DETECTION OF POTENTIAL BACTERIAl PATHOGENS AND AFLATOXIGENIC FUNGI PATHOGENS FROM GRAIN SAMPLE

A THESIS

BY

HINDA ABDUKADIR MOHAMED

REGISTRATION NO. 1605581

SEMESTER: JULY-DECEMBER, 2017

SESSION: 2016

MASTER OF SCIENCE (MS)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

DECEMBER, 2017

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Approved as to style and content by

_____	_____
Research Supervisor	**Research Co-supervisor**
Dr. Farzana Afroz	Prof. Dr. Md. Khaled Hossain
Assistant Professor	Chairman
Department of Microbiology	Department of Microbiology
HSTU, Dinajpur	HSTU, Dinajpur

_____

(Prof. Dr. Md. Khaled Hossain)
Chairman
Department of Microbiology and
Examination committee

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

DECEMBER, 2017

DEDICATED

TO

MY BELOVED PARENTS, SISTER AND BROTHERS

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ABSTRACT

The present research work was carried out for the Detection of potential bacterial pathogen and aflatoxigenic fungi *Aspergillus spp.* from grain samples during the period from January to June 2017. A total 25 grain samples comprising {Rice (5), Maize (5), Wheat (5), Khessari dal (5) and Anchora dal (5)} were collected from 3 different local markets of Dinajpur district, Bangladesh. 15 bacterial isolates comprising 4 genara of bacteria were found from a total of 25 samples. The Isolated bacteria were *Staphylococcus spp., Escherichia coli, Klebsiella spp and Salmonella spp*. Among the 25 grain samples, 4 *Staphylococcus spp.,* 7 *Salmonella spp.,* 4 *Escherichia coli* and 4 *Klebsiella spp*. were isolated with 16%, 28%, 16% and 16% prevalence respectively. Antibiogram studies revealed that overall effective drugs against isolated bacteria were ciprofloxacin followed by Gentamycine. But resistant drugs were Penicillin, Vancomycin, Erythromycin, Kanamycin and Amoxicilline. The variation in the sensitivity of common antibiotic could be result of extensive and indiscriminate use of these antibiotics. *Aspergillus spp* was isolated from 4 grain samples with 16% prevalence. But Aflatoxigenic *Aspergillus spp* was isolated from 3 samples with 12% prevalence. From the wheat samples and maize the Aflatoxigenic fungus was isolated and their prevalence in maize, wheat was 40% and 20% respectively. Their early detection can help to take preventive measures to combat economic and health losses. The study showed that earlier detections can be made by simple traditional identifications using macro and micro morphological fungal features rather than adopting the time and cost consuming molecular identification techniques.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	: Negative
%	: Percentage
@	: At the rate of
+	: Positive
<	: Less than
>	: Greater than
µg	: Microgram
µl	: Microliter
^{0}C	: Degree of Celsius
A	: Acid
BD	: Bangladesh
CFM	: Cefixime
CIP	: Ciprofloxacin
CN	: Cefalexin
CXM	: Cefuroxime sodium
E	: Erythromycin
EMB	: Eosin Methylene Blue
et al.	: Associated
etc	: Etcetera
GEN	: Gentamycin
Gm	: Gram
H_20_2	: Hydrogen peroxide
H_2S	: Hydrogen sulfide
HSTU	: Hajee Mohammad Danesh Science and Technology University
i.e.	: That is
Interp	: Interpretation
K	: Alkaline reaction
K	: Kanamycin
Lid	: Limited
M. S	: Master of Science

LIST OF ABBREVIATIONS AND SYMBOLS (Contd.)

MC	: MacConkey Agar
MIU	: Motility Indole Urease
Ml	: Milliliter
mm	: Millimeter
MR	: Methyi Red
MSA	: Manito Salt Agar
N	: Neomycin
NA	: Nutrient Agar
NB	: Nutrient Broth
No.	: Number
PBS	: Phosphate Buffer Saline
PDA	: Potato Dextrose Agar
Prof	: Professor
PSS	: Physiological Saline Solution
RPM	: Rotation Per Minute
SDA	: Sabroud Dextrose Agar
SE	: Standard Error
SL	: Serial number
Spp	: Species
SSA	: *Salmonella Shigella* Agar
v/v	: Volume by volume
VP	: Voges-Proskauer
w/v	: Weight by volume
YEP	: Yeast Extract Powder

CHAPTER I

INTRODUCTION

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INTRODUCTION

Cereals and cereal products are significant and important human food resources and livestock feeds worldwide. The main cereal grains used for foods include corn (maize), wheat, barley, rice, oats, rye, millet, and sorghum. Soybeans are not a cereal product, but rather, are legumes or a pulse, but are often considered with cereals because of their importance as a food source. Examples of cereal products derived from cereal grains include wheat, rye, and oat flours and semolina, cornmeal, corn grits, dough's, breads, breakfast cereals, pasta, snack foods, dry mixes, cakes, pastries, and tortillas. In addition, cereal products are used as ingredients in numerous products, such as batters and coatings, thickeners and sweeteners, processed meats, infant foods, confectionary products, and beverages such as beer.

High production of crops requires using high quality of seed. Contamination of agricultural products, cereals and oil seeds in particular are the main sources of diseases in the human and animal food chains. Cereal crops can potentially become contaminated with pathogenic micro-organisms during growth and at the time of harvesting. There is small but unquantified potential risk of transfer of enteric pathogens to humans and animals from contaminated plants and grains. The quality and safety of agricultural product are of major concern due to increasing occurrence of chronic diseases associated with consumption of contamination.

Fungi are a major cause of spoilage in stored food grain. *Aspergillus* is a saprophytic fungus ubiquitously present in the environment. It is