት ለሆድ ህመም የሚያጋልጡ ምክንያቶችን ለማረጋገጥ::

ዋና ተመራማሪ

ስም:- እሸቱ ችሎ

የተቋሙ ስም:- ጅማ ዩኒቨርስቲ የጤና ሳይንስ ት/ቤት የህክምና ላቦራቶሪ ሳይንስ ትምህርት ክፍል

ረጅ ድርጅት :- ጅማ ዩኒቨርስቲ

መግቢያ

ይህ ጥናት ጅማ ዩኒቨርስቲ በሚገኘው ጅማ ዩኒቨርስቲ የአትክልት ብክለት ልየስከትሉ የሚችሉ የሆድ ህመም ተህዋስያን (ባክቴሪያዎች) ለሚሰጣቸው መድሀኒቶች ያላቸው ተጋላጭነትና ለአትክልት ብክለት የሚያጋልጡ ምክንያቶችን ለማረጋገጥ በጅማ ከተማ የተመሬጡ የገበያ ስፈራዎች የተወስደ የአትክልት ናሙና ተወስዶ ለሚሰራዉ ጥናት ለህመሙ የሚያጋልጡ ምክንያቶችን ለማወቅ የሚካሄድ ጥናት ሲሆን ጥናቱ የሚካሄደው ጅማ ዩኒቨርስቲ የጤና ሳይንስ ት/ቤት የህክምና ላቦራቶሪ ሳይንስ ትምህርት ክፍል የሁለተኛ ዲግሪ ተማሪ በሆነው በአቶ እሸቱ ችሎ እና በአማካሪዎቿ ነው::

የጥናቱ አላማ

የጥናቱ አላማ በጅማ ዩኒቨርስቲ የአትክልት ብክለት ልየስከትሉ የሚችሉ የሆድ ህመም ተህዋስያን (ባክቴሪያዎች) ለሚሰጣቸው መድሀኒቶች ያላቸው ተጋላጭነትና ለአትክልት ብክለት የሚያጋልጡ ምክንያቶችን ለማረጋገጥ በጅማ ከተማ የተመሬጡ የገበያ ስፈራዎች የተወስደ የአትክልት ናሙና ተወስዶ ለሚሰራዉ ጥናት ላይ ለሚሳተፉ የተዘጋጀ መረጃ፡፡

በጥናቱ ላይ በመሳተፍ የሚገኝ ጥቅምና ሊደርስ የሚችል ጉዳት

እርስዎ በጥናቱ ላይ በመሳተፍዎ የሚደርስብዎት ምንም አይነት ጉዳት ወይም ችግር የለም ነገር ግን የእርስዎ በጥናቱ ላይ መሳተፍ ለጥናቱ ከፍተኛ ጥቅም ይሰጠዋል፡፡

በጥናቱ ላይ ያለመሳተፍ መብት

በጥናቱ ላይ ያለመሳተፍ መብትዎ የተጠበቀ ነው፡፡ በጥናቱ ላይ መሳተፍ በፈቃደኝነት ላይ የተመሰረተ ስለሆነ በጥናቱ ላይ ያለመሳተፍ መብተዎ እንደተጠበቀ ሆኖ ነገር ግን ለመሳተፍ ፈቃደኛ ሆነው በመካከል ላይ ማቋረጥ ቢፈልጉም መብትዎ ነው፡፡ በዚህ ጥናት አለመሳተፍ ወይንም በማንኛውም ጊዜና ሁኔታ ማቋረጥ ሆነ ለአንዳንድ ጥያቄዎች መልስ አለመስተጥ ይችላሉ፡፡ ለመሳተፍ ፍቃደኛ በመሆንዎም ምንም አይነት ተጽኖ እንደማይደርስብዎ ልናረጋግጥልዎ እንወዳለን፡፡

የመረጃ ሚስጥራዊነት፡- ለዚህ ጥናት ስኬታማነት የሚሰጡት ማንኛውም መረጃ ሚስጥራዊነቱ የተጠበቀና ስምዎትም የማይፃፍ ከመሆኑም ባሻገር መልስ የሰጡበትም ወረቀት የራሱ ኮድ ተሰጥቶት ተቆልፎ የሚቀመጥ ነው፡፡

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ስም፡- እሸቱ ችሎ

ስልክ ቁጥር:-+251-09-32-48-20-41 ኢሜይል eshetuchilo@gmail.com

በጥናቱ ለመሳተፍ ፈቃደኛ ነዎት?

