Molecular Characterization of Bacterial Pathogen Isolated from Raw Salad Vegetables

A THESIS

BY

MD. SADEKUZZAMAN

REGISTRATION NO. 1805359

SEMESTER: JULY-DECEMBER, 2019

SESSION: 2018-2019

MASTER OF SCIENCE (MS)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

DECEMBER, 2019

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HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

DECEMBER, 2019

DEDICATED

TO MY

BELOVED PARENTS

ABDUL ZAHUR

AND

RUKHSHANA BEGUM

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The Author

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Abstract

Vegetables salad are considered as a high-risk food because they do not require any heating, washing or cleaning prior to consumption. The study was conducted to examine microbiological quality of raw salad vegetables and their role as a source of antibiotic resistant bacteria. Seven types of vegetables which are commonly used for salad i.e Cucumber, Carrot, Green Chilli, Peeper Mint, Lemon, Onion, Corriandar Leaves were collected from two Open markets and four different hotel/ restaurants of Dinajpur town. All the vegetables were contaminated with *Klebsiella* spp, *E.coli* spp, *Salmonella* spp, *Shigella* spp, *Staphylococcus* spp, *Proteus* spp, *Vibrio* spp, *Pseudomonas* spp, *Streptococcus* spp. The isolated bacteria were identified by studying staining characteristics, cultural properties on different selective media, biochemical tests, their antimicrobial resistance by Kirby-Bauer disc diffusion method and finally molecular characterization by PCR by 16S rRNA gene region was amplified with the universal primers, Forward primer 27F (5'-AGAGTTTGATCCTEGGCTCAG3) and Reverse primer 1492 R (5'-TACCTTGTTACGACTT3). From market samples, the bacterial loads were : *Klebsiella* spp. (10.90%), *E.coli* (12.73%), *Salmonella* spp (7.27%), *Shigella* spp (18.18%), *Staphylococcus* spp (1.82%) ,*Proteus* spp (16.36%), *Vibrio* spp (12.73%), *Pseudomonas* spp (18.18%), *Streptococcus* spp (1.82%). On the other hand, In hotel/ restaurant samples, the bacterial loads were : *Klebsiella* spp (6.38%), *E.coli* (29.79%), *Salmonella spp* (10.64%), *Shigella* spp (23.40%), *Staphylococcus* spp (6.38%), *Proteus* spp (2.13%), *Vibrio* spp (8.51%), *Pseudomonas* spp (8.51%) and *Streptococcus* spp (4.26%). Antibiotic sensitivity test showed that all bacterial isolates were resistant to Amoxicillin, Piperacillin, Bacitracin, Cloxacillin, Novobiocin, Methicillin, Cefixime and Vancomycin and sensitive to Nalidixic acid, Azithromycine, Chloramphenicol; Intermediate sensitive to Kanamycin, Tetracycline, and Norfloracin. Therefore, we saw that a great risk towards public health is posed by the organic fertilizers applied in the fields. Vegetables grown with untreated fertilizers may play a significant role in showering pathogens to the consumers. Hence, it is recommended that a more close supervision of such food type should be carried out by relevant authorities to avoid any future pathogen outbreaks. Thus the results suggest the necessity to follow the hygienic practices in salad preparation because salad might have an important role as a source of multiple antibiotic resistant bacteria.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	=	Negative
%	=	Percentage
&	=	And
+	=	Positive
≤	=	Less-than or equal to
≥	=	Greater-than or equal to
°C	=	Degree Celsius
MDROs	=	Multi Drug Resistant Organisms
mg	=	Milligram
ml	=	Millilitre
MR	=	Methyl Red
n	=	Number of isolates from each source
NA	=	Nutrient Agar
NB	=	Nutrient Broth
OIE	=	Office International Des Epizooties
PBS	=	Phosphate Buffer Saline
R	=	Resistant
S	=	Sensitive
Sl. No.	=	Serial Number
spp.	=	Species
SS	=	Salmonella-*Shigella* Agar
TE	=	Tetracycline
V-P	=	Voges-Proskauer
WHO	=	World Health Organization
µg	=	Microgram
µl	=	Microlitre
CLSI	=	Clinical and Laboratory Standards Institute
Contd.	=	Continuation
E	=	Erythromycin
E. coli	=	*Escherichia coli*
e.g.	=	Example

EMB	=	Eosine Methylene Blue
et al.	=	Et alia (associates)
etc.	=	Etcetra
FAO	=	Food and Agricultural Organization
Fig.	=	Figure
gm	=	Gram
GN	=	Gentamicin
H_2O_2	=	Hydrogen peroxide
H_2S	=	Hydrogen Sulphide
I	=	Intermediate
K	=	Kanamycin
KOH	=	Potassium hydroxide
HSTU	=	Hajee Mohammad Danesh Science and Technology University
i.e.	=	That is
M.S	=	Master of Science
MSA	=	Mannitol Salt Agar
Prof.	=	Professor
PCR	=	Polymerase Chain Reaction
kb	=	kilo base
bp	=	base pair

CHAPTER-I

INTRODUCTION

Now a days salads have got much attention due to health-related beneficial features. Raw vegetables have become main constitutive. They provide for a great variety of fiber contents, vitamins, minerals and other phytochemicals, which are very much essential for human health. Demand for fresh, minimally processed vegetables has driven to an increase in the content and diversity of ready-to-eat products available to the consumer. These products embed a suitable meal for today's lifestyles, because they require minimal preparation. Minimally processed fresh (MPF) vegetables may be simply chopped vegetables or may consist of trimmed, peeled, sliced/shredded, washed and/or disinfected vegetables. The bacterial species on the outer plant surface are more related to soil bacteria whereas specific typical plant–bacteria associations are often determined by molecular factors. Differences in bacterial communities in soil come from many factors, possibly also as a consequence of farming methods. Little is known about microbes on salad, from the point of field harvesting to the consumers table. (Brocklehurst *et al*., 2006).

Vegetables serve a major part of our food supply. Raw vegetables harbor a number of pathogenic microorganisms, which may be dispersed over the plants or appear as microcolonies embedded in the plant tissues (Beuchat *et al*., 2002). During harvesting and transportation, raw vegetables may be bruised resulting in the release of plant nutrients, and thereby, providing substrates for microorganisms present on the surface of the vegetables to grow. In addition, the processing of fresh salad vegetables may alter or increase the number and type of pathogens present on the surface of the product. With a view of such exposure to pathogens, vegetables have been associated with the outbreaks of food borne disease in many countries (Alice *et al.,* 1997). Therefore, a great risk towards public health is posed by the organic fertilizers applied in the fields. The major bacterial diseases shaded are the various enteric diseases, diarrhoea, anthrax, salmonellosis, listeriosis, Crohn's disease, thrombocytopenic purpura, neurological disorders, arthritis, etc. (Cray and Moon 1995, Snowdon *et al.,* 1989, Starutch *et al.,* 1991). Food borne illnesses can be caused mainly by microorganisms and/or their toxins. Cultivation of vegetables may largely account for such

pathogenic contamination. Manures used to promote the growth of crops and vegetables contain a large number of pathogenic microorganisms including *Salmonella*, *Escherichia coli* O157:H7, *Bacillus anthracis*, *Mycobacterium* spp., *Brucella* spp., *Listeria monocytogenes*, *Yersinia enterolytica*, *Clostridium perfringens*, *Klebsiella* spp. and *M. paratuberculosis* (Alice *et al.*, 1997). Bacteria involved in spoilage of vegetables are usually pectinolytic species of the Gram negative genera of *Erwinia*, *Pseudomonas*, *Clostridium*, and *Xanthomonas* and the non-sporing Gram positive organisms like *Corynebacterium* (Adams and Moss, 1999). Salads containing raw vegetables have been identified as vehicles of traveller's diarrhea, an illness sometimes experienced by visitors to developing countries (Beuchat and Larry, 1996). Due to the high nutritional value, vegetables are considered as important components in every healthy human diet. Regular consumption of vegetables can reduce risk of some important disease such as cancers, stroke and cardiovascukar diseases (Van Duyn and Pivonka, 2000). In Bangladesh, food and water have often been pointed as the principal causes for the transmission of various enteric diseases (Ahmed *et al.*, 2014; Khan *et al.*, 2014; Feroz *et al.*, 2013; Rahman and Noor, 2012; Nawas *et al.*, 2012; Nipa *et al.*, 2011). Several suggestive data let us assume that the plantation soils, fertilizers applied into the agricultural lands and the irrigation waters could disseminate harmful microorganisms like *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus, Salmonella* spp., *Aeromonas* spp., *Streptococcus* spp., *Staphylococcus* spp., *Pseudomonas* spp. and *Vibrio* spp. into the vegetables and fruits grown (Ahmed *et al.*, 2014; Feroz *et al.*, 2013; Rahman and Noor, 2012; Nipa *et al.*, 2011).

Nevertheless, no experimental demonstration still exists from the perspective of Bangladesh to bring about the logical explanation behind the vegetable contamination. In addition, the microbial contamination may be attributed to the unhygienic environments during the crop cultivation, production, processing, harvesting and storage (Nawas *et al.*, 2012; Telias *et al.*, 2011). Indeed, along with especially the organic fertilizers, the plantation soils are widely known to harbor a range of pathogenic microorganisms (Galitskaya *et al.*, 2015; Bao *et al.*, 2012; Uddin *et al.*, 2012). The waters originating from various sources also play a vital role in propagating microorganisms not only into the plantation soils but also directly into the vegetables during irrigation or while moistening (Acharjee *et al.*, 2013; Bassan *et al.*, 2013; Chigor *et al.*, 2013; Hawkins *et al.*, 2013; Dufour *et al.*, 2012; Hanjra *et al.*, 2012; OECD,

2012; Oliveira and von Sperling, 2011; Qadir *et al.*, 2010). Microorganisms accessing the vegetables from all these sources are likely to affect the shelf life and the nutritional quality of the fresh produces (Feroz *et al.*, 2013). According to World Health Organization more than 5,000 children die every day due to the consumption of contaminated food and water (Hannan *et al*., 2014). The condition is worse in the underdeveloped countries due to poverty, overcrowding, unhygienic and inadequate sanitary condition and illiteracy (Balter *et al.*, 2006).There are various types of microorganisms and their associated toxins that causes food borne diseases such as *Salmonella* spp., *Shigella* spp., *Escherichia coli, Bacillus anthracis, Klebsiella* spp., *Brucella* spp., *Listeria monocytogenes, Yersenia enterolytica, Clostridium perfringens* (Dan *et al*., 2015). Development of resistant bacteria in food animals can result from chromosomal mutations but is more commonly associated with the horizontal transfer of resistance determinants borne on mobile genetic elements which promote resistance in natural and opportunistic conditions such as vegetal surfaces or human colon. Therefore, the occurrence of antimicrobial resistant pathogens in fresh vegetables creates an additional worry for consumer safety (Aarestrup *et al*., 2008). It is clearly evident that a large number of vegetables are a good source of antioxidants and phytonutrients, and have health protecting properties (Meng *et al.*, 2002; Heo and Lee, 2006; Vrchovska *et al.*, 2006; Adjrah *et al.*, 2013), to improve human well being. In contrast with these advantages, the salads containing raw vegetables may be unsafe, mainly because of the environment under which they are prepared and consumed (Taban and Halkman, 2011; Adjrah *et al.*, 2013) and also of the lake of personal hygiene (Martinez-Tomé *et al.*, 2000; Cuprasitrut *et al.*, 2011; Adjrah *et al*., 2013).

With a great consideration given to the above facts in view, the goal of the present study were-

- To isolate and identify bacteria in different types of raw salad vegetables collected from markets and hotels/ restaurents in Dinajpur town.
- To determine antimicrobial susceptibility patterns of identified bacteria.
- To identify the molecular pattern by using PCR to know the identified organisms are whether harmful or not.

CHAPTER-II

REVIEW OF LITERATURE

Caldera *et al.*, (2016) collected sixty-six putative *Pseudomonas* strains isolated from different food matrices (ready-to-eat vegetables, meat, milk and dairy products) were examined for their phenotypic features and enzymatic spoilage activities. Their genotype was studied by BOX-PCR, *Pseudomonas* specific 16S PCR, *aprX* and housekeeping genes sequencing (16S rRNA gene, *gyrB* and *rpoB*). The majority of the isolates are very versatile as shown by their wide ranges in growth temperature (4–45 °C), pigment production and production of enzymes. The BOX-PCR clustering showed a high genetic diversity among the isolates and phylogenetic analysis of the *rpoB* gene allowed a first putative identification at the species level. Thirteen isolates were provisionally classified as *Pseudomonas gessardii*-like, but probably belong to a yet unknown *Pseudomonas* species in the *Pseudomonas fluorescens* group. Protease-activity was qualitatively and quantitatively verified. A large variation in proteolytic activity measured in UHT-milk was observed amongst the protease positive isolates. Several isolates provisionally classified as *P. gessardii*-like showed the highest activities. An *aprX* gene based phylogenetic dendrogram showed five different groups and two sub-groups, for which a correlation with the matrix of origin could be demonstrated. An insertion of 15 bp was observed in the *aprX* gene sequences of isolates of mainly dairy origin.

Ilyas *et al.*, (2016) evaluated the frequency and antimicrobial susceptibility pattern of pathogens present in ready-to-eat salads available at a local market. A 100 salad samples were collected aseptically. Each sample (25 g) was homogenized in 225 ml of sterile peptone water and was serially diluted up to 1×106. Dilutions were inoculated on nutrient agar by surface spread plate technique. Aerobic colony count (ACC) was estimated by counting the colonies. Bacterial isolates were cultured on blood and MacConkey agar and identified on the basis of their morphology, culture characteristics and confirmed by API 20E and 20NE. Antimicrobial susceptibility was determined as per CLSI 2014.ACC range was 1.1×103 cfu/g to 5.8×109 cfu/g. Among these the highest ACC was found in channa chat (4.9×104 to 5.8×109 cfu/g). A total of 127 microorganisms were identified; 73 were gram negative rods (GNRs) and 24 were gram positive cocci (GPC). Among

GNRs; *Klebsiella* spp. (*n*=18) was the predominant whereas among GPC, *Staphylococcus aureus* (*n*=6) were the chief pathogen. *Klebsiella* spp. showed 100 percent resistance to ampicillin, 89-78 percent to amoxicillin/clavulanic acid and 33 percent to imipenem, however, *Enterobacter* spp. were resistant to ampicillin (100 percent) amoxicillin/clavulanic acid (77 percent) and imipenem (23 percent). *Staphylococcus aureus* showed resistance to co-amoxiclav (83 percent) and penicillin (75 percent).

M.A. Islam *et al.*, (2016) intended for molecular detection of *E. coli* isolated from raw cow's milk. A total of 20 milk samples were collected from different upazila markets of Jamalpur, Tangail, Kishoreganj and Netrokona districts of Bangladesh. Milk samples were cultured onto various culture media for the isolation of bacteria. The isolated bacteria were identified by studying staining characteristics, cultural properties on different selective media, biochemical tests, catalase and coagulase test, and finally by PCR. Out of 20 samples, 15 (75%) milk samples were found positive for *E. coli*. 15 *Escherichia coli* isolates were amplified by *16S rRNA* gene based PCR. Antimicrobial sensitivity test was carried out to ascertain the susceptibility of the organism to various antibiotics. Its results showed that the *E. coli* isolates were resistant to amoxycillin (86.67%) and erythromycin (73.33%) but sensitive to azithromycin (53.33%), ciprofloxacin (86.67%), gentamicin (86.67%), norfloxacin (80%) and streptomycin (66.67%).

Kim *et al.*, (2016) reported that organic foods have risen in popularity recently. However, the increased risk of bacterial contamination of organic foods has not been fully evaluated. In this study, 100 samples each of organic and conventional fresh vegetables (55 lettuce samples and 45 sprout samples) sold in South Korea were analyzed for aerobic bacteria, coliforms, *Escherichia coli*, and *Bacillus cereus*. Although the aerobic bacteria and coliform counts were not significantly different between the two farming types ($p > 0.05$), the occurrence rate of *B. cereus* was higher in organically cultivated vegetables compared with those grown conventionally (70% vs. 30%, respectively). The mean contamination level of *B. cereus*-positive organic samples was also significantly higher (1.86 log colony-forming unit [CFU]/g vs. 0.69 log CFU/g, respectively) ($p < 0.05$). In addition, six samples of organic vegetables were found to be contaminated with *B. cereus* at over 4 log CFU/g categorized as unsatisfactory according to Health Protection Agency guideline. The

relatively higher occurrence rate of *B. cereus* in organic vegetables emphasizes the importance of implementing control measures in organic vegetable production and postharvest processing to reduce the risk of food poisoning.

Tan *et al.*, (2016) conducted water vending machines provide an alternative source of clean and safe drinking water to the consumers. However, the quality of drinking water may alter due to contamination from lack of hygienic practices and maintenance of the machines. Hence, this study was conducted to determine the microbiological quality of water from vending machines and associated contact surfaces. Seventeen water samples and 85 swab samples (nozzles, drip trays, coin slots, buttons and doors) from 3 locations in Kelantan were collected. Polymerase chain reaction amplification and 16S ribosomal ribonucleic acid (rRNA) sequencing were carried out and sequences obtained were compared against the sequences available in the National Centre for Biotechnology Information database using the basic local alignment search tool programme. Coliform counts were observed in 94.12 % of water samples, 76.47 % of nozzles and 82.35 % of drip tray swabs. Furthermore, results of 16S rRNA sequence analysis indicated that two gram-negative isolates were identified as *Escherichia coli* U 5/41 (Accession no. NR_024570.1) and *E. coli* O157:H7 EDL933 (Accession no. CP008957.1) with similarity value of 100 %, respectively. The results from this study further improve our understanding of the potential microorganisms in drinking water. Regular maintenance and cleaning of water vending machines are important to reduce bacterial growth and the presence of waterborne pathogens.

Cheah *et al.*, (2015) examined *Escherichia coli* and *Escherichia coli* O157 were identified from "selom" *(Oenanthe stolonifera)*, "pegaga" *(Centella asiatica)*, beef, chicken, lamb, buffalo, "ulam Raja" *(Cosmos caudatus)* and "tenggek burung" *(Euodia redlevi)*. The bacteria were recovered using chromagenic agar. Isolated *Escherichia coli* and *Escherichia coli* 0157 were further characterized by plasmid profiling and enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). The virulence genes of the isolates *(VT1, VT2, LT, ST, eaeA, inV)* that produces pathogenic *Escherichia coli* and *16S rRNA* gene were screened by a multiplex PCR assay. The plasmid profiling analysis showed that out of 176 isolates, only 103 isolates contained plasmids. ERIC-PCR analysis generated

amplified products in the range of ~150 bp to > 1000 bp categorizing isolates into a total of 52 different profiles. Multiplex PCR showed that 20 (32.3%) of the isolates carried *eaeA* gene, 6 (9.7%) isolates possessed *inV* genes, only 1 (1.6%) have *VT2* genes and 1 (1.6%) as well carried *VT1* genes, 2 (3.2%) of the isolates harboured *LT* genes, and only 1 (1.6%) isolate possessed *ST* genes. There were no correlation between plasmid, ERIC-PCR and virulence genes profiles.

Gurler *et al.*, (2015) conducted to the microbiological safety of ready-to-eat (RTE) foods is of special concern as they are not exposed to further processing before consumption. In the present study, *Listeria monocytogenes* and *Salmonella* spp. were isolated from 15(6%) and 21(8%) samples respectively out of 261 RTE foods commercialized in Turkey. *Escherichia coli* was present in 10(4%) samples analyzed. Psychrotrophic aerobic populations > 6 log CFU/g were found in 36 (14%) of the samples, while total coliforms were detected in 155 (59%) of samples analyzed. All of the *Salmonella* spp. and *L. monocytogenes* isolates tested, exhibited resistance to one or more antimicrobial agents used. For *Salmonella* spp. isolates, resistance to penicillin (69%), erythromycin (38%), gentamicin (36%), tetracycline (36%) neomycin (33%), ampicillin (33%), amikacin (33%), vancomycin (33%), streptomycin (29%) cefotaxime (9%) and oxacillin (9%) was observed. For *L. monocytogenes* isolates, resistance to erythromycin (23%) and cephalothin (20%) was evident. The presence of pathogens and the relatively high resistance among the bacteria tested in RTE foods could pose public health and therapeutic problems in consumers. These results indicate the need of implementing hygienic rules in the production chain of RTE foods to ensure microbiological safety and to improve shelf life.

Md. Sajjad Alam *et al.*, (2015) emphasized on contamination sources of freshly cultivated vegetables commonly consumed by the Bangladeshi people. Several local studies have been conducted to detect the microbial contamination within fresh vegetables, plantation lands and the irrigation waters separately; however, the correlation of microbial contamination between the fresh produces and the surrounding environment has not been clarified.Bottle gourd (*Lagenaria siceraria*), pumpkin (*Cucurbita pepo*), radish (*Raphanus sativus*) and eggplant (*Solanum melongena*); their plantations soils and the fertilizers applied across the agricultural lands; and, finally, the irrigation waters used were analyzed from nine districts

of Bangladesh using conventional microbiological and biochemical methods. Almost all vegetable samples studied were found to be immensely contaminated with bacteria and fungi. Among the pathogens, *Klebsiella* spp., *Staphylococcus* spp. And *Pseudomonas* spp. were found to be dominant. Besides, massive microbial growth was also observed in the plantation soils and fertilizers, including *Klebsiella* spp., *Pseudomonas* spp., *Bacillus* spp., *Listeria* spp., *Escherichia coli* and *Vibrio* spp. Existence of the fecal coliforms, *E. coli*, *Klebsiella* spp., *Salmonella* spp. and *Listeria* spp., was noticed in the irrigation waters. Although the present study revealed the combined results connecting the vegetable contamination aspect with the knowledge on microbiology ultimately in the food chain, implementation of molecular studies detecting the virulence genes both in the fresh produces and the plantation soils, fertilizers and the irrigation waters would further clarify the microbial dissemination mechanism. Earlier studies demonstrated the ability of water bodies to disseminate numerous microorganisms into the plantation soils, and to some extent unraveled the ability of organic fertilizers to propagate pathogenic bacteria into the vegetation objects. These microorganisms may pose as a threat to vegetables, particularly by limiting crop production as well as the shelf life of the fresh produces.

Hannan *et al.*, (2014) reported food-borne diseases are the global public health problem. These play a significant role in human morbidity, mortality and economic loss. Ready-to-eat salads are considered as a high-risk food because they do not require any heating, washing or cleaning prior to consumption. Therefore, we aimed to determine the microbiological quality of ready-to-eat salads in our locality. A total of 50 different salads were collected aseptically from different vendors and restaurants of Lahore, Pakistan. Each sample (10 g) was homogenized in stomacher. The homogenized material was serially diluted up to 10⁻⁶ using 0.1% peptone water as diluent. The dilutions were inoculated on blood, nutrient and MacConkey agar by Surface-Spread Plate technique and plates were incubated at 35°C for overnight. Aerobic colony count (ACC) was determined by counting the colonies on nutrient agar plates. The identification of the organisms was determined by their morphology, culture characteristics and biochemical profile. The ACC range of salad samples was found to be 1.0 x 10^3 cfu/g to 5.8 x 10⁸ cfu/g. Among these, 22% samples showed unsatisfactory level of ACC and 20% were at borderline. The highest CC (cfu/g) was found in dry vegetables salads (5.8 x 10⁸) and least microbial loads

(1.0 x 10^3) were observed in vinegar-containing vegetable salads. Among Gram-negative rods *Klebsiella* spp. (16%) were isolated most frequently followed by *Enterobacter* spp. (11%). Whereas among Gram-positive cocci, *Enterococcus* spp. (13%) was foremost followed by *Staphylococcus aureus* (7.5%). This study revealed the potential hazard of ready-to-eat salads and it is the need of the hour to perform a surveillance study at national scale.

Muhammad *et al.*, (2014) evaluated the frequency and antimicrobial susceptibility pattern of pathogens present in ready-to-eat salads available at a local market. A 100 salad samples were collected aseptically. Each sample (25 g) was homogenized in 225 ml of sterile peptone water and was serially diluted up to 1×106. Dilutions were inoculated on nutrient agar by surface spread plate technique. Aerobic colony count (ACC) was estimated by counting the colonies. Bacterial isolates were cultured on blood and MacConkey agar and identified on the basis of their morphology, culture characteristics and confirmed by API 20E and 20NE. Antimicrobial susceptibility was determined as per CLSI 2014.ACC range was 1.1×103 cfu/g to 5.8×109 cfu/g. Among these the highest ACC was found in channa chat (4.9×104 to 5.8×109 cfu/g). A total of 127 microorganisms were identified; 73 were gram negative rods (GNRs) and 24 were gram positive cocci (GPC). Among GNRs; *Klebsiella* spp. (n ¼ 18) was the predominant whereas among GPC, *Staphylococcus aureus* (n ¼ 6) were the chief pathogen. *Klebsiella* spp. showed 100 percent resistance to ampicillin, 89-78 percent to amoxicillin/clavulanic acid and 33 percent to imipenem, however, *Enterobacter* spp. were resistant to ampicillin (100 percent) amoxicillin/clavulanic acid (77 percent) and imipenem (23 percent). *Staphylococcus aureus* showed resistance to co-amoxiclav (83 percent) and penicillin (75 percent).

