ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA FROM SONALI CHICKEN MEAT AND STUDY OF THEIR ANTIBIOTIC SENSITIVITY PATTERN

A THESIS

BY

MAHBUBA ZANNAT

REGISTRATION NO. 1605131

SEMESTER: JULY - DECEMBER, 2017

SESSION: 2016

MASTER OF SCIENCE (MS)

IN

MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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Approved as to style and content by

Research Supervisor **Dr. Mst. Deloara Begum** Assistant Professor Department of Microbiology HSTU, Dinajpur	**Research Co- Supervisor** **Dr. Mir Rowshan Akter** Associate Professor Department of Microbiology HSTU, Dinajpur

Dr. Md. Khaled Hossain
Chairman of
Examination committee and
Chairman, Department of Microbiology

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

DECEMBER, 2017

Dedicated

To

My Beloved Parents

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The author, *December, 2017*

ABSTRACT

The present research work was conducted with a view to isolation and identification of pathogenic bacteria from sonali chicken raw meat and study of their antibiotic sensitivity pattern. The sample were collected aseptically from local market of Dinajpur city in Bangladesh and brought to the bacteriology laboratory, Department of Microbiology, HSTU, Dinajpur, during the period from July to December, 2017. A total of 50 samples were collected from the breast and thigh muscle of dressed sonali chicken. All of them were found to be contaminated with different microbes isolated as *Salmonella spp*. (100%), *Escherichia coli* (100%), *Vibrio spp*. (10%), *and Staphylococcus spp*. (92%). Isolated bacteria were identified using the biochemical tests namely Indole test, Methyl red test, Voges - Proskauer test, Citrate utilization test, Triple sugar iron test and MIU test. The study of antibiotic sensitivity pattern showed a number of pathogenic isolates were drug-resistant. All of the isolates were 100% sensitive to Gentamycin. It may be concluded from the study that, the sonali chicken meat usually contaminated with some bacteria. Therefore, it is suggested that, proper hygiene and sanitary practices should be maintained to make sonali chicken meat safe.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	:	Negative
%	:	Percentage
/	:	Per
<	:	Less than
>	:	Greater than
+	:	Positive
µg	:	Microgram
µl	:	Micro liter
^{0}C	:	Degree of Celsius
CFU	:	Colony forming units
D_x	:	Dextrose
E. coli	:	*Escherichia coli*
e.g.	:	Example
EMB	:	Eosin Methylene Blue
et al.	:	Associated
etc.	:	Etcetera
FAO	:	Food and Agricultural Organization
Fig.	:	Figure
gm.	:	Grams
H_2S	:	Hydrogen sulfide
hrs.	:	Hours
HSTU	:	Hajee Mohammad Danesh Science and Technology University
lb.	:	Pound
Kg	:	Kilogram
KOH	:	Potassium hydroxide
L	:	Lactose
MC	:	MacConkey Agar
mg	:	Milligram
min	:	Minutes
MI	:	Milliliter
MIU	:	Motility Indole Urease

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

ml	:	Milliliter
ML	:	Maltose
mm	:	Millimeter
MN	:	Manitol
MR	:	Methyl Red
MSA	:	Manitol Salt Agar
N	:	Number
NA	:	Nutrient agar
NB	:	Nutrient broth
ND	:	Not done
-	:	Negative
No.	:	Number
NS	:	Non-significant
PBS	:	Phosphate Buffer Saline
R	:	Resistant
S	:	Sucrose
S	:	Sensitive
S	:	Significance
Sec	:	Second
SEM	:	Standard error means
SL.	:	Serial
spp.	:	Species
Sq.	:	Square
SSA	:	Salmonella Shigella Agar
v/v	:	Volume by volume
w/v	:	Weight by volume
TSI	:	Triple sugar iron
VP	:	Voges Proskaur

CHAPTER 1

INTRODUCTION

Bangladesh is an agro based country where most of the population lives on agriculture. Since livestock plays a vital role in the economy of Bangladesh, therefore its contribution bears significant strain on maintaining domestic resources for promotion of lifestyle and livelihood of landless and marginal farmers. It is evident that livestock wealth provides about 9% of the Gross National Product (GNP), which is 6.5% of Gross Domestic Product (GDP) (Ali *et al.,* 1998). A total of about 20% of the population is now engaged in raising livestock and poultry, particularly in layer and broiler production but now a days Sonali (meat type chicken) plays a great role in this sector (Ali *et al.,* 1998).

Poultry comes from the French word Poule, itself derived from the Latin word Pullus, which means small animal. Poultry is the second most widely eaten meat in the world. Sonali chicken, the crossbred of Fayoumi female and RIR (Rhode Island Red) male developed in 1986, has been reported to perform better with respect to egg and meat production, rapid growth and low mortality under scavenging, semi-scavenging and intensive farming system. It has been taking its place besides the indigenous hens due to its adaptability and acceptability in the climatic conditions of Bangladesh. Sonali, with a phenotypic appearance similar to local chicken has higher market demand than exotic breed. As an important segment of livestock production, the Sonali chicken industry in Bangladesh is considered a great avenue for the economic growth and simultaneously creates numerous employment opportunities. About 76 percent of Sonali beneficiary has improved their conditions by rearing this type of poultry (Hossen *et al.,* 2012).

Meat is considered an important source of proteins, essential amino acids, B complex vitamins and minerals. Due to this rich composition, it offers a highly favorable environment for the growth of pathogenic bacteria. The microbiological contamination of carcasses occurs mainly during processing and manipulation, such as skinning, evisceration, processing, storage and distribution at slaughterhouses and retail establishments (Gill, 1988). Fecal matter is a major source of contamination and can reach carcasses through direct deposition, as well as by indirect contact through

contaminated and clean carcasses, equipment, workers, installations and air (Borch and Arinder, 2002).

Poultry provides an excellent medium for the growth of microorganisms. Various pathogenic microbes, such as *Escherichia coli, Salmonella spp., Bacillus spp., Streptococcus spp*. and *Staphylococcus spp.,* have been implicated to reduce the growth of poultry (Duke, 1986). *Enterobacteriaceae are* common contaminants of meat and meat products (Ayhan *et al.,* 2000). The presence of *Enterobacteriaceae* in meat has its importance due to its public health significance (Mira, 1989). *Enterobacteriaceae* are the significant causes of serious infections, and many of the most important members of this family are becoming increasingly resistant to currently available antimicrobials (Paterson, 2006). Contamination of the meat comes from different sources that may be originated from the environment, human handling and manipulation or the animal itself. (EL-Mossalami, 1988) mentioned that dirty floors, clothes, butchers hands and knives may constitute. (Fournaud *et al.,* 1978) also reported that animal sources of carcasses contamination include hide, gastrointestinal tract and respiratory tract. Potential causes of contamination of poultry during the slaughtering and processing procedures include contact of carcass with body parts that contain a high microbial load (e.g. feathers, feet, intestinal contents), use of contaminated equipment and physical manipulation of the meat (e.g. deboning, grinding). After slaughtering poultry carcass has to be chilled to reduce and then maintain the temperature of the meat below a value that will ensure a high quality, safe product. Chilling successfully reduce the rate of growth of spoilage microorganisms. They also have an effect on the major quality indicators of flavor, appearance and meat texture (James *et al.,* 2006).

The modern poultry industry can produce market-ready broiler chickens in less than six weeks. This accomplishment has been achieved through genetic selection, improved feeding and keen health management practices including usage of antibiotics as therapeutic agent's to treat bacterial diseases in intensive farming systems (Ahmed *et al.,* 2009). In Bangladesh similar types of managemental operation is followed in the Sonali chicken production which is marketed in less than 9 weeks. In Bangladesh, the economic aspect of poultry disease and their mortality and morbidity Resistant against frequently used antibiotics has been observed in bacteria present in poultry since the introduction of these antimicrobial agents in poultry. The rise in antibiotic resistant has been reported in the past two decade in many countries including Bangladesh (Kapil, 2004). Due to

bacterial infection is a matter of great concern to the livestock owners. The antibiotic resistant pattern increases the incidence of disease in poultry and subsequently affects the economy of Bangladesh.

Considering the above points the present study was undertaken with the following objectives-

i) To isolate and identify the bacterial pathogens from chicken (sonali) meat samples collected from local market of Dinajpur city.
ii) To detect the antibiotic sensitivity pattern of the identified bacteria.

CHAPTER 2

REVIEW OF LITERATURE

Islam *et al*., (2016) performed an investigation for the isolation and characterization of the Listeria monocytogenes from meat samples collected from different local markets in Bangladesh entitled by "Prevalence of Listeria monocytogenes in Beef, Chevon and Chicken in Bangladesh". A total of 36 meat samples were collected from cattle, goat and chicken. After preparation the samples were inoculated into different selective media such as Oxford Agar, Manito Salt Agar and Blood Agar for isolation and identification of *L. monocytogenes*. In Oxford Agar*, Listeria spp*. produced black zone around the colonies. Gram staining, motility test, blood hemolysis, Christe Atkins Munch Peterson (CAMP) test and biochemical test were performed to confirm *L. monocytogenes. The L. monocytogenes* fermented dextrose and maltose with the production of only acid and no gas was observed in Durham′s tube. The *L. monocytogenes* were found Methyl-Red and Voges-Proskauer (MR-VP) test positive but indole negative. *L. monocytogenes* were catalase, CAMP test positive and motile. Among 36 meat samples, 4 (11.11%) were *L. monocytogenes*. Among animal species, the distribution of *L. monocytogenes* was 8.33% (1/12) in chicken, 16.66% (2/12) in beef and 8.33% (1/12) in Chevon. The *L. monocytogenes* isolate was resistant to ampicillin and penicillin; sensitive to the ciprofloxacin, vancomycin and gentamicin. They stated that "multidrug resistant *L. monocytogenes* may be transmitted to the human through consumption of contaminated meat and may lead to public health hazard".

Al-Salauddin *et al.,* (2015) performed a study namely "Isolation, identification, and antibiogram studies of *Salmonella* species and *Escherichia coli* from boiler meat in some selected areas of Bangladesh". A total of 60 samples were identified by using cultural, biochemical, and polymerase chain reaction assays. They revealed that *E. coli* was isolated from 50 (83.33%) samples and *Salmonella* spp. from 18 (31.66%) samples by using standard bacteriological techniques. Furthermore, the isolates were subjected to antibiogram studies by disk diffusion method using eight commonly used antibiotics. Antibiogram studies revealed that gentamicin, ciprofloxacin, and norfloxacin were highly sensitive against all the isolated bacteria, whereas most of the isolates were resistant to amoxicillin, erythromycin, and tetracycline. Out of all the isolates, 5 isolates

of *Escherichia coli* and 3 isolates of *Salmonella* were found multidrug resistant. They revealed that presence of multidrug resistant *Salmonella* and *E. coli* were isolated from broiler meat sold in live bird market of different upazilla.

Akbar *et al.,* (2014) performed a study in Pakistan entitled by "Presence of *Escherichia coli* in poultry meat: A potential food safety threat". They investigated the presence of *E. coli* and its pathogenic strain O157 in raw poultry meat and its antimicrobial sensitivity pattern to common antibiotics. Total number (n = 152) of samples were studied, out of which 25% (38/152) were found contaminated with *E. coli*. and the prevalence of pathogenic strain O157 was 2% (3/152). In the antibiogram study, 92% (35/38) isolates showed resistant to ampicillin and tetracycline. The resistant against kanamycin were 15.8% (6/38), whereas 23.7% (9/38) against streptomycin. Several *E. coli* isolates were found resistant to multiple antibiotics. One *E. coli* isolate showed resistant to seven antibiotics (ampicillin, tetracycline, sulfamethoxazole/trimethoprim, gentamicin, chloramphenicol, nalidixic acid and kanamycin) out of nine antibiotics used in the study. The antibiotic resistant of *E. coli* to common commercial antibiotic is a potential threat to food safety and public health.

Bhaisare *et al.,* (2014) studied that chicken is a nutritious, healthy food which is low in fat and cholesterol compared to other meats but an excellent source of protein. Meat must be of a high microbiological quality in order to ensure that the consumer receives a product that is not spoilt or does not carry food-borne disease. Food borne diseases associated with the consumption of poultry meat and its processed products are of public health significance worldwide. This paper reviewed information on the sources of microbial contamination, contamination of poultry with major pathogenic microorganisms, the consequences of this contamination to human health, prevalence of microbes in poultry meat and products in the world and in India.

Nossair *et al.,* (2014) performed an investigation in Egypt entitled by "Detection of some Enteric Pathogens in Retailed Meat". They stated that contamination of meat at the retail level is very common. The presence of members of the family *Enterobacteriaceae* on the meat surface will render the meat unsafe to the consumer as they are encountered in causing food poisoning and also they reflect the hygienic standard of the butchers' shops. The current study was done to evaluate the microbiological status of retailed meat of cattle and buffaloes with special concern to *Escherichia coli* and *Salmonella*. A total

of 100 random samples of meat of cattle and buffaloes (50 of each) were collected from different butchers' shops at Behera province. The samples were evaluated bacteriologically with a trial to isolate some enteric pathogens of public health significance. The obtained results revealed that the mean values of total *Enterobacteriaceae* count in examined meat samples of cattle and buffaloes were 1.4 X 104±1.4 X 103 and 2.7 X 104± 3.3 X 103 cfu/g, respectively. Also, the mean values of total Coliforms count in cattle and buffaloes meat samples were 1.0 X 104±1.4 X 103, 1.8 X104± 1.8 X 103 cfu/g, respectively. In addition, isolation and identification of enteric bacteria revealed that the isolation *of E. coli, Salmonella species*, *Enterobacter aerogenes, E. intermedium, E. gergoviae, Citrobacter amalonaticus, C. diversus, C. freundii, Serratia marcescens, S. ficaria, S. fonticola, S. liquefaciens. S. rubidaea Edwardsiclla ictalori, E. hoshinae, Providencia alcalifaciens, P. stuartii, Klebsiella pneumonia subspecies ozaenae,* and *Proteus mirabilis.* Concerning *E. coli*, it was detected in 32% and 40% of the examined meat samples of cattle and buffaloes, respectively. Furthermore, the serological identification of the obtained isolates of *E. coli* revealed the presence of the following serotypes O55: K59, O111:K58, O114:K96, O111:K69, O125:K70, O126:K71, O127:K63.