አዎ ---------- አይደለሁም----------

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ቀን ---------- አመሰግናለሁ!

Assurance of principal investigator

The undersigned agrees to accept responsibility for the scientific ethical and technical conduct of the research project and for provision of required progress reports as per terms and conditions of the college of Health Science in effect at the time of grant is forwarded as the result of this application.

Name of the student: _____

Date._____ Signature _____

APPROVAL OF ADVISORS

This proposal is submitted with my approval as University advisor.

Name of the first advisor: _____

Date._____ Signature _____

Date of submission: _____

Name of the second advisor: _____

Date._____ Signature _____

Date of submission: _____

Annex-II: Questionnaire

Jimma University College of Health Sciences, Department Of Medical Laboratory Sciences and Pathology

Questionnaire will be filled by the data collector by interviewing each vender during the study period. When the participant answered from choice please thick on the answer or write in the space provided.

Code number _____ Interviewer_____Date of interview _____

Questionnaires for assessment knowledge of the vegetable venders and potential sources of contamination

Section I: Socio demographic variables

1. Age _____ gender _____
2. Educational level of vendors.
    A. No formal education
    B. Primary education
    C. Secondary education
3. The selected market place in the study area.
    A. Kochi
    B. Agip
    C. Hirmata merkato
    D. Bishishe merkato
4. The type of vegetables in the retail area.
    A. Green pepper
    B. Carrot
    C. Lettuce
    D. Cabbage
    E. Tomato
5. Description of vegetable status at the time of purchase
    A. Ripened
    B. Un ripened
    C. Injured or bruising
6. The type of seller of vegetable

A. Farmer

B. Merchant

7. If the answer for Q6 is “B”, what is place of display?

    A. Grocery

    B. Open market

8. How do you Store the vegetables in your vending area?

    A. Refrigerator

    B. On table next to other vegetable

    C. On the same shelf with other vegetables

9. Is there waste container around the vending area?

    A. Yes

    B. No

10. If yes to Q9 waste container

    A. has lid

    B. has no lid

    C. leaking

Section II: knowledge and practice related to fresh product for all vegetable venders

11. Do you think contaminated vegetable is a sources of foodborne disease?

    A. Yes

    B. No

12. If Q11 is yes what do you think are the sources of vegetable contamination?

    A. Contaminated hands

    B. Using polluted water for washing

    C. Contaminated container

    D. Others (specify)_____

13. Do you use a toilet or latrine to urinate and defecate?

    A. Yes

    B. No

14. Do you wash and dry your hands after toileting?
    A. Yes
    B. No
15. Do you wash and dry your hands after diapering a child?
    A. Yes
    B. No
16. Do you wash and dry your hands after contact with animals?
    A. Yes
    B. No
17. Do you change your clothes and bathe regularly?
    A. Yes
    B. NO
18. If yes to Q 17 How often?
    C. Daily
    D. Once in a week
    E. Twice a week
19. Do you cover cuts, lesions and wounds before contact with vegetables?
    A. yes
    B. No
20. Do you eat fresh vegetable salad?
    A. Yes
    B. No
21. If yes to Q19 do you wash it before eating?
    A. Yes
    B. No
22. If yes to Q20 how often?
    A. Always
    B. Sometimes
23. Is there any harm of eating fresh vegetable?
    A. Yes
    B. No
    C. I do not know
24. If yes to Q22 what type of harm do you know?
    A. Diarrhea
    B. Vomiting
    C. Others specify _____
25. Do you wash your hand before handling vegetables?