Owda *et al.*, (2014) aimed to investigate the bacteriological quality of fresh vegetables salad sold in the local school canteens and restaurants inGaza strip. Methodology: Samples examined in the present investigation included different types of fresh vegetables salad. A total of 200 random samples were collected from UNRWA and Palestinian authority school canteens (100 samples) and different restaurants (100 samples) in Mid Zone, Khan Younis and Rafah governorates. All microbiological examinations were carried out at the Public Health Laboratory for Food and Water, Gaza-Palestine. Chi square test was used to detect

significant difference or correlation among variables. Results: In this study it was found that 88% of vegetables salad samples failed to comply with standards, the percentage distributed as follows; 79.5% (159/200) with Total Plate Count, 60.5% (121) with Total Coliform bacteria, 53.5% (107) with *E. coli*, 21.5% (43) with *S. aureus*, 14% (28) *B. cereus*, 7.5% (15) *Listeria* spp., 2% (4) L. monocytogenes, 5% (10) *Salmonella* spp., 1% (2) *Shigella* spp., and 1% (2) *E. coli* O157:H7. In this study the mixed vegetables salad samples showed the highest non-compliant. Moreover, other isolated bacteria included; *Cronobacter sakazakii* (formerly known as *Enterobacter sakazakii*) (12.5%; 25), *Pasturella* spp. (3%; 6) and *Aeromonas hydrophila* (0.5%; 1). The results revealed that there was statistically significant correlation between the compliance and non compliance in different governorates (P= 0.025).

Tango *et al.*, (2014) stated that foods grown in organic production systems have been described as representing an increased risk to public health compared with foods from conventional production. Leafy vegetables (spinach, romaine lettuce, and green sesame leaves) grown in organic and conventional systems were collected from various areas in Korea and examined using standard culture methods to compare the microbiological quality of the produce grown in the two agricultural systems. The 354 samples of these leafy vegetables were analyzed for levels of indicator bacteria (aerobic bacteria, coliforms, and *Escherichia coli*) and the prevalence of the pathogens *Staphylococcus aureus*, *E. coli* O157:H7, *Listeria monocytogenes*, *Bacillus cereus*, and *Salmonella*. Aerobic bacteria and coliforms were detected in all vegetable types, but nonpathogenic *E. coli* was below the limit of detection in all samples. *B. cereus* was the most prevalent pathogen, found on 7 (11.1%) of the 63 organic spinach samples. The prevalence of *S. aureus* was highest in organic sesame leaves; it was found on 5 (8.0%) of the 63 samples. The prevalence of *L. monocytogenes* was highest on organic romaine lettuce and spinach; it was found in 4 (6.4%) of 63 samples of each type of vegetable. *E. coli* O157:H7 found on only 1 (1.58%) of 55 conventional spinach samples. These results suggest that farming type at most only slightly affects the hygienic quality of leafy vegetables, and no effect was found for sample collection area. *Salmonella* was not isolated from any of the conventional or organic leafy vegetables. These results do not support the hypothesis that organic produce poses a substantially greater risk of pathogen contamination than does conventional produce.

Tasnia *et al.*, (2014) mapped a complete pathogenic profile of the salad vegetables in Dhaka Metropolis, Bangladesh. In addition to a huge bacterial load found previously in lettuce, tomato, cucumber and carrot, current study further detected microbial contamination in chili, onion, capsicum and coriander samples. While *Vibrio* spp., *Salmonella* spp. and *Shigella* spp. fecal coliform and *Eshcherichia coli* were found to be absent within these vegetable samples; a colossal burden of *Aeromonas* spp. (>106 cfu/g) was observed in chili, capsicum, coriander, whereas *Staphylococcus aureus* (1.2×108 cfu/g) and *Klebsiella Pneumoniae* (104 cfu/g) were detected in onion. Fungal growth was also observed in all samples. Most of the pathogens from all 8 samples were resistant against erythromycin (15 µg), amoxicillin (30 µg) and ampicillin (10 µg) while susceptible against ciprofloxacin (5 µg), kanamycin (30 µg) and gentamicin (10 µg). Interestingly, lettuce and cucumber samples were found to exhibit the anti-bacterial activity against *Staphylococcus aureus* and *Aeromonas* spp.

Avazpour *et al.*, (2013) essessed that vegetables, used in preparing salads, are most important part of the diet. These materials are often irrigated by untreated urban wastewater. Wastewater, contaminated with ova of parasites, bacteria and protozoa, are used as fertilizers and it can cause a variety of infectious diseases. The purpose of this study is detection of microbial contamination of salad used in Ilam's restaurants. In this study, 42 samples were collected from all restaurants placed in Ilam city and transferred to the laboratory. Brilliant Green Medium, Trypton water and Coax reagent used for detection of Escherichia coli. Water broth, Selenit systein, Tetrationat, *Salmonella-shigella* agar and Briliant green was used for identification of salmonella. For detection of *Enterococcus*, KF agar medium containing a diphenyl Tetrazolium chloride was used. Sabro dextrose agar medium (SDA) was used for detection of mold and yeast and wet mount and concentration methods used for parasitology investigations. The results of this study indicate that about 66.66% of samples were infected with *Enterococcus*, 69% had *E. coli* contamination and 83.33% of samples were contaminated with yeast. Samples were negative for presence of *Salmonella* and mold (mold not more than 103). Parasites contamination of samples was (4 cases) 9.5% for *Giardia lamblia*, (10 cases) 23.8% for *Taenia* eggs, 31% (13 cases) for *Hymenolepis nana* 16.6% (7 cases) for *Entamoeba coli*. The results of this study showed that salads are contaminated with infectious agent and the use of appropriate disinfectants

and washing the vegetables used in salad preparation is essential for controlling infectious diseases.

Emerenini *et al*., (2013) investigated the diversity and identities of Lactic Acid Bacteria (LAB) isolated from different fresh fruits and vegetables using Molecular Nested PCR analysis with the view of identifying LAB with anti-microbial potentials. Nested PCR approach was used in this study employing universal 16S rRNA gene primers in the first round PCR and LAB specific Primers in the second round PCR with the view of generating specific Nested PCR products for the LAB diversity present in the samples. Biotechnology Centre of Federal University of Agriculture, Abeokuta, Ogun State, Nigeria, between January 2011 and February 2012.Forty Gram positive, catalase negative strains of LAB were isolated from fresh fruits and vegetables on Man Rogosa and Sharpe agar (Lab M) using streaking method. Standard molecular methods were used for DNA extraction (Norgen Biotek kit method, Canada), Polymerase Chain Reaction (PCR) Amplification, Electrophoresis, Purification and Sequencing of generated Nested PCR products (Macrogen Inc., USA).The partial sequences obtained were deposited in the database of National Centre for Biotechnology Information (NCBI). Isolates were identified based upon the sequences as *Weissella cibaria* (5 isolates, 27.78%), *Weissella kimchi* (5, 27.78%), *Weissella paramensenteroides* (3, 16.67%), *Lactobacillus plantarum* (2, 11.11%), *Pediococcus pentosaceus* (2, 11.11%) and *Lactobacillus pentosus* (1, 5.56%) from fresh vegetable; while *Weissella cibaria* (4, 18.18%), *Weissella confusa* (3, 13.64%), *Leuconostoc paramensenteroides* (1, 4.55%), *Lactobacillus plantarum* (8, 36.36%), *Lactobacillus paraplantarum* (1, 4.55%) and *Lactobacillus pentosus* (1, 4.55%) were identified from fresh fruits.This study shows that potentially LAB can be quickly and holistically characterized by molecular methods to specie level by nested PCR analysis of the bacteria isolate genomic DNA using universal 16S rRNA primers and LAB specific primer.

Feroz *et al*., (2013) attempted to examine the growth and subsequent survival of the common spoilage bacteria in vegetable samples collected from Dhaka, Bangladesh. Carrot, cucumber, tomato and lettuce samples were obtained from local markets and rendered free of contaminants. Each sample was then inoculated separately with an array of 9 test bacteria, resulting in the initial load of 105 cfu/g. The results revealed more than 6-log reduction of

Salmonella spp. in carrot and tomato samples, *Shigella* spp. in carrot, lettuce and cucumber samples, *Aeromonas* spp. in tomato samples, *Pseudomonas* spp. in lettuce samples, and *Listeria* spp. in cucumber samples. No significant reduction in *E. coli* was observed in the cucumber samples, while in carrots and tomato samples, approximately 2- log reductions was found. Demonstration of the capacity of vegetables to influence microbial growth would further aid in the maintenance of the food quality and stability as well as their shelf life. Conducting such experiments after the quantification of spoiling microorganisms thus imparts a complete bacteriological profile, which is of public health significance.

Goja *et al.*, (2013) carried out to investigate the microbiological quality of some vegetables sold in ED DueimTwon, Sudan. Four species of vegetables were used, Arugula (*Eruca sativa*), Mloukhia (*Corchorus olitorius*), Tomato (*Lycopersicon esculentum*) and Green pepper (*Capsicum annuum*). The samples were collected and examined according to standardized methods for total viable bacteria, coliforms and fecal coliform count. The average of total viable count ranged from 1.2x105-5.6x105 CFU mL^{-1} for Arugula; 2.1x105-2.8x107 CFU mL^{-1} for Mloukhia; 3.4x105-4.8x105 for Tomato and 2.3x105-8.0x106 CFU mL^{-1} for Green pepper. However, the maximum level of total and fecal coliform were (93, 21); (28, 11); (75, 15) and (150, 20) MPN 100 mL^{-1}, respectively. Twelve bacteria belonging to five genera were isolated. *Staphylococcus* (33%) was the most predominant isolated followed by *Enterobacteriaceae* (25%), *Bacillus* (17%) and *Streptococcus* (17%). *Micrococcus* (8%) was the least dominant isolated. The results of microbial counts of these vegetable samples in this study indicate that, the agricultural practices, harvesting, hygiene, transporting and selling points are poor and therefore, the higher microbial load could be risked for public health.

Nma *et al.*, (2013) evaluated vegetables promote good health but harbor a wide range of microbial contaminants. To assess the microbial quality of street-vended ready-to-eat fresh vegetables, fourteen samples of cabbage and lettuce vegetable were purchased from different markets. Samples of salad vegetables were analyzed using standard bacteriological methods. The bacteria loads as reflected by the total aerobic count ranged from 3.1 x 105 to 7.8 x 105 CFU/g for cabbage and 3.1 x 105 to 6.9 x 105 CFU/g for lettuce. The total

coliform counts ranged from 3.4 x 105 to 5.6 x 105 CFU/g for cabbage and 3.4 x 105 to 4.0 x 105 CFU/g for lettuce. The total *Salmonella-Shigella* counts ranged from no significant growth (0.0 x 105) to 3.6 x 105 CFU/g for cabbage and no significant growth (0.0 x 105) to 3.4 x 105 CFU/g for lettuce. A total number of twelve genera of bacteria were isolated and identified as *Staphylococcus* (7.6%), *Proteus* spp. (5.1%), *Bacillus* spp. (3.4%), *Shigella* spp. (2.5%), *Micrococcus* spp. (1.7%), *Pseudomonas* spp. (7.6%), *Enterobacter* spp. (1.7%), *Serratia* spp. (1.7%), *Citrobacter* spp. (2.5%) *Klebsiella* spp. (6.8%), *Salmonella* spp. (13.6%) and *Escherichia coli* (45.8%). This showed that *Escherichia coli* (45.8%) were most predominant, followed by *Salmonella* spp. (13.6%) while *Micrococcus* spp. (1.7%), *Enterobacter* spp. (1.7%) and *Serratia* spp. (1.7%) were least predominant. Since the vegetables are ready-to-eat and will not be subjected to heat treatment, it could be a source of food poisoning to consumers. However, regular inspections of food premises and education of food vendors has been recognised as one of the measures to ensure improvement of the quality of street foods. Thus, government should placed emphasis on educating vendors on simple preventive steps of keeping food hygienically safe.

Osamwonyi *et al.*, (2013) worked on salad is a term broadly applied to many food preparations that have mixture of chopped or sliced ingredients which may be mostly fruits or vegetables. Eighteen samples of vegetable salads sold from three restaurants located at Okada town, Edo State were collected and their bacteriological attributes were investigated using routine methods. The mean heterotrophic and coliform counts recorded for the salad samples ranged from 1.46×104 to 2.80×104 CFU/g and 1.46×104 to 2.84×104 CFU/g for food centre A. THC and TCC counts (1.74×104 to 2.36×104 CFU/g and 1.36×104 to 2.10×104 CFU/g, respectively were obtained for vegetable salads collected from Eatery B. Microbial counts for salads obtained from food service center C varied from 2.08×104 to 2.60×104 CFU/g and 1.12×104 to 2.90×104 CFU/g for THC and TCC, respectively. The differences between the mean bacterial counts were statistically insignificant (P>0.01). Twelve non hemolytic bacterial isolates were identified; *Acinetobacter* sp., *Bacillus* sp., *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens*, *Staphylococcus epidermidis*, *Micrococcus leutus*, *Enterobacter aerogenes*, *Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumoniae* and *Klebsiella oxytoca*. *E. aerogenes* had the highest percentage of occurrence (56%) amongst the isolates. The high microbial load of the foods

was indicative of the fact that the microenvironments within these salads provided favourable conditions for the growth and proliferation of diverse groups of bacteria. It is recommended that sellers and food handlers within the respective restaurants should make conscious efforts to decontaminate and properly handle the vegetables prior to its salad preparation.

Farjana *et al.*, (2012) assessed microbial quality of common salad vegetables (*viz.* carrot, cucumber, tomato and lettuce) collected from Dhaka metropolis was analysed to detect the presence of bacterial pathogens. The occurrence of huge numbers of fecal coliforms (1.0×104 - 4.09×106 cfu/g), *Escherichia coli* (1.0×104 - 5.0×108 cfu/g), *Staphylococcus aureus* (2.0×105 - 5.95×107 cfu/g), and *Listeria* spp. (1.5×106 6.5×107 cfu/g) were detected in all the tested samples. Interestingly, occurrence of viable but non-culturable (VBNC) bacteria was also noticed.

Hossain *et al.*, (2012) Estimated food-borne diseases are the global public health problem. These play a significant role in human morbidity, mortality and economic loss. Vegetables salad are considered as a high-risk food because they do not require any heating; washing or cleaning prior to consumption. However, the aim of this study is isolation & identification of pathogenic bacteria from Salad sample which collected from Noakhali, Bangladesh. Carrot, cucumber and tomato samples were obtained from different kind of Hotel & Restaurant and rendered free of contaminants. Each sample was then inoculated separately with an array of 9 test bacteria, resulting in the initial load of 105 cfu/g. The results revealed more than 6-log reduction of *Salmonella spp*. in carrot and tomato samples, *Shigella spp*. in carrot, cucumber samples, *Pseudomonas spp.* in tomato samples, *Pseudomonas spp*. and *Listeria spp*. in cucumber sample. Therefore, we saw that a great risk towards public health is posed by the organic fertilizers applied in the fields. The major bacterial diseases shaded are the various enteric diseases, diarrhoea, anthrax, salmonellosis, listeriosis, Crohn's disease, thrombocytopenic purpura, neurological disorders, arthritis, etc. Pathogens associated with untreated manure are assumed to enter into the food chain through crop. Thus, vegetables grown in such assistance of untreated fertilizers may play a significant role in showering pathogens to the consumers. Therefore, an attempt was taken to assess the bacteriological

quality, particularly pathogenic bacteria of fresh salad vegetables collected from several retail shops in Noakhali city.

Minna *et al.*, (2012) characterized the bacteria causing decay of carrots during storage and marketing. Spoilage strains were identified by 16S–amplified rDNA restriction analysis and intergenic transcribed spacer–PCR–restriction fragment length polymorphism (ITS-PCR-RFLP). Genotypic fingerprinting by RFLP–pulsed-field gel electrophoresis was used to assess the genetic diversity of the isolates. A total of 252 Pseudomonas isolates from carrots were identified and classified into eight separate groups. Most strains belonged to group A (*Pseudomonas fluorescens, Pseudomonas marginalis*, and *Pseudomonas veronii*) and group B (*Pseudomonas putida*). The strains identified as *Pectobacterium carotovorum* subsp. *carotovorum, Pectobacterium atrosepticum, Dickeya chrysanthemi*, and *Erwinia rhapontici* were distinguished by ITS-PCR-RFLP. All isolates belonging to the genera *Pectobacterium* and *Erwinia* were responsible for carrot spoilage. This work has led to the development of new strategies for the identification and genotyping of vegetable-spoiling strains of Pseudomonas, *Pectobacterium*, and *Erwinia*. This is also the first report describing the occurrence of carrot-spoiling *E. rhapontici*. Early recognition of spoilage bacteria in vegetables is important for the implementation of effective handling strategies. Pectolytic bacteria may cause considerable financial losses because they account for a large proportion of bacterial rot of fruits and vegetables during storage, transit, and marketing.

Najafi *et al.*, (2012) aimed to determine the microbiological quality of mixed fresh-cut vegetable salads and mixed ready-to-eat fresh herbs produced in Mashhad, Iran. A total of 174 samples including 89 mixed fresh-cut vegetable salads and 85 mixed ready-to-eat fresh herbs were collected between July 2010 and March 2011. Samples were analyzed for aerobic plate counts, coliforms, *enterobacteriaceae*, *Escherichia coli, Escherichia coli* O157:H7, *Salmonella* spp., *Staphylococcus aureus*, and yeast and mold counts. The incidence levels of aerobic plate count bacteria indicated that 50.6% of mixed ready-to-eat fresh herbs and 49.4% of mixed fresh-cut vegetable salads contained less than 107 cfu/g. *Enterobacteriaceae* and total coliform levels ranged from 3 log cfu/g to 8.3 log cfu/g. Lactic acid bacteria were present in mixed fresh-cut vegetable salads and mixed ready-to-eat fresh herbs at 5.9 log cfu/g and 4.88 log cfu/g respectively. Yeasts and molds were found in mixed

fresh-cut vegetable salads and mixed ready-to-eat fresh herbs at 5.68 log cfu/g and 5.78 log cfu/g respectively. Yeasts and molds at ≤ 5 log cfu/g were recovered from 42.7% (38 of 89 samples) and 40% (34 of 85 samples) of mixed fresh-cut vegetable salads and mixed ready-to-eat fresh herbs respectively. While 19.1% of mixed fresh-cut vegetable salads and 27.8% mixed ready-to-eat fresh herbs contained E. coli, only 6.3% of all samples were contaminated with the microorganism at ≥2 log cfu/g. *E. col*i O157:H7 was detected in mixed fresh-cut vegetable salads and mixed ready-to-eat fresh herbs with an incidence of 6.5% and 11.4% respectively. *Staphylococcus aureus* was found in 94.9% of samples, whereas coagulase-positive staphylococci were detected in 23.6% of samples. Our results also exhibited that 9.4% of mixed ready-to-eat fresh herbs and 5.6% of mixed fresh-cut vegetable salads were contaminated with Salmonella spp.

T. Nawas *et al.*, (2012) aimed at examining the microbial quality of restaurant salad and the water used for salad preparation and their role as a source of antibiotic resistant bacteria. Samples were collected from 15 different restaurants located in Chittagong city. The range of Total Viable Count was 1.86×104 to7.28×105 CFU/g and 1.60×104 CFU/ml to 4.38×105 CFU/ml for salad and water respectively. Total colifrm and fecal coliform count > 1100 CFU/100 ml were found in 73.33% of salad and 33.33% water samples. *Salmonella* spp was present in 46.67% of restaurants salad and water. *Vibrio* spp. was present in 66.67% of salad and 53.33% of water. A total of 102 isolates belonging to genus *Vibrio, Salmonella* and *E. coli* were subjected to antibiotic sensitivity test by disc diffusion method by using nine different types of antibiotic discs. *Salmonella* spp. from salad and water showed resistance against Amoxicillin (75%), Cephradine and Cephalexin (68.75%). 85.71% *Vibrio* spp. isolated from salad and water were resistant to Amoxicillin respectively. Multiple drug resistance was seen in 39 and 51 isolates of *Salmonella* and *Vibrio* isolates, respectively. The results suggest the necessity to follow the hygienic practices in salad preparation and salad might have an important role as a source of multiple antibiotic resistant bacteria.

Puspanadan *et al.*, (2012) investigated that *Klebsiella pneumoniae* (*K. pneumoniae*) is one of the most important members of *Klebsiella* genus in *Enterobacteriacae* family, which is responsible for pneumonia (the destructive lung inflammation disease). Vegetables are known as source of contamination with K. pneumonia. Raw vegetables are usually

consumed in salads and other dishes. The aim of this study was to investigate the occurrence of *K. pneumoniae* in raw vegetables marketed in Malaysia. Two hundred commonly used salad vegetables (lettuces, parsley, cucumber, tomato and carrot) from hypermarkets and wet markets were investigated for presence of *K. pneumoniae* using Most Probable Number-Polymerase Chain Reaction (MPN-PCR). *K. pneumoniae* was found to be significantly more frequent (100%) and (82.5%) in lettuce and cucumbers, respectively. *K. pneumoniae* contamination was lowest in carrot samples (30%).

Farzana *et al.*, (2011) evaluated the food safety status of a street vended fruit product locally known as fruit chat. Sample collection was carried out from four groups of vendors which were discriminated based on their mobility, vending practice and storage facility. The extent of prevalence for *Enterobacter species*, *Escherichia coli*, *Klebsiella* species, *Salmonella* species, *Staphylococcus aureus* and *S. epidermidis* was assessed in fruit chat samples. Bacterial isolates were identified through biochemical characterization. The degree of susceptibility of these isolates was evaluated against six different antibiotics that is, amikacin, ampicillin, ciprofloxacin, gentamycin, ceftriaxone and co-trimoxazol using disc diffusion method. Results indicated a higher (105 to 1010 CFU/g) total plate count (TPC) and total coliforms count (TCC) ranged from 104 to 109 CFU /g in samples collected from mobile vendors without covering. Mobility of vendors and, use of no covering, resulted in significant ($P \leq 0.05$) contamination. However, fruit chat samples collected from stationary vendors with refrigeration facilities exhibited considerably lower bacterial count. The study clearly indicated that consumption of fruit chats from street vendors can be a potential risk for food borne outbreaks because of their contamination level by pathogenic bacteria. Bacterial species had shown variable susceptibility and resistance patterns in response to different antibiotics used in the study. However, all bacterial species had shown greater sensitivity for amikacin, whereas, bacterial strains appeared to develop resistance against other commonly used antibiotics. The mounting resistance against antibiotics is currently one of the foremost challenges to treat food borne infections. Therefore, both preventative and effective curative measures should be adopted. Firstly, the safety status of street vended food products can be enhanced by improving hygienic conditions during the preparation of food stuff. Secondly, the use of effective antibiotics will be of significant importance to cure food borne infections as well as to avoid growing resistance in pathogenic bacteria.

Itohan *et al.*, (2011) worked on salad vegetables are essential part of people's diet all around the world. They are usually consumed raw and often without heat treatment or thorough washing; hence have been known to serve as vehicles for the transmission of pathogenic microorganism associated with human diseases. Fresh samples of lettuce, carrot and cucumber collected from different markets and vendors in Abuja Municipal Area Council, Federal Capital Territory, Nigeria were evaluated for bacterial loads using spread plate agar dilution method. Bacterial loads ranged from 1.6 x 106 to 2.9 x 108 cfu/g. *Escherichia coli*, *Klebsiella* and *Enterobacter* were amongst the coliforms (lactose fermenters), while *Proteus*, *Pseudomonas aeruginosa*, *Salmonella* and *Shigella* were non-lactose fermenters associated with the samples. *Staphylococcus aureus* was isolated from majority of the samples.

Khiyami *et al.*, (2011) investigated fresh salad samples (Tabbouleh, Fattoush, Hummus, Mutabbel and Caesar) collected from various restaurants located in five different areas (west, north, south, east and center) in Riyadh (the capital city of Saudi Arabia). Isolated colonies found were identified via molecular methods. Total number of identified isolates in the vegetable salads Tabbouleh, Fattoush, Mutabbel, Hummus and Caesar were 24, 20, 18, 16 and 12, respectively. *Pseudomonas* sp., *Bacillus cereus* and *Enterobacter aerogenes* were recorded in the five types of vegetable salads. *Escherichia coli*, *Klebsiella* sp., *Kluyvera cryocrescens* and *Kluyvera ascorbata* were recorded in all types of vegetable salads except Caesar. Vegetable salads collected from all sites were contaminated with *E. coli*, *Enterobacter* sp. and *Enterobacter aerogenes*. Elevated level of contamination with *E. coli* was recorded in the samples collected from the north and east areas, while high level of contamination with *Enterobacter aerogenes* was recorded in the samples collected from central area. Estimation of bacterial genetic relationships was determined using DNA sequencing, phylogenetic tree and bioinformatic techniques. The 36 isolates were arranged in one main cluster including 34 bacterial species and two bacterial species *Pseudomonas* and *Bacillus cereus* were out groups with similarity index range of 0.008- 0.063 and 0.008-0.125, respectively, to the main group. This cluster was subdivided into two subgroups: subgroup A included *Acinetobacter* sp. and *Stenotrophomonas* sp.; subgroup B included 32 bacterial species. Subgroup B contains one major subgroup C comprising 31 bacterial species with similarity index range of 0.061-0.019 to *Hafnia alvei*. Subgroup C was

subdivided into subgroup D including 15 bacterial species and subgroup E including 16 bacterial species.