Nimri *et al.*, (2014) performed a surveillance program in Jordan entitled by "Foodborne bacterial pathogens recovered from contaminated shawarma meat in northern Jordan". Bacterial contamination of 100 shawarma sandwiches with pathogenic bacteria was studied by culture on selective media, serology, PCR assay, and antimicrobial susceptibility testing. One hundred and forty-five bacterial isolates were identified. The predominant species was *Escherichia coli* (28.3%), with six isolates of serotype O157:H7, followed by *Salmonella spp*. (25.5%). Higher contamination rates were found in chicken sandwiches. The majority of these bacteria expressed high resistant to several antimicrobials, especially tetracycline and streptomycin. *Citrobacter freundii* was isolated from 15.9% and *Staphylococcus aureus* was isolated from 8.3% of the sandwiches. The presence of these pathogens is of primary concern because some strains are capable of producing a heat-stable enterotoxin that causes food poisoning in humans, and should therefore be taken into account in risk assessment. The results signify the importance of sustained surveillance of foodborne pathogens in shawarma sandwiches to minimize the risk of contamination. Availability of data on the isolated pathogens and

modes of transmission in food from different countries would provide a common ground for reaching international agreement on food safety regulations

Jarallah *et al*., (2014) conducted a study for detection of pathogenic bacterial species that contaminate meat in the butchers shops and kebab restaurants in Al-Kut city entitled by "Isolation and Identification of some pathogenic Bacterial Species Contaminated from Meats in Butchers Shops and Kebab Restaurants in AL-Kut city". Ten samples from 10 butcher shops and 10 restaurants were collected. These samples were suspended in sterile normal saline in order to using for isolation and identification of pathogenic bacteria. The cultural properties and biochemical tests results revealed the bacterial isolates return to two+ bacterial species: *Escherichia coli* (40%) and *Staphylococcus aureus* (29%) in butchers' shops, in front of *E. coli* (19%), *S.aureus* (28%) and *Klebsiella sp*. (9%) in restaurants. The antibiotics susceptibility pattern results showed all of these isolates were resist to most traditional antibiotics but in different ratios

Momtaz *et al.,* (2013) PCR-based assay was developed to detect the occurrence of *Yersinia* virulence genes. This feasible and informative method was able to provide a rapid and reliable characterization of field isolates. A total of 720 chicken meat samples were collected randomly from abattoirs in western Iran and tested by culturing and PCR methods. Of these, 132 (18.33%) were found to be positive for *Y. enterocolitica* by both methods. Isolates included biotypes 1A (0%), 1B (0%), 2 (18.18%), 3 (52.27%), 4 (17.42%), and 5 (12.12%), and serotypes included O:3 (36.84%), O:5,27 (59.84%), O:8 (5.30%), and O:9 (0%). Of the 46 *Y. enterocolitica* serotype O: 3 isolates, the prevalence of virulence genes included yadA (82.60%), inv (100%), ail (95.65%), ystA (93.47%), and virF (58.69%). This study highlighted the importance of chicken meat as potential sources of *Y. enterocolitica* infection in Iran.

Bhandari *et al.,* (2013) observed cross sectional study included 26 fresh broiler meat samples from registered retail shops. They found that mean count log+/- SE colony forming units per gram(cfu/g) for Bharatpur, Ratnanagar and Institute of Agriculture and science (iaas).vicinity were obtained as 11.1±0.3, 11.5±0,3 and12.2±0.5 TVC; 8.5±0.2, 9.2±0.3 and 10.2±0.4 TEC; 6.5±0.3 and 8.4±0.5 TCC; 6.5±0.2, 6.8±0.3 and 7.7±0.4 TSC respectively.

Rothrock *et al.,* (2013) investigated the effects of sampling times and sampling methods on the cultural and molecular (via qPCR) quantification of dominant zoonotic pathogens

within a poultry processing facility. The results show that ddPCR detected pathogen-specific genes from more pathogen: sampling time combinations than either qPCR or culturing methods from the final scolder and chiller tanks at three stages of processing (Start, Mid and End). In fact, both ddPCR and qPCR substantially outperformed culture methods commonly used in poultry processing food safety-related studies, with *Salmonella* recovered only from the Mid and End sampling times from the scalder tank. While neither *C. jejuni, L. monocytogenes* were recovered culturally.

Abraham *et al.*, (2012) investigated 27 chicken thigh samples collected from the retail outlets. *Escherichia coli* were determined. Mean total viable counts for the supermarkets, local markets and farms were 6.46, 6.91 and 6.57 log10 cfu/g respectively. Mean total coliform counts for the supermarkets, local markets and farms were 3.80, 3.46 and log10cfu/g respectively and the mean *S. aureus* counts also 2.32, 2.28 and 2.70 log10 cfu/g respectively. There were no significant differences (p> 0.50) between the mean total viable count, total coliform counts and *S. aureus* count for the supermarkets, local markets and the farms. Mean counts of *E. coli* detected at the supermarket, local markets and farms were 1.27, 2.59 and 2.74 log10 cfu/g respectively. *Salmonella spp*. was detected in 2 out of the 27 samples. Fifty-two percent and 70% of samples respectively had total viable counts and total coliform counts within the microbial safety standards.

Svobodova *et al.*, (2012) evaluated the impact of four processing steps (plucking, evisceration, washing and chilling) on the total viable counts(TVC), counts of *Escherichia coli, Salmonella spp*. and *Listeria spp*. a total of 160 broiler carcasses originating from one farm were collected during one year period at a Czech slaughter house and examined. Both TVC and *E. coli* counts decreased during processing from 4.6 log cfu/cm² and 3.5 cfu/cm to 3.7 log cfu/cm and 1.8 log cfu/cm, respectively with a major impact of washing on TVC and washing and chilling on *E. coli* decrease (p 0.001). A decreasing trend was observed in *Salmonella* counts and *Listeria spp*. incidence during processing.

Rumni Sengupta *et al.*, (2012) studied to determine the microbial quality of chicken meat and its public health implications. They found mean standard plate count (SPC), coliform count and *Staphylococcus* count of chicken meat obtained from semi-urban markets was higher as compared to urban markets.

Shareef *et al*., (2012) observed 60 samples (30 samples of local origin and 30 imported ones) of frozen thighs kept in deep-freezers at-18C .samples were then tested for total count of aerobic mesophilic bacteria (APS) and *Staphylococcus aureus*. Study showed that microbiological quality and contamination of frozen chicken thighs, APC was found within the acceptable limits of satisfactory products. *Staphylococcus aureu*s were isolated in 16.66% from imported thighs versus 33.33% in local ones, with 10 CFU/cm in both thigh types.

Rani Roy *et al.,* (2012) performed a research work for the isolation and identification of bacterial flora from internal organs of broiler. Ten Hubbard classic broiler bird were purchased from retail market in Mymensingh, Bangladesh. The birds were sacrificed and their liver, lung, esophagus, duodenum and tracheal swab samples were collected (n=50). Using standard bacteriological techniques, *Escherichia coli* were isolated from 26 (52%) samples. Similarly, *Salmonella spp., Staphylococcus spp., Bacillus spp*., and *Pasteurella spp.* were isolated from 15 (30%), 10 (20%), 9 (18%) and 4 (8%) samples, respectively. On the basis of individual sample type*, E. coli* could be isolated from 8 (80%) duodenum samples being the most prevalent organism. On the other hand, *Salmonella spp*., *Staphylococci spp., Bacillus spp*. and *Pasteurella spp.* were identified in 5 (50%) lungs, 5 (50%) liver, 4 (40%) duodenum and 2 (20%) lungs samples, respectively. Among these isolated bacteria, *E. coli* was found to be pathogenic for mice. Antibiogram studies revealed that Ciprofloxacin was highly sensitive against all the isolated bacteria. Diversified bacterial species are prevalent in broiler. However*, E. coli* and *Salmonella* spp. infection might make the bird vulnerable for easy access of infection. Proper vaccination and use of selective antibiotics are crucial in protecting broilers from these pathogens.

Sharaf *et al.,* (2012) carried out on 40 random samples of cooked chicken products represented by chicken luncheon and Shawerma (20 of each) in KSA were subjected to the microbiological investigations which revealed the presence of some microbiological investigations, which revealed the presence of *S. aureus*, *E. coli,* mould, yeast in 10.0, 25.0, 50.0 and 65.0% in Luncheon and zero, 20.0, 65.0 and 70.0% in Shawerma, respectively.

Daoud *et al*., (2012) microbial quality of 50 frozen chicken meat samples from grocery stores in Qena city, Egypt was assessed and the mean values for total aerobic, total

coliform, total faecal coliform and total *E. coli* counts for locally produced chicken meat were 2.1 x103, 5.1 x10, 4.9x10 and 3.5 cfu/g for breast and 2.7x 103, 6.4x10, 6.4x10 and 1.5 x10 cfu/g for thigh samples respectively.

Wilfred Ruban *et al*., (2012) studied on the prevalence of common food borne pathogens (*Salmonella*, *Staphylococcus* and *E. coli*) in chicken meat obtained from wet market in Bangalore under different processing conditions was carried out. Results revealed higher prevalence of *Salmonella* in the range of 25 to 65 per cent and *E. coli in the range of* 42 to 88 percent.

Arul kumar *et al.,* (2011) contamination of chicken meat, by *S. aureus* sold at various retail markets of Namakkal of Tamil Nadu was assessed by culture of meat samples and colonial count. Out of 210 meat samples collected, 6.67% of the meat samples were positive by culture and the colony count was 1.03 ± 0.08 log10 cfu/g.

DeGiusti *et al.,* (2011) stated that Verocytotoxin *Escherichia coli* is a frequent and important cause of diarrhea and hemolytic uremic syndrome all over the world. Consumption of ground beef, lettuce, and other kinds of food have been associated with outbreaks. They performed a study entitled by "Detection of *Escherichia coli* O157 in raw and cooked meat: comparison of conventional direct culture method and Enzyme Linked Fluorescent Assay (ELFA)".Three hundred and ten food samples (80 of cooked and 230 of raw meat) were screened for *E. coli* O157 by ISO culture method and by enzyme-linked-fluorescent-assay (ELFA)-based methods (VIDAS®system, bio-Mérieux). All isolates obtained were tested for VT1 and VT2 genes by PCR. The statistical analysis considered absolute frequencies and percentages. The K statistic was applied to assess agreement between direct culture method and the VIDAS system. A total of 6 (1.9%) *E. coli* O157 isolates were recovered from raw meat samples by the culture method; of these only four were identified by PCR as VTEC producers. A total of 9 (2.9%) *E. coli* O157 isolates were recovered from raw meat samples by the VIDAS® system. No *E. coli* O157 was detected in cooked products. All comparisons between the direct culture method and the VIDAS system were statistically significant (K= 0,795; $p<0.001$). They concluded that ELFA-based methods are highly specific and rapid for the detection of *E. coli* O157 in food samples compared with the direct culture method. ELFA method is useful to verify the effectiveness of the HACCP system in the risk

management of potential contaminating hazards during the preparation of foods for susceptible persons.

Kumar *et al.,* (2011) studied to find the differences in quality of fresh chicken obtained from different sources with different processing practices viz., market/road side chicken shop (MSC), Retail outlets (RSC) and semi-automatic processing plant (Scientifically Slaughtered Chicken) (SSC). The sources of meat had no significant effect ($p<0.05$) on pH and tyrosine value of fresh chicken meat. However, SSC samples had significantly ($p<0.05$) higher water holding capacity, extract release volume and lower thiobarbituric acid value compared to other samples. Similarly, SSC samples harboured significantly ($p<0.05$) lower total viable count, coliform count, psychrophilic count and yeast and mould counts. Sensory evaluation of cooked samples did not reveal any difference in organoleptic attributes viz., appearance, flavour, juiciness and texture but overall palatability scores of SSC meat was significantly ($p<0.05$) higher than meat from other two sources. It was concluded that SSC meat was of better quality than MSC and RSC meat.

Ammar *et al.,* (2010) identified the most common *Salmonella* serovars in broiler and laying breeding reproducer eastern Algeria according to the ISO 6579 method. A total of 294 samples obtained from two flocks of the 10,000 broiler and laying breeding reproduces. Sample included livers and spleens, drag swabs of bottom boxes of young chickens, cloacal swabs, and fecal samples of chickens. Additional sample wear also taken form water, feed and dusty surfaces, then got the result that only the cloacal swabs, poultry face and samples from dusty surfaces were positive for *Salmonella typhimurium* and *Salmonella* Livingstone with a detection rate of 12% and 16% respectively

Ruban *et al.,* (2010) studied to isolate and identify *Salmonella* spp. from chicken slaughtered under different processing conditions viz. wet market, super market and modern processing units in Karnataka was carried out. A total of 450 (225 breast and 225 thigh muscle) samples were tested by PCR and shown that prevalence of *Salmonella* spp. was higher in thigh meat (31.99 %) compaired to breast muscles (24.88%)

Granić *et al.,* (2009) conducted at five poultry slaughterhouses in Medimurje country in Croatia which included a total of 75 poultry meat swabs, 15 of which carcass cooling water samples and 15 samples of poultry meat collected in retail shops. Of which 10 samples of poultry meat were found positive for *Campylobacter spp*. (66.6%), out of

these which *C. jejuni* was isolated from six samples (40%), and *C. coli* from four sam6ples (26,6%) of poultry meat

Rahimi *et al.*, (2008) studied 800 poultry meat samples from raw chicken (280), quail (248), turkey (212) and ostrich (60) were procured from the Esfahan city of Iran and analyzed for the prevalence of *Campylobacter spp*. The highest prevalence (68.4%) of *Campylobacter* spp. was recorded in quail meat, followed by chicken meat (56.1%), turkey meat (27.4%) and ostrich meat (11.7%). The overall prevalence of *Campylobacter* in studied samples was 47.1% (377 from 800). Out of which, 76.4% were identified as *C. jejuni* and 23.6% as *C. coli*.

Marcia de *et al.*, (2007) tested the microbiological quality of beef and meat products under a study named "Identification of main contamination points by hygiene indicator microorganisms in beef processing plants". They found that without proper hygienic control, the environment in slaughterhouses and butcher shops can act as an important source of microbiological contamination. To identify the main points of microbiological contamination in the beef processing chain, 443 samples of equipment, installations and products were collected from 11 establishments (1 slaughterhouse and 10 butcher shops) located in the state of Paraná, Brazil. The microbiological quality of all the samples was evaluated using Petri dishes to obtain counts of mesophilic aerobes (AC), total *coliforms, Escherichia coli* (EC), yeasts and molds (YM). The main contamination points identified in butcher shops, in decreasing order, were stainless steel boxes, beef tenderizers, grinders, knives, mixers, sausage stuffers, plastic boxes, floors and drains. In the slaughterhouse, these points were sausage stuffers, platforms, floors and drains. The most severely contaminated products were fresh sausages and ground beef. This information about the main points of microbiological contamination in the beef processing chain is expected to aid professionals responsible for hygiene in similar establishments to set up proper hygienic procedures to prevent or reduce microbiological contamination of beef and meat products.

Kozacinski *et al.*, (2006) performed bacteriological analysis on 66 samples of fresh, retail-cut chicken meat (21 samples of chicken breasts without skin - "fillet", and 19 samples of chicken breasts with skin) and 26 samples of frozen ground chicken meat and found the presence of *Salmonella* spp. (10.60%), *S. aureus* (30.30%), *L. monocytogenes* (3.03%), *Enterobacter* spp. (34.84%) and sulphite-reducing clostridia (1.50%)

Franchin *et al*., (2005) observed possible sources of contamination of *Campylobacter* spp. in poultry meat before slaughter found to be examining samples of feathers, cloaca swabs, litter swabs, transport coops, rinse water from coop washing equipment, and chicken breast supports in the slaughter line just before stunning The samples were collected from eight broiler houses and from eight different producers, from a poultry integration system in southern Brazil. The study was carried out over a 12-month period, and each broiler house was sampled in three consecutive flocks, for a total of 24 flocks/broiler house. *Campylobacter* was found in 79.2% of the feather samples, followed by cloacal swabs (75.0%) and transport coop (50.0%), litter (37.5%), breast support (33.3%) and coop rinse water (25.0%) samples.