A. Yes

B. No

26. If yes to Q24 how often?

A. Always

B. Sometimes

27. Are the vegetables washed before you display?

A. Yes

B. No

28. If so, what type of water source used for washing purpose?

A. Pipe water

B. Well water

C. River water

29. Is there direct hand contact with vegetables during selling, harvesting and trimming?

A. Yes

B. No

Section III: Questionnaire for farmer respondent

30. Do you put animals in a fenced area to prevent them from entering the growing fields?

A. Yes
B. No

31. Do you use harvest and storage containers for carrying materials other than vegetables?

A. Yes
B. No

32. What types of fertilizers are used?

A. Raw manure

B. Decayed manure

C. Chemical fertilizer

33. What type of water source are used for irrigation purpose?

A. Pipe water

B. Well water

C. River water

34. Are the equipment used for harvesting and transportation washed and dried?

A. Yes

B. No

35. Do you have hand washing habit before harvesting?

A. Yes

B. No

36. Do you keep containers off the ground before, during and after harvesting?

A. Yes

B. No

37. Are there insects near vegetable display areas? (answered after observation)

A. Yes

B. No

38. The health condition of the venders or presence of any illness in the last two weeks?

A. Coughing

B. Diarrhea

C. Vomiting

D. Others Specify _____

Annex VIII: Modified Kirby-Bauer susceptibility testing technique

REQUIRED

● *Mueller Hinton agar*

Prepare and sterilize the medium as instructed by the manufacturer. The pH of the medium should be 7.2–7.4. Pour into 90 mm diameter sterile petri dishes to a depth of 4 mm (about 25 ml per plate). Care must be taken to pour the plates on a level surface so that the depth of the medium is uniform. **Note:** If the medium is too thin the inhibition zones will be falsely large and if too thick the zones will be falsely small. Store the plates at 2–8 _C in sealed plastic bags. They can be kept for up to 2 weeks. For use, dry the plates with their lids slightly raised in a 35–37 _C incubator for about 30 minutes.

● *Antimicrobial discs*

The choice of antimicrobials to be included in susceptibility tests will depend on the pathogen, the specimen, range of locally available antimicrobials, and local prescribing policies. Consultation between laboratory, medical, and pharmacy staff is required. The range of first choice drugs should be limited and reviewed at regular intervals. Additional drugs should be included only by special request. Where there is cross-resistance, only one member from each group of related antimicrobials need be selected. An oxacillin disc is representative of the whole group of *beta*-lactamase resistant penicillins when testing staphylococci. *Note*: Paper antimicrobial discs are commercially available from most manufacturers of culture media. Stable antimicrobial susceptibility testing tablets are available from Rosco Diagnostica. Most paper discs can be used for 1 year or longer from the date of manufacture providing they are stored correctly (–20 _C, or working stock at 2–8 _C in an airtight container with an indicating desiccant. Discs that have expired should not be used. Quality control of discs is essential. About 1 hour before use, the working stock of discs should be allowed to warm to room temperature, protected from direct sunlight. *Important*: Decreasing control zone sizes with a particular antimicrobial disc is often an indication of deterioration of the antimicrobial due to moisture or heat.

● *Turbidity standard equivalent to McFarland 0.5* This is a barium sulphate standard against which the turbidity of the test and control inocula can be compared. When matched with the standard, the inocula should give confluent or almost confluent growth. Shake the standard immediately before use.

Preparation of turbidity standard

1 Prepare a 1% v/v solution of sulphuric acid by adding 1 ml of concentrated sulphuric acid ***to*** 99 ml of water. Mix well. ***Caution*****:** Concentrated sulphuric acid is hygroscopic and highly corrosive, therefore do not mouth pipette, and *never add the water to the acid*. 2 Prepare a 1% w/v solution of barium chloride by dissolving 0.5 g of dihydrate barium chloride (BaCl2.2H2O) in 50 ml of distilled water. 3 Add 0.6 ml of the barium chloride solution to 99.4 ml of the sulphuric acid solution, and mix. 4 Transfer a small volume of the turbid solution to a capped tube or screw-cap bottle of the same type as used for preparing the test and control inocula. When stored in a well-sealed container in the dark at room temperature (20–28 _C), the standard can be kept for up to 6 months.

Control strains

Control strains are used to test the performance of the method. The following strains of bacterial species are recommended.

● *Staphylococcus aureus* ATCC 25923.

● *Escherichia coli* ATCC 25922.

Sources of control strains

Reference Laboratories should supply local laboratories. The control strains should be grown on slopes of nutrient agar or tryptone soya agar and stored refrigerated at 2–8 _C. They should be subcultured every 3–6 months. At the beginning of each week a nutrient broth or agar culture should be prepared for daily use.

Method

1 Using a sterile wire loop, touch 3–5 well-isolated colonies of similar appearance to the test organism and emulsify in 3–4 ml of sterile physiological saline or nutrient broth. 2 In a good light match the turbidity of the suspension to the turbidity standard (mix the standard immediately before use). When comparing turbidities it is easier to view against a printed card or sheet of paper.

3 Using a sterile swab, inoculate a plate of Mueller Hinton agar. Remove excess fluid by pressing and rotating the swab against the side of the tube above the level of the suspension Streak the swab evenly over the surface of the medium in three directions, rotating the plate approximately 60_ to ensure even distribution .