Nipa *et al.*, (2011) conducted to examine microbiological quality of raw salad vegetables and their role as a source of antibiotic resistant bacteria. Eight types of vegetables which are commonly used for salad i.e. Tomato, Cucumber, Carrot, Green chilli, Lemon, coriander leaf, Pepper mint, Beet root were collected from two Open markets and two Super shops of Chittagong City. All the vegetables were highly contaminated with Coliform and fecal Coliform (> 1100 CFU/100ml). Range of microbial count of Tomato was 9.0×104 CFU/ml to 3.8×105 CFU/g, Cucumber was 5.5×104 CFU/g to 1.9×106 CFU/g, Carrot was 1.2×104 to 2.6×106 CFU/g, Green chilli was 1.0×104 to 4.0×105 CFU/g, Lemon was 1.5×105 to 1.2×106 CFU/g, Coriander leaf was 5.87×105 to 1.8×106 CFU/g, Peppermint was 2.2×105 to 7.7×105 CFU/g and it was 5.0×103 to 5.4×105 CFU/g for Beet root. Yeast and mold was not detected in most of the vegetables. A total of 266 bacterial isolates of ten genera and three fungi *Rhizopus*, *Penicilium* and *Aspergillus* were identified. *Enterobacter* spp. (21.80%) was the most dominant followed by *Pseudomonas* spp. (19.17%), *Vibrio* spp. (16.92%), *Lactobacillus* spp. (15.04%), *Staphylococcus* spp. (10.15%), *Klebsiella* spp *(9.04%), E. coli* (4.89%), *Citrobacter* spp. (2.26%), *Serratia* spp. (0.37%) *and Salmonella* spp. (0.37%). Fifty-one selected isolates from Karnafully market were tested for antibiotic susceptibility. Multiple drug resistance was observed in 98.06% isolates with a resistance to two to seven antibiotics. These results suggest the necessity to follow the hygienic practices in handling the vegetables in open markets as well as the super shops and vegetables might have an important role as a source of multiple antibiotic resistant bacteria.

Abdullahi *et al.*, (2010) collected some ready to eat leafy vegetables on sale at Sabon-gari market, Zaria were analysed for their bacterial flora and counts. Lettuce had aerobic plate count range of 2.0 x 107 to 5.7 x 108 cfu/g, cabbage had a count range of 1.3 x 107 – 5.6 x 108 cfu/g and cucumber had a range of count of 3.0 x 105 to 1.9 x 106. The coliform index showed lettuce to have a count of 8.8 x 106 – 1.3 x 109, cabbage was 2.1 x 106 to 8.0 x 107 cfu/g and cucumber was 8.0 x 105 to 1.9 x 106. Bacillus species and *Staphylococcus aureus* were the predominant bacteria isolated from these vegetables. The counts were obviously above the recommended standards for ready to eat

vegetables especially coliforms which should be less than 10 coliform bacteria per gram(FAO, 1979). There is the need for hygiene officials to take interest on what is offered to consumers and specify acceptable handling practices.

Russell *et al.*, (2010) tested of Jarjeer/rocket (*Eruca sativa L*.) salad greens after multiple washings in water and mild disinfectant revealed a significant number of sequestered total coliforms and *E. coli* remained in the fresh greens. Presumptive tests of 64 locally purchased fresh jarjeer greens resulted in finding 100% of the samples were contaminated by coliforms and *E. coli.* Jarjeer greens had 2,509,273 CFU/g and 224,250 E. coli/g when washed once. Washing the greens three times reduced the number of CFU by 95% and E. coli by 83%, but E. coli counts remained high 9,741 / g or 292,230 per 30 g normally eaten portion. After macerating the thrice washed jarjeer the number of CFU increased to 2,129,774 / g and E. coli 56,292/g, which indicated the bacteria are sequestered in the leaves and could not be washed off. Disinfection with diluted chlorine bleach reduced CFU by 68% and E. coli by 84%, but upon maceration CFUs increased from 42,059/g to 833,812/g and E. coli from 5/g to 2,150/g, which indicates washing with a disinfectant cannot rid the greens of coliforms. Counts on parsley and lettuce were significantly lower than on jarjeer. Results indicate there is sequestered fecal contamination of fresh jarjeer salad greens that remained on and inside epidermal cells even after multiple washings. These data show that there is probably a persistent health threat when eating these fresh salad greens, but further testing for the presence of Salmonella and other pathogens is required.

Oni *et al.*, (2010) reported that there were several cases of bacterial food poison in the student community of Ambrose Alli University, Ekpoma. However, there were no studies that Isolated the common bacteria causes of food poison in this environment. The objective of this study was to isolate and characterize the common bacteria food poison in Ekpoma, Nigeria. METHOD: Ten prepared vegetable salad samples obtained at various sales points in Ekpoma were investigated for the isolation of Bacteriausing standard cold and non-cold enrichment method. RESULT: The result obtained demonstrated the presence of *Staphylococcus aureus* (50%), *Bacillus* (30%), *Proteus* (20%), Yeast (10%). The commonly diagnosed *Listeria monocytogene* was not isolated. CONCLUSION: Appropriate

hygienic measures for the consumption of raw food products, canned foods and vegetables should be practiced.

Uzeh *et al.*, (2009) stated that pre-packed mixed vegetable salad and salad ingredients-carrots, cucumber, cabbage, and lettuce were analyzed for their microbial quality. The salads were obtained from fast food outlets (well packaged at 4 oC) and open markets (exposed at 35 oC) within Lagos metropolis. The analysis was both qualitative and quantitative. Microorganisms isolated from salad samples from fast food outlets include *Aspergillus fumigatus*, *Trichoderma* spp, *Staphylococcus aureus* and *Proteus mirabilis*, while those isolated from open market samples include *Mucor* spp, *A. fumigatus*, *Aspergillus niger*, *Trichoderma* spp, *Neurospora crassa*, *Proteus vulgaris*, *S. aureus*, *Citrobacter freundii*, *Proteus mirabilis*, and *Corynebacterium* spp. Those from salad ingredients include Mucor spp, *A. fumigatus*, *Trichoderma spp*, *N. crassa*, *Rhizopus spp*, *A. niger*, *P. vulgaris*, *P. mirabilis*, *S. aureus*, *Pseudomonas aeruginosa* and *C. fruendii*. The total viable count was highest in salad samples from open markets (5.9×10 6 cfu/g) and lowest in salad samples from fast food outlets (2.6 × 10 4 cfu/g). The total viable counts obtained from the salad ingredients were generally lower than those obtained from salads. Among the salad ingredients the highest count was however obtained from carrot (3.0 × 10 2 cfu/g) and lowest count from cucumber (1.3 × 10 2 cfu/g). Gentamicin, chloramphenicol, cotrimoxazoleoflaxacin were most effective against the bacterial isolates yielding greater zones of inhibition. The storage temperature and the dirty nature of the open markets must have been responsible for the occurrence of more microorganisms in salad samples from open markets than those from fast food outlets. The need for safe salad cannot be overemphasized.

Balter *et al.*, (2006) essessed Foodborne pathogens can create a considerable amount of work at state and local health departments. Between foodborne outbreaks, restaurant inspections, environmental testing, botulism reports, customer complaints, and confirmation of isolates referred for testing, many health department resources are directed toward these pathogens and preventing illness from them. Moreover, the mass media are increasingly interested in food safety, particularly after large, multistate outbreaks caused by *Escherichia coli* O157:H7 and *Salmonella*, among other pathogens, and increasing public interest in raw

and unpasteurized foods that are perceived as more natural or healthy. The audience for Foodborne Pathogens: Microbiology and Molecular Biology appears to be public health practitioners working on epidemiologic, environmental, and laboratory aspects of foodborne illness. One of the book's strengths is that it attempts to include reference material on epidemiology and on the molecular and microbiologic aspects of the various pathogens. However, as the title suggests, the emphasis is on molecular and microbiologic aspects, and much of the information is extremely technical and primarily for the laboratory scientist. The book includes a range of food pathogens, from bacteria and viruses to mycotoxins. The primary omission is bovine spongiform encephalopathy. Chronic wasting disease is included briefly in a chapter on potential food pathogens, which makes the omission of bovine spongiform encephalopathy all the more striking. In addition to separate chapters on individual pathogens or groups of pathogens, the book covers laboratory issues, including animal and cell culture models, molecular approaches for detection, and stress responses of foodborne pathogens. Other chapters are based on more sensational topics, such as bioterrorism and food, although this chapter discusses the subject in general terms. In a chapter on biosensor-based detection of foodborne pathogens, the authors conclude, not convincingly, that biosensors will soon be as widespread as glucose kits and home pregnancy tests.

Łaniewska *et al.*, (2006) estimated antibiotic resistance of 114 strains of the *Enterobacteriaceae* family bacteria isolated from vegetables, originating from retail, was investigated in the study. The highest number of the strains isolated were resistant to ampicillin (81.9%), whereas a lower number of the strains exhibited resistance to the followingantibiotics: neomycin (29.3%), streptomycin (28.4%), rifampicin (21.5%), chloram phenicol (19.8%), colistin (12.9%), and nitrofurantoin (11.%). All the isolated strains appeared to be susceptible to vancomycin, kanamycin, doxycycline, nalidixic acid and gentamicin.

CHAPTER-III

MATERIALS AND METHODS

The present research work was conducted during the period from January to December, 2019 in the Bacteriology Laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh.

3.1 Materials

The materials used in this study were comprised of the sample materials and laboratory materials.

3.1.1 Sample Collection Site

Samples were collected in two categories. Firstly,7 types of Raw Salad Vegetables are collected from two different markets in Dinajpur Town. Secondly, 4 types of Raw Salad Vegetables are collected from four different hotels in Dinajpur Town.

3.1.2 Study Site and period

The study was carried out in the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh during the period from January 2019 to December 2019 for laboratory analysis.

3.1.3 Sample collection

A total 30 samples comprising cucumber (06), carrot (02), green chilli (06), peeper mint (02), lemon (06), onion (06). Corriandar leaves (02) were collected from different markets and hotels using hand gloves, sealed poly bags and then the samples were sent to the Microbiology laboratory for analysis.

Fig. 1: Samples Collected from different Markets and Hotels

3.1.4 Glassware and Appliances

The different types of sterilized equipment used for this work.

1. Distilled water
2. Sterile bent glass or plastic spreader rods.
3. Micropipette
4. Spirit lamp
5. Labeling tape
6. Experimental test tube
7. Stopper of test tube
8. Petri dish
9. Conical flask.
10. Durham's tube
11. Slide
12. Microscope
13. Cotton, Immersion Oil, Toothpick
14. Autoclave ,thermometer
15. Incubator
16. Jar ,Beaker, Cylinder
17. Electric Balance
18. Filter paper
19. Cover slips and
20. Bacteriological loop (straight and coiled)
21. Stop watch
22. Test tube stand
23. Water bath, Detergent powder
24. Sealed poly bags
25. Alluminium foil roll
26. Refrigerator
27. Hot air oven
28. Biosafety cabinet type ii
29. ice box

3.1.5 Media

3.1.5.1 Media used for Culture

Different bacteriological culture media and biochemical reagents were used for isolation, identification and propagation of bacteria from different raw salad vegetables in this experiment are as follows:

1. Plate Count Agar (PCA)
2. Nutrient Agar (NA)
3. Mac Conkey agar
4. Salmonella-Shigela Agar
5. Brillant Green Agar
6. Manitol Salt Agar
7. Mueller Hinton agar

3.1.5.2 Liquid Culture Media

Nutrient Broth (NB)

Nutrient Broth (NB) was used to preliminary growth and also propagation of the microorganisms from collected the samples (Cheesbrough, 1985).

3.1.5.3 Solid Culture Media

Plate Count Agar (PCA)

Plate Count Agar (PCA), also called Standard Methods Agar (SMA), is a microbiological growth medium commonly used to assess or to monitor "total" or viable bacterial growth of a sample. PCA is not a selective medium (Cheesbrough, 1985).

Nutrient Agar (NA)

Nutrient agar is used for cultivating of non-fastidious microorganisms (Cheesbrough, 1985).

Mac Conkey Agar

A differential medium for the isolation of coliforms and intestinal pathogens in water and biological specimens (Cheesbrough, 1985).

Eosin Methylene Blue (EMB) agar

Eosin Methylene Blue (EMB) agar medium was used for the purpose of selective growth of Escherichia coli (Cheesbrough, 1985).

***Salmonella Shigella* Agar (SS)**

Salmonella Shigella Agar is used as a selective medium for *Salmonella spp* which causes enhancement of the growth of *Salmonella spp* (Cheesbrough, 1985).

Brilliant Green Agar (BGA)

Brilliant Green Agar is used as a selective medium for *Salmonella spp* which causes enhancement of the growth of *Salmonella spp* (Cheesbrough, 1985).

Mannitol Salt Agar (MSA)

Each isolate was streaked on MSA and incubated at 37 ^{0}C for overnight. Next day demonstrated morphological characteristics of the bacterial colonies .When the *Staphylococcus spp* is present then the plate was yellow color (Cheesbrough, 1985).

3.1.5.2 Media for Biochemical test

i. Sugar Fermentation Broth
ii. Indole Broth
iii. Methyl Red Broth
iv. Voges-proskauer Broth
v. Simmon's citrate Agar
vi. Triple sugar iron (TSI) agar
vii. Motility Indole Urease (MIU)
viii. Catalase Test

3.1.6 Chemicals, Reagents and Solutions

i. Crystal violet dye
ii. Grams iodine
iii. Acetone Alcohol
iv. Safranin
v. Saline solution
vi. Iodine solution
vii. Phosphate Buffer Saline Solution (PBS)
viii. Kovac's reagent
ix. Methyl- red solution
x. 3% H_2O_2
xi. P – Amino dimethylanilin oxalate
xii. Phenol red solution
xiii. Alpha-napthanol
xiv. Potassium hydroxide solution
xv. Distilled water

3.1.7 Materials Required for Antibiogram Study

Mueller Hinton Agar (MBA)

Mueller Hinton Agar plates were specially used for the antibiotic sensitivity test (Cheesbrough, 1985)

3.1.8 Materials used for bacterial genomic DNA isolation

i. TE buffer
ii. 10% (w\v) Sodium dodecyl sulfate (SDS)
iii. 20 mg\ml protinase k (stored in small single-use aliquots at -200C)
iv. 3 M Sodium Acetate, pH 5.2
v. 25:24:1 Phenol/Chloroform/Isoamyl alcohol
vi. Isopropanol
vii. 70% Ethanol
viii. 95% Ethanol
ix. 1.5 ml microcentrifuge tubes

3.1.9 Materials used for Polymerase Chain Reaction

Table 1: PCR Reaction Mixture for specific Forward & Reverse primer.

Buffer	2.5 µl
dNTP	2.5 µl
MgCl2	2.5 µl
Forward Primer (E1)	1.0 µl
Reverse Primer (E2)	1.0 µl
Nano Pure Water	12.5 µl
DNA	2.0 µl
Taq DNA Polymerase	1.0 µl
Final Volume	25 l

3.1.10 Primers used for PCR:

Forward primer**:** 27F (5'-AGAGTTTGATCCTEGGCTCAG3) [**Ref.:** Tsen *et al*-1998].
Reverse primer**:** 1492 R (5'- TACCTTGTTACGACTT3). [**Ref.:** Gautam *et al*-2012 Indian j ani. Sci.,82(2):204-208]

- Product size: 1492 bp
- Thermal Cycler (Thermo cycler, ASTEC, Japan)
- 2% agarose gel
- Gel casting tray with gel comb
- TAE buffer
- Microwave oven
- Conical flask
- Electrophoresis apparatus (Biometra standard power pack P 2T)
- 100 bp DNA size marker
- Bromphenicol blue of loading bufter.
- Ethidium bromide (0.5 µg/ml)
- Distilled water
- UV trans-illuminator

3.1.11 Antibiotic Sensitivity Test against Isolated Microbes

To determine the drug Sensitivity and resistance patterns of isolated organisms used different types of commercially available antimicrobial discs, (Mast diagnostics Mersey side, UK.) which were showed in (Table 1). The antibiotic resistance was determined by Kirby-Bauer disc diffusion technique using Mueller-Hinton agar (Difco), according to the recommendations of National Committee for Clinical Laboratory Standards (CLSI 2011). After overnight incubation at 37°C, the diameter in millimeters of the zones of inhibition around each of the antimicrobial discs was recorded and categorized as resistant or sensitive in accordance with company recommendations. (Cappuccino 2005). *E.coli*, *Salmonella* spp, *Staphylococcus* spp, and *Bacillus* spp isolates were tested for sensitivity to (15 of routine and practical antibiotics) Kanamycin (30µg), Amoxicillin (30µg), Piperacillin (110µg), Bacitracin (10µg), Tetracyclin (30µg), Nalidixic Acid (30µg), Azithromycine (30µg),

Cloxacillin (1µg), Chloramphenicol (30µg), Norflaracin (10µg), Novobiocin (30µg), Methicillin (5µg), Cefixim (5µg) and Vancomycine (30µg). The disks were purchased from national company. The results were interpreted by special manufacturer's tables

Table 2: Antimicrobial Sensitivity Discs

Antimicrobial Agent	Symbol	Disc Concentration (µg/disc)
Kanamycin	**K**	30µg
Amoxicillin	**AMX**	30µg
Piperacillin	**TZP**	110µg
Bacitracin	**B**	10µg
Tetracycline	**TE**	30µg
Nalidixic Acid	**NA**	30µg
Azithromycine	**AZM**	30µg
Cloxacillin	**COX**	1µg
Chloramphenicol	**C**	30µg
Norflaracin	**NX**	10µg
Novobiocin	**NV**	30µg
Methicillin	**MET**	5µg
Cefixime	**CFM**	5µg
Vancomycine	**VA**	30µg

Note : µg = mirogram

3.1.9 Recording and Interpreting Results

The zones of growth inhibition was compared with the zone-size interpretative table standard for *Klebsiella, E. coli, Salmonella, Shigella, Staphylococcus, Proteus, Pseudomonas, Vibrio, Streptococci*

Table 3: Zone Diameter Interpretative Standards for *Salmonella spp*

Antimicrobial Agent	Zone of Diameter		
	Resistant ≤(mm)	Intermediate (mm)	Sensitive ≥(mm)
Kanamycin	13	14-17	18
Amoxicillin	14	-	15
Piperacillin	20	21-22	23
Bacitracin	8	9-12	13
Tetracyclin	19	20-23	24
Nalidixic Acid	17	-	18
Azithromycine	18	-	19
Cloxacillin	10	-	13
Chloramphenicol	20	-	21
Norflaracin	16	17-19	20
Novobiocin	-	-	-
Methicillin	15	16-18	19
Cefixime	19	-	20

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

[Legands: K=Kanamycin, AMX=Amoxicillin, TZP=Piperacillin, B=Bacitracin, TE=Tetracyclin, NA=Nalidixic Acid, AZM=Azithromycine, COX=Cloxacillin, C=Chloramphenicol, NX= Norflaracin, NV=Novobiocin, MET=Methicillin, CFM=Cefixim, VA=Vancomycine,]

Table 4: Zone Diameter Interpretative Standards for *Shigella spp*

Antimicrobial Agent	Zone of Diameter		
	Resistant ≤(mm)	Intermediate (mm)	Sensitive ≥(mm)
Kanamycin	13	14-17	18
Amoxicillin	14	-	15
Piperacillin	20	21-22	23
Bacitracin	8	9-12	13
Tetracyclin	19	20-23	24
Nalidixic Acid	17	-	18
Azithromycine	18	-	19
Cloxacillin	10	-	13
Chloramphenicol	20	-	21
Norflaracin	16	17-19	20
Novobiocin	-	-	-
Methicillin	15	16-18	19
Cefixime	19	-	20

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

[Legands: K=Kanamycin, AMX=Amoxicillin, TZP=Piperacillin, B=Bacitracin, TE=Tetracyclin, NA=Nalidixic Acid, AZM=Azithromycine, COX=Cloxacillin, C=Chloramphenicol, NX= Norflaracin, NV=Novobiocin, MET=Methicillin, CFM=Cefixim, VA=Vancomycine,]

Table 5: Zone Diameter Interpretative Standards for *Klebsiella spp*

Antimicrobial Agent	Zone of Diameter		
	Resistant ≤(mm)	Intermediate (mm)	Sensitive ≥(mm)
Kanamycin	13	14-17	18
Amoxicillin	14	-	15
Piperacillin	20	21-22	23
Bacitracin	8	9-12	13
Tetracyclin	19	20-23	24
Nalidixic Acid	17	-	18
Azithromycine	18	-	19
Cloxacillin	10	-	13
Chloramphenicol	20	-	21
Norflaracin	16	17-19	20
Novobiocin	-	-	-
Methicillin	15	16-18	19
Cefixime	19	-	20

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

[Legands: K=Kanamycin, AMX=Amoxicillin, TZP=Piperacillin, B=Bacitracin, TE=Tetracyclin, NA=Nalidixic Acid, AZM=Azithromycine, COX=Cloxacillin, C=Chloramphenicol, NX= Norflaracin, NV=Novobiocin, MET=Methicillin, CFM=Cefixim, VA=Vancomycine,]

Table 6: Zone Diameter Interpretative Standards for *Staphylococcus spp*

Antimicrobial Agent	Zone of Diameter		
	Resistant ≤(mm)	Intermediate (mm)	Sensitive ≥(mm)
Kanamycin	13	14-17	18
Amoxicillin	14	-	15
Piperacillin	20	21-22	23
Bacitracin	8	9-12	13
Tetracyclin	19	20-23	24
Nalidixic Acid	17	-	18
Azithromycine	18	-	19
Cloxacillin	10	-	13
Chloramphenicol	20	-	21
Norflaracin	16	17-19	20
Novobiocin	-	-	-
Methicillin	15	16-18	19
Cefixime	19	-	20

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

[Legands: K=Kanamycin, AMX=Amoxicillin, TZP=Piperacillin, B=Bacitracin, TE=Tetracyclin, NA=Nalidixic Acid, AZM=Azithromycine, COX=Cloxacillin, C=Chloramphenicol, NX= Norflaracin, NV=Novobiocin, MET=Methicillin, CFM=Cefixim, VA=Vancomycine,]

Table 7: Zone Diameter Interpretative Standards for *Proteus spp*

Antimicrobial Agent	Zone of Diameter		
	Resistant ≤(mm)	Intermediate (mm)	Sensitive ≥(mm)
Kanamycin	13	14-17	18
Amoxicillin	14	-	15
Piperacillin	20	21-22	23
Bacitracin	8	9-12	13
Tetracyclin	19	20-23	24
Nalidixic Acid	17	-	18
Azithromycine	18	-	19
Cloxacillin	10	-	13
Chloramphenicol	20	-	21
Norflaracin	16	17-19	20
Novobiocin	-	-	-
Methicillin	15	16-18	19
Cefixime	19	-	20

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

[Legands: K=Kanamycin, AMX=Amoxicillin, TZP=Piperacillin, B=Bacitracin, TE=Tetracyclin, NA=Nalidixic Acid, AZM=Azithromycine, COX=Cloxacillin, C=Chloramphenicol, NX= Norflaracin, NV=Novobiocin, MET=Methicillin, CFM=Cefixim, VA=Vancomycine,]

Table 8: Zone Diameter Interpretative Standards for *Psedomonas spp*

Antimicrobial Agent	Zone of Diameter		
	Resistant ≤(mm)	Intermediate (mm)	Sensitive ≥(mm)
Kanamycin	13	14-17	18
Amoxicillin	14	-	15
Piperacillin	20	21-22	23
Bacitracin	8	9-12	13
Tetracyclin	19	20-23	24
Nalidixic Acid	17	-	18
Azithromycine	18	-	19
Cloxacillin	10	-	13
Chloramphenicol	20	-	21
Norflaracin	16	17-19	20
Novobiocin	-	-	-
Methicillin	15	16-18	19
Cefixime	19	-	20

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

[Legands: K=Kanamycin, AMX=Amoxicillin, TZP=Piperacillin, B=Bacitracin, TE=Tetracyclin, NA=Nalidixic Acid, AZM=Azithromycine, COX=Cloxacillin, C=Chloramphenicol, NX= Norflaracin, NV=Novobiocin, MET=Methicillin, CFM=Cefixim, VA=Vancomycine,]

Table 9: Zone Diameter Interpretative Standards for *Vibrio spp*

Antimicrobial Agent	Zone of Diameter		
	Resistant ≤(mm)	Intermediate (mm)	Sensitive ≥(mm)
Kanamycin	13	14-17	18
Amoxicillin	14	-	15
Piperacillin	20	21-22	23
Bacitracin	8	9-12	13
Tetracyclin	19	20-23	24
Nalidixic Acid	17	-	18
Azithromycine	18	-	19
Cloxacillin	10	-	13
Chloramphenicol	20	-	21
Norflaracin	16	17-19	20
Novobiocin	-	-	-
Methicillin	15	16-18	19
Cefixime	19	-	20

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

[Legands: K=Kanamycin, AMX=Amoxicillin, TZP=Piperacillin, B=Bacitracin, TE=Tetracyclin, NA=Nalidixic Acid, AZM=Azithromycine, COX=Cloxacillin, C=Chloramphenicol, NX= Norflaracin, NV=Novobiocin, MET=Methicillin, CFM=Cefixim, VA=Vancomycine,]

Table 10: Zone Diameter Interpretative Standards for *Streptococcus* spp.

Antimicrobial Agent	Zone of Diameter		
	Resistant ≤(mm)	Intermediate (mm)	Sensitive ≥(mm)
Kanamycin	13	14-17	18
Amoxicillin	14	-	15
Piperacillin	20	21-22	23
Bacitracin	8	9-12	13
Tetracyclin	19	20-23	24
Nalidixic Acid	17	-	18
Azithromycine	18	-	19
Cloxacillin	10	-	13
Chloramphenicol	20	-	21
Norflaracin	16	17-19	20
Novobiocin	-	-	-
Methicillin	15	16-18	19
Cefixime	19	-	20

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

[Legands: K=Kanamycin, AMX=Amoxicillin, TZP=Piperacillin, B=Bacitracin, TE=Tetracyclin, NA=Nalidixic Acid, AZM=Azithromycine, COX=Cloxacillin, C=Chloramphenicol, NX= Norflaracin, NV=Novobiocin, MET=Methicillin, CFM=Cefixim, VA=Vancomycine,]

Table 11: Zone Diameter Interpretative Standards for *E.coli spp*

Antimicrobial Agent	Zone of Diameter		
	Resistant ≤(mm)	Intermediate (mm)	Sensitive ≥(mm)
Kanamycin	13	14-17	18
Amoxicillin	14	-	15
Piperacillin	20	21-22	23
Bacitracin	8	9-12	13
Tetracyclin	19	20-23	24
Nalidixic Acid	17	-	18
Azithromycine	18	-	19
Cloxacillin	10	-	13
Chloramphenicol	20	-	21
Norflaracin	16	17-19	20
Novobiocin	-	-	-
Methicillin	15	16-18	19
Cefixime	19	-	20

3.2 Methods

The experimental layout is schematically presented in figure 1. The entire study is divided into three steps. The first step includes the total viable counts of the collected samples. The second steps includes isolation and identification of the bacteria from the sample by cultural, morphological and biochemical test. Third step includes evaluation of antibiotics sensitivity against the isolated bacteria molecular characterization of that bacteria.

3.2.1 Experimental Layout

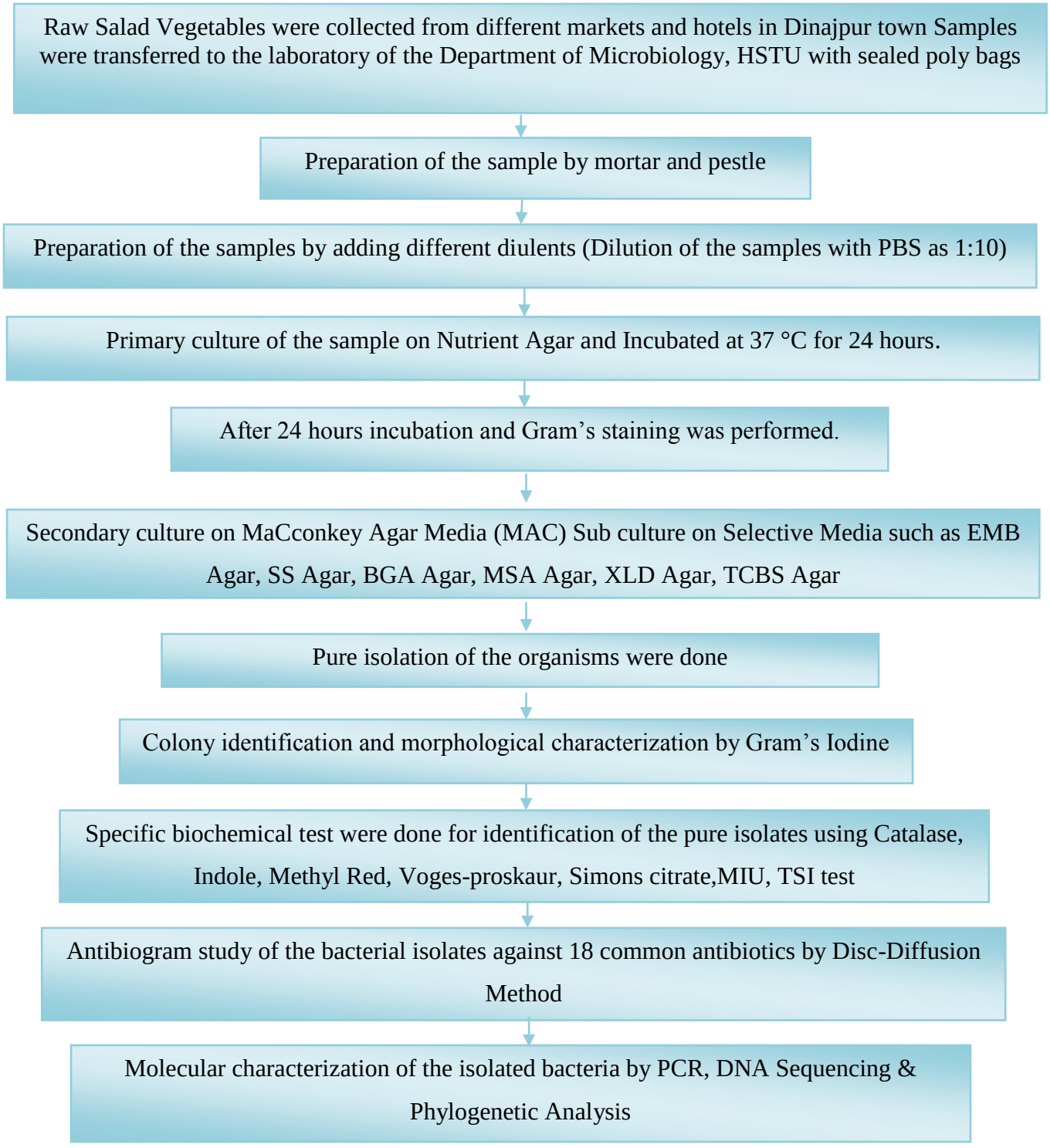

Fig. 02: Schematic illustration of the experimental layout.

3.2.2 Laboratory Preparation

All items of glassware including test tubes, pipettes, cylinder, flasks, conical flasks and other necessary instruments cleaned by brushing, washed thoroughly and finally sterilized by autoclaving for 15 minutes at 121 °C under 15 lbs pressure per square inch. Autoclaved items were dried in a Hot Air Oven over 50 °C. All the glass wares kept in oven at 50 °C for future use.

3.2.3 Preparation of Culture Media

All the media, broth and reagents used in this experiment were prepared according to instruction of the manufacturer.

3.2.3.1 Nutrient Broth Media

Thirteen grams of dehydrated nutrient broth was suspended into 1000 ml of distilled water and boil to dissolve it completely. The solution was then distributed in tubes, stopper with cotton plugs and sterilized in autoclaving at 121 °C and 1.2 kg/cm^2 pressure for 15 minutes.

The sterility of the medium was checked by incubating at 37 °C for overnight and stored at 4 °C in aerator for further use (Cater 1979).

3.2.3.2 Plate Count Agar (PCA)

Add 17.5g to 1 liter of distilled water. Dissolve by bringing to the boil with frequent stirring, mix and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was poured into each sterile petri dish and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use (Cater 1979).

3.2.3.3 Nutrient Agar (NA) Media

28 grams of nutrient agar powder was dissolved in 1000 ml of cold distilled water in a flask. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petri dish and allowed to solidify. After solidification of the medium in the

petri dishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use (Cater 1979).

3.2.3.4 Eosin Methylene Blue (EMB) Agar

Thirty six grams of EMB agar base was added to 1000 ml of water in a flask and boil to dissolve the medium completely. The medium was sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121°C for 15 minutes and I to 50° C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile Petri dish sized and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37° C for overnight to check their sterility and petri dishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.5 Mac Conkey agar

51.5 grams Mac Conkey agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 450- 500C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.6 *Salmonella Shigela* (SS) Agar

Suspend 50g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by boiling for 5 minutes. After boiling the medium was put into water bath at 450- 500C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri

dishes (medium sized) and in 15 ml quantities in sterile glass petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.7 Brilliant Green Agar (BGA)

Suspend 50g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. After autoclaving the medium was put into water bath at 450- 500C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.8 Mannitol Salt Agar (MSA)

111 grams Mannitol Salt Agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 450- 500C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural

characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.4 Preparation of Reagents

3.2.4.1 Methyl- Red solution

The indicator MR solution was prepared by dissolving 0.1 gm of Bacto methyl- red in 300 ml of 95% alcohol and diluted to 500 ml with the addition of distilled water.

3.2.4.2 Methyl Red

A quantity of 17 gm of MR-VP medium was dissolved in 1000 ml of distilled water, dispensed in 2 ml amount in each tube and the tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37oC for overnight o check their sterility and then in refrigerator for future use.

3.2.4.3 Alpha- naphthol solution

Alpha- naphthol solution was prepared by dissolving 5 gm of Alpha- naphthol in 100 ml of 95% ethyl alcohol.

3.2.4.4 Potassium Hydroxide Solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of Potassium hydroxide crystals in100 ml of cooled water.

3.2.4.5 Phosphate Buffered Saline Solution

Eight grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate Na2HPO4, 12H2O), 0.2 gram of potassium chloride (KC1) and 0.2 gram of potassium hydrogen phosphate (KH2PO4) were suspended in 1000 ml of distilled for the preparation of phosphate buffered saline solution. The solution was heated to dissolve completely. Then the solution was sterilized by autoclaving at 1.2 kg / cm2 pressure and 121° C for 15 minutes and stored for future use.

3.2.4.6 Indole Reagent (Kovac's Reagent)

This solution was prepared by dissolving 25 ml of concentrated Hydrochloride acid in 75 ml of amyl alcohol and to the mixture 5 grams of paradimethyl –amino- benzyldehyde crystals were added. This was then kept in a flask equipped with rubber cork for future use.

3.3.1 Cultivation and isolation of organisms

Samples were collected and each of the samples diluted with distilled water as 10-1, 10-2,10-3,10-4,10-5 and 10-6 and inoculated into nutrient agar. Then the petri dishes were marked properly and incubated at 37℃ for 24hours aerobically in bacteriological incubator. then sub-cultured onto the Mac Conkey, SS agar and MSA agar by streak plate method (Cheesbrough, 1985) to observe the colony characteristic colony morphology of *E. coli, Salmonella spp, Staphylococcus spp and Bacillus spp* was repeatedly sub-cultured onto Mac-Conkey, SS agar and MSA agar and Soft agar until the pure culture morphology (shape, size, surface texture, edge and elevation, color, opacity etc). The organisms showing with homogenous colonies were obtained.

3.3.2 Morphological characterization by Gram's staining method

The most widely used staining procedure in microbiology is the gram stain ,discovered by the Danish scientist and physician Hans Christian Joachim Gram in 1884,Gram staining is a differential staining technique that differentiates bacteria into two groups :gram- positives and gram-negatives. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram-negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple .Gram-positive bacteria are not decolorized by alcohol and will remain as purple .After decolorization step, and a counterstain is used to impart a pink color to the decolorized gram- negative organisms.

3.3.3 Preparation of Gram,s Staining Solution

The four solutions needed for the Gram staining procedure.

- Crystal violet
- Gram's iodine
- 95% alcohol
- Safranin

3.3.4 Gram's Staining Procedure

1) Obtain clean glass slides were taken.

2) A sterile technique was used, a smears of each of the organisms was prepared. Smear made of a drop of water on the slide was placed then each organism separetly to the drop water with a sterile was transferred. A circular motion of the inoculating loop of organism was mixed and spreads.

3) Air –dry the smears were allowed then heat fixed in the usual manner.

4) Smears were flooded with crystal violet and let stood for 2 min gently washed with tap water.

5) Smears were flooded with Grams iodine mordant and let stood for 1 min .Gently washed with tap water.

6) Smears were decolorized with 95% ethyl alcohol .Wait it for 15 seconds.

7) Gently washed with tap water.

8) Smears were counter stain with safranin for 30 sec.

9) Slides ware washed with tap water.

10) Slides ware examine under oil immersion.

3.3.5 Biochemical Examination by Different Tests

Biochemical tests are the tests used for the identification of bacteria species based on the differences in the biochemical activities of different bacteria. Bacterial species differs from one species to the other.

Isolated organism with supporting growth characteristics of suspected identified by biochemical test are performed Catalase test, Indole test , Methyi-Red (MR Test), Voges-Proskauer test, Simmon's Citrate Test (Citrate Utilization Test), Triple Sugar Iron Agar(TSI), Mortility Indole Urease **(**MIU) test.

3.3.5.1 Catalase test

The presence of catalase is determined by its ability to break down peroxide into water and oxygen, releasing bubbles of oxygen. This test was used to differentiate bacteria which produce the enzyme catalase, such as staphylococci fom non-catalase producing bacteria such as streptococci.

Procedure:

i. To perform this test, a small colony of good growth pure culture of test organism was smeared on a slide .
ii. Then one drop of catalase reagent (3%H_2O_2) was added on the smear.
iii. The slide was observed for bubbles formation. Formation of bubble within few seconds was the indication of positive test while the absence of bubble formation indicated negative result. (Cheesbrough, 1985)

3.3.5.2 Indole test

The Test was cultured in a medium containing tryptophan. The organisms break down tryptophan and indole is released. I was detected by the action of Kovac's reagent or Ehrlich reagent (formation of red colored compound). This test was important for the identification of *Enterobacteria* such as *E.coli, Pseudomonas vulgaris,* etc. (Cheesbrough, 1985)

Procedure:

i. Tryptophan containing broth was inoculated with bacteria.
ii. The tube was incubated at 37 °C for 24 hours.
iii. Added 0.5 ml of the Kovac'c reagent after the bacterial growth.
iv. Shaked well and examined after 1 minute.
v. If indole positive, within 30 second a red color ring appeared at the junction of medium in the tube.
vi. In case of negative result, no color development or slightly pink color.

3.3.5.3 Methyl Red Test (MR)

This test was performed to differentiate *Enterobacteria*. Some *Enterobacteria* when cultured in buffered glucose peptone water, ferment glucose to produce sufficient acidity, which gives red color with methyl red indicator (pH range: 4.4-6.2, Color change: red-yellow.

Procedure:

i. Sterile MR-VP broth was inoculated with the test organism and following incubation at 37 °C for 24 hours.

ii. Few drops of methyl red solution were added.

iii. A distibct red color indicated MP positive test while yellow or orange color indicated a negative result. (Cheesbrough, 1985).

3.3.5.4 Voges-Proskauer test (VP)

The test organisms were cultured in glucose phosphate peptone water for 24 hours. Aceton formed was converted to diacetyl. It was converted into a pink compound by the action of creatine. This test was used to assist in the differentiation of *Enterobacteria* such as *Vibrio cholerae*, *Klebsiella* spp and some strains of *Enterobacter* (Cheesbrough, 1985).

Procedure:

i. Sterile MR-VP broth was inoculated with the test organism and following incubation at 37°C for 24 hours.

ii. After incubation, 5 drops of napthol solution and 5 drops of KOH solution were added.

iii. The development of a bright red or pink-red color was recorded as a positive result.

3.3.5.5 Triple Sugar Iron Agar (TSI)

TSI slants are useful in the identification of *Enterobacteria* by their specific reaction on the slants alkaline reaction (red color) was shown by the organisms, who fail to ferment any one of the sugar. Fermentation of the sugars was indicated by yellow color since pH range of phenol red is 6.8 and color change from yellow to red.

Since the glucose (Dextrose) present on the surface of the medium was used up and since the surface of the slant was exposed to atmosphere, under aerobic conditions, the acid reaction on the surface reverts to alkaline (red color) in 18 to 24 hours.(This is a critical duration for this observation). In the butt, since anaerobic condition exists, the color of the butt remains yellow. Gas production (carbon dioxide) was indicated by reacting with ferrous ions. It was an indication of H_2S producing organisms. TSI slants are useful in the identification of *Enterobacteria* by their specific reaction on the slants (Cheesbrough, 1985).

Procedure:

i. A loop of bacteria was spread across the surface of the agar.
ii. A needle of bacteria was inserted (stabbed) into the bottom (butt) of the tube.
iii. Kept the tubes at 37°C for 24 hours for incubation.
iv. Examined the tubes for result.

Examination of Result:

This tube medium is used to identify Gram negative enteric bacilli based on the following biochemical characteristics (Cheesbrough, 1985):

- Glucose fermentation – indicated by yellow butt
- Lactose fermentation – indicated by yellow slant
- Hydrogen sulfide production – indicated by blackening of the medium
- Gas production – indicated by presence of a crack, bubble or gas space
- pH indicator – phenol red
- Hydrogen sulfide indicator – ferric ammonium citrate with sodium thiosulfate.

3.3.5.6 Motility Indole Urease (MIU) test

MIU medium is a semisolid medium used in the qualitative determination of motility, production of indole and ornithine decarboxylase. MIU medium is used for the differentiation of the Family Enterobacteriaceae. The organisms tested must ferment glucose for proper performance of the medium. MIU medium contains dextrose as fermentable sugar, ornithine as an amino acid, bromcresol purple as pH indicator, casein peptone as a source of tryptophan, and other essential nutrients for growth. The medium contains a small amount of agar allowing for detection of motility (Cheesbrough, 1985).

Procedure:

i. Motility was observed by growth extending from the line of inoculums or diffuse turbidity of the medium. Non-motile organisms grow only along the line of inoculation.
ii. Urease activity was observed by a change of color to red.
iii. Indole production is indicated by the formation of a pink to red color after the addition of three or four drops of Kovac's reagent to the surface of the medium. A negative reaction is indicated by the development of a yellow color. The red color of phenol red in alkaline pH didi nit interfere because of the activity of Kovac's reagent.

3.3.5.7 Citrate Utilization Test

The test organism was cultured in a medium containing sodium citrate, an ammonium salt and bromothymol blue indicator. The organisns use citrate (the only source of nitrogen). The Citrate Utilization is by alkaline reaction (change of the color from light to blue) and growth in the medium was indicated by appearance of turbidity. This test was performed in the identification of *Enterobacteria.* (Cheesbrough, 1985).

Procedure:

i. i.A loop of bacteria was sread across the surface of the agar.
ii. Kept the tubes at 37°C for 24 hours for incubation.
iii. Examined the tubes for result.
iv. Positive case – produce blue color
v. Negative case – no color

CHAPTER- IV

RESULT

The present study was conducted to molecular characterization of bacterial pathogen isolated from different markets and hotels/restaurants. Samples were collected from different markets and hotels/restaurants of Dinajpur town in Bangladesh for their antibiogram study. A total of 30 samples were collected for various bacteriological, biochemical examinations in the laboratory of the Department of Microbiology, HSTU, Dinajpur.

4.1 Results of Isolation of organism

The major contaminants were Gram-negative bacteria namely *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Shigella* spp. *Vibrio* spp., *Proteus* spp., *Pseudomonas* spp., But Gram-positive also present namely *Staphylococcus* spp. and *Streptococcus* spp. in the isolation of bacteria From different samples collected from different markets which are shown on Table No 1.

Table 12: Summary of Isolation of Bacteria from Different Samples Collected from Different Markets

Samples	No. of Isolated Bacteria (n=55)									Total No. of Isolates
	Klebsiella spp.	*E.coli*	*Salmonella* spp.	*Shigella* spp.	*Staphylococcus* spp.	*Proteus* spp.	*Vibrio* spp.	*Pseudomonas* spp.	*Streptococcus* spp.	
Cucumber	1	1	-	2	-	1	1	2	-	8
Onion	1	1	1	2	-	1	1	2	-	9
Tomato	1	1	-	2	-	1	-	1	-	6
Lemon	1	1	1	1	-	1	1	2	-	8
Corriander Leaves	-	1	-	1	-	1	1	1	1	6
Green Chilli	1	1	-	2	1	2	1	1	-	9
Carrot	1	1	2	-	-	2	2	1	-	9
Total	6(10.90%)	7(12.73	4(7.27%)	10(18.18%	1(1.82%)	9(16.36%)	7(12.73%	10(18.18%	1(1.82%)	55

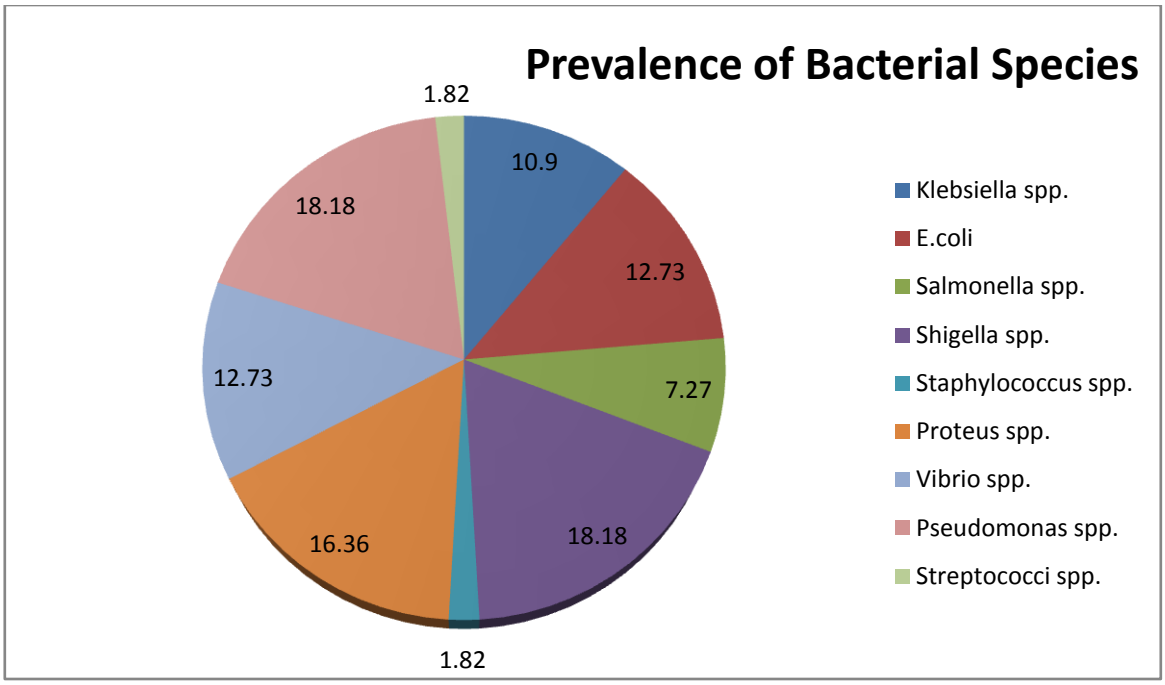

Fig 3: Prevalence of Bacterial Species Isolated From Different Samples Collected from Different Markets

Table 13: Summary of Isolation of Bacteria from Different Samples Collected from Different Hotels/Restaurants

Samples	No. of Isolated Bacteria (n=47)								Total No. of Isolates	
	Klebsiella spp.	*E.coli*	*Salmonella* spp.	*Shigella* spp.	*Staphylococcus* spp.	*Proteus* spp.	*Vibrio* spp.	*Pseudomonas* spp.	*Streptococcus* spp.	
Cucumber	-	4	2	2	-	1	1	2	1	13
Onion	-	4	1	3	1	-	-	2	1	12
Lemon	1	3	1	4	1	-	1	-	-	11
Green Chilli	2	3	1	2	1	-	2	-	-	11
Total (Percentage)	3(6.38%)	14(29.79%)	5(10.64%)	11(23.40%)	3(6.38%)	1(2.13%)	4(8.51%)	4(8.51%)	2(4.26%)	47

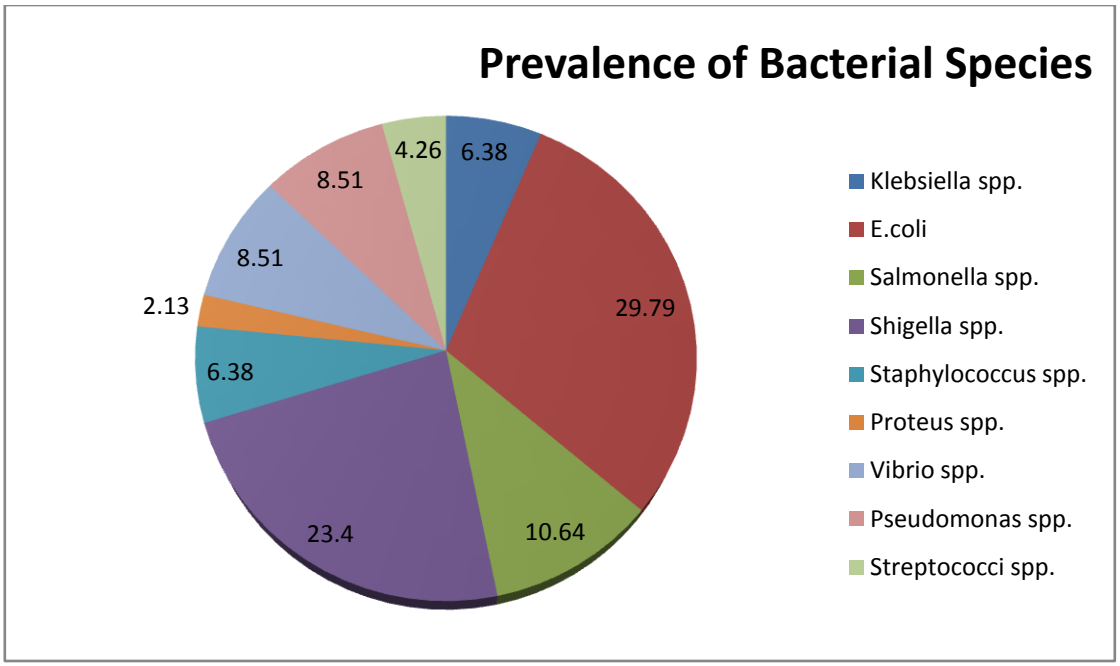

Fig 4: Prevalence of Bacterial Species Isolated from Different Samples Collected from Different Hotels/Restaurants

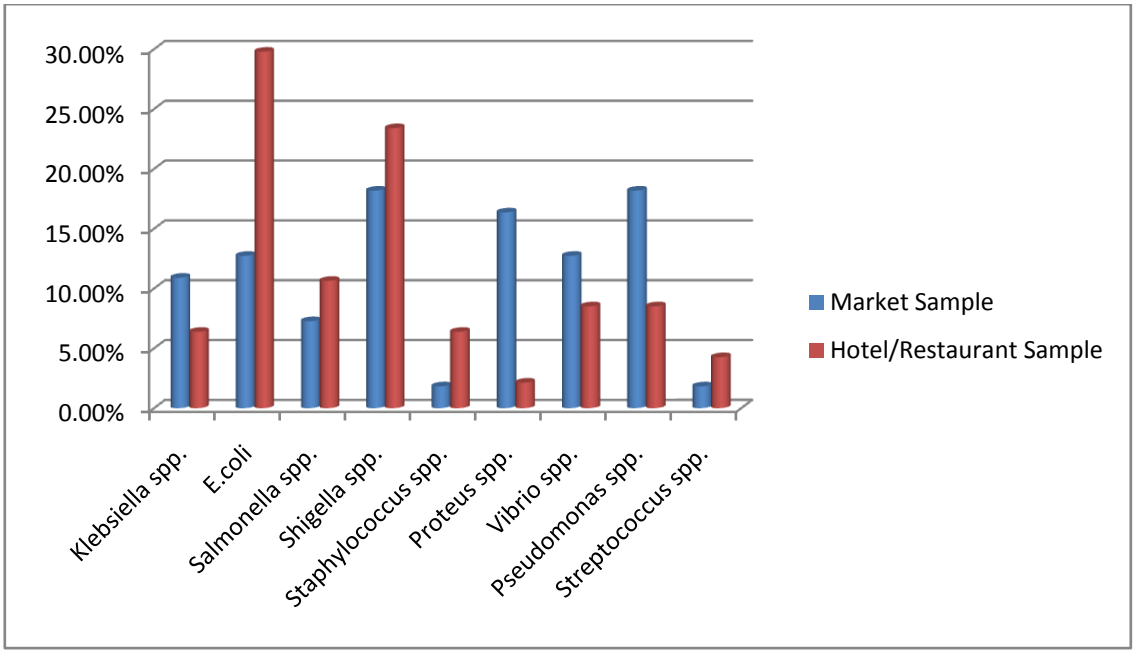

Fig.5: Distribution of Bacterial species Isolated from Market and Hotel/Restaurant Samples

4.2 Isolation and Identification of Bacteria by Different Bacteriological Methods

4.2.1: The Cultural Characteristics of *Klebsiella* spp., *E. coli, Salmonella* spp., *Shigella* spp., *Staphylococcus* spp., *Proteus* spp., *Vibrio* spp., *Pseudomonas* spp., *Streptococcus* spp.