Adwan *et al*., (2005) performed a research work entitled by "Prevalence of foodborne pathogens in meat samples in Palestine to investigate the prevalence of enterotoxigenic *Staphylococcus aureus*, *Salmonella* and *Escherichia coli* patho-types in different meat types. Forty meat samples fresh (n=35) and frozen (n=5) were purchased from local markets in Jenin district, Palestine. Multiplex PCR was used to detect enterotoxigenic *S. aureus, Salmonella* and *E. coli* pathotypes. Total mesophilic aerobic bacterial count ranged between 4.3 log10 to 5.7 log10 cfu/g for frozen meat and 6.95 log10 to 7.78 log10 cfu/g for fresh meat. The prevalence of S. aureus, *Salmonella* and E. coli was 30%, 25% and 95%, respectively. Among tested S. aureus strains 75% were enterotoxigenic. Two other samples of non *S. aureus* (FemA-) were enterotoxigenic; one was sec+ and the other was seen. The results also showed that 89.5% of meat samples contaminated with *E. coli* that belong to enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enter aggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), diffuse adherent *E. coli* (DAEC) pathotypes. According to these results, it is recommended to establish a suitable surveillance program for microbial contamination with all foodborne pathogens.

Biswas *et al.*, (2004) analyzed 335 organ samples collected from 6 upazilla of 4 districts namely Gaibandha, Sirajonj, Sherpur and Netrokona to identify the endemic bacterial, viral and fungal diseases affecting semi-scavenging chickens. Analyses of the organ samples revealed that three viral, three bacterial and one fungal disease were prevalent in the study areas namely New Castle disease, Fowl pox, Infectious bursal disease; Salmonellosis, Colibacillosis, Fowl cholera and Aspergillosis with the prevalence of 15.5%, 9.3%, 3.9%, 3.0%, 8.7%, 5.1% and 1.2% respectively.

Saleque *et al.,* (2003) conducted a survey from January 2000 to December, 2001 on both breeding flocks, commercial broiler and layer flocks in major poultry raising belts in and around Dhaka and Gazipur districts of Bangladesh. Prevalence of 45%, 17%, 12.2%, 6.6%, 4.5%, 1.5% and 12.4% of bacterial, viral, mycoplasmas, protozoan, parasitic, fungal were reported in birds examined.

Derakhshantar *et al.,* (2002) studied avian cellulitis in broiled chickens. The authors identified 71.80% of *E. coli* infection from cellulites of broiler by bacteriological investigation.

Mishra *et al*., (2002) isolated *E. coli* from domestic poultry. They isolated fifty strains of *E. coli* from 250 specimens like heart blood, intestinal contents, liver, lung, ovaries, peritoneal fluids, spleen and unabsorbed yolk of poultry. Majority was isolated from heart blood and serotype O78 was the most predominant and prevalence was 61.22%.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1 Experimental site and duration

The present studies were conducted during the period from July-December, 2017 in the Microbiology laboratory of the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur.

3.1.2 Sample size

A total of 50 Sonali chickens were collected from the local market (Bahadur Bazar) of Dinajpur sadar after collection bacteriological analysis of the samples were performed under two major principal assessments. Firstly isolation and identification of various bacteria containing the meat sample by using different biochemical tests. Secondly, antibiotic sensitivity test.

3.1.3 Laboratory preparation

All items of glass wares including test tubes, pipettes, cylinder, flasks, conical flasks, glass plate, slides, vials and agglutination test tubes soaked in a household dishwashing detergent solution ('Trix, Recket and Colman Bangladesh Ltd.) for overnight, contaminated glassware's were disinfected in 2% sodium hypo chloride solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly and finally sterilized either by dry heat at 160^0 C for 2 hours or by autoclaving for 15 minutes at 121^0 C under 15 lbs pressure per square inch. Autoclaved items were dried in a hot air oven over at 50^0 C. Disposable plastic were (micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50^0 C for future use.

3.1.4 Instrument and apparatus

At research laboratory, Department of microbiology, Hajee Mohammad Danesh Science and Technology University. We used various kinds of apparatus, which are given below-Test tube, Petri dish, Conical flask, Pipette, Micro pipette, Slide, Microscope, Cotton,

Immersion oil, Toothpick, Autoclave, Jar, Beaker, Cylinder, Ice box, Balance, Laminar flow, Spirit lamp, Refrigerator, Marking pen, Bacteriological loops etc. were used.

3.1.5 Media for culture

3.1.5.1 Liquid media

- Nutrient broth.
- 1% peptone water (Hi-media, India).

3.1.5.2 Solid media

- Nutrient agar base (Hi-media, India)
- Salmonella-Shigella agar (Hi-media, India).
- Eosin methylene blue (EMB) agar (Hi-media, India).
- MacConkey agar medium (Hi-media, India).
- Thiosulfate-citrate-bile salts-sucrose agar (Hi-media, India).
- Manitol salt agar (Hi-media, India)

3.1.5.3 Media for biochemical tests

- Triple sugar iron (TSI) agar slant (Hi-media, India).
- Motility, Indole, Urease (MIU) medium (Hi-media, India).
- Methyl Red (MR) media
- Simmons's Citrate agar media
- Voges-Proskauer (VP) media

3.1.5.4 Reagents

- Gram's staining reagent: Crystal violet, Gram's iodine, Acetone and Safranine.
- Alpha-naphthol solution.
- Kovac's reagent.
- Ethyl alcohol (70% and 95%).
- Phosphate Buffered Saline (PBS)
- Physiological Saline Solution (PSS)

- Methylene Blue stain
- Voges-Proskauer (VP) Solution
- Indole Solution
- Methyl Red Solution
- Potassium-di-hydrogen phosphate (0.2M, $KH_2PO_4\,2H_2O$)
- Di-sodium hydrogen phosphate (0.2M, $Na_2HPO_4 12H_2O$)

3.1.6 Antimicrobial Sensitivity Discs

To determine the drug sensitivity pattern of different isolated bacteria different types of commercially available antibiotic discs (Oxoid Ltd., UK) were used. The method allowed for the rapid determination of the efficacy of the drug by measuring the diameter of the zone of inhibition that result from different diffusion of the agent into the medium surrounding the disc. The followings are the antibiotics that were tested against the selected organism.

Table 1. Antimicrobial agents with their disc concentration

S/N	Name of antibiotics	Disc concentration (µg/disc)
1	Amoxicillin (AMX)	30 µg/disc
2	Cefixime (CFM)	5 µg/disc
3	Chloramphenicol (CH)	25 µg/disc
4	Colistin (CL)	10 µg/disc
5	Cloxacilin (COX)	1 µg/disc
6	Erythromycin (E)	15 µg/disc
7	Gentamycin (GEN)	10 µg/disc
8	Penicillin G (P)	10 µg/disc
9	Vancomycin(VA)	30 µg/disc

Legend: µg = Microgram

3.2 Methods

3.2.1 Design of experiment

The meat samples were collected directly from local market of Dinajpur city (Bahadur bazaar) for the bacteriological analysis with antibiogram study of the isolated bacteria. The experimental layout illustrated in figure 1.

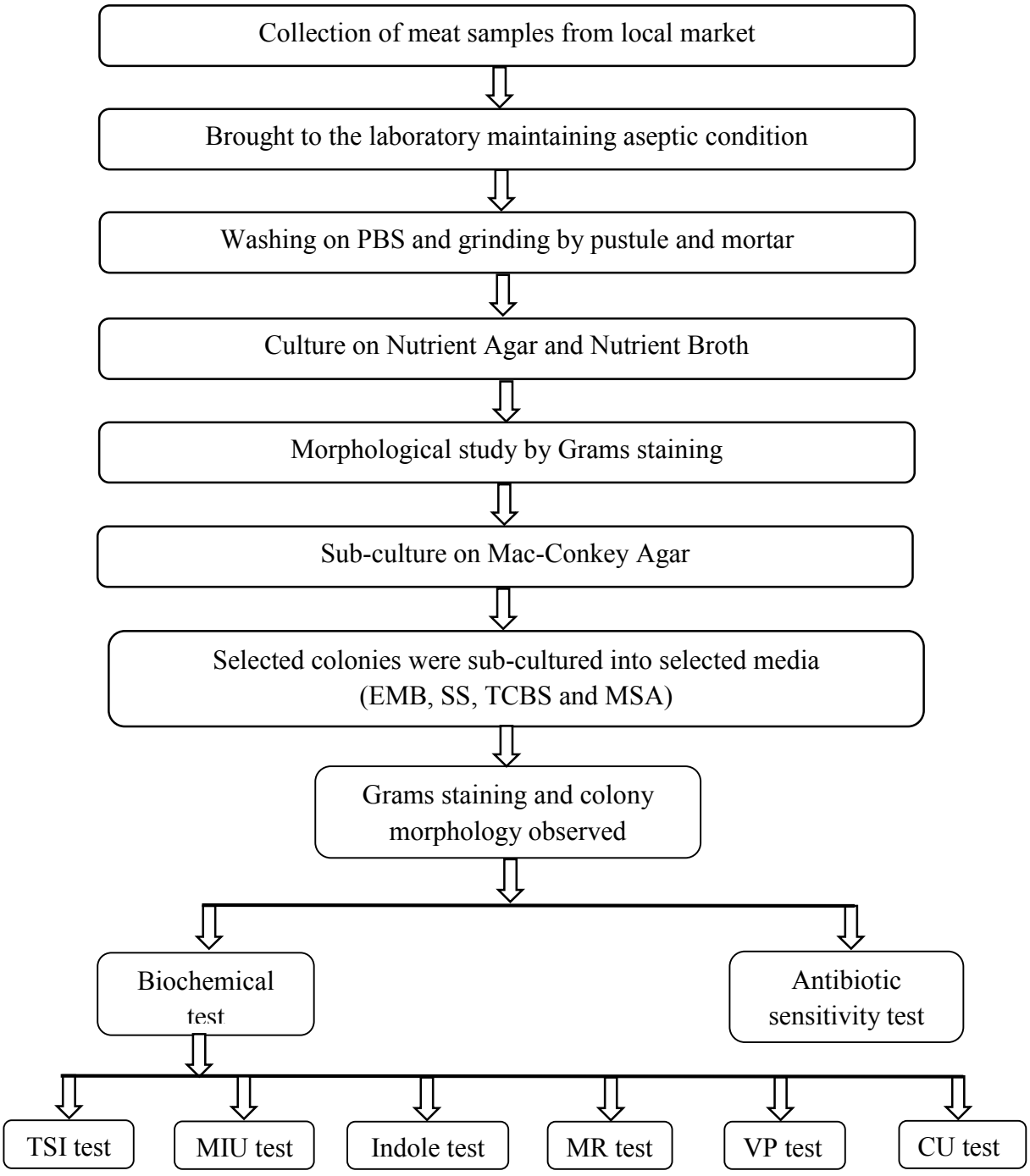


Figure 1: Experimental layout

3.2.2 Preparation of culture media

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

3.2.2.1 Liquid Media

3.2.2.1.1 Nutrient Broth

Nutrient broth (NB) was used to grow the organisms from the samples collected from the study areas before performing biochemical test (Cheesebrough, 1985). 13 gram of Bacto-nutrient broth (Difco) was dissolved in 1000 ml of cold distilled water and heated up to boiling to dissolve it completely. The solution was then distributed in tubes, stopper with cotton plugs and sterilized in the autoclave machine at l21°C and 15 pounds pressure per square inch for 15 minutes. The sterility of the medium was judged by incubating overnight at 37°C and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.2.2 Solid Media

3.2.2.2.1 Nutrient Agar

28.0 grams of nutrient agar powder (Hi-media, India) was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile Petridis and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37^0C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.2.2.2.2 Salmonell*a* Shigella Agar

Salmonella Shigella (SS) agar is the selective medium for the isolation of *Salmonella* and *Shigella*. 63.0 grams SS agar powder was dissolved in 1000 ml of distilled water. It was mixed well until a homogeneous suspension is obtained. It was heated with frequent agitation and boiled for one minute. It did not sterilized by autoclaved. It was cooled to 45ºC and 50° C and distributed in Petri plates and allow the medium to solidify partially uncovered. (Leifson, 1935)

3.2.2.2.3 Eosin Methylene Blue Agar

Eosin methylene blue (EMB) agar medium was used to observe the growth of *Escherichia coli* (Cheesbrough, 1985). 36 gram of EMB agar base (Hi-media, India) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in to sterile glass. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged and used or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.2.2.4 MacConkey Agar

51.50 grams of dehydrated Bacto-MacConkey agar (Difco) was suspended in 1000 ml of cold distilled water taken in a conical flask and was heated up to boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured sterile glass petridishes. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.2.2.5 Thiosulfate-citrate-bile salts-sucrose (TCBS) agar

TCBS Agar is recommended for the selective isolation and cultivation of *Vibrio cholera* and other enteropathogenic Vibrio's causing food poisoning.89.08 grams of TCBS agar powder was suspended in 1000 ml distilled water. It was heated to dissolve the medium completely. It did not autoclaved. It was cooled to 50°C.

3.2.2.2.6 Manitol Salt Agar (MSA)

MS agar is used as a selective media for the isolation of pathogenic Staphylococci. Suspend 111.02 grams in 1000ml distilled water. Then it was heated to boiling and dissolve the medium completely. Sterilized the medium by autoclaving at 15lbs pressure (121°C) for 15 minutes and then cooled to 45-50°C.

3.2.2.2.7 Mueller Hinton Agar

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method. 38 grams of Mueller Hinton agar powder was suspended in 1000 ml of distilled water. It was mixed well. It was heated agitating frequently and boiled for about one minute. It was dispensed and sterilized in autoclave at 116 - 121°C (15 lbs. sp) for 15 minutes. It was cooled to 45° or 50° C (Carter, 1979).

3.2.2.2.8 Motility Indole Urease Test Medium (MIU)

18 grams of MIU agar (Difco) was suspended in 950 ml of cold distilled water taken in a conical flask and heated up to boiling to dissolve the medium completely. Ninety five ml was dispensed into flasks and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then was Cooled to about 50-55°C and aseptically add 5ml was added of sterile 40% basal medium. After mixing were dispensed into sterile test tubes. Allow to cool in an upright position. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.3 Reagents preparation

3.2.3.1 Methyl Red-Voges Proskauer broth

A quantity of 3.4 grams of Bacto MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube and then the test tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and used for biochemical characterization or stored at 4°C in refrigerator for future use (Cheesbrough, 1985).

3.2.3.2 Methyl red solution

The indicator methyl red (MR) solution was prepared by dissolving 0.1 gm of Bacto methyl red (Difco) in 300 ml of 95% alcohol and diluting this to 500 ml with the addition of 200 ml of distilled water.

3.2.3.3 Alpha-naphthalin solution

Alpha-naphthol solution was prepared by dissolving 5 grams of 1-naphthol in 100 ml of 95% ethyl alcohol.

3.2.3.4 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of potassium hydroxide crystals in 100 ml of cold distilled water.

3.2.3.5 Kovac's reagent

This solution was prepared by mixing 25 ml of concentrated Hydrochloric acid in 75 ml of amyl alcohol and to this mixture 5 grams of paradimethyl-aminohenzyldehide crystals were added. This was then kept in a flask equipped with rubber cork for future use (Merchant and Packer, 1967).

3.2.3.6 Phosphate buffered saline solution

For preparation of Phosphate buffered saline (PBS) solution, 8 gram of sodium chloride (NaCl), 2.89 gram of disodium hydrogen phosphate (Na_2HPO_4, $12H_2O$), 0.2 gram of potassium chloride (KCl) and 0.2 gram of potassium hydrogen phosphate were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclave at 121 °C maintaining a pressure of 15 pounds per square inch for 15 minutes and stored at refrigerator until use. The pH of the solution was measured by a pH meter and maintained at 7.0-7.2 (Cheesbrough, 1985).

3.2.4 Sample collection and sample processing

Meat (Sonali) samples (50) from local market of Dinajpur city (Bahadur bazaar) were collected aseptically and brought to the bacteriology laboratory, Department of Microbiology, HSTU, Dinajpur with necessary precautions for bacteriological examination. At first, Samples were rinsed thoroughly with sterile distilled water. Then 10 g of all part of the samples were homogenized through blending with 90 ml peptone water (Cappuccino and Sherman, 1996). Then 1-10 fold dilutions were performed.

3.2.4.1 Serial dilution of Sample

Serial 10 fold dilutions of each of the samples in a series of dilution tubes were prepared. At first for each of the processed samples 10 sterile test tubes were placed on a test tube holder rack containing 9 ml of 2% buffered peptone water. 1 ml processed sample was mixed with 9 ml of Phosphate buffer solution in the 1^{st} test tube in order to make 10^{-1} dilution. Then 1ml solution from 1^{st} test tube mixed with 2^{ndt} test tube, then from 2^{nd} test

tube to 3rd test tube and finally 9th to 10th test tube and 1ml discard from 10th test tube by the help of pipette and in every steps mixing was done properly.

3.2.4.2 Isolation and identification of bacteria

3.2.4.2.1 Culture of meat sample

Media such as Nutrient agar, Nutrient broth, MacConkey agar, Eosin Methylene Blue agar (EMB), Salmonella-Shigella (SS) agar, Thiosulfate-citrate-bile salts-sucrose (TCBS) agar and Manitol Salt Agar (MSA) were used.

3.2.4.2.2 Culture in ordinary media

Samples were inoculated separately into ordinary media like nutrient agar, nutrient broth and were incubated at $37^{O}C$ for overnight. The colonies on primary cultures were repeatedly sub-cultured by streak plate method (Cheesbrough, 1985) until the pure culture with homogenous colonies were obtained.

3.2.4.2.3 Isolation of bacteria in pure culture

For isolation of bacteria in pure culture, the mixed culture was inoculated into nutrient agar media by streak plate technique to obtain isolated colonies as per:

Step-1: An inoculum was picked up with a sterile loop and spread on an area of the medium in the Petridis.

Step-2: The loop was sterilized by being heated as red hot in a flame.

Step-3: The inoculum was spread over the reminder of the plate by drawing the cooled parallel line.

This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more times to ensure purity.

3.2.5 Morphological characterization of organisms by Gram's staining methods

The grams staining was followed to study the morphological and staining characteristics of bacteria and to provide information about the presumptive bacterial identification as per recommendation of Cowan and Steel (1985).

Technique:

A drop of sterile normal saline was taken on the middle of the clear slide. Then a loopful bacterial suspension (young culture) was transferred to the sterile drop of normal saline and a very thin film was prepared on the slide by spreading uniformly. The film was fixed by passing it gently over flame for two or three times.

- The slide was flooded with crystal violet solution for up to one minute. Wash off briefly with tap water (not over 5 seconds). Drained.
- The slide was flooded with Gram's Iodine solution, and allow to act (as a mordant) for about one minute. Wash off with tap water. Drained.
- Excess water was removed from slide and blotted, so that alcohol used for decolonization was not diluted. Slide was flooded with 95% alcohol for 10 seconds and washed off with tap water. (Smears that are excessively thick may require longer decolonization. This is the most sensitive and variable step of the procedure, and requires experience to know just how much to decolorize). Drained.
- The slide was flooded with Safranine solution and allowed to counter stain for 30 seconds. Washed off with tap water. Drained and blotted with bibulous paper.
- All sides of bacteria were examined under the oil immersion lens.

3.2.6 Culture into differential media

3.2.6.1 Mac-Conkey agar

Samples were sub-culture on Mac-conkey agar media and incubated at 37°C for overnight. After that both lactose fermenter bacteria (rose pink color colony) and lactose non fermenter bacteria (pale color colony) were selected.

3.2.7 Culture on selective media

3.2.7.1 Eosin Methylene Blue (EMB) agar

Samples of positive lactose fermenter were taken and sub-culture on EMB agar media and incubated at 37°C for overnight. Some EMB agar plate showed slightly circular colonies with dark center metallic sheen. Also in some EMB agar, the growth was indicated by smooth, characteristics mucoid and pink colored colonies which are a consequence of the organism's abundant polysaccharide capsule.

3.2.7.2 *Salmonella* -Shigella agar

Sample of non-lactose fermenter were taken and sub-culture on SS agar media and incubated at 37°C for overnight, which after inoculation, raised, black centered, smooth colorless colonies.

3.2.7.3 Thiosulfate-citrate-bile salts-sucrose (TCBS) agar

Samples were inoculated into TCBS agar plates which after incubation, the growth was indicated by smooth, Characteristics yellow colonies**.**

3.2.7.4 Manitol salt agar (MSA)

Samples were inoculated into MSA plates and incubated at 37°C for overnight, the growth was indicated smooth yellow color colonies.

3.2.8 Microscopic study for identification of (*E. coli. Salmonella* spp*., Vibrio* spp*., staphylococcus spp.*) Suspected colonies by Gram's staining methods

Gram's staining was performed by taking colony from selected media to determine the size, shape, and arrangement of bacteria according to the methods described by Merchant and Packer (1967). Stained slides were examined under light microscope at 100 x magnification.

3.2.9 Identification of isolated bacteria by different Biochemical Tests

Isolated organisms with supported growth characteristics of *E. coli, Salmonella* spp*., Vibrio* spp., *Staphylococcus* spp*.,* were maintained in pure culture and subjected to biochemical tests.

3.2.9.1 Procedure of Indole test

2 ml of peptone water was inoculated separately with 5 ml of culture of each of the isolated bacteria and incubated for 48 hours. 0.5 ml Kovac's reagent was added, shakes well and examined after 1 minute. A red color ring at the top of the reagent indicated production of the indole by the organisms (Cowan and Steel, 1985).

3.2.9.2 Procedure of MR test

The test was performed by inoculating separately a colony of the each of the isolated test organisms in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37^0C, a drop of methyl red solution was added. A positive methyl red test was shown by the appearance of a bright red color. A yellow or orange color was a negative test (Cowan and Steel, 1985).

3.2.9.3 Procedure of VP test

2 ml of sterile glucose phosphate peptone water were inoculated separately with 5ml of each of the isolated organisms and incubated at 37°C for 48 hours. A very small amount (knife point) of creatine was added and mixed. 3 ml of 40% potassium hydroxide were added and shacked well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of a pink color for positive cases. In negative cases there was no development of pink color (Cowan and Steel, 1985).

3.2.9.4 Procedure of Motility Indole Urease test (MIU)

MIU media were prepared in test tubes. Then the isolated organisms were inoculated separately into the media by stabbing method with the help of sterile straight wire. Then the test tubes were incubated 37°C overnight. Single stick that is no turbidity throughout the medium indicate gram negative organism (non-motile) and turbidity throughout the medium indicate gram positive case (Cowan and Steel, 1985)

3.2.9.5 Procedure of Triple Sugar Iron Test (TSI)

Triple sugar iron contains three sugars (Glucose, Sucrose and Lactose). At first TSI agar slant were prepared in a test tube. Then the isolated organisms were inoculated separately into the butt with a sterilized wire and on the slant with a wire loop producing zigzag streaking. The tubes were incubated for 24 hours at 37°C.Yellow color of butt and slant of the test tube indicate fermentation of Glucose, Sucrose and Lactose fermentation and butt shows blacking indicate H_2S production (Cowan and Steel, 1985).

3.2.9.6 Procedure of Citrate Utilization test

Simmons citrate agar slants of 2 ml in each vials were prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of each of the isolated bacteria from 24-hours old pure culture were inoculated separately into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37°C (Cappuccino and Sherman, 1996).

3.2.10 Antibiotic susceptibility tests

3.2.10.1 The Kirby-Bauer disc diffusion methods

All aspects of the Kirby-Bauer procedure are standardized to ensure consistent and accurate results. Because of this, a laboratory must adhere to these standards. The media used in Kirby-Bauer testing must be Mueller-Hinton agar at only 4 mm deep, poured into either 100m or 150mm Petri dishes. The pH level of the agar must be between 7.2 and 7.4. Using the cotton swab streaked the entire agar surface horizontally, vertically and around the outer edge of the plate to ensure a heavy growth over the entire surface.

3.2.10.2 Reading Plates and Interpreting Results

After 24 hours of incubation, each plate was examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones oh inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies were apparent, the inoculum was too light and the test must be repeated. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter, using sliding calipers or a ruler, which was held on the back of the inverted Petri plate. The Petri plate was held a few inches above a black, nonreflecting background and zones are measured in millimeter (mm) from the upper surface of the agar illuminated with reflected light, with the cover removed (EUCAST, 2015).

CHAPTER 4

RESULTS

4.1 Isolation and identification of *E. coli, Salmonella* spp*., Vibrio* spp*. Staphylococcus* spp. by different bacteriological methods

E. coli (100%), *Salmonella* spp. (100%), *Vibrio spp*. (10%), *Staphylococcus spp*. (92%) were frequently isolated from almost all samples.

Table 2: Frequency of occurrence of bacteria isolated from meat (Sonali) samples

Bacterial isolates	**Examined**	**Positive**	**Rates (%)**
E. coli	50	50	100%
Salmonella spp.	50	50	100%
Vibrio spp.	50	5	10%
Staphylococcus spp.	50	46	92%

4.2 Differential media

4.2.1 MacConkey agar

MacConkey agar plates streaked separately with the organisms from nutrient agar revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically. The growth of lactose fermenting organisms was indicated by bright pink colored colonies of on MacConkey agar**.** The growth of non-lactose fermenting organisms was indicated by pale colored colonies of on MacConkey agar**.**

4.3 Selective media

4.3.1 Eosin Methylene Blue (EMB) Agar

EMB agar plates streaked separately with the lactose fermenter organisms from MacConkey agar revealed the growth of *E. coli bacteria* after 24 hours of incubation at 37^0C aerobically. The growth of *E. coli* was indicated by smooth, circular, black color colonies with metallic sheen on the agar plate

4.3.2 Salmonella- Shigella (SS) Agar

SS agar plates streaked separately with the non-lactose fermenting organisms from MacConkey agar revealed the growth of *Salmonella* spp. after 24 hours of incubation at 37^0C aerobically. The growth of *Salmonella* spp. was indicated by smooth, colorless, usually with black center.

4.3.3 Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar

TCBS agar plate streaked separately with the non-lactose fermenting organisms from MacConkey agar revealed the growth of *Vibrio* spp. after 24 hours of incubation at 37^0C aerobically. The growth of *Vibrio* spp. was indicated by yellow colonies.

4.3.4 Manitol Salt Agar (MSA)

MSA plates streaked separately with the non-lactose fermenting organisms from Mac-Conkey agar revealed the growth of *Staphylococcus* spp. after 24 hours of incubation at 37^0C aerobically. The growth of *Staphylococcus spp*. was indicated by golden yellow color colonies.

Table 3: Cultural properties of isolated bacteria

Bacteria	**Name of the media**	**Colony characteristics**
E. coli	Eosin Methylene Blue (EMB) agar	smooth, circular, black color colonies with metallic sheen
Salmonella spp.	Salmonella-Shigella (SS) agar	Smooth, colorless, usually with black center.
Vibrio spp.	Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar	Yellow color colonies
Staphylococcus spp.	Manitol Salt Agar (MSA)	Golden yellow color colonies.

4.4 Results of biochemical tests

The isolated organisms were confirmed by different biochemical tests.

Table 4: Identification of *E. coli* by different biochemical tests

Biochemical test	**Change of the media**	**Results**
Citrate utilization test	No color change	Negative
Indole test	Pink color ring at the top of the media	Positive
Triple sugar iron (TSI) test	S-yellow, B-yellow	S-A, B-A, gas (+), H_2S (-)
MR test	Red color	Positive
VP test	No color change	Negative
MIU test	Diffuse, hazy growth, slightly opaque media	Positive

Legends: (S=Slant, B=Butt, A = Acid, MR = Methyl-Red test, VP = Voges-Proskauer test, MIU= Motility Indole Urease**,** + = Positive reaction, - = Negative reaction)

Table 5: Identification of *Salmonella* spp. by biochemical test

Biochemical test	**Change of the media**	**Results**
Citrate utilization test	Prussian blue color	Positive
Indole test	No color change	Negative
Triple sugar iron (TSI) test	S-Red, B-Black	S-Al, B-A, gas(+), H_2S (+)
MR test	Red color	Positive
VP test	Red color	Positive
MIU test	No color change	Negative

Legends: (S=Slant, B=Butt, A = Acid, Al- Alkaline, MR = Methyl-Red test, VP = Voges-Proskauer test, MIU= Motility Indole Urease**,** + = Positive reaction, - = Negative reaction)

Table 6: Identification of *Vibrio* spp. by biochemical test

Biochemical test	**Change of the media**	**Results**
Citrate utilization test	Prussian blue color	Positive
Indole test	Pink color ring at the top of the media	Positive
Triple sugar iron (TSI) test	S-Red, B-BLACK	S-Al, B-A, gas(+), H_2S (+)
MR test	Red color ring	Positive
VP test	Red color	Positive
MIU test	Diffuse, hazy growth, slightly opaque media	Positive

Legends: (S=Slant, B=Butt, A = Acid, Al- Alkaline, MR = Methyl-Red test, VP = Voges-Proskauer test, MIU= Motility indole urease, + = Positive reaction, - = Negative reaction)

Table 7: Identification of *Staphylococcus* spp. by biochemical test

Biochemical test	**Change of the media**	**Results**
Citrate utilization test	Prussian blue color	Positive
Indole test	No color change	Negative
Triple sugar iron (TSI) test	S-yellow, B-yellow	S-A, B-A, gas (+), H_2S (-)
MR test	Red color	Positive
VP test	Red color	Positive
MIU test	Yellow color	Positive

Legends: (S=Slant, B=Butt, A = Acid, Al- Alkaline, MR = Methyl-Red test, VP = Voges-Proskauer test, MIU= Motility indole urease**,** + = Positive reaction, - = Negative reaction)

4.5 Results of Antimicrobial susceptibility tests

Antimicrobial susceptibility testing was performed using Muller-Hinton agar (Mumbai, India) plates as recommended by the Clinical and Laboratory Standards Institute.

4.5.1 Antibiotic sensitivity test of *E. coli*

The antibiotic sensitivity test revealed that all of the isolated *E. coli* were resistant to Cefixime, Chloramphenicol, Penicillin, Cloxacilin, Vancomycin, Amoxicillin and Erythromycin. The isolates were sensitive to Gentamycin and Colistin.

Table 8: Results of antibiotic sensitivity test of *E. coli*

Antibacterial agents	**Disc concentration (mcg /disc)**	**Diameter of zone of inhibition (mm)**			**Interpretation**
		Sensitive	**Intermediate**	**Resistant**	
Cefixime	5 mcg/disc	-	-	-	R
Gentamycin	10mcg/disc	20	-	-	S
Chloramphenicol	25mcg/disc	-	-	-	R
Penicillin	10mcg/disc	-	-	-	R
Erythromycin	15 cg/disc	-	-	-	R
Colistin	10mcg/disc	18	-	-	S
Cloxacilin	1mcg/disc	-	-	-	R
Amoxicillin	30mcg/disc	-	-	-	R
Vancomycin	30mcg/disc	-	-	-	R

4.5.2 Antibiotic sensitivity test of *Salmonella spp.*

The antibiotic sensitivity test revealed that all of the isolated *Salmonella spp.*were found resistant to Chloramphenicol, Penicillin, Cloxacilin, Vancomycin, and Amoxicillin. Colistin and Erythromycin. The isolates were sensitive to Gentamycin and Cefixime.

Table 9: Results of antibiotic sensitivity test of *Salmonella* spp.