4 With the petri dish lid in place, allow 3–5 minutes (*no longer than 15 minutes*) for the surface of the agar to dry.

5 Using sterile forceps, needle mounted in a holder, or a multidisc dispenser, place the appropriate antimicrobial discs, evenly distributed on the inoculated plate. ensure the discs are correctly placed.

Note: The discs should be about 15 mm from the edge of the plate and no closer than about 25 mm from disc to disc. No more than 6 discs should be applied (90 mm dish). Each disc should be lightly pressed down to ensure its contact with the agar. It should not be moved once in place.

6 Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35 _C for 16–18 h (temperatures over 35 _C invalidate results for oxacillin).

7 After overnight incubation, examine the control and test plates to ensure the growth is confluent or near confluent. Using a ruler on the underside of the plate measure the diameter of each zone of inhibition in mm. The endpoint of inhibition is where growth starts.

Sulphonamides and co-trimoxazole Ignore any slight growth within the inhibition area. *Beta-lactamase producing staphylococci* A zone of inhibition can be formed by penicillin resistant

staphylococci when the amount of *beta*-lactamase (penicillinase) is insufficient to inactivate the penicillin close to the disc. Such a zone, however, has a heaped up clearly defined edge with no gradual fading away of growth towards the disc as seen with susceptible strains. Colonies may sometimes be seen growing within the inhibition zone. Report all strains showing a heaped up zone edge, regardless of the size of the inhibition zone, as '*Resistant*'. *Proteus strains* Some *Proteus* strains may swarm into the area of inhibition but the actual zone of inhibition is usually clearly outlined.

Interpretation of zone sizes

Using the Interpretative Chart, interpret the zones sizes of each antimicrobial, reporting the organism as 'Resistant', 'Intermediate/Moderately susceptible', 'Susceptible'.

Resistant: A pathogen reported as 'resistant' implies that the infection it has caused will not respond to treatment with the drug to which it is resistant irrespective of dose or site of infection.

Intermediate: A pathogen reported as intermediately susceptible suggests that the infection it has caused is likely to respond to treatment when the drug is used in larger doses than normal or when the drug is concentrated at the site of infection, e.g. in the urinary tract. Consideration should be given to using other drugs that may provide more optimal therapy.

Susceptible: A pathogen reported as susceptible suggests that the infection it has caused is likely to respond to treatment when the drug to which it is susceptible is used in normal recommended doses and administered by an appropriate route.

ANNEX: IV Procedure for plasmid isolation

1. Inoculate10 ml of rich medium (tryptic soya Broth) Incubate the culture overnight at 37°C.
2. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at maximum speed for 30 seconds in a microfuge. Store the unused portion of the original culture at 4°C.
3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
4. Resuspend the bacterial pellet in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing.
5. Add 200 µl of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents well by inverting the tube . Do not vortex! Store the tube in ice.
6. Add 150 µl of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube in ice for 3-5 minutes.
7. Centrifuge the bacterial lysate for 5 minutes at maximum speed in a microfuge. Collect the supernatant to a fresh tube.
8. (Optional) Add equal volume of phenol: chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 2 minutes in a microfuge. Transfer the aqueous upper layer to a fresh tube.
9. Precipitate nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature.
10. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes in a microfuge.
11. Discard the supernatant by aspiration. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kim wipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.
12. Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for 2 minutes in a microfuge.
13. Remove all of the supernatant by aspiration. Take care with this step, as the pellet sometimes does not adhere tightly to the tube.
14. Remove any beads of ethanol from the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5-10 minutes).
15. Dissolve the nucleic acids in 50 µl of TE (pH 8.0) Vortex the solution gently for a few seconds and detect plasmid by gel electrophoresis.

Recipes for Buffers, Solutions and Media:

Alkaline Lysis Solution I :

50 mM glucose.
25 mM Tris-Cl (pH 8.0).
10 mM EDTA (pH 8.0).

Prepare Solution I from standard stocks in batches of approx. 100 ml, sterilize by autoclaving and store at 4°C.
(For plasmid preparation.)

Alkaline Lysis Solution II:

0.2 N NaOH (freshly diluted from a 10 N stock).
1% (w/v) SDS.
Prepare Solution II fresh and use at room temperature.
(For plasmid preparation.)

Alkaline Lysis Solution III:

5 M potassium acetate, 60.0 ml.
Glacial acetic acid, 11.5 ml.
H2O, 28.5 ml.
The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.
(For plasmid preparation.)

EDTA:

To prepare 0.5 M EDTA (pH 8.0): Dissolve 186.1 g of disodium EDTA•$2H_2O$ in 800 ml of Distilled $2H_2O$. Stir well on a magnetic stirrer. EDTA will not dissolve into solution until the pH of the solution is reached to ~ 8.0 . So the pH should adjust to 8.0 with NaOH (~ 20 g of NaOH pellets) and make up the final volume to 1000ml with distilled water. Prepare the aliquots and sterilize by autoclaving.

NaCl:

To prepare 5 M NaCl : Dissolve 292 g of NaCl in 800 ml of sterile H_2O and the volume is make up to to 1 liter with deionized H_2O. Prepare the aliquots and sterilize it by autoclaving.

NaOH:

To 800 ml of H_2O, add 400g of NaOH pellets slowly, stirring continuously. After dissolving the pellets,completely, make up the final volume to 1 liter with sterile H_2O. Store the solution at room temperature.

Potassium Acetate:

5 M potassium acetate, 60 ml.
Glacial acetic acid, 11.5 ml.
H_2O, 28.5 ml.
The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at room temperature

SDS:

Also called sodium lauryl sulfate. To prepare a 20% (w/v) solution, dissolve 200 g of SDS in 900 ml of H2O. Heat to a temperature of 68°C and stir with a magnetic stirrer to help dissolution. Adjust the volume to 1 liter with distilled H_2O. Store at room temperature. Autoclaving not necessary.

STE:

10 mM Tris-Cl (pH 8.0).
0.1 M NaCl.
1 mM EDTA (pH 8.0).
Sterilize the solution by autoclaving and store at 4°C.

TE:

100 mM Tris-Cl (desired pH).
10 mM EDTA (pH 8.0).
(10x Tris EDTA) Sterilize the buffer by autoclaving and store at room temperature.

Tris-Cl:

Dissolve 121.1 g of Tris base in 800 ml of H2O. Adjust the pH by adding concentrated HCl , to the desired value.The volume of the solution is make up to 1 liter with distilled H_2O. Prepare the aliquots and sterilize by autoclaving.

10M NaOH
Dissolve 400g NaOH in 450ml ddH_2O
Add H_2O to 1 liter

3M Sodium acetate
Dissolve 408g sodium acetate·$3H_2O$ in 800ml ddH_2O
Adjust pH to 5.2 with glacial acetic acid
Add ddH_2O to 1 liter

1M Tris·Cl [tris(hydroxymethyl)aminomethane]
Dissolve 121g Tris base in 800ml ddH_2O

Adjust to the desired pH with concentrated HCl
Mix and add ddH_2O to 1 liter

250ml GTE	LB-Ampicilin
2.25g glucose	Stock: 100mg/ml
6.25ml 1M Tris (pH 8.0)	Working: 100µg/ml
5.0ml 500mM EDTA	

50ml NAOH/SDS	5M KAc (pH 4.8)
45ml H_2O	73.5g Potassium Acetate
1.0ml 10N NaOH	28.75ml Glacial Acetic Acid
5ml 10% SDS	H_2O to bring volume to 250ml
(make fresh every 2 weeks)	

Procedure for Operating the Virtual Lab:

Check whether you have done all the steps listed below:

- Prepare the culture containing the desired plasmid.
- Incubate the culture for 24 hours at 37°C.
- Take the culture from the incubator.
- Transfer 1.5ml of the culture to a microfuge tube.
- Centrifuge the tube for 30seconds at maximum speed
- Remove the supernatant.
- Add 100µl alkaline lysis solution I.
- Vortex the sample.
- Add 200µl of alkaline lysis solution II.
- Mix the sample by inverting the tube.
- Store in ice for 1 minute.
- Add alkaline lysis solution III.
- Mix the contents by inverting the tube.
- Store in ice for 3-5 minutes.
- Centrifuge the solution at maximum speed for 5 minute .
- Collect the supernatant to a fresh tube.
- Precipitate the nucleic acid by adding 2 volumes of ethanol.
- Mix by vortexing.
- Stand the tubes for 2 minutes.
- Centrifuge for 5 minutes.
- Collect the precipitated DNA.
- Discard the supernatant by aspiration.
- Stand the tube as inverted to drain the fluid away.
- Add 1ml 70% ethanol.
- Mix by inverting.

- Centrifuge the mixture for 2 minutes.
- Discard the supernatant by aspiration.
- Allow to dry for 3-5 minutes.
- Add TE buffer
- Mix by flickering.
- Detect the plasmid by doing agarose gel electrophoresis.