Table -14 : Results of Cultural Examination

Sl. No.	Suspected case of Bacteria	Name of media	Colony characteristics
01.	*Klebsiella* spp.	Nutrient Agar	Mucoid, opaque
		MacConkey agar	Large,mucoid,bright pink lactose fermented colony
		EMB agar	Large,mucoid,bright pink lactose fermented colony but no metallic green sheen colony
		XLD	Mucoid,yellow colony
02.	*E. coli*	Nutrient agar	Circular, smooth, white to grayish or white colony
		MacConkey agar	Bright pink colored colony
		EMB agar	Metallic sheen (greenish black) colony
03.	*Salmonella* spp.	Nutrient agar	Small, white, translucent dew drop like colony(Pale colored colony)
		SS agar	Opaque, smooth, round with black centered
		XLD Agar	Red colonies, Black centers
04.	*Shigella* spp.	Nutrient agar	2-3 mm in diameter circular,grayish or colorless, smooth and translucent colonies
		SS agar	Pale colony
		XLD Agar	Red colonies
05.	*Staphylococcus* spp.	Nutrient agar	Black colour/ non-colour smooth, glistening colony
		Staphylococcus Agar No.110	Golden yellowish colony
		Blood Agar	β-hemolytic colony
		MSA Agar	Yellow colonies with yellow zone or red colonies with red zone

06.	*Proteus* spp.	Nutrient agar	Smooth, creamy, shiny,convex and swarming colony
		XLD Agar	Yellow colonies
07.	*Vibrio* spp.	TCBS Agar	Flat yellow colonies, 2-3 mm in diameter
08.	*Pseudomonas* spp.	Nutrient agar	Colonies are surrounded by bluish green coloration
		MacConkey agar	Pale colored colony
		Cetrimide agar	Yellow-green to blue colony
		XLD Agar	Pink,flat,rough colony
		TCBS Agar	Blue colony
09.	*Streptococcus* spp.	Blood Agar	Round, translucent or mucoid with alpha-hemolysis
		MSA Agar	No growth

Here,

EMB Agar = Eosin Methylene Blue Agar

XLD Agar = Xylose Lysine Deoxycholate Agar

SS agar = Slamonella-Shigella Agar

MSA Agar = Manitol Salt Agar

TCBS Agar = Thiosulphate Citrate Bile Salts agar

4.2.1.1 Nutrient Agar (NA)

Nutrient agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 370C aerobically and were indicated by the growth of circular, small smooth, convex and gray white or yellowish colonies.

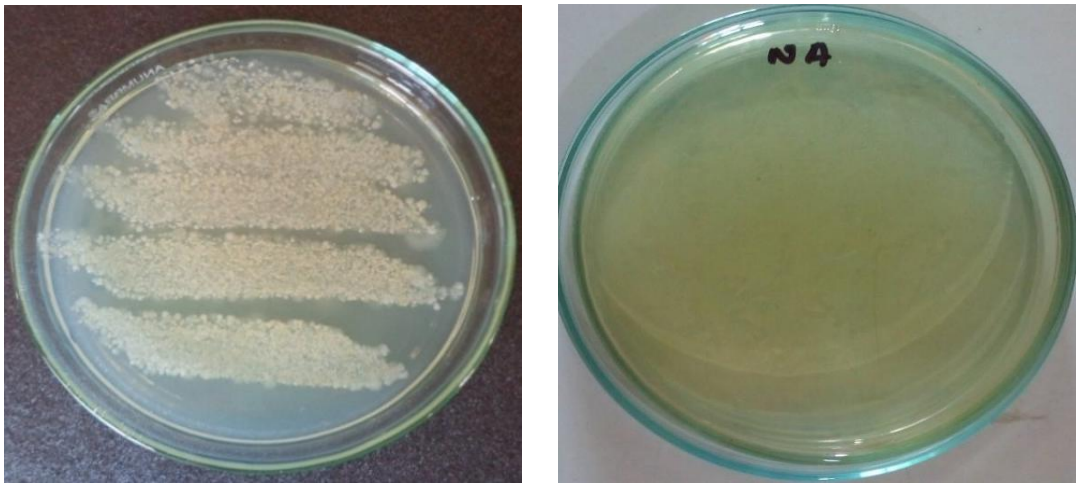

Plate 1: Culture of organism on Nutrient's Agar (left); Control of Nutrient's Agar (right).

4.2.1.2 Mac Conkey Agar

Mac Conkey Agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 370C aerobically and were indicated the colorless colonies after prolonged incubation pink color colonies.

Plate 2: Culture of organism on Mac Conkey Agar (left); Control of Mac Conkey Agar (right).

4.2.1.3 Eosin Methioline Blue Agar (EMB)

Eosin Methioline Blue Agarplates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 370C aerobically and were indicated the Large,mucoid,bright pink lactose fermented colony but no metallic green sheen colony colonies after prolonged incubation.

Plate 3: *Klebsiella* spp on Eosin Methioline Blue Agar (left); Control of Eosin Methioline Blue Agar (right).

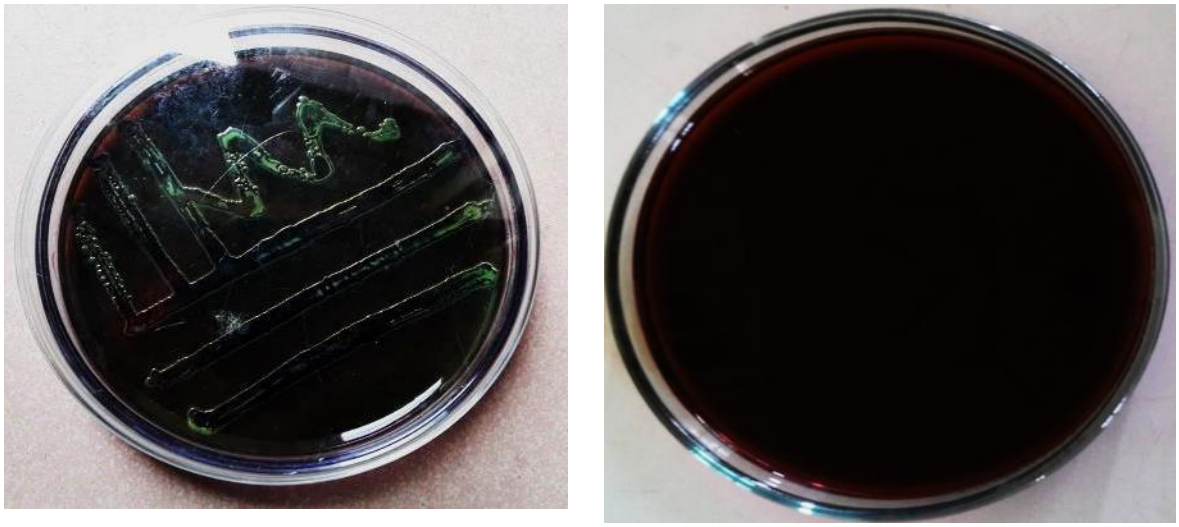

Plate 4: *E. coli* on Eosin Methioline Blue Agar (left); Control of Eosin Methioline Blue Agar (right).

4.2.1.4 *Salmonella-Shigella Agar* (SS)

Salmonella-Shigella Agarplates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 370C aerobically and were indicated by the clear, black center transparent colony.

Plate 5: *Salmonella* spp. on Salmonella-Shigella Agar (left); Control of Salmonella-Shigella Agar (right).

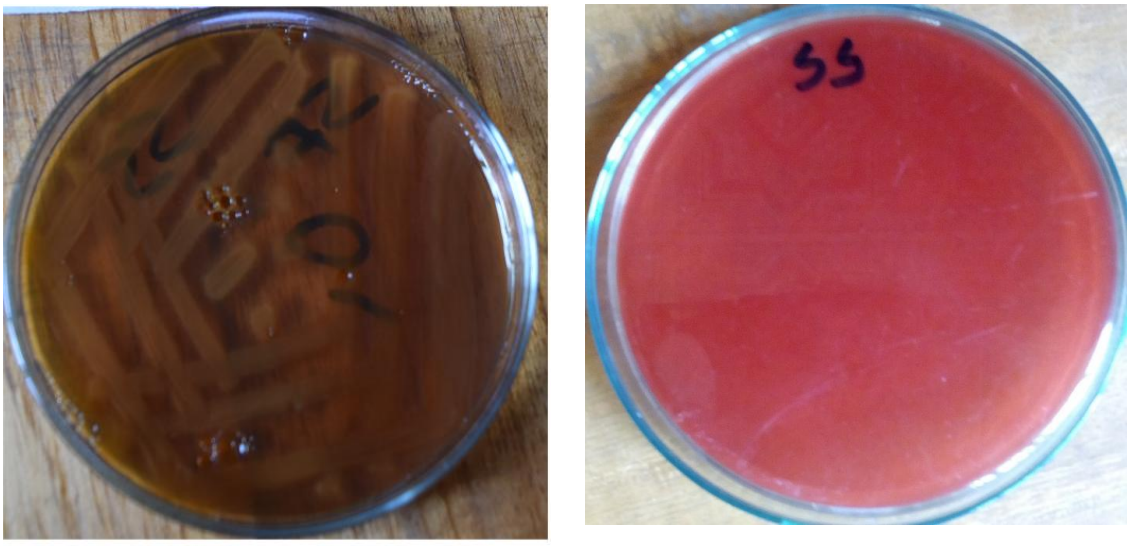

Plate 6: *Shigella* spp. on Salmonella-Shigella Agar (left); Control of Salmonella-Shigella Agar (right).

4.3.1.4 Brilliant Green Agar (BGA)

Brilliant Green Agarstreaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 370C aerobically and observed red, pink white colonies.

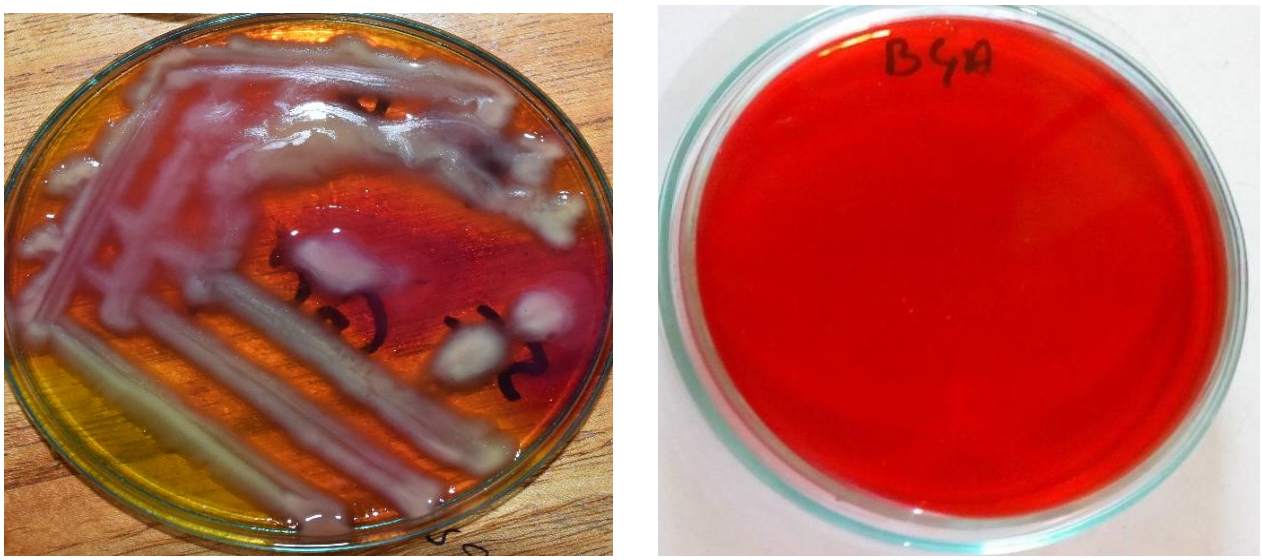

Plate 7: *Salmonella spp*. on Brilliant Green Agar (left); Control of Brilliant Green Agar (right).

4.3.1.5 Manitol Salt Agar (MSA)

Manitol salt agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 370C aerobically and were indicated by the pink color.

Plate 8: *Staphylococcus* spp. on Manitol Salt Agar (left); Control of Manitol Salt Agar (right).

4.3.1.6 Xylose Lysine Deoxycholate Agar (XLD)

Xylose Lysine Deoxycholateagar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 370C aerobically and were indicated by the red colonies with black centers, red colonies or pink, flat, rough, mucoid, yellow color colonies.

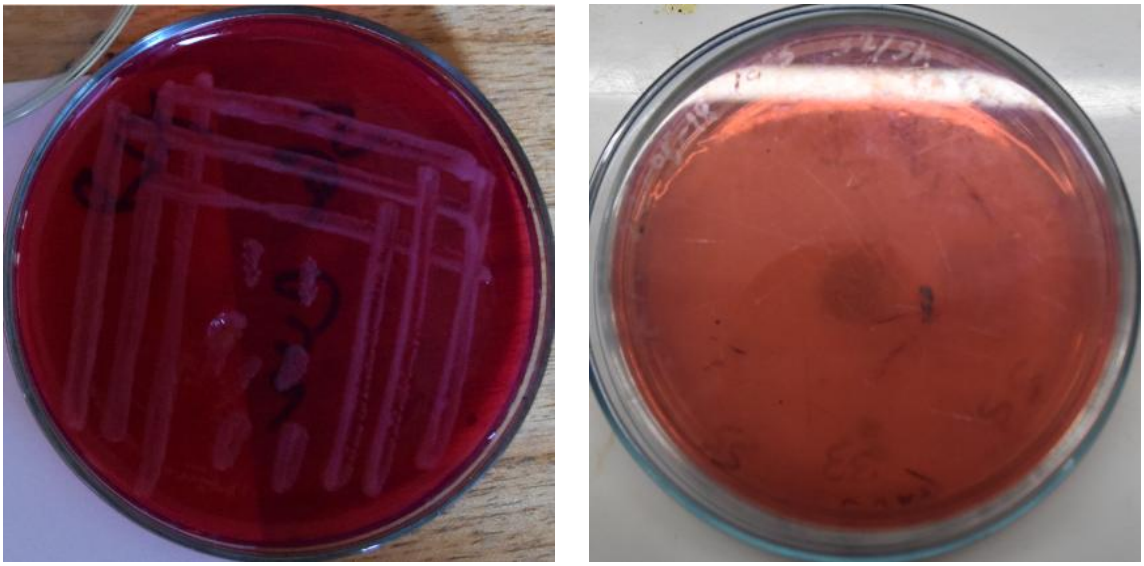

Plate 9: *Pseudomonas* spp. on Xylose Lysine Deoxycholate Agar (Left); Control of Xylose Lysine Deoxycholate Agar(Right)

4.3.1.7 Thiosulfate Citrate Bile Salts Agar (TCBS)

Thiosulfate Citrate Bile Salts agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 370C aerobically and were indicated by the flat yellow colonies, 2-3 mm in diameter, blue colonies or tiny transparent colonies.

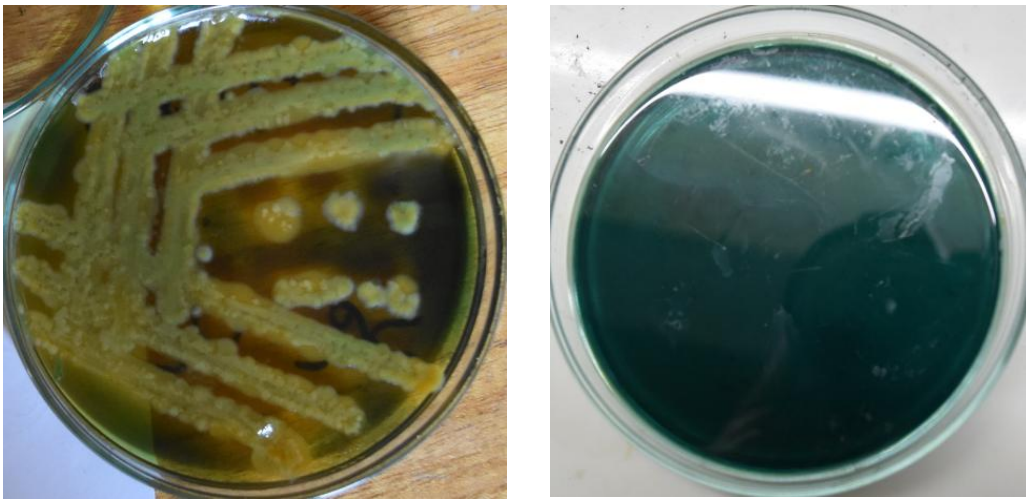

Plate 10: *Vibrio* spp. on Thiosulfate Citrate Bile Salts Agar (Left); Control of Thiosulfate Citrate Bile Salts Agar (Right)

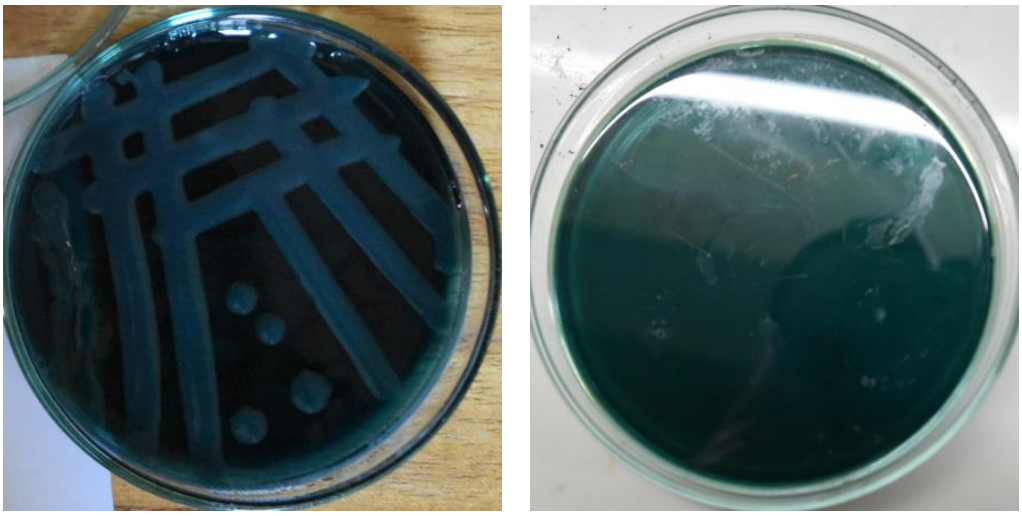

Plate 11: *Vibrio* spp. on Thiosulfate Citrate Bile Salts Agar (Left); Control of Thiosulfate Citrate Bile Salts Agar (Right)

4.3.2 Microscopic Examination

Microscopic observation was performed to observe shape and gram reaction of the isolates. Both of the isolates were found to be gram positive and gram negative, curved, comma and rod shape.

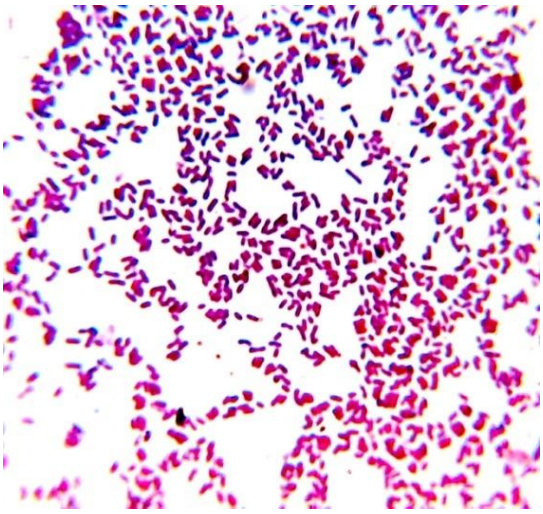

Plate 12: Grams staining of *Klebsiella* spp. isolates showing gram negative, mucoid or pink coloured, yellow colonies, small rod-shaped, single or paired organisms.

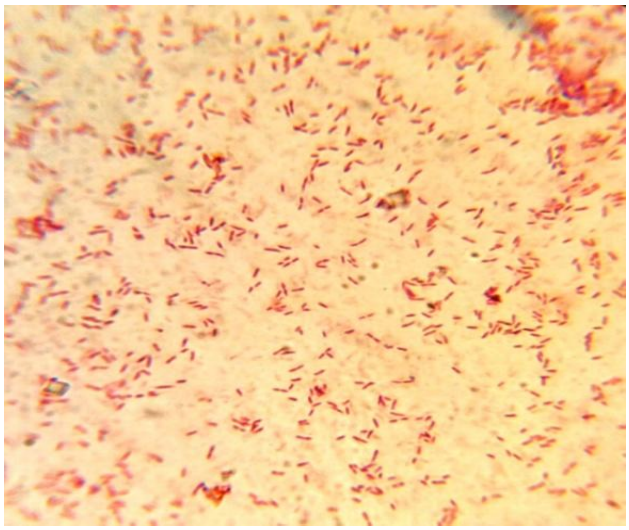

Plate 13: Grams staining of *E. coli* isolates showing gram negative, pink coloured, small rod-shaped, single or paired organisms.

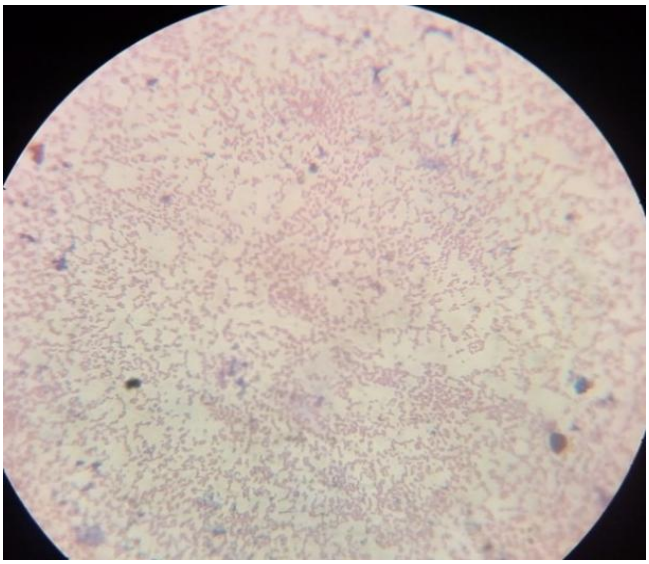

Plate. 14 .Gram-negative single very short rods of *Salmonella* spp

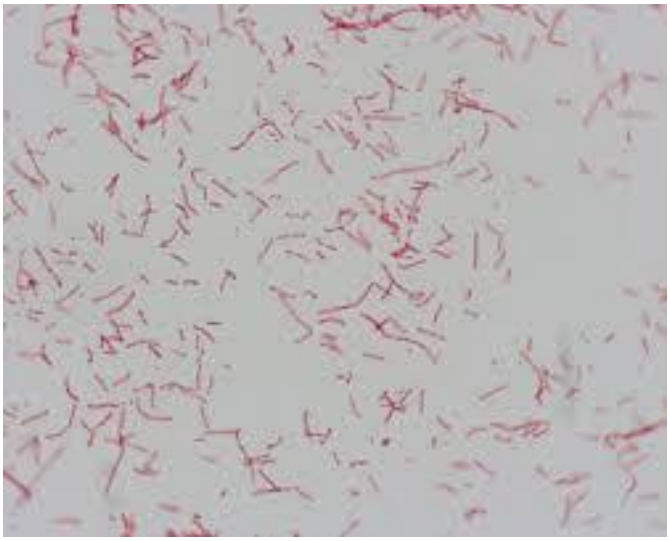

Plate.15: Gram-negative, very short rod-shaped, single of *Shigella* spp.

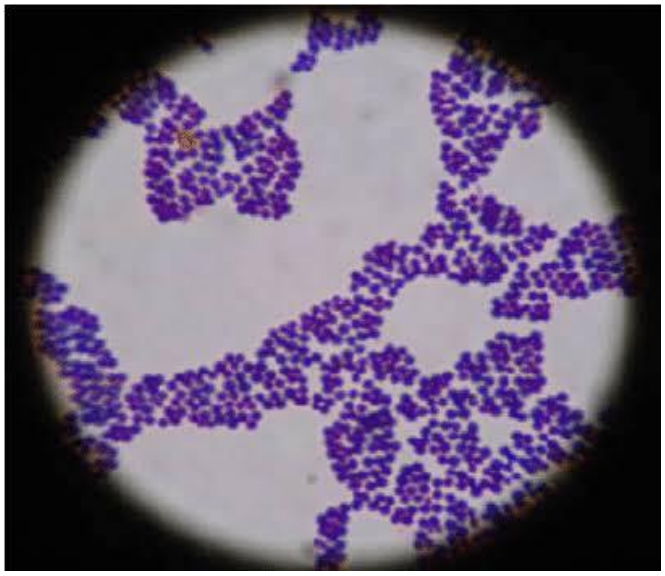

Plate. 16: Gram-positive, appear spherical (cocci), and form in grape-like clusters. of *Staphylococcus* spp.

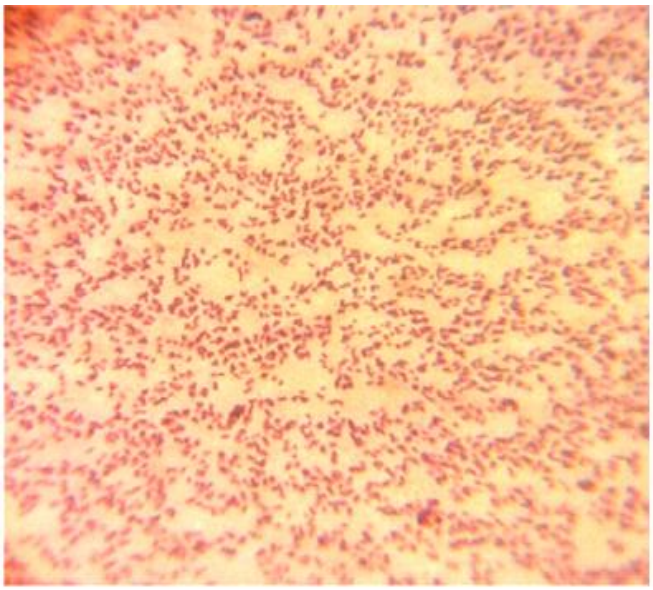

Plate. 17: Gram-positive, rod shaped, motile has a characteristic "swarming" ability that allows it to migrate across catheter surfaces *Proteus* spp.

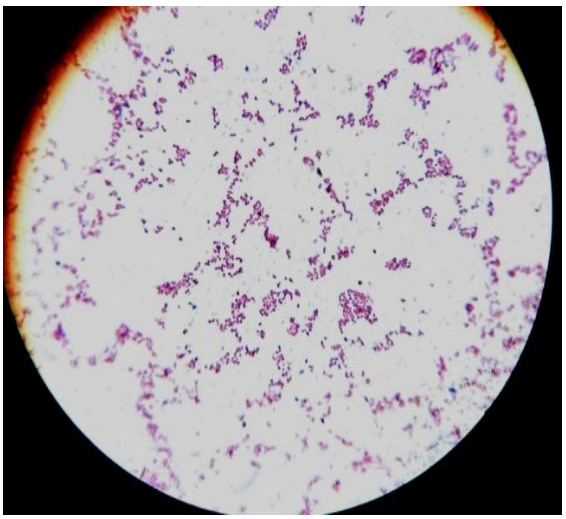

Plate. 18: Gram positive purple colour short chain *Streptococcus* spp

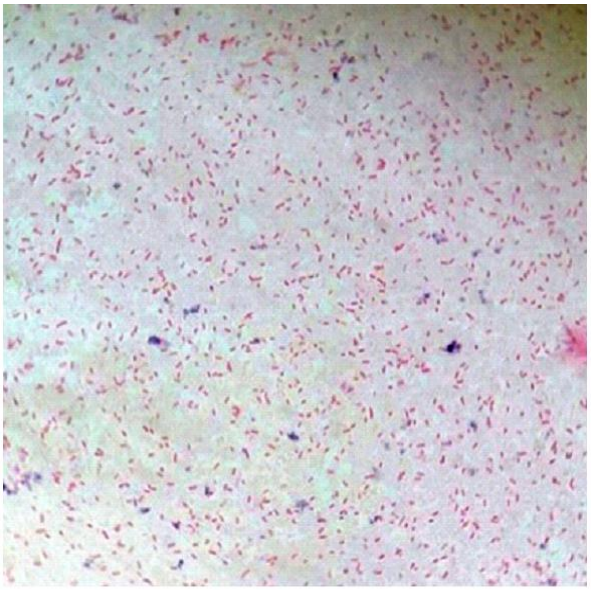

Plate. 19: Gram negative (pink colour) small rod shaped of *Pseudomonas* spp

4.2.3 Results of Biochemical Test

Isolated organisms were confirmed by different biochemical tests. Result of biochemical test is presented on Table 15.

Table 15: Result of biochemical test for the representative isolates

SL No.	MR	VP	Ind	TSI				MIU			SC	LC	CT	OX	Result
				Butt	Slant	Gas	H_2S	M	I	U					
1	-	+	-	Y	Y	-	-	-	-	-	+	+	+	-	*Klebsiella* spp.
2	+	-	+	Y	Y	+	-	+	+	-	-	+	+	-	*E.coli*
3	+	-	-	Y	R	+	+	+	-	-	+	-	+	-	*Salmonella* spp.
4	+	-	+	Y	R	-	V	-	-	-	-	-	+	-	*Shigella*spp.
5	+	-	-	Y	Y	-	-	+	-	+	-	+	+	-	*Staphylococcus* spp.
6	+	-	-	Y	R	+	+	+	+	+	-	-	+	-	*Proteus* spp.
7	-	-	-	Y	Y	+	-	+	-	-	+	-	+	-	*Pseudomonas* spp.
8	+	-	-	Y	Y	-	-	+	+	-	+	+	+	-	*Vibrio* spp.
9	+	-	-	Y	R	-	V	-	-	-	+	-	-	-	*Streptococcus* spp.

[Legands: + = positive, - = negative, OX= Oxidase, CT= Catalase, Ind= Indole, MR= Methyl Red, VP= Voges-Proskaur, SC= Simmon's Citrate, TSI= Triple Sugar Iron, MIU= Motility Indole Urease,Y=Yellow, R=Red, V=Variable]

4.3.3.1 Oxidase Test

All isolates were negative for oxidase test with no colour change.

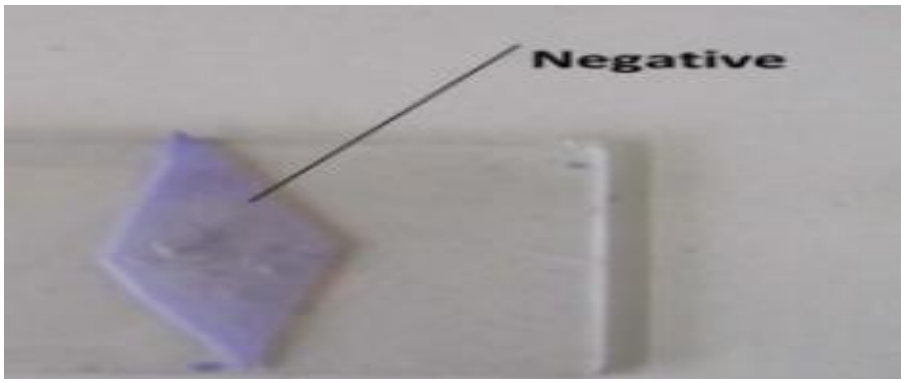

Plate 20: Oxidase Test

4.3.3.2 Catalase Test

All isolates were positive for catalase test with gas bubble formation.

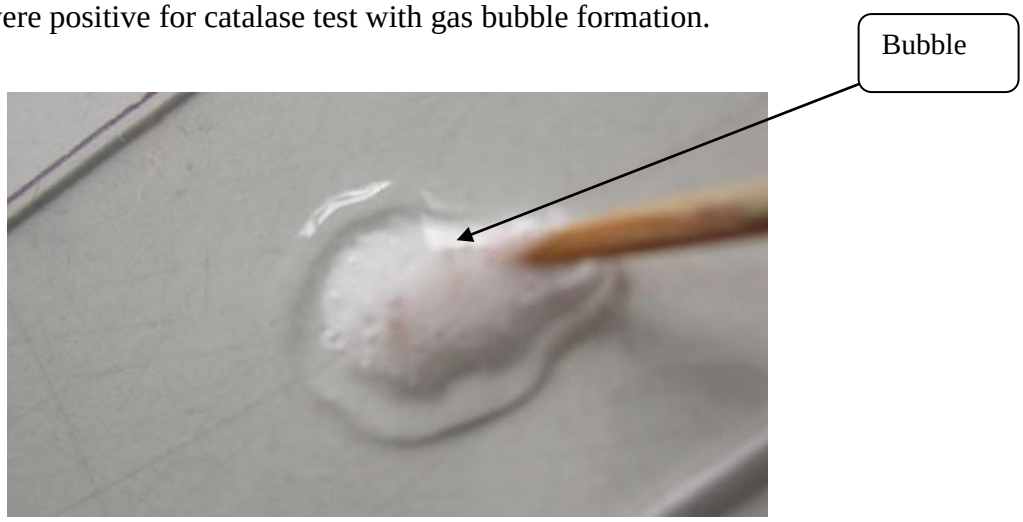

Plate 21: Catalase Test

4.3.3.3 Methyl Red Test

The *E.coli, Salmonella*spp.,*Shigella*spp., *Staphylococcus*spp., *Proteus*spp.,*Vibrio*spp.and *Streptococcus* spp.were positive and *Klebsiella and Pseudomonas* spp.were negative formethyl red test.

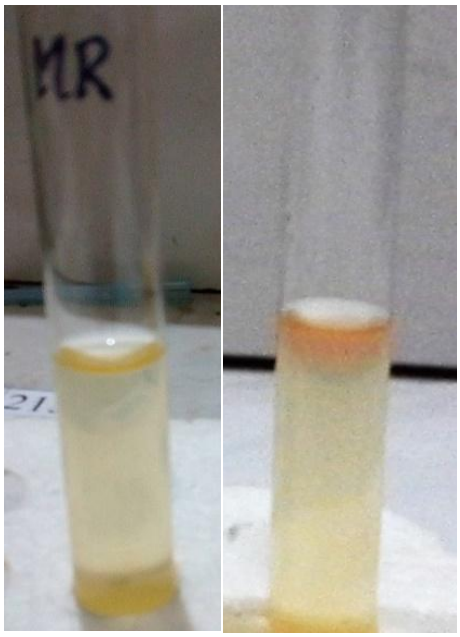

Plate. 22: *Klebsiella* spp. showing negative result by no color change (right) and control (left).

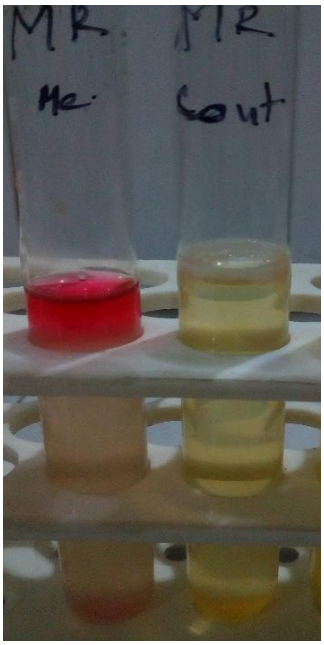

Plate. 23: *E. coli* showing positive result by bright red coloration (left) and control (right).

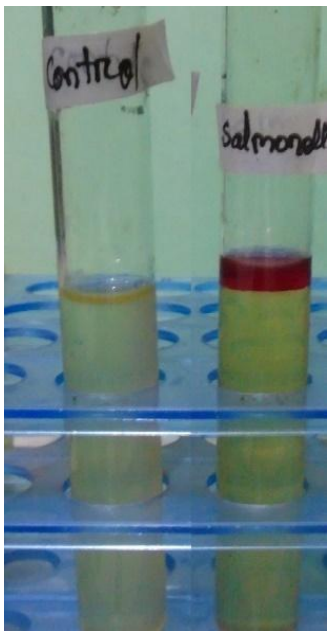

Plate. 24: *Salmonella* spp. showing positive result (right) and control

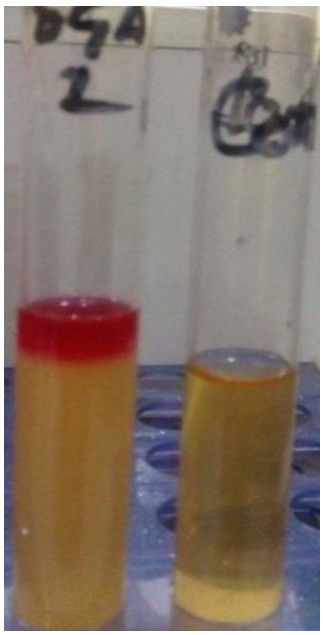

Plate. 25: *Shigella* spp. showing positive result by bright red coloration (right) and control (left).

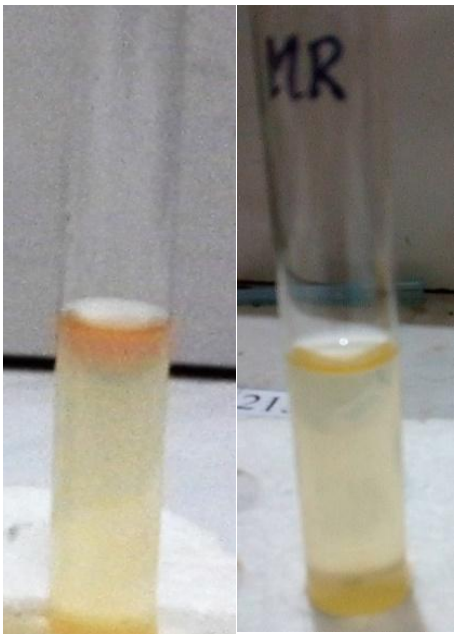

Plate. 26: *Staphylococcus* spp. indicated positive by changing the medium into bright red colour (right) and control (left).

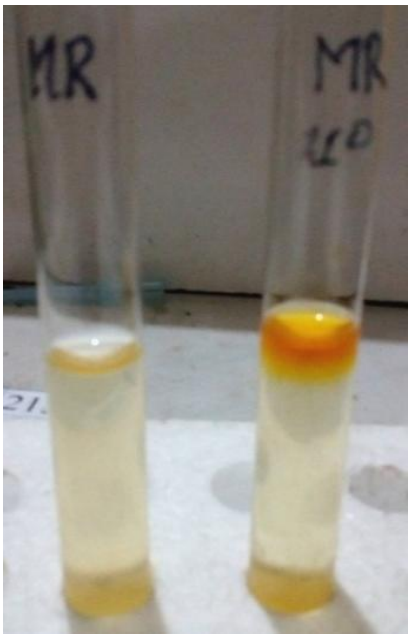

Plate. 27: *Pseudomonas* spp. indicated negative by no colour change of the medium (right) and control (left).

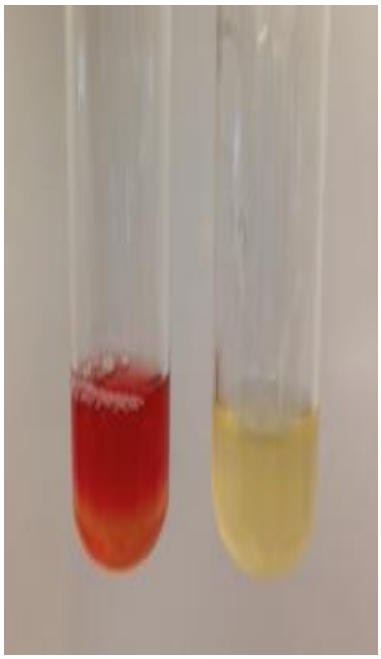

Plate. 28: *Proteus* spp. indicated positive by changing of the medium (left) and control (right).

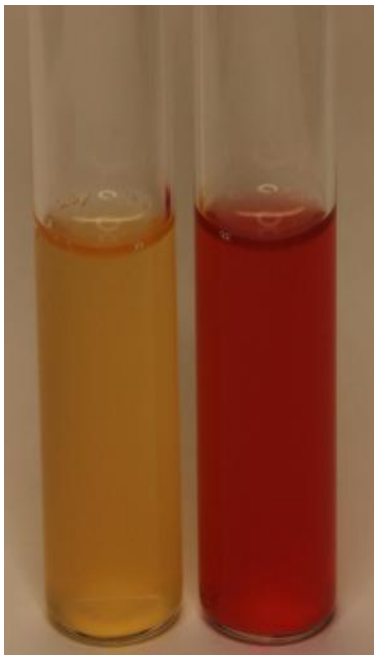

Plate. 29: *Vibrio* spp. indicated positive by changing of the medium (right) and control (left).

4.2.3.3 Voges-Proskauer Test

The *Klebsiella* spp. positive and *E. coli* spp., *Salmonella* spp., *Shigella* spp., *Staphylococcus* spp., *Proteus* spp., *Vibrio* spp., *Streptococcus* spp. *and Pseudomonas* spp. were negative for Voges-Proskauer Test.

Plate. 30: *E. coli, Salmonella* spp., *Staphylococcus* spp., *Proteus* spp., *Vibrio* spp., *Streptococcus spp. and Pseudomonas* spp. showing VP negative result by no changed of medium to rose red colour (right) and control (left).

Plate. 31: *E. coli, Salmonella* spp., *Staphylococcus* spp., *Proteus* spp., *Vibrio* spp., *Streptococcus spp. and Pseudomonas* spp. showing VP negative result by no changed of medium to rose red colour (right) and control (left).

4.2.3.1 Indole Test

The *E.coli and Shigella* spp. were positive and *Klebsiella* spp., *Salmonella* spp., *Staphylococcus*s pp., *Proteus* spp., *Vibrio* spp., *Streptococcus* spp. *and Pseudomonas* spp. were negative for Indole Test.

Plate. 32: *E. coli* showing positive result by red coloration (left) and control (right).

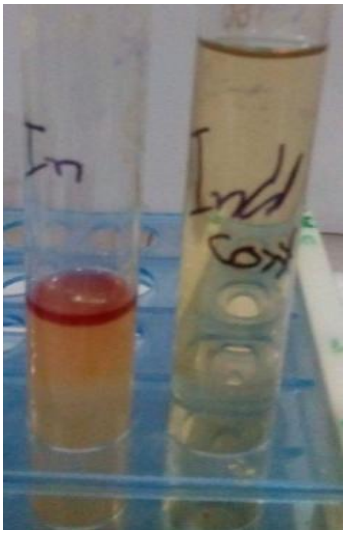

Plate. 33: *Shigella* spp. showing positive result by red coloration (left) and control (right).

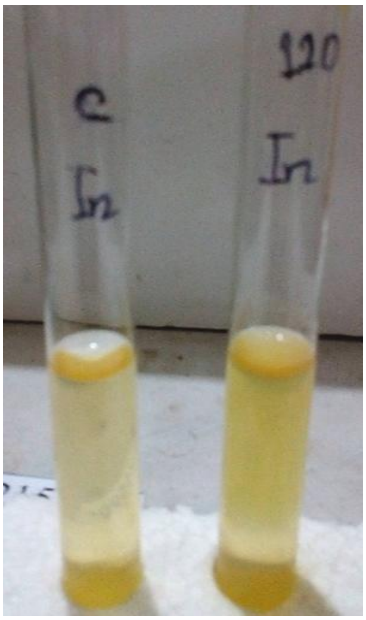

Plate. 34: *Klebsiella* spp., *Salmonella* spp., *Staphylococcus* spp., *Proteus* spp., *Vibrio* spp., *Streptococcus* spp. *and Pseudomonas* spp. showing negative result by no colour change of the medium (right) and control (left).

4.2.3.5 Triple Sugar Iron (TSI) Test

Plate 35: Triple Sugar Iron Test for *Klebsiella* spp. showing yellow colour butt & yellow colour slant inoculated (left) and control (right).

Plate. 36: Triple Sugar Iron Test for *E. Coli* showing yellow colour butt & yellow colour slant inoculated (right) and control (left).

Plate. 37: Triple Sugar Iron Test for *Salmonella Spp.* showing yellow colour butt & red colour slant with gas and H_2S production inoculated (left) and control (right).

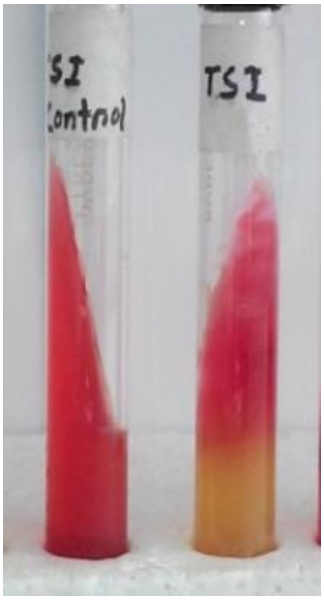

Plate. 38: Triple Sugar Iron Test for *Shigella spp.*showing yellow colour butt & red colour slant inoculated (left) and control (right).

Plate. 39: Triple Sugar Iron Test for *Staphylococcus* spp. showing yellow colour butt & yellow colour slant inoculated (left) and

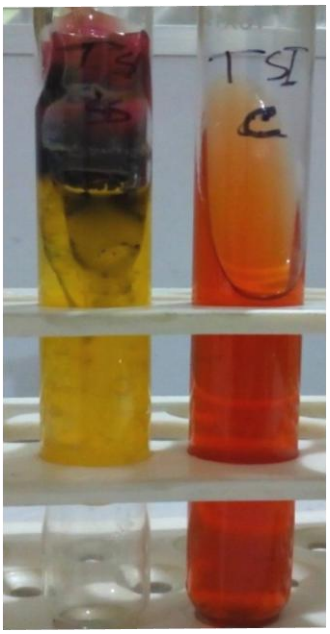

Plate. 40: Triple Sugar Iron Test for *Proteus* spp.showing yellow colour butt & red colour slant with gas and H_2S production inoculated (left) and control (right).

Plate. 41: Triple Sugar Iron Test for *Proteus* spp. showing yellow colour butt & red colour slant with gas and H_2S production inoculated (left) and control (right).

Plate. 42: Triple Sugar Iron Test for *Vibrio* spp. showing yellow colour butt & yellow colour slant inoculated (right) and control (left).

Plate. 43: Triple Sugar Iron Test for *Streptococcus* spp. showing yellow colour butt & red colour slant inoculated (right) and control (left).

4.2.3.6 Simmons Citrate Test

The *E.coli; Shigella* spp., *Staphylococcus spp.*& *Proteus spp.* were negative and *Klebsiella* spp., *Salmonella* spp., *Pseudomonas* spp., *Vibrio* spp. and *Streptococcus* spp. were positive for Simmons Citrate Test.

Plate. 44: Simmon's Citrate Test for *E. coli; Shigella* spp., *Staphylococcus spp. & Proteus spp.* showing negative result by no changed of medium to blue colour inoculated (left) and control (right).

Plate. 45: Simmon's Citrate Test for *Klebsiella* spp. showing positive result by colour change of the medium into blue colour (right) and control (left)

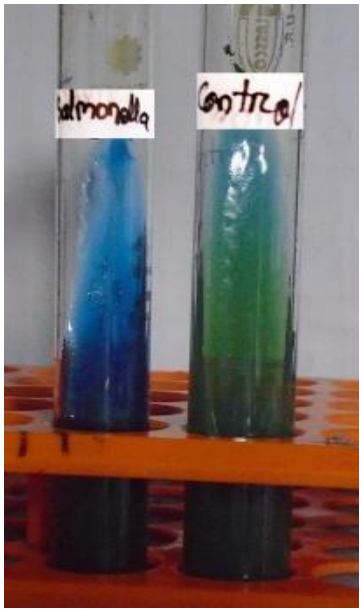

Plate. 46: Simmon's Citrate Test for *Salmonella* spp. showing positive result (left) and control (right).

Plate. 47: Simmon's Citrate Test for *Pseudomonas* spp. showing positive result by colour change of the medium into blue colour (left) and control (right).

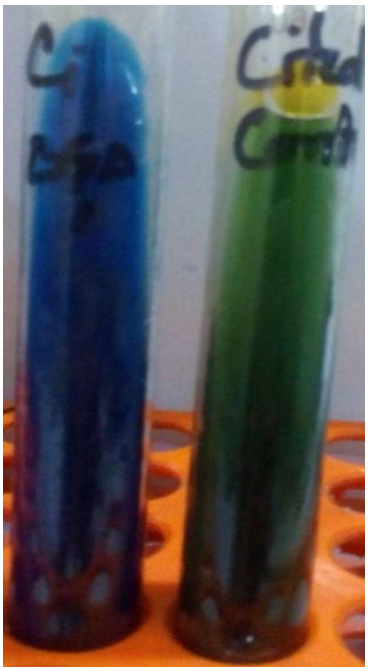

Plate. 48: MIU Test for *E. coli, Salmonella* spp., *Staphylococcus* spp., *Proteus* spp., *Vibrio* spp *.and Pseudomonas* spp. showing positive result by the diffuse, hazzy growth and slightly opaque media (right) and control (left).

Plate. 49: Simmon's Citrate Test for *Streptococcus spp.*showing result by colour changed of medium to blue colour inoculated (left) and control (right).

4.2.3.4 MIU Test

The *E.coli, Salmonella* spp., *Staphylococcus* spp., *Proteus* spp., *Vibrio* spp. *and Pseudomonas* spp. were positive and *Shigella* spp. , *Klebsiella* spp. and *Streptococcus* spp. were negative for MIU Test.

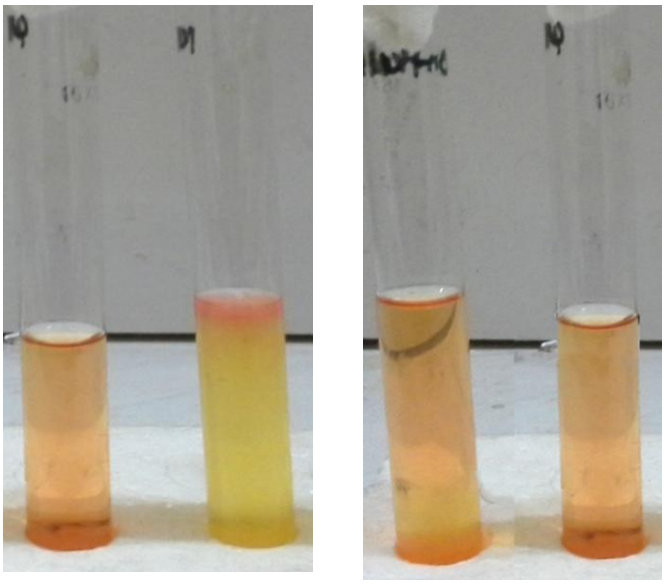

Plate. 50: MIU Test for *Shigella* spp. ,*Klebsiella* spp .and *Streptococcus* spp. showing negative result by no colour change of the media (left) and control (right).

4.2.4 Results of Antibiotic Sensitivity Test

Results of antibiotic sensitivity test of *Klebsiella* spp., *E. coli, Salmonella* spp., *Shigella* spp., *Staphylococcus spp., Proteus* spp., *Vibrio* spp. *and Pseudomonas* spp., and *Streptococcus* spp. against commonly used antibiotics are shown in table 6,7,8,9.

Table 16: Antimicrobial profile of *Klebsiella* spp.

Antimicrobial Agent	Diameter of Zone of Inhibition(mm)	Interpretation
Kanamycin (K)	14	I
Amoxicillin (AMX)	-	R
Piperacillin (TZP)	-	R
Bacitracin (B)	-	R
Tetracyclin (TE)	20	I
Nalidixic Acid (NA)	18	S
Azithromycine (AZM)	25	S
Cloxacillin (COX)	-	R
Chloramphenicol (C)	20	I
Norflaracin (NX)	18	I
Novobiocin (NV)	-	R
Methicillin (MET)	-	R
Cefixime (CFM)	-	

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

Note: Legends: S= Sensitive, R= Resistant, I=Intermediate

Table 17: Antimicrobial profile of *E. coli* spp.

Antimicrobial Agent	Diameter of Zone of Inhibition(mm)	Interpretation
Kanamycin (K)	**15**	I
Amoxicillin (AMX)	**-**	R
Piperacillin (TZP)	**-**	R
Bacitracin (B)	**-**	R
Tetracyclin (TE)	**22**	I
Nalidixic Acid (NA)	**18**	S
Azithromycine (AZM)	**26**	S
Cloxacillin (COX)	**-**	R
Chloramphenicol (C)	**23**	S
Norflaracin (NX)	**17**	I
Novobiocin (NV)	**-**	R
Methicillin (MET)	**-**	R
Cefixime (CFM)	**-**	R

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

Note: Legends: S= Sensitive, R= Resistant, I=Intermediate

Table 18: Antimicrobial profile of *Salmonella* spp.

Antimicrobial Agent	Diameter of Zone of Inhibition(mm)	Interpretation
Kanamycin (K)	**15**	I
Amoxicillin (AMX)	**-**	R
Piperacillin (TZP)	**-**	R
Bacitracin (B)	**-**	R
Tetracyclin (TE)	**24**	S
Nalidixic Acid (NA)	**18**	S
Azithromycine (AZM)	**19**	S
Cloxacillin (COX)	**-**	R
Chloramphenicol (C)	**21**	S
Norflaracin (NX)	**21**	S
Novobiocin (NV)	**-**	R
Methicillin (MET)	**-**	R
Cefixime (CFM)	**-**	R

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

Note: Legends: S= Sensitive, R= Resistant, I=Intermediate

Table 19: Antimicrobial profile of *Shigella* spp.

Antimicrobial Agent	Diameter of Zone of Inhibition(mm)	Interpretation
Kanamycin (K)	**18**	S
Amoxicillin (AMX)	**-**	R
Piperacillin (TZP)	**-**	R
Bacitracin (B)	**-**	R
Tetracyclin (TE)	**20**	I
Nalidixic Acid (NA)	**19**	S
Azithromycine (AZM)	**19**	S
Cloxacillin (COX)	**-**	R
Chloramphenicol (C)	**22**	S
Norflaracin (NX)	**23**	S
Novobiocin (NV)	**-**	R
Methicillin (MET)	**-**	R
Cefixime (CFM)	**-**	

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

Note: Legends: S= Sensitive, R= Resistant, I=Intermediate

Table 20: Antimicrobial profile of *Staphylococcus* spp.

Antimicrobial Agent	Diameter of Zone of Inhibition(mm)	Interpretation
Kanamycin (K)	**21**	S
Amoxicillin (AMX)	**-**	R
Piperacillin (TZP)	**-**	R
Bacitracin (B)	**-**	R
Tetracyclin (TE)	**21**	I
Nalidixic Acid (NA)	**18**	S
Azithromycine (AZM)	**25**	S
Cloxacillin (COX)	**-**	R
Chloramphenicol (C)	**21**	S
Norflaracin (NX)	**17**	I
Novobiocin (NV)	**-**	R
Methicillin (MET)	**-**	R
Cefixime (CFM)	**-**	R

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

Note: Legends: S= Sensitive, R= Resistant, I=Intermediate

Table 21: Antimicrobial profile of *Proteus* spp.

Antimicrobial Agent	Diameter of Zone of Inhibition(mm)	Interpretation
Kanamycin (K)	**15**	I
Amoxicillin (AMX)	**-**	R
Piperacillin (TZP)	**-**	R
Bacitracin (B)	**-**	R
Tetracyclin (TE)	**20**	I
Nalidixic Acid (NA)	**18**	S
Azithromycine (AZM)	**19**	S
Cloxacillin (COX)	**-**	R
Chloramphenicol (C)	**21**	S
Norflaracin (NX)	**18**	I
Novobiocin (NV)	**-**	R
Methicillin (MET)	**-**	R
Cefixime (CFM)	**-**	R
Vancomycine (VA)	**-**	R

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

Note: Legends: S= Sensitive, R= Resistant, I=Intermediate

Table 22: Antimicrobial profile of *Vibrio* spp.

Antimicrobial Agent	Diameter of Zone of Inhibition(mm)	Interpretation
Kanamycin (K)	**14**	I
Amoxicillin (AMX)	**-**	R
Piperacillin (TZP)	**-**	R
Bacitracin (B)	**-**	R
Tetracyclin (TE)	**20**	I
Nalidixic Acid (NA)	**17**	I
Azithromycine (AZM)	**19**	S
Cloxacillin (COX)	**-**	R
Chloramphenicol (C)	**20**	I
Norflaracin (NX)	**21**	S
Novobiocin (NV)	**-**	R
Methicillin (MET)	**-**	R
Cefixime (CFM)	**-**	

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

Note: Legends: S= Sensitive, R= Resistant, I=Intermediate

Table 23: Antimicrobial profile of *Pseudomonas* spp.

Antimicrobial Agent	Diameter of Zone of Inhibition(mm)	Interpretation
Kanamycin (K)	**14**	I
Amoxicillin (AMX)	**-**	R
Piperacillin (TZP)	**-**	R
Bacitracin (B)	**-**	R
Tetracyclin (TE)	**21**	I
Nalidixic Acid (NA)	**18**	S
Azithromycine (AZM)	**24**	S
Cloxacillin (COX)	**-**	R
Chloramphenicol (C)	**21**	S
Norflaracin (NX)	**17**	I
Novobiocin (NV)	**-**	R
Methicillin (MET)	**-**	R
Cefixime (CFM)	**-**	R

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

Note: Legends: S= Sensitive, R= Resistant, I=Intermediate

Table 24: Antimicrobial profile of *Streptococcus* spp.

Antimicrobial Agent	Diameter of Zone of Inhibition(mm)	Interpretation
Kanamycin (K)	**20**	S
Amoxicillin (AMX)	**-**	R
Piperacillin (TZP)	**-**	R
Bacitracin (B)	**-**	R
Tetracyclin (TE)	**20**	I
Nalidixic Acid(NA)	**18**	S
Azithromycine (AZM)	**19**	S
Cloxacillin (COX)	**-**	R
Chloramphenicol (C)	**21**	S
Norflaracin (NX)	**17**	I
Novobiocin (NV)	**-**	R
Methicillin (MET)	**-**	R
Cefixime (CFM)	**-**	R

[Source: ECUCAST - European Committee on Antimicrobial Susceptibility Testing]

Note: Legends: S= Sensitive, R= Resistant, I=Intermediate

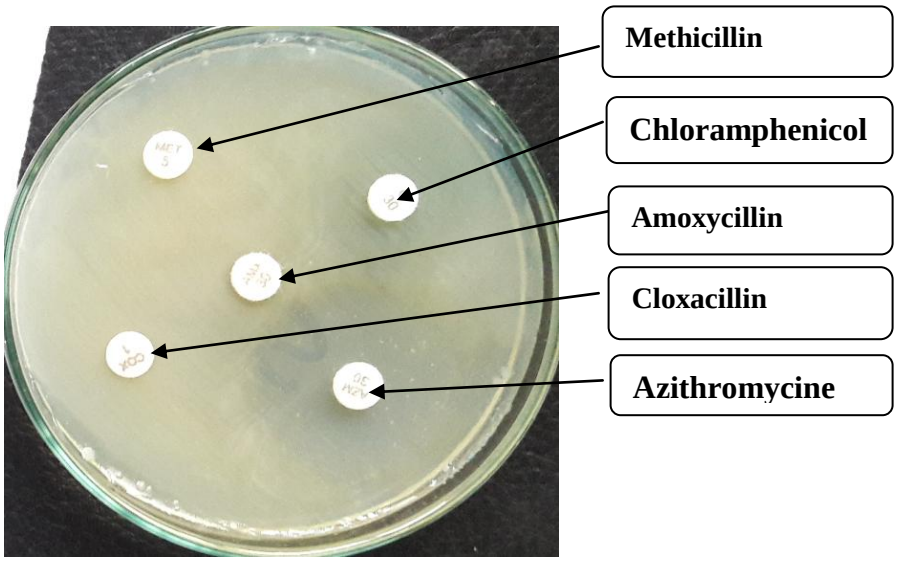

Plate 51: Antibiogram Test of *Klebsiella* spp.

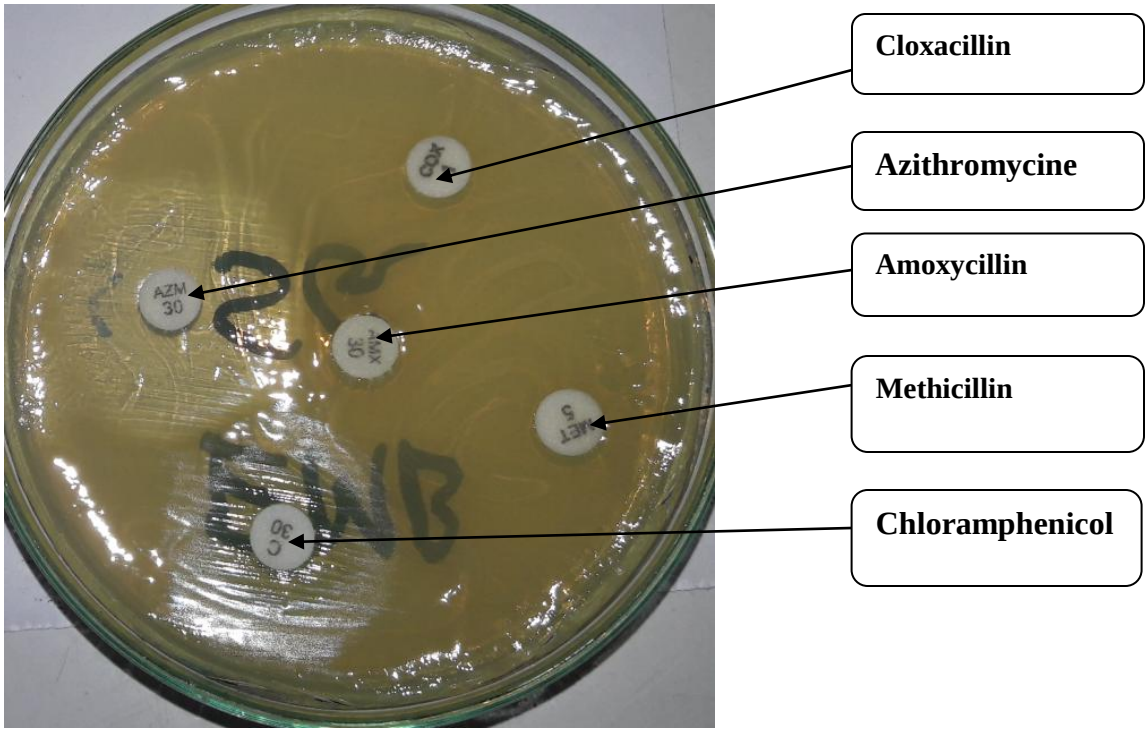

Plate 52: Antibiogram Test of *E. coli.* spp.

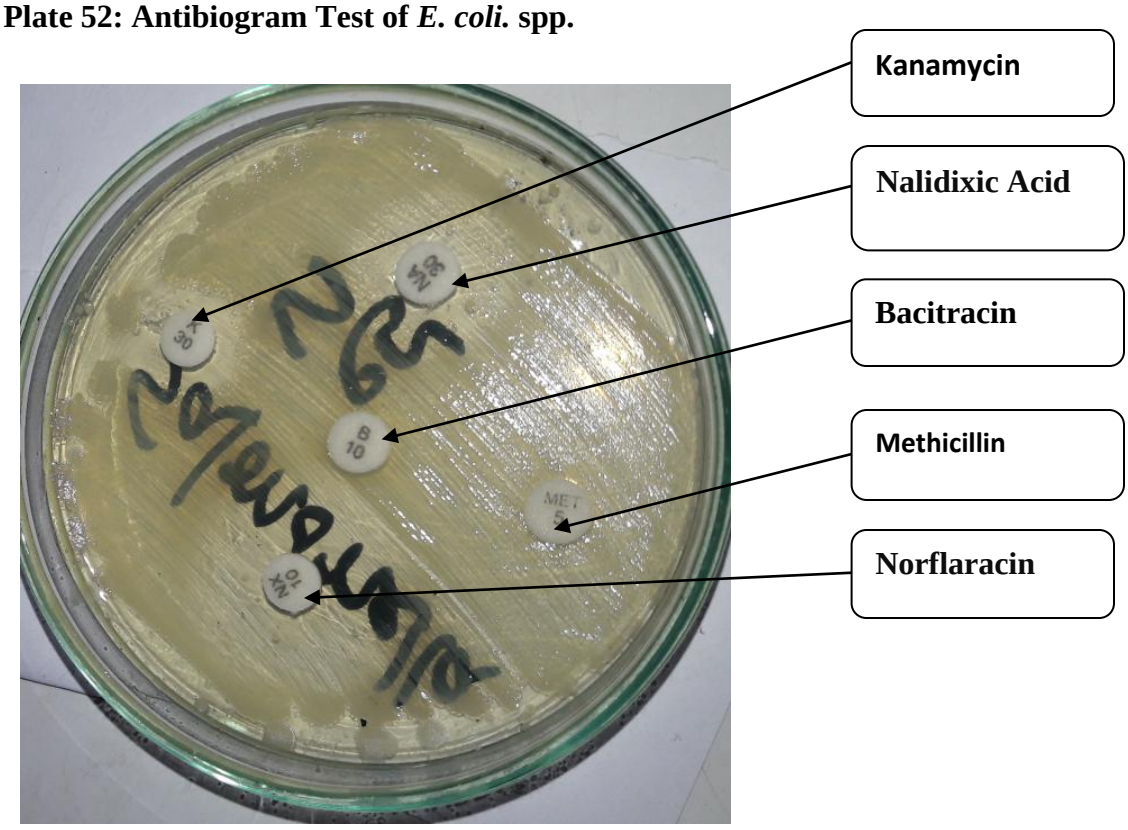

Plate 53: Antibiogram Test of *Salmonella* spp.

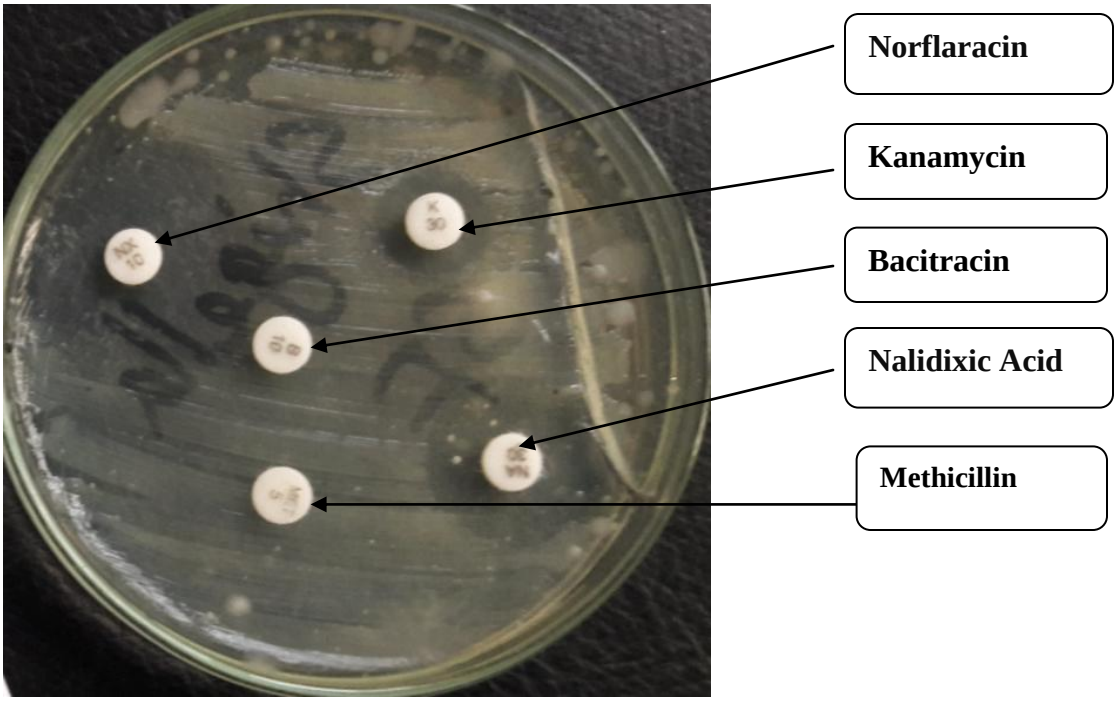

Plate 54: Antibiogram Test of *Shigella* spp.

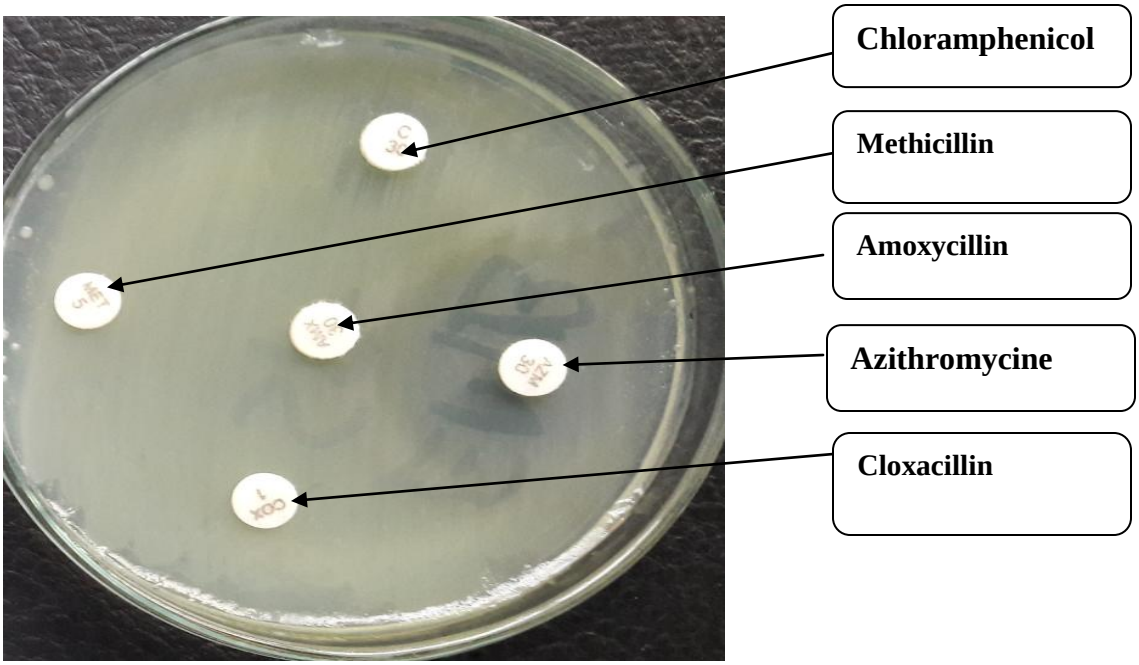

Plate 55: Antibiogram Test of *Staphylococcus* spp.

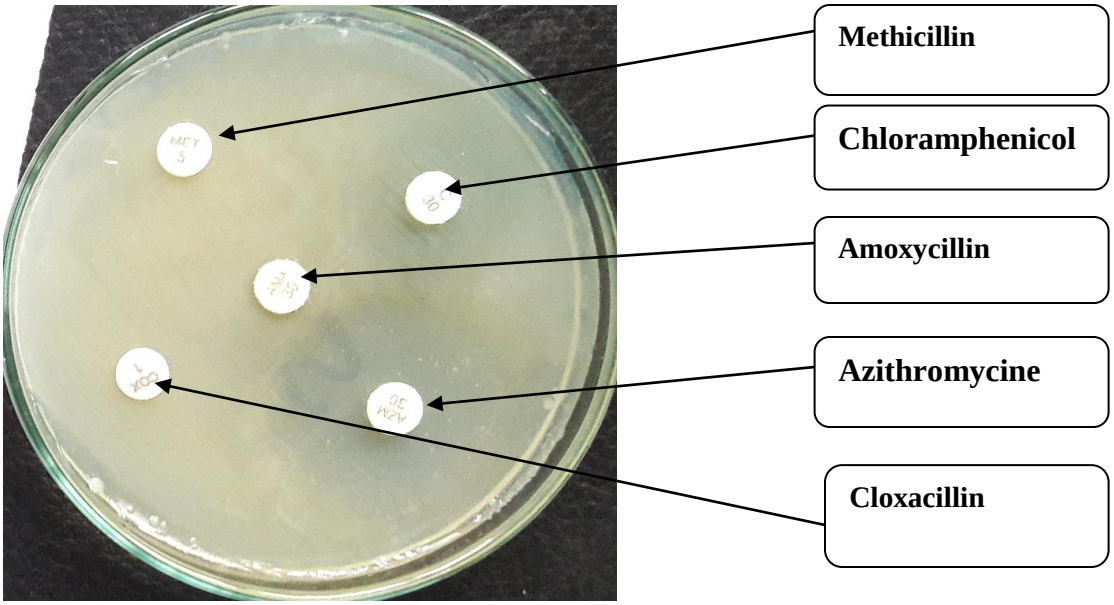

Plate 56: Antibiogram Test of *Pseudomonas* spp.

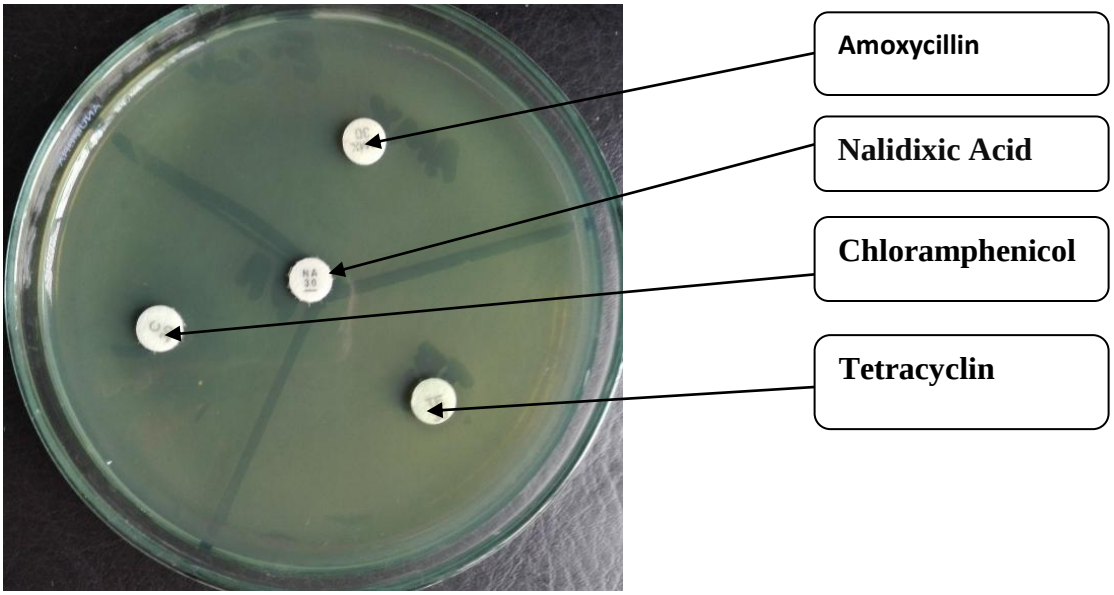

Plate 57: Antibiogram Test of *Proteus* spp.

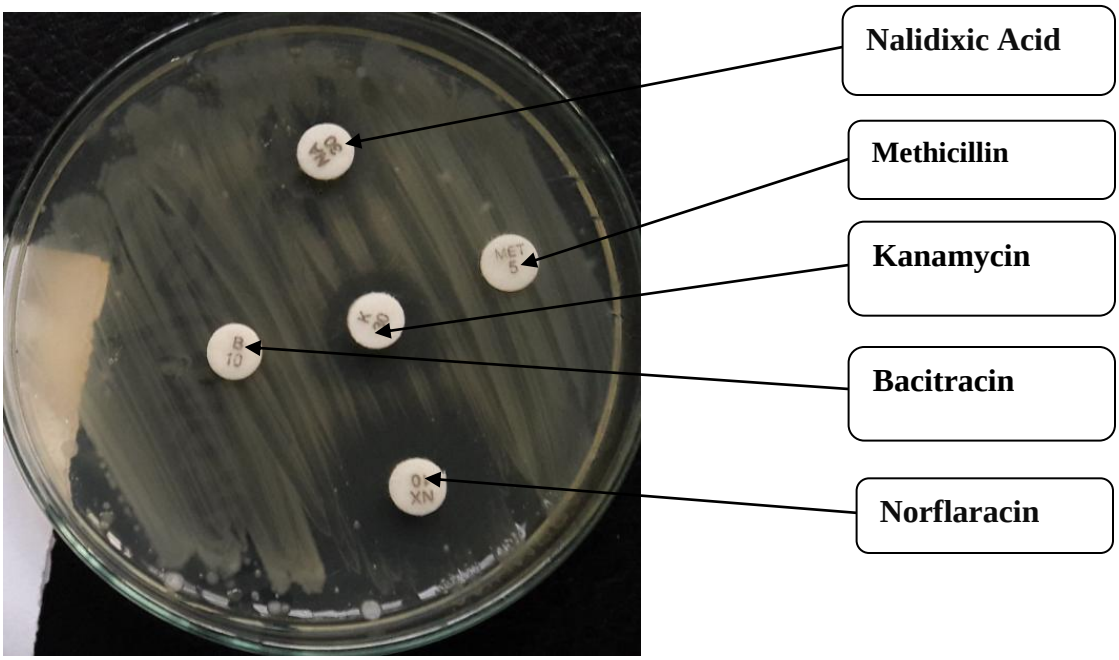

Plate 58: Antibiogram Test of *Vibrio* spp.

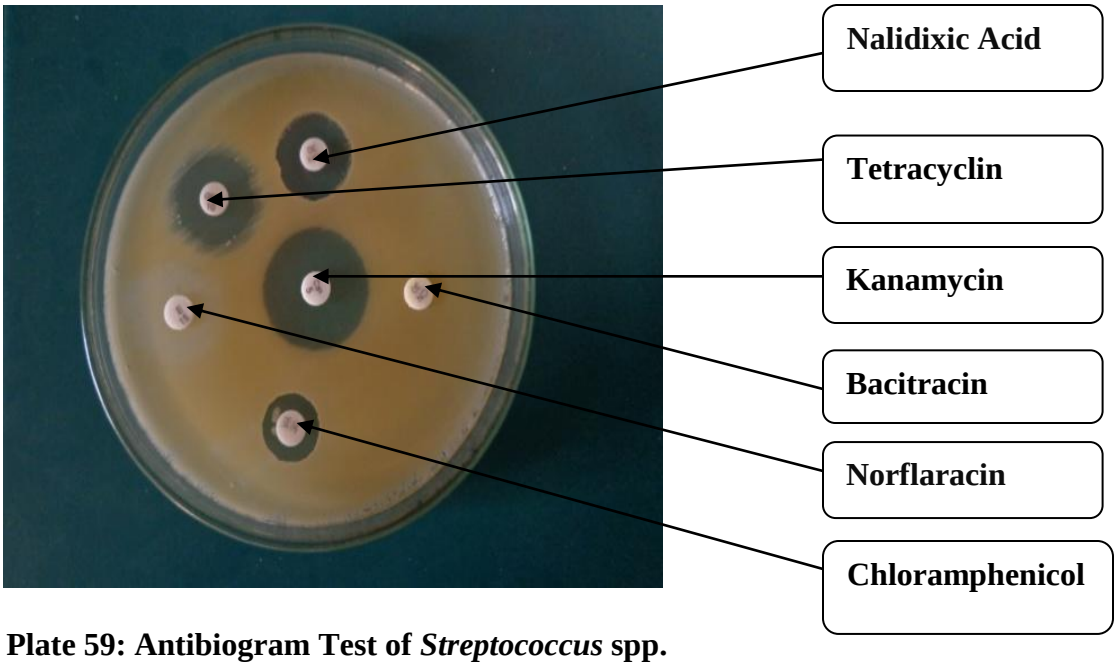

Plate 59: Antibiogram Test of *Streptococcus* spp.

4.2.5 Result of PCR Amplification Sequencing of 16s rRNA Genes with universal Primers and phylogenetic analysis of *Pseudomonas spp.*

16S rRNA gene region was amplified with the universal primers, Forward primer 27F (5'-AGAGTTTGATCCTEGGCTCAG3) and Reverse primer 1492 R (5'-TACCTTGTTACGACTT3). PCR Amplification band was found at 1466 bp.

Gel Image:

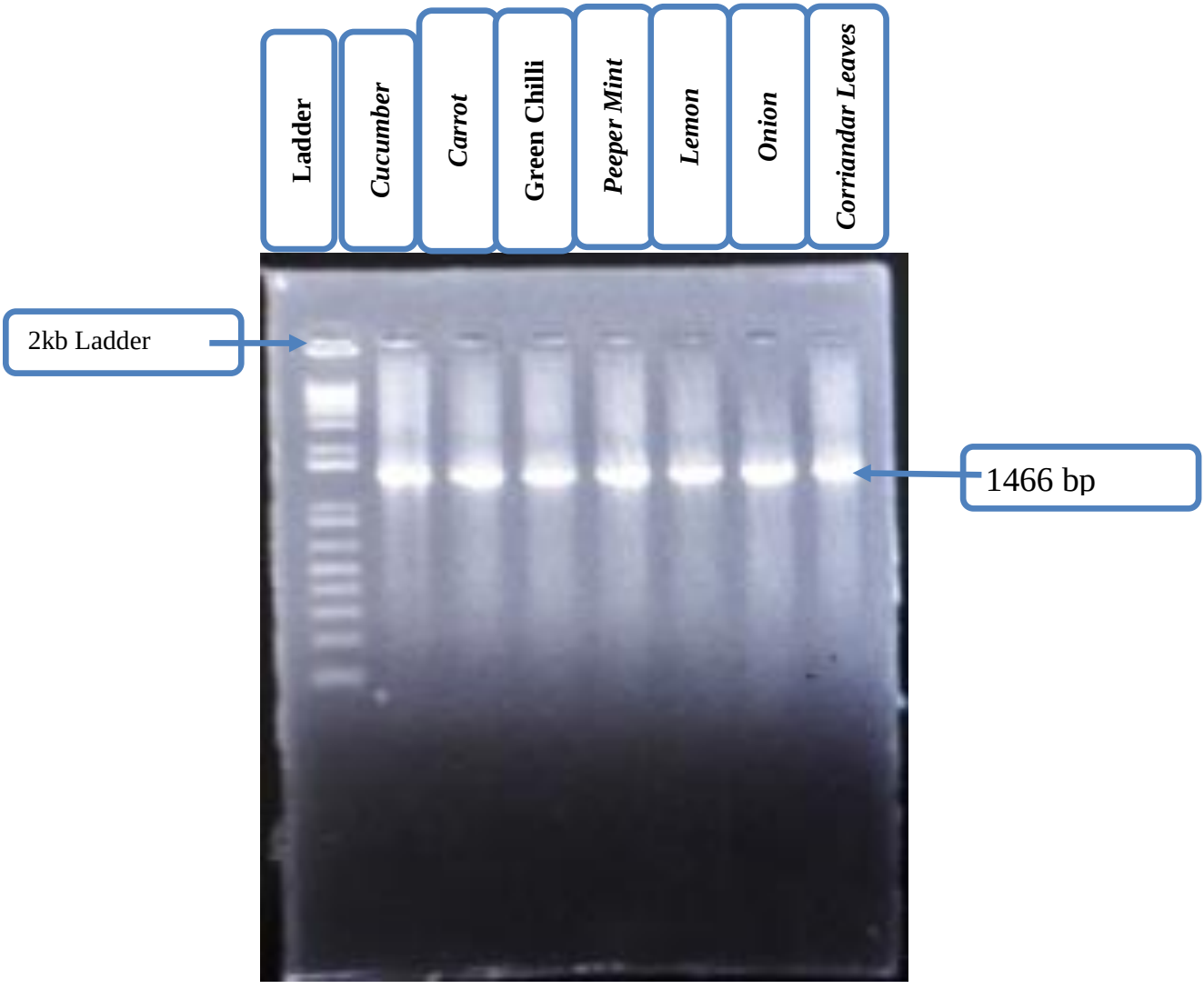

Fig 6: Result of amplification of 16S rRNA gene region of *Pseudomonas spp.* by PCR.

Sample 1- *Cucumber*

Sample 2- *Carrot*

Sample 3- *Green Chilli*

Sample 4- *Peeper Mint*

Sample 5- *Lemon*

Sample 6- *Onion*

Sample 7- *Corriandar Leaves*

Note: PCR= Polymerase Chain Reaction, kb= kilo base, bp= base pair

4.2.5.1 Electropherogram

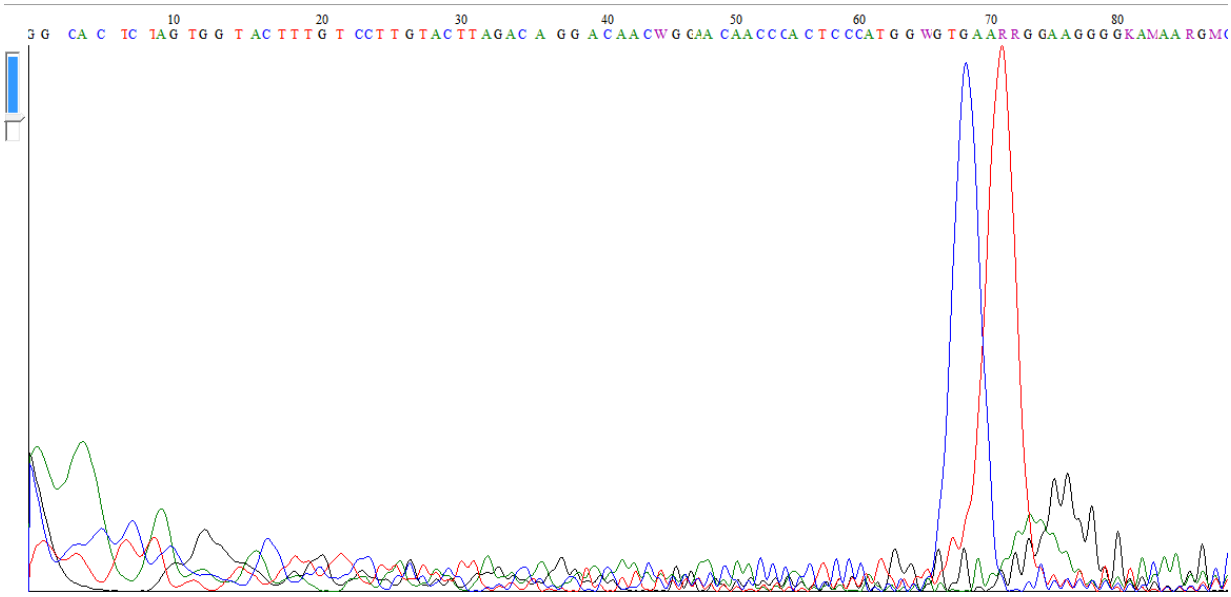

Fig 7: Electropherogram of 16s rRNA Gene Sequence of *Pseudomonas aeruginisa*

4.2.5.2 Phylogenic tree analysis of *Pseudomonas aeruginisa*

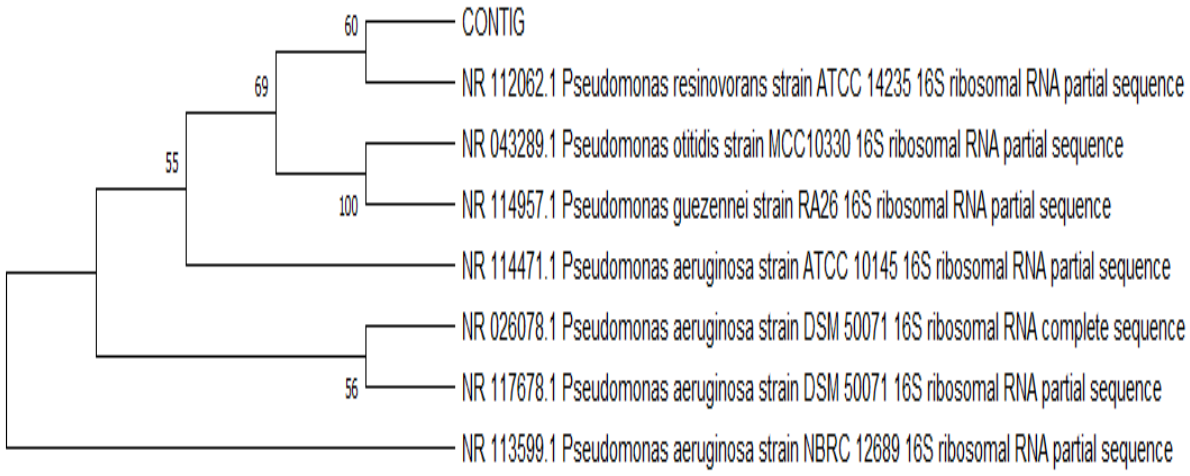

Fig. 8: Phylogenic tree analysis of of *Pseudomonas aeruginisa*

4.2.5.4 Coting Sequence of *Pseudomonas aeruginisa*

GMCCGGGAACGTATTCACCGTGCCCTTYWGAKTCACGATTACTARCGATDNAT
CGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCC
GTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGC
AGTCTCCTTAGAGTGCCCACCCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGC
TCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCA
GCACCTGTGTCTGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCAGC
ATGTCAAGGCCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTC
CACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTAC
TCCCCAGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCC
CAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGT
TTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTC
GCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCA
CCACCCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGC
CCGGGGATTTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGT
AATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTA
GCCGGTGCTTATTCTGTTGGTAACGTCAAAACAGCAAGGTATTAACTTACTGCC
CTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACMCGCGG
CATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCG
TAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAGACCA
GTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACTAGCTAATCCGACCT
AGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCTCAGGACG
TATGCGGTATTAGCSCCCRTTKCCGGACGTTATCCCCCMCTACCAGGCAG

CHAPTER-V

DISCUSSION

Vegetables serve a major part of our food supply. Raw vegetables harbor a number of pathogenic microorganisms, which may be dispersed over the plants or appear as microcolonies embedded in the plant tissues (Beuchat *et al*., 2002). The present study was conducted to molecular characterization of bacterial pathogen isolated from different markets and hotels/restaurants. Samples were collected from different markets (suihari market, bahadur bazar) and hotels/restaurants of Dinajpur town in Bangladesh for their antibiogram study. A total no. of 30 samples were collected to subjected for various bacteriological, biochemical examinations to test for the presence of different microorganisms. From the total 60 samples, 28 samples were from two different markets and the rest 32 samples were from 4 different hotels/ restaurants. All the samples were inoculated into Nutrient Agar (NA) and then MacConkey agar. After the inoculation into Nutrient Agar (NA) and MacConkey agar the samples were inoculated into various selective media such as Eosin Methylene Blue (EMB) agar, Salmonella Shigella Agar (SS), Brilliant Green Agar (BGA), Mannitol Salt Agar (MSA), Xylose Lysine Deoxycholate Agar (XLD) and Thiosulfate Citrate Bile Salts Agar (TCBS). Among the 60 samples, all samples (98%) had bacterial contamination. A total no. of 9 bacterial species were identified from 55 isolates. Findings of the research work were nearly similar with the findings of Hossain *et al*.(2012). The identified bacterial species were *Klebsiella* spp., *E. coli, Salmonella spp., Shigella* spp., *Staphylococcus* spp., *Proteus* spp., *Vibrio* spp., *Pseudomonas* spp., *Streptococcus* spp. From the isolation it is found on the market samples that *Klebsiella* spp. were 10.90%, *E. coli were* 12.73%, *Salmonella spp. were* 7.27%, *Shigella* spp. 18.18%, *Staphylococcus* spp. were 1.82% ,*Proteus* spp. were 16.36% , *Vibrio* spp. were 12.73% , *Pseudomonas* spp. were 18.18%, *Streptococcus* spp. were 1.82% and from hotel/ restaurant samples, *Klebsiella* spp. were 6.38%, *E. coli were* 29.79%, *Salmonella spp. were* 10.64%, *Shigella* spp. 23.40%, *Staphylococcus* spp. were 6.38% ,*Proteus* spp. were 2.13% , *Vibrio* spp. were 8.51% , *Pseudomonas* spp. were 8.51%, *Streptococcus* spp. were 4.26%. Findings of the research work were more or less similar with the findings of Meher *et al.*, (2011),

Osamwonyi *et al.*,(2013), Nawas *et al.*,(2012)., Sajjad et al., (2015), Owda *et al.*,(21014), Odu *et al.*,(2013)., Goja *et al.*, (2013), Meldrum *et al*., (2009). From the isolations we have found that *E.coli* and *Shigella* spp. are very much available on both market samples and hotel/ restaurant samples. *Pseudomonas* spp., *Proteus* spp. and *Klebsiella* spp. were comparatively higher on market samples than hotel/ restaurant samples and they were more available bacterial species. Antimicrobial sensitivity test was performed according to the procedure Kirby-bauer disk diffusion susceptibility test protocol suggested by janet Hudzicki (2009). Antibiotic sensitivity test showed that *Klebsiella* spp., *E. coli, Salmonella* spp., *Shigella* spp. and *Vibrio* spp. were resistant to Kanamycin. *Staphylococcus* spp. and *Streptococcus* spp. were sensitive to Kanamycin. *Pseudomonas* spp. and *Proteus* spp. were intermediate sensitive to Kanamycin. *Klebsiella* spp., *E. coli, Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Pseudomonas* spp.,*Proteus* spp. and *Streptococcus* spp. all were resistant to Nalidixic acid except *Staphylococcus* spp. It was sensitive to Nalidixic acid. *Klebsiella* spp., *Shigella* spp., *Staphylococcus* spp. *Vibrio* spp., *Pseudomonas* spp.,*Proteus* spp. and *Streptococcus* spp. all were resistant to Chloramphenicol. *E.coli* and *Salmonella* spp. were sensitive to Chloramphenicol. *Salmonella* spp., *Vibrio* spp., *Proteus* spp. and *Streptococcus* spp. were resistant to Azithromycine whereas *Klebsiella* spp., *E.coli*, *Shigella* spp., *Staphylococcus* spp. *and Pseudomonas* spp. were sensitive to Azithromycine. Norfloracin is resistant by *Staphylococcus* spp. ,*Pseudomonas* spp., *E.coli* and *Proteus* spp. Norfloracin is sensitive to *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. and was intermediate sensitive by *Klebsiella* spp and *Streptococcus* spp. Moreover, All the isolates were resistant to Tetracycline, Amoxicillin Piperacillin, Bacitracin, Cloxacillin, Novobiocin, Methicillin, Cefixime and Vancomycine Findings of the research work were more or less similar with the findings of Gurler *et al.*, (2015), Jeki *et al*., (2015), Goja *et al.*, (2013),Ilyus *et al.*, (2016), Laniewska *et al.*, (2006), Tasnia *et al*., (2014), Nawas *et al.*,(2012), Meher *et al.*, (2011), Islam *et al.*, (2016). This study indicated that the different raw salad vegetables having very much contamination of different types of bacterial pathogens. The bacterial pathogens were very much health hazardous as they were mostly resistant to most of the antibiotics. Hence, it is recommended that a more close supervision of such food type should be carried out by relevant authorities to avoid any future pathogen outbreaks.

CHAPTER -VI

CONCLUSION

In this study, we have found a large number of contaminations of *Klebsiella* spp., *E. coli*, *Salmonella* spp, *Shigella* spp., *Pseudomonas* spp, *Proteus* spp, *Staphylococcus* spp, *Streptococcus* spp and *Vibrio* spp. Our study thus imparted not only a complete picture on pathogenic profile of the salad vegetables but also presented a hopeful result on the antibacterial activity of different raw salad vegetables. The study demonstrated the occurrence of multiple antibiotic resistances among bacterial isolates vegetables sold on markets and hotel/ restaurants in Dinajpur, Bangladesh. According to the results of this study, raw vegetable salads may contain pathogenic bacteria; food pathogens can survive in vegetable salads, and thereby represent a risk to the consumers in regard to food borne disease and hygiene practice must be improved in all types of restaurants. As preparation of salads does not require further heat treatment, it is important to thoroughly wash vegetables and dip them in food grade antibacterial chemicals for a good time to eliminate pathogens and significantly reduce the microbial load.

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APPENDICES

APPENDIX 1

Composition of Media

1. Nutrient broth

Ingredients	**g/L**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH(at25°C)	7.4±0.2

2. Nutrient agar

Ingredients	**g/L**
Beef extract	3.0
Peptone	5.0
Sodium chloride	5.0
Agar	20.0
Final pH	7.1±0.1

3. *Salmonella Shigella* agar

Ingredients	5.00 gm
Peptic digest of animal tissue	
Beef extract	5.00 gm
lactose	10.00 gm
Bile salts mixture	8.50 gm
Sodium citrate	10.00 gm
Sodium thiosulphate	8.50 gm
Ferric citrate	1.00 gm
Brilliant green	0.00033 gm
Neutral red	0.025 gm

	Agar	15.00 gm
	Distilled water	1000 ml
	Final pH(at25°C)	7.0±0.2 gm

4. Brilliant green agar

Ingredients	gtL
Lab- Lemco power	5.0
Bacteriological pepton	10.0
Yeast extract	3.0
Disodium hydrogen phosphate	1.0
Lactose	10.0
Sucrose	10.0
Phenol red	0.09
Brilliant areen	0.007
Agar	12.0
Final pH~-O?	6.9±0.2

5. MacConkey Agar

Ingredients	**g/L**
peptone	17.0
Protease peptone	3.0
Lactose	10
Bile salt	1.5
Sodium cholride	5.0
Agar	13.5
Neutral Red	0.03
Crystal violet	0.001
Final pH	7.1±0.2

6. Eosine methylene blue agar

Ingredients	**g/L**
Peptone	100
Lactose	10.0
K2HP04	2.0
Eosin	0.4
Methylene blue	0.065
Agar	20.0
Final pH	6.8±0.2

7. MR VP medium (Himedium, India)

Composition	7.0
Buffered peptone	
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH(at 25°CO	6.9±0.2

8. Sugar media

a. Peptone water	10.0 gm
Bacto-peptone	
Sodium chloride	5.0 gm
0.5% phenol red	0. 1 ml
Distilled water	1000 ml
b. Sugar solutions	5 gm
Individul sugar	100 ml
Distilled water	
c. Sugar mediua preparation	4.5 ml
Pepton water	
Sugar solution	0.5 ml

9. Simmons citrare agar

Ingredients	**g/L**
Magnessium sulphate	0.20
Ammunium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothvmol blue	0.08
Agar	15.0

10. TSI Agar slant

Ingredients	3.00 gm
Lab Lamco Powder	
Yeast extract	3.00 gm
Peptone	20.00 gm
Sodium chloride	5.00 gm
Lactose	10.00 gm
Sucrose	10.00 gm
Glucose	1.00 gm
Ferric citrate	0.3 gm
Sodium thiosulphate	0.3 gm
Phenol red	0.3 gm
Agar	12.00 gm
Distilled water	1000 ml

11. Mueller Hinton Agar

Component	Amount (g/L)
Beef infusion	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25 ^{0}C)	7.3±0.1

12. Thiosulfate-citrate-bile salts-sucrose agar

Yeast extract	10.0
Protease Peptone	
Sodium thiosulfate	10.0
Sodium citrate	10.0
Ox gall	5.0
Sodium cholate	3.0
Saccharose	20.0
Saccharose	10.0
Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15.0

APPENDIX 2

Preparation of reagents

1. **Peptone water** I gm

peptone

Distilled water 1000 ml

Conc. HCL 25 ml

2. V-P reagent-1

5% alpha- naphthanol in absolute ethyl alcohol

3. **V-P reagent-2**

40% potassium hydroxide containing 0.3% creatine. The ingredient was dissolved by heating gently over a steam bath.

When in solution, added 0.052 gm of cotton blue dye.

4. Methyl red Solution

Methyl red 0.05 gm

Ethanol(absolute) 28 ml

Distilled water 22 ml

5. Phenol red solution

0.2^{0}o aqueous solution of phenol red

6. Gram stain solutions

a. **Stock crystal violet**

crystal violet 10 gm

Ethy1 alcohol 1000m1

b. Stock oxalate

Ammonium oxalate 1 gm

Distilled water 1000 ml

Crystal violet working solution: 20 ml of solution no. I mixed with 80 ml of solution no. 2. Additional dilution was made when desired.

c. Lugol's Iodine solution

Iodine crystal I gm

Potassium iodide 2gm

Dissolved completely in 10 ml of distilled water, then added to distilled water to make 300 ml. stored in ambar bottle.

d.	**Ethyl alcohol**	250 ml
e.	**Acetone**	250 ml
f.	**Counterstain**	2.5 ml
	Safranine	
	Ethyl alcohol (95%)	100 ml

Safranine working solution:
The stock safranine is usually diluted as 1:4 with distilled water.