Antibacterial agents	**Disc concentration**	**Diameter of zone of inhibition(mm**			**Interpretation**
		Sensitive	**Intermediate**	**Resistant**	
Cefixime	5 mcg/disc	24	-	-	S
Gentamycin	10mcg/disc	18	-	-	S
Chloramphenicol	25 mcg/disc	-	-	-	R
Penicillin	10 mcg/disc	-	-	-	R
Erythromycin	15 mcg/disc	-	-	-	R
Colistin	10mcg/disc	-	-	-	R
Cloxacilin	1mcg/disc	-	-	-	R
Amoxicillin	30mcg/disc	-	-	12	R
Vancomycin	30mcg/disc	-	-	-	R

4.5.3 Antibiotic sensitivity test of *Vibrio* spp.

The antibiotic sensitivity test revealed that all of the isolated *Vibrio spp.* were found resistant to Chloramphenicol, Penicillin, Cloxacilin, Cefixime, Amoxicillin, Colistin and Erythromycin. The isolates were sensitive to Gentamycin and Vancomycin.

Table 10: Results of antibiotic sensitivity test of *Vibrio* spp.

Antibacterial agents	**Disc concentration**	**Diameter of zone of inhibition (mm)**			**Interpretation**
		Sensitive	**Intermediate**	**Resistant**	
Cefixime	5 mcg/disc	-	-	-	R
Gentamycin	10mcg/disc	22	-	-	S
Chloramphenicol	25 mcg/disc	-	-	-	R
Penicillin	10 mcg/disc	-	-	-	R
Erythromycin	15 mcg/disc	-	-	-	R
Colistin	10mcg/disc	-	-	-	R
Cloxacilin	1mcg/disc	-	-	-	R
Amoxicillin	30mcg/disc	-	-	-	R
Vancomycin	30mcg/disc	18	-	-	S

4.5.4 Antibiotic sensitivity test of *Staphylococcus spp.*

The antibiotic sensitivity test revealed that all of the isolated *Staphylococcus spp.* was resistant to Chloramphenicol, Penicillin, Cloxacilin, Cefixime, Amoxicillin and Erythromycin. The isolates were sensitive to Gentamycin and Vancomycin. The isolates were intermediate to Colistin.

Table 11: Results of antibiotic sensitivity test of *Staphylococcus spp.*

Antibacterial agents	**Disc concentration**	**Diameter of zone of inhibition (mm)**			**Interpretation**
		Sensitive	**Intermediate**	**Resistant**	
Cefixime	5 mcg/disc	-	-	-	R
Gentamycin	10mcg/disc	23	-	-	S
Chloramphenicol	25 mcg/disc	-	-	-	R
Penicillin	10 mcg/disc	-	-	-	R
Erythromycin	15 mcg/disc	-	-	-	R
Colistin	10mcg/disc	-	10	-	I
Cloxacilin	1mcg/disc	-	-	-	R
Amoxicillin	30mcg/disc	-	-	-	R
Vancomycin	30mcg/disc	25	-	-	S

Plate 1: Typical chicken shop

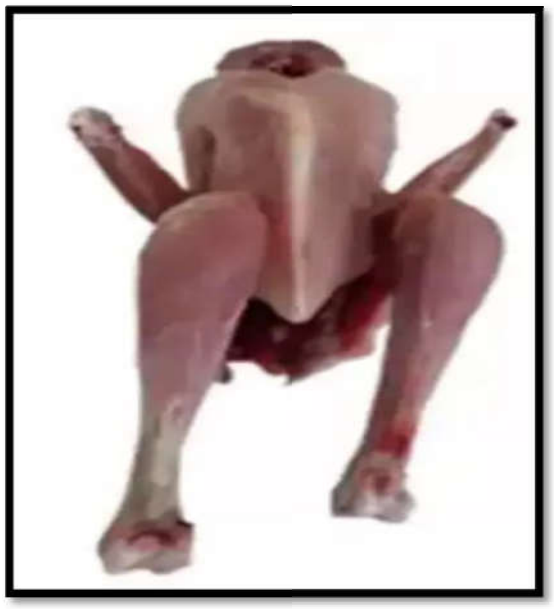


Plate 2: Samples collected from dressed chicken

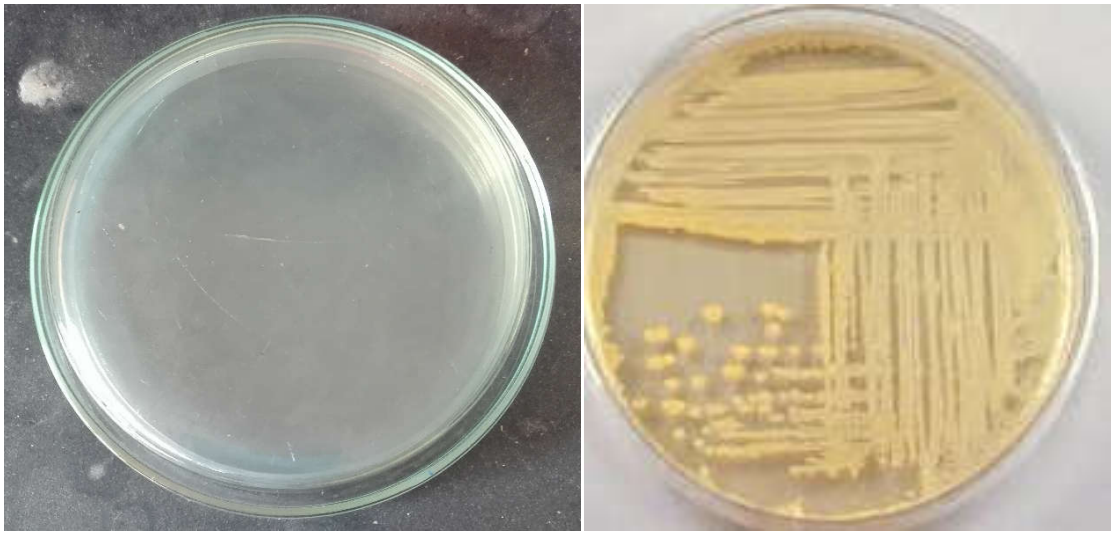


Plate 3: Pale colored colonies on nutrient agar (right) and un-inoculated control (left).

Plate 4: Pink colored colonies on MacConkey agar (right) indicating lactose fermenting bacteria and un-inoculated control (left)

Plate 5: Pale colored colonies on MacConkey agar (right) indicating non-lactose fermenting bacteria and un-inoculated control (left)

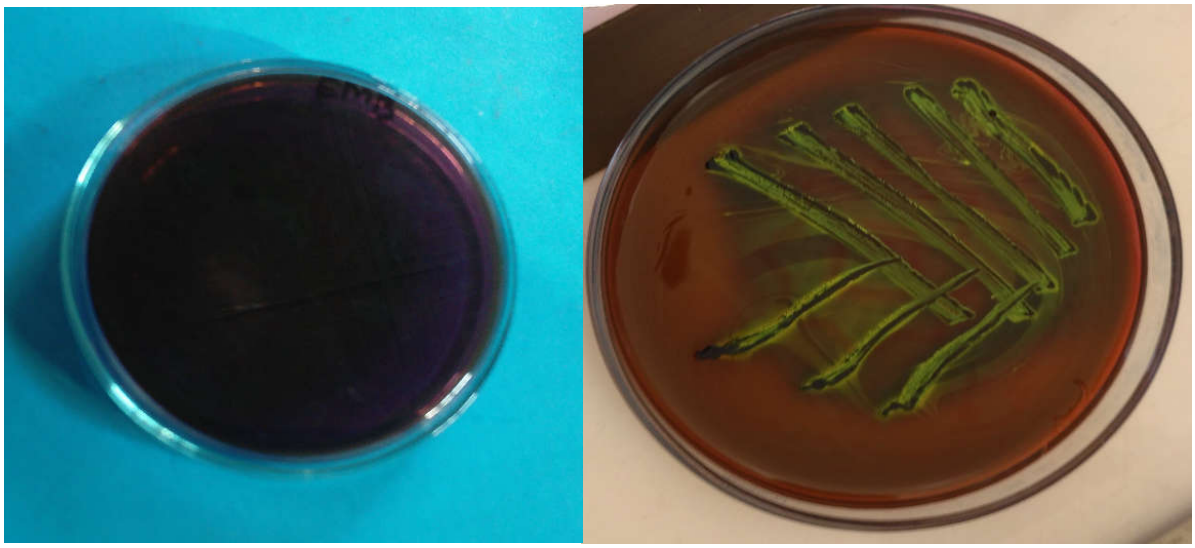


Plate 6: Metallic sheen produced by *E. coli* on EMB agar (right) and un-inoculated control (left).

Plate 7: Golden yellow color colony produced by *Staphylococcus sp*. on MS agar (right) and un-inoculated control (left).

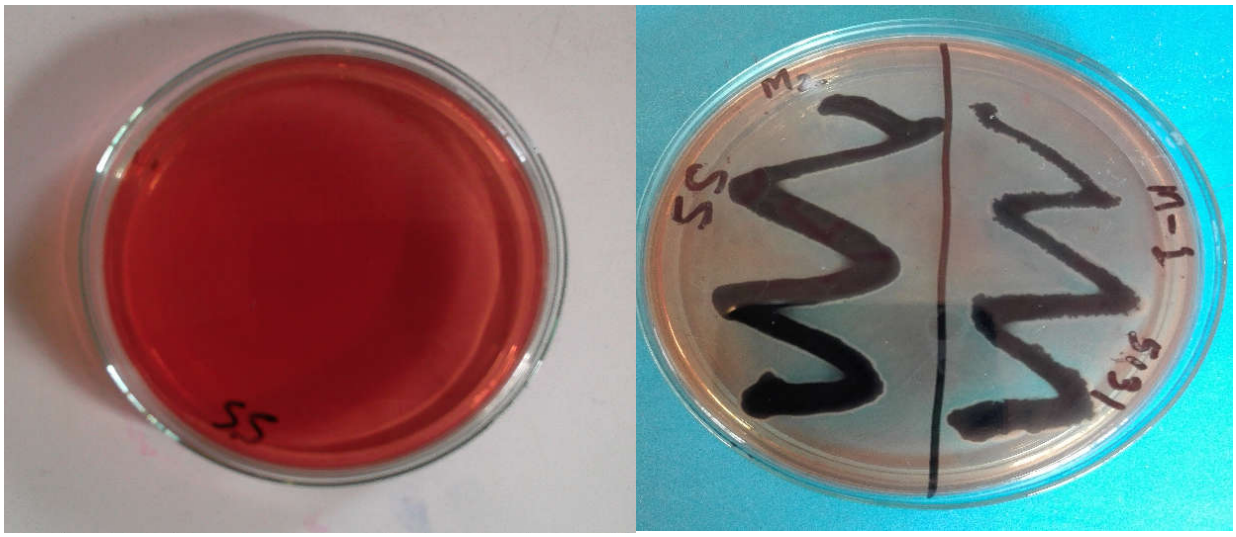


Plate 8: Black centered colonies produced by *Salmonella* spp. on SS agar (right) and un-inoculated control (left).

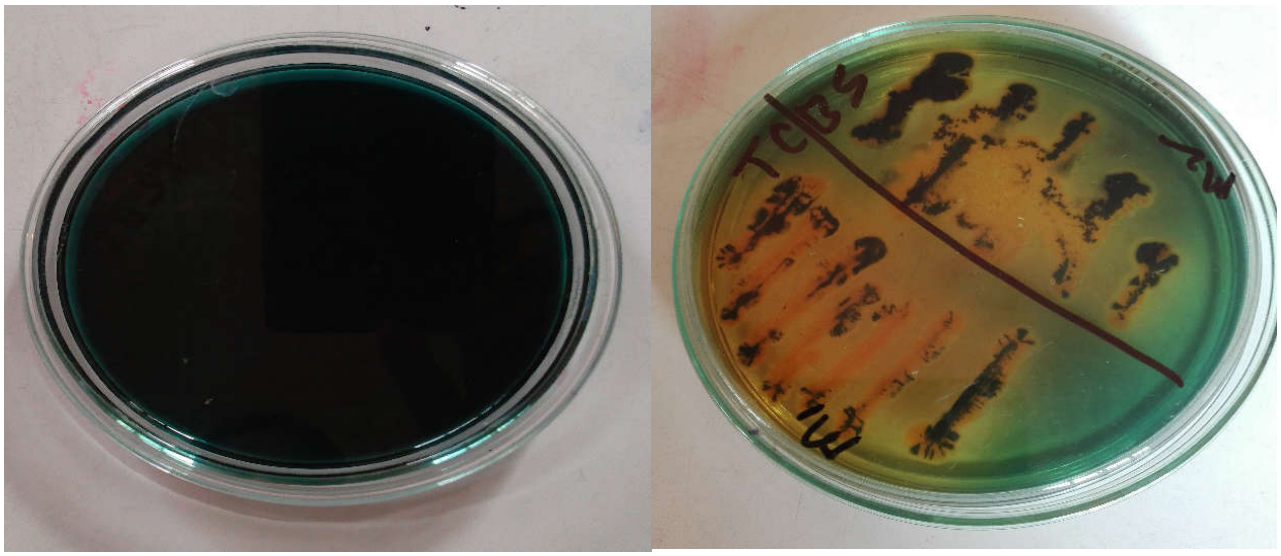


Plate 9: Yellow colonies produced by *Vibrio* spp. on TCBS agar (right) and un-inoculated control (left).

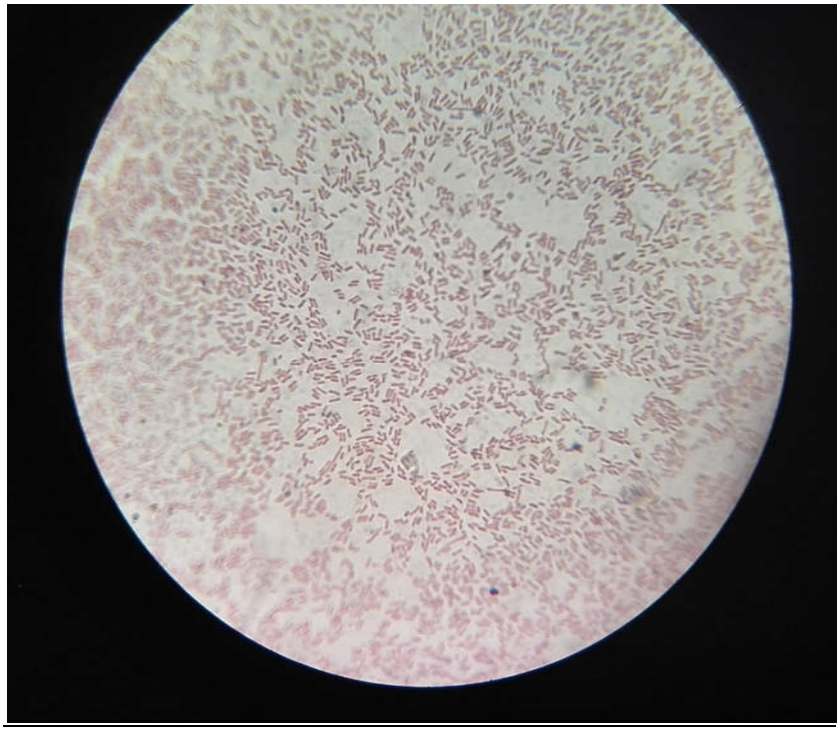


Plate 10: Light microscopic image of *E. coli* at 100x magnification (Gram's staining).

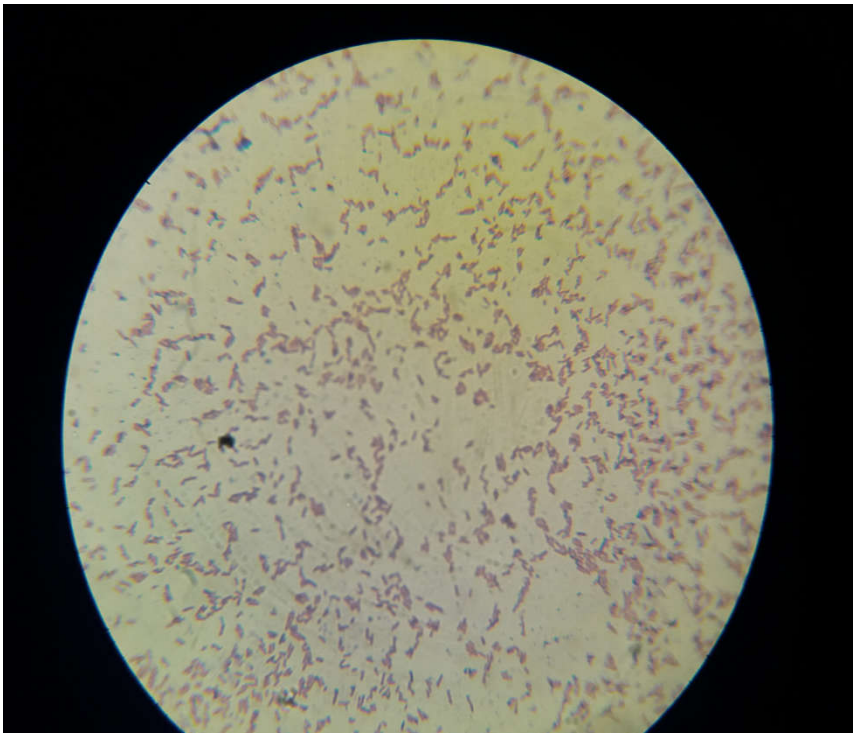


Plate 11: Light microscopic image of *salmonella spp.* at 100x magnification (Gram's staining)

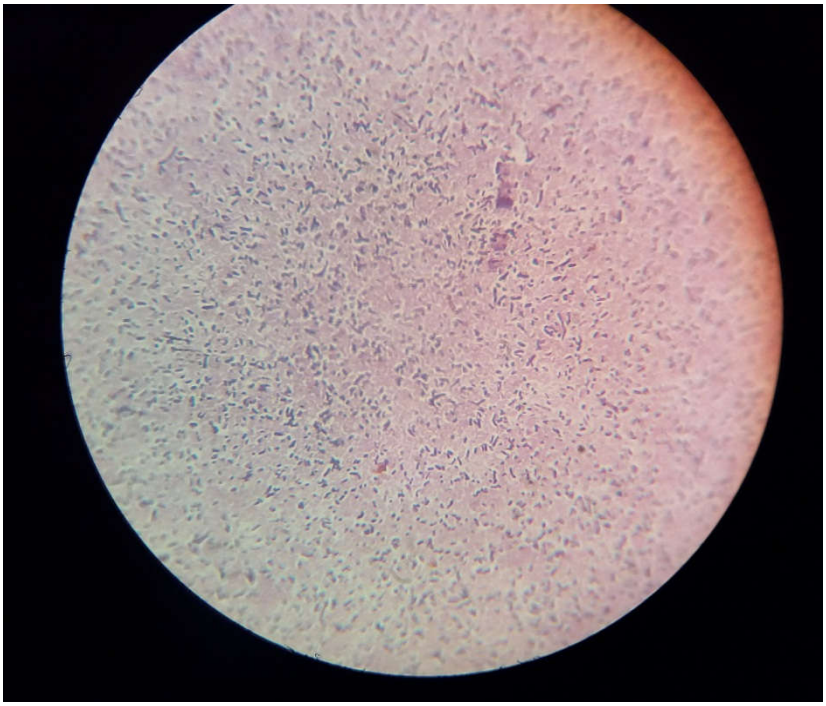


Plate 12: Light microscopic image of *vibrio spp*. at 100x magnification (Gram's staining).

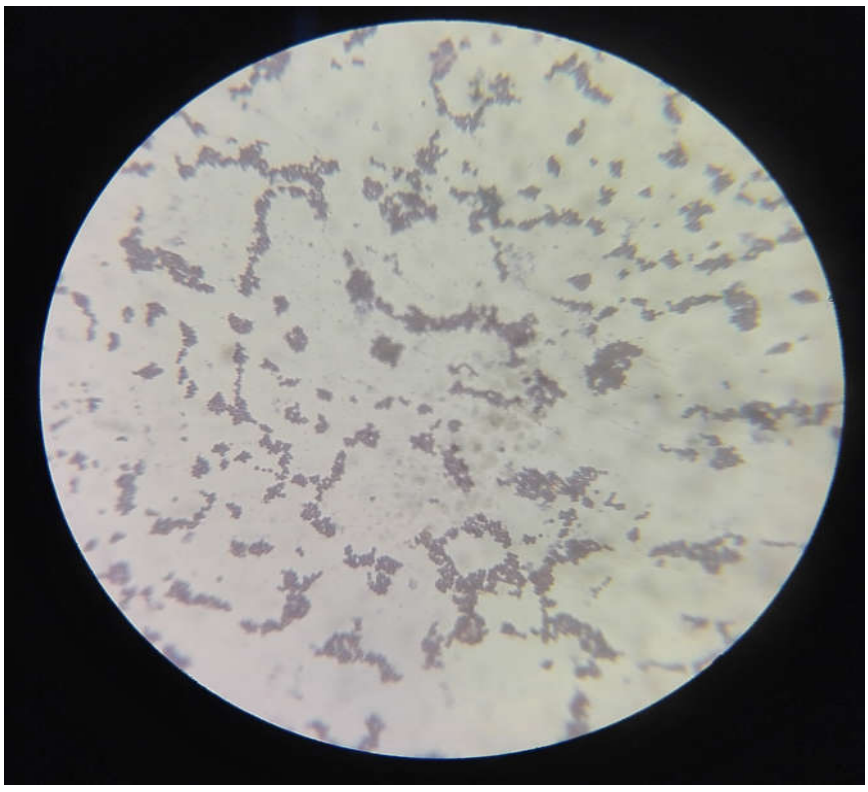


Plate 13: *Staphylococcus* spp. showing at 100x magnification (Gram's staining)

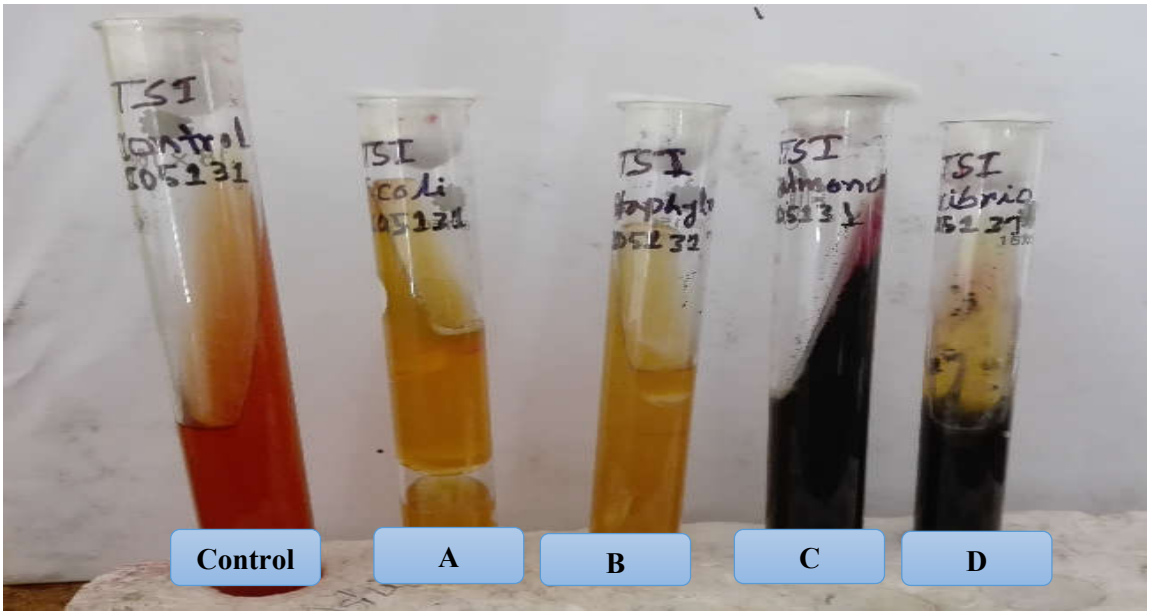


Plate 14: TSI test results (right) A= *E.* coli [S-A, B-A, gas (+), H_2S (-)], B= *Staphylococcus* spp. [S-A, B-A, gas (+), H_2S (-)] C=*Salmonella* spp.[S-Al, B-A, gas(+), H2S(+)], D= *Vibrio* spp. [S-Al, B-A, gas(-), H2S (+)]

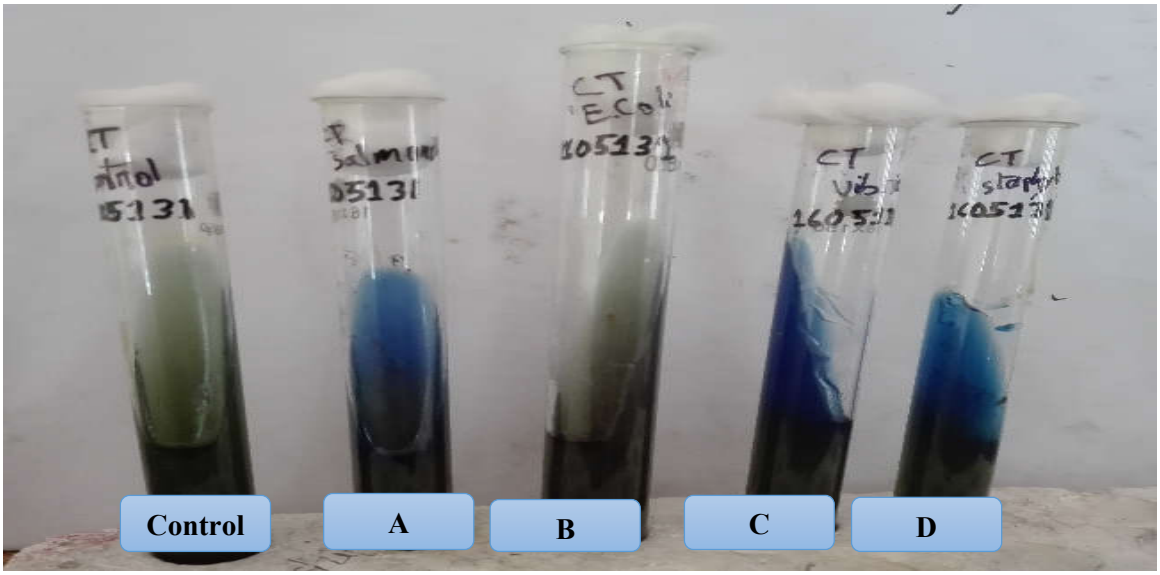


Plate 15: Citrate utilization test results; A=*Salmonella* spp.(positive), B=*E. coli* (negative), C=*Vibrio spp*. (positive), D=*Staphylococcus spp.* (positive) and un-inoculated control(left).

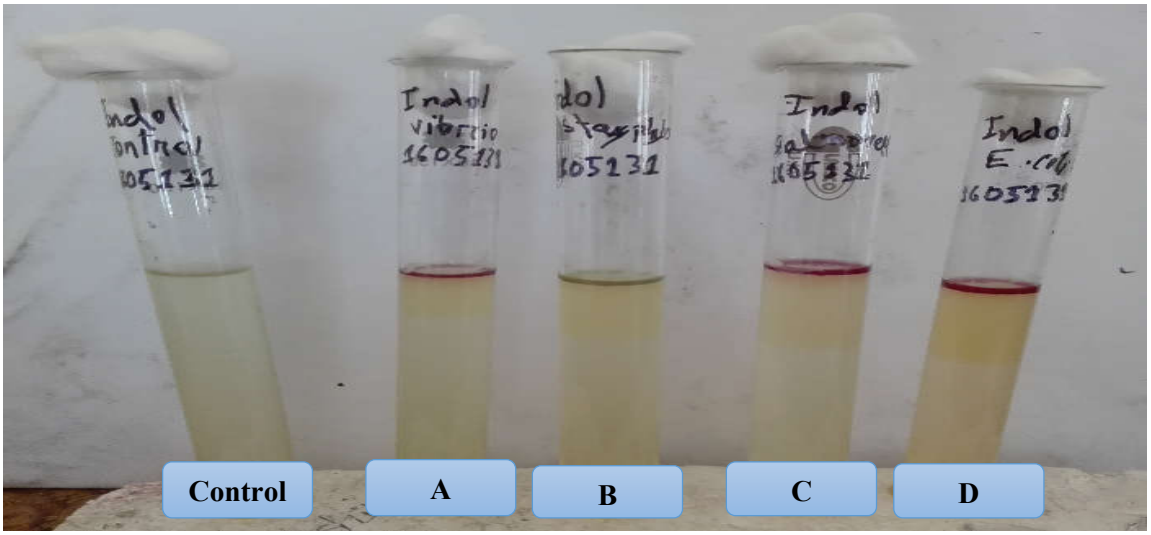


Plate 17: Indole test results;A= *Vibrio* spp. (positive), B=*Staphylococcus* spp. (negative), C= *Salmonella* spp. (positive), D= *E. coli* (positive) and un-inoculated control (left).

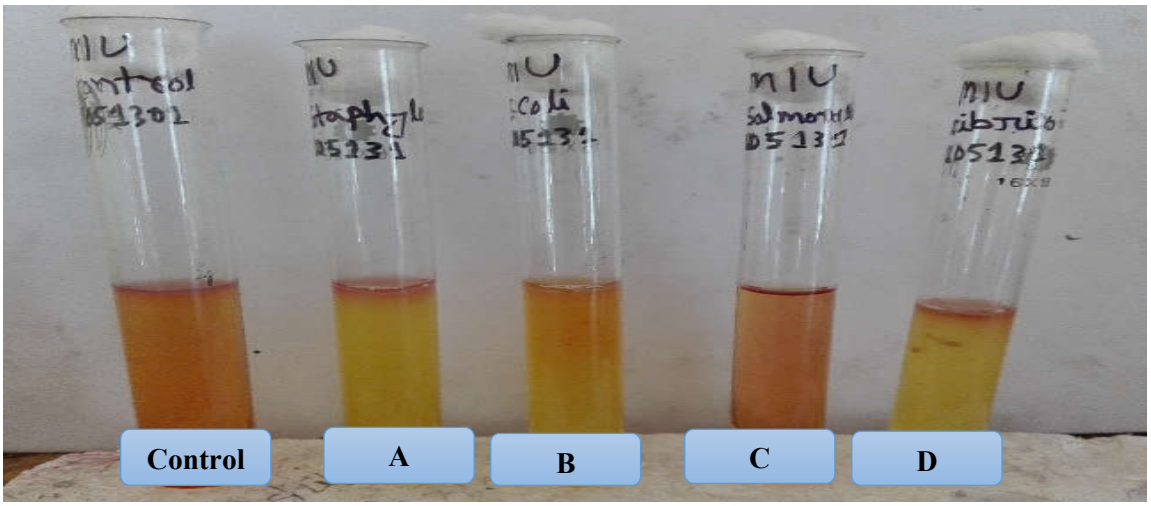


Plate 18: MIU test results; A= *Staphylococcus* spp. (positive), B= *E. coli* (positive), C= *Salmonella* spp. (negative), D= *Vibrio* spp. (positive), and un-inoculated control (left).

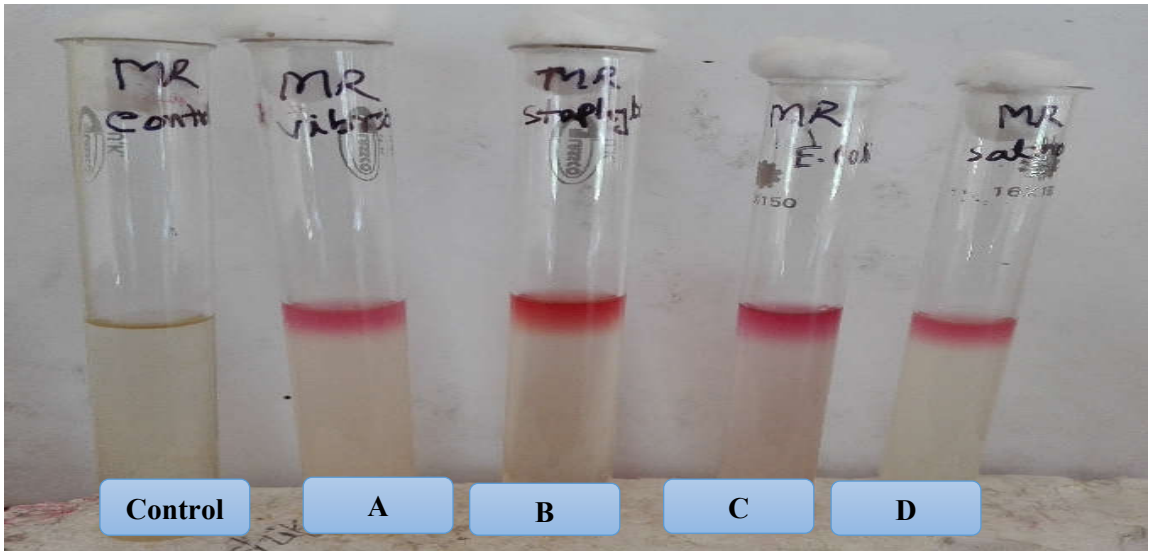


Plate 19: MR test results (right) A=*Vibrio spp.* (positive), B= *Staphylococcus* spp. (positive), C= *E. coli.* (Positive*)*, D= *Salmonella* spp. (positive) and un-inoculated control (left).

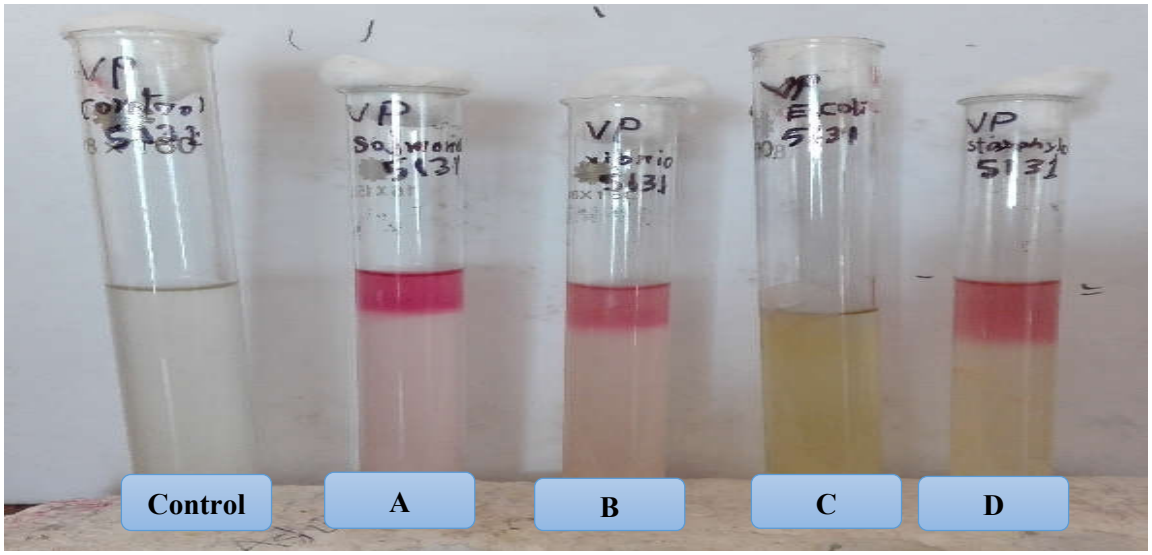


Plate 20: VP test results (right) A=*Salmonella spp.* (positive) B=*Vibrio* spp. (positive), C= *E. coli.* (Negative*)*, D= *Staphylococcus* spp. (positive), and un-inoculated control (left).

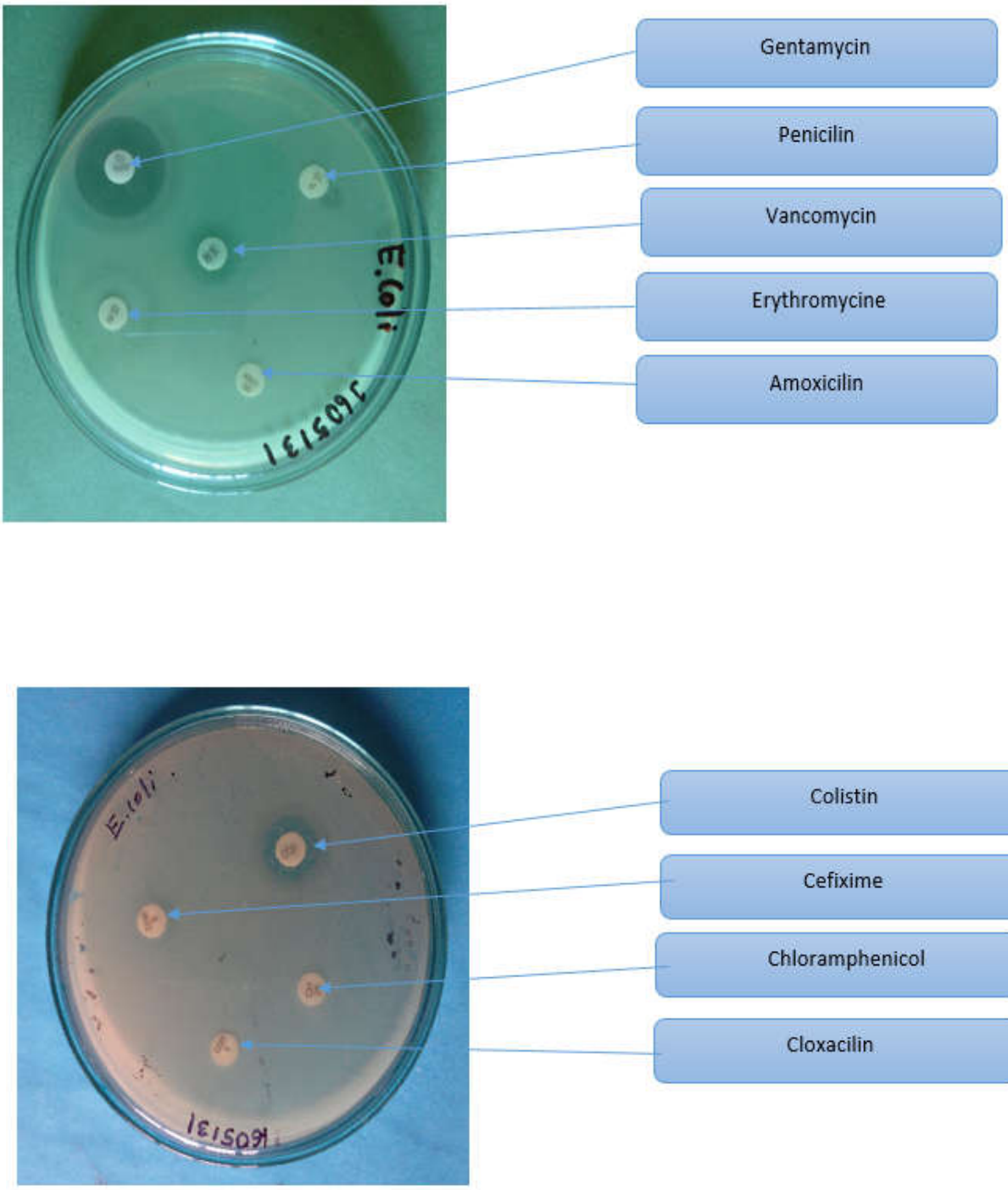


Plate 21: Antibiotic sensitivity test results of *E. coli* on Mueller-Hinton agar.

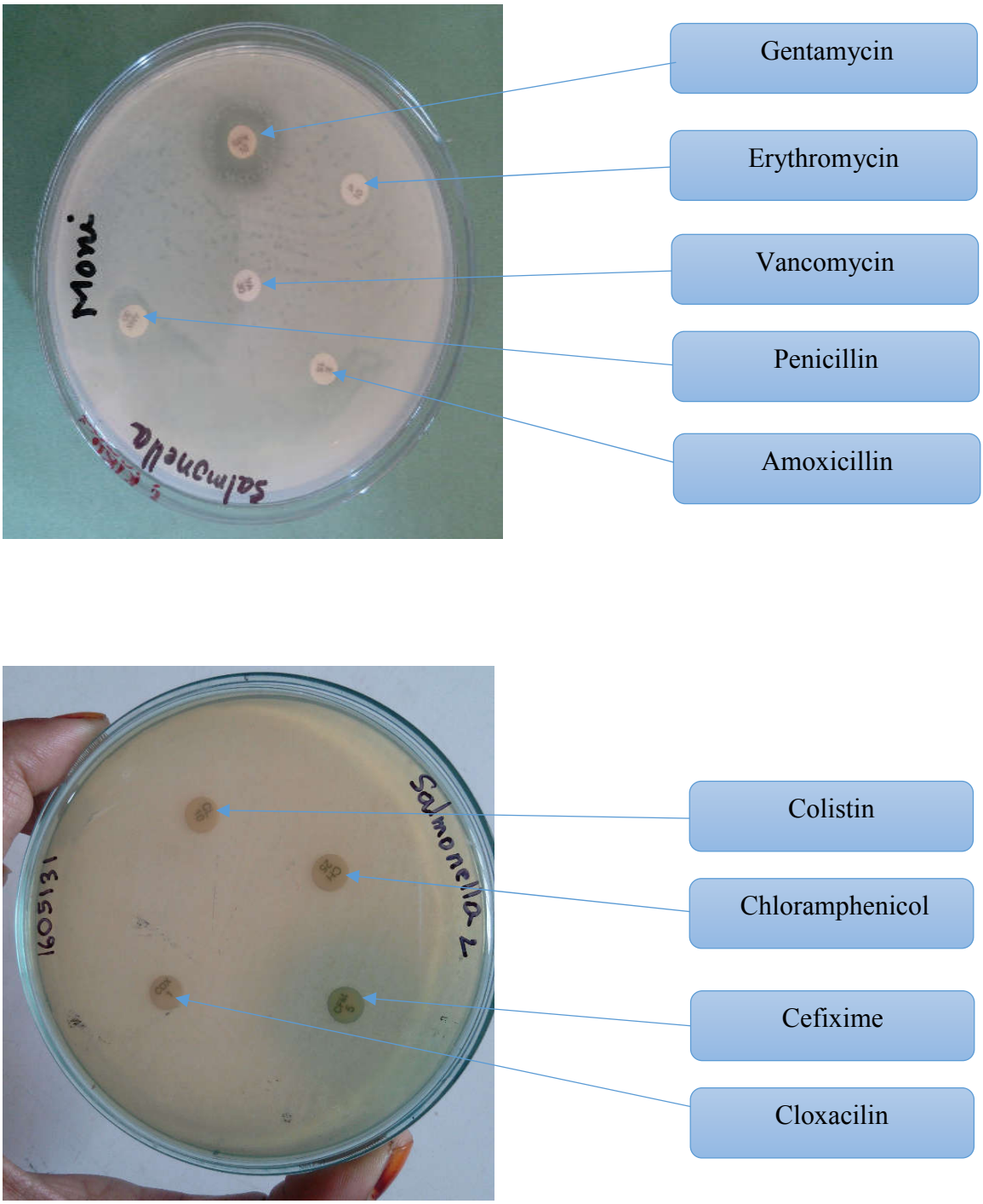


Plate 22: Antibiotic sensitivity test results of *Salmonella spp*. on Mueller-Hinton agar.

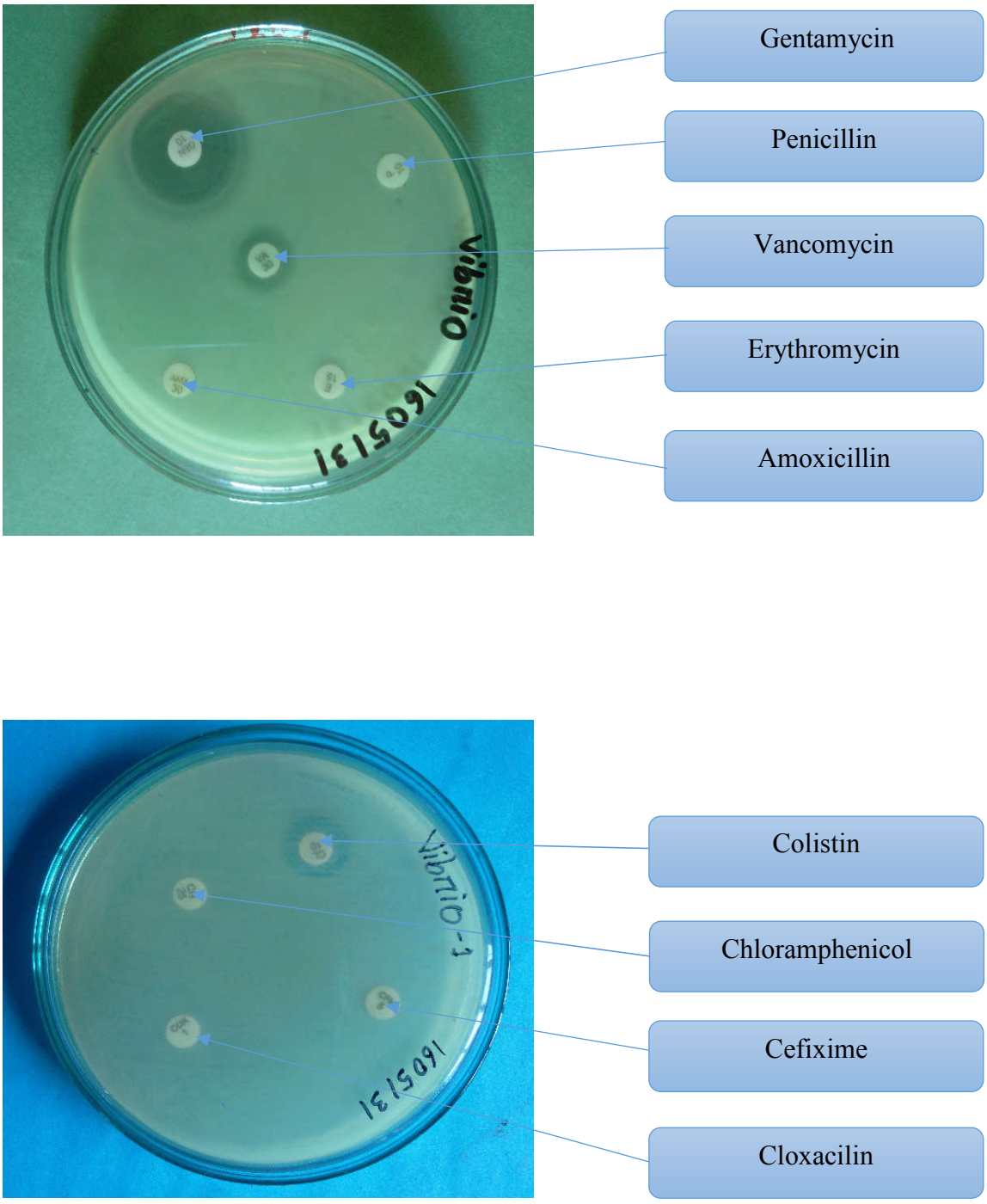
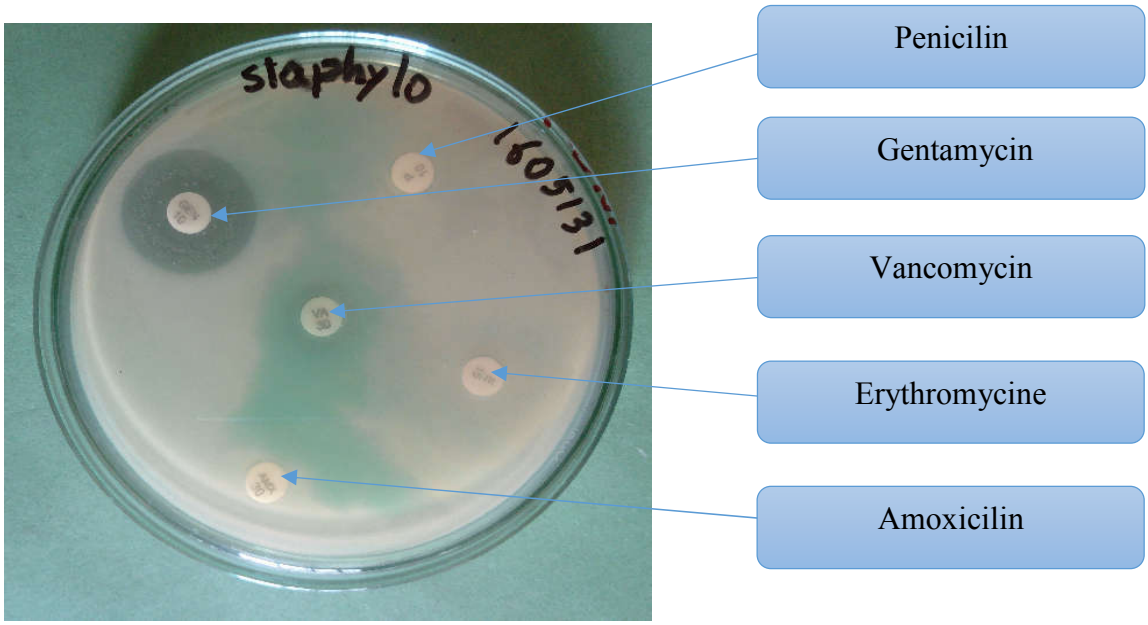


Plate 23: Antibiotic sensitivity test results of *Vibrio* spp. on Mueller-Hinton agar



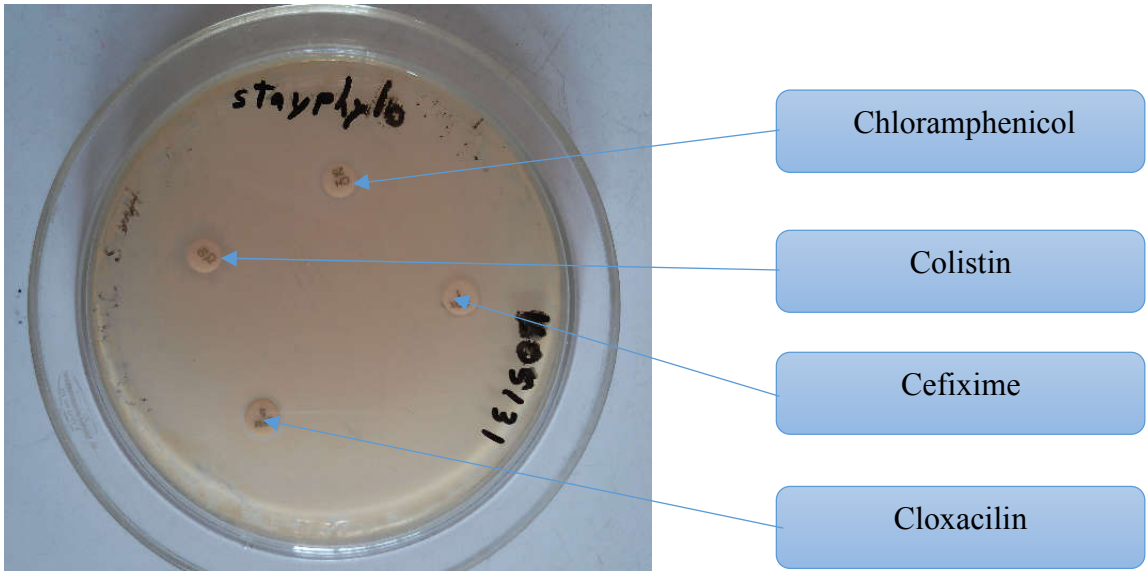


Plate 24: Antibiotic sensitivity test results of *Staphylococcus* spp. on Mueller-Hinton agar

CHAPTER 5

DISCUSSION

The present study was designed to isolate and identify the bacteria and to find out the effective antibiotics against the bacteria through antibiogram studies in sonali chicken meat collected from local market of the Dinajpur city (Bahadur bazaar), Bangladesh. A total number of 50 meat samples were collected and studied from July-December, 2017.

Poultry provides an excellent medium for the growth of microorganisms. Various pathogenic microbes, such as *Escherichia coli, Salmonella spp., Bacillus spp., Streptococcus spp*. and *Staphylococcus spp.,* have been implicated to reduce the growth of poultry (Duke, 1986).

In the present study, four different bacteria (*E. coli, Salmonella spp., Vibrio spp.,* and *Staphylococcus spp*.) were isolated from the raw meat of Sonali chicken. This is in line of findings in broiler meat by Malmuthuge *et al.,* 2012 and Voidarou *et al.,* 2011. From the 50 samples *E. coli* were isolated 100% but Awad-Alla *et al.,* 2010 and Aguirre *et al.,* 1992 were described a prevalence of 51% in broiler and 52% in black-billed ducks. *Salmonella spp*. was isolated 50% from meat samples but *Salmonella* spp. was described in other findings, such as 17.9% by Tibaijula *et al.,* 2003, 14.37% by Petrovic *et al.,* 2011 and 13% by Ellerbroek *et al.,* 2010. *Staphylococcus spp*. was 92% (46 out of 50 sample) but Hanning *et al.,* 201 and Alfonso and Barnes 2006 found 20%, *Vibrio spp*. was 10% (5 out of 50 sample). These variations might be due to variation of sample size, geographical location and type of birds.

In this present study, the meat pathogenic bacteria *Escherichia coli, Salmonella spp. Staphylococcus spp.* and *Vibrio spp*. were isolated from the breast and thigh muscle of the sonali chicken. *E. coli* and *Salmonella* are the most common and frequent pathogens responsible for food poisoning and food related infections. *Escherichia coli* is responsible for 25% of the infant diarrhoea in developing countries (WHO, 2000). Food poisoning caused by *Staphylococcus* species is one of the most common causes of foodborne illness due to the widespread occurrence of *S. aureus* and the ability of many strains to produce enterotoxins (Jay *et al.,* 2005).

In this investigation, *Escherichia coli, Salmonella spp. staphylococcus spp. Vibrio spp.* were isolated from sonali chicken *(Gallus gallus domesticu*s) meat. This indicates that, these pathogenic bacteria might cause food poisoning and diarrhoea, vomiting, enterotoxaemia in human body and leading to considerable economic losses.

In this study nine different antibiotics were used to observe the antibiotic sensitivity pattern against identified bacterial species isolated form Sonali chicken meat. The antibiotics were Amoxicillin, Colistin, Cefixime, Chloramphenicol, Erythromycin, Penicillin, Gentamycin and Vancomycin.

Escherichia coli isolated from Sonali were found to be sensitive to Gentamycin and Colistin but resistant to Amoxicillin, Cefixime, Cloxacilin, Chloramphenicol, Erythromycin, Penicillin and Vancomycin. Similar findings were reported by Jeyasanta *et al.,* (2012) and Akond *et al*., (2009).

The *Salmonella sp.* was found to be sensitive to Gentamycin and Cefixime and resistant to Amoxicillin, Colistin, Cloxacilin, Chloramphenicol, Erythromycin, Penicillin and Vancomycin, and this is in line with the findings of (Jawahar, 2011) whose findings were similar with bacterial human pathogens highly sensitive to ciprofloxacin, gentamycin and chloramphenicol.

The *Vibrio spp.* were resistant to Amoxicillin, Cefixime, Colistin, Cloxacilin, Chloramphenicol, Erythromycin and Penicillin but found to be sensitive to Gentamycin and Vancomycin. Among the Gram positive bacteria, *Staphylococcus spp*. was found to be sensitive to Gentamycin and Vancomycin and intermediate sensitive to Colistin. In the present study all the isolated bacteria were resistant against the common using antibiotics and among them only Gentamycin was sensitive. A possible cause of this variation could be due to random use of antibiotic resulting resistant against different antibiotics and rapid chromosomal mutation and the specific plasmid DNA.

The study indicates that, the present antibacterial resistant condition is a great threat to the poultry farmers and consumers because it play an important role to produce various diseases in poultry and human body.

From research interest point of view the following task may be scheduled for future study:

1. Molecular and antigenic characterization of *E. coli. Salmonella spp., Vibrio spp.,* and *Staphylococcus spp.*
2. Serotyping of the isolated *E. coli. Salmonella spp., Vibrio spp.,* and *Staphylococcus spp*. and identification of immunogenic variation.
3. Extraction of toxin and development of vaccine from identified field isolates
4. Rescheduling the management system of poultry and searching the way to produce safe poultry by focusing FDA, OIE standard and "One Health" concept.

CHAPTER 6

SUMMARY AND CONCLUSION

In this study we found that the Sonali chicken (meat) sample of local markets which contain huge amount of pathogenic microorganism especially *E. coli, Salmonella spp., Vibrio spp.* and *Staphylococcus spp*. were isolated from most of the sample. It is clear that the meat samples have not been protected from microbial spoilage during handling, slaughtering, dressing, storage and other operation. Appropriate maintenances need to control microbial contamination. The antibiogram study revealed that the most of the pathogen were found to be resistant to commonly used antibiotics which is very threat full for future human and animal health but only found that all the isolates were sensitive to gentamycin. So it is high time to take necessary attention to maintain the quality of safe meat and meat product during rearing, handling, slaughtering and processing. If proper cooking time and temperature not maintained organism can survive in the cooked meat and caused hazard to the consumers.

From all the findings of this study it may be concluded that-

- The presence of pathogenic microorganism (*E. coli, Salmonella, Vibrio*, and *Staphylococcus*) in the most of the sample is public health concern.
- It also found that the antibiotic resistant is common in every organism it is a big threat and serious health issue.

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APPENDICES

APPENDIX 1

Composition of Different Media

1. Nutrient agar (Hi Media)

Ingredients:	**g/L**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25^{0}C)	7.4 ± 0.2

2. Eosine methylene blue Agar (Hi Media)

Ingredients:	**g/L**
Peptic digest of animal tissue	10
Lactose	5.0
Sucrose	5.0
Dipotassium phosphate	2.0
Eosin - Y	0.40
Methylene blue	0.065
Agar	20.0
Final pH (at 25^{0}C)	7.2 ± 0.2

3. MacConkey agar (Hi-media)

Ingredients:	**g/L**
Peptic digest of animal tissue	17.0
Protease peptone	3.0
Lactose monohydrate	10
Bile salt	1.5
Sodium chloride	5.0
Agar-agar	15.0
Neutral red	0.03
Final pH (at 25^{0}C)	7.1 ± 0.2

4. Thiosulfate-citrate-bile salts-sucrose agar

Ingredients:	**g/L**
Yeast extract	10.0
Protease Peptone	10.0
Sodium thiosulfate	10.0
Sodium citrate	10.0
Ox gall	5.0
Sodium cholate	3.0
Saccharose	20.0
Sodium chloride	10.0
Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15.0

5. Simmon's Citrate Agar

Component	**Amount (g/L)**
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

6. TSI agar (Hi Media)

Ingredients:	**g/L**
Peptic digest of animal tissue	10.00
Casein enzymic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Sodium chloride	5.00

Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	12.00
Final pH (at 25°C)	7.4 ± 0.2

7. MIU medium base (Hi Media)

Ingredients:	**g/L**
Casein enzymic hydrolysate	10.00
Dextrose	1.00
Sodium chloride	5.00
Phenol Red	0.01
Agar	2.00
Final pH (at 25°C)	6.8 ± 0.2

8. MR-VP medium (Hi Media)

Ingredients:	**g/L**
Buffered peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00
Final pH (at 25°C)	6.9 ± 0.2

9. Sugar media

Ingredients:	**g/L**
a. Peptone water	
Bacto-peptone	10.0 gm.
Sodium chloride	5.00 gm.
0.5% phenol red	0. 10 ml
Distilled water	1000 ml
b. Sugar solutions	
Individual sugar	5.00 gm.
Distilled water	100 ml

c. Sugar media preparation

Peptone water	4.50 ml
Sugar solution	0.50 ml

10. Peptone water

Ingredients:	**g/L**
Peptone	1.00 gm.
Distilled water	1000 ml

APPENDIX 2

Preparation of reagents

1. Kovacs reagent

P-dimethyl aminobenzal dehyde	5 gm.
Amyl alcohol	l75 gm.
Conc.HCL	25 ml

2. V-P reagent 1

5% alpha –naptholin absolute ethyl alcohol

3. V-P reagent 2

40%potassium hydroxide containing 0.3 creatine. The ingredients were dissolved by heating gently over steam bath. When in solution add 0.05gm of cotton blue dye.

4. Phosphate buffered solution

Sodium chloride	8 gm.
Disodium hydrogen phosphate	2.8 gm.
Potassium chloride	0.2 gm.
Potassium hydrogen phosphate	0.2 gm.
Distilled water to make	1000 ml

5. Methyl red solution

Methyl red	0.05 gm.
Ethanol (absolute)	28 ml
Distilled water	22 ml

6. Phenol red solution

0.2% aqueous solution of phenol red

7. Potassium hydroxide solution

40% aqueous solution of KOH

8. Gram stain solution

a) Stock crystal violet

Crystal violet	10 gm.
Ethyl alcohol (95%)	1000 ml

b) Stock oxalate solution

Ammonium oxalate	1 gm.
Distilled water	1000 ml

c) Lugoles iodine solution

Iodine crystal	1 gm.
Potassium iodide	2 gm.

d) Ethyl alcohol 250 ml

e) Acetone 250 ml

f) Counterstain

Safranine	2.5 ml
Ethyl alcohol (95%)	100 ml

g) Safranine working solution

The stock Safranine is diluted 1:4 with distilled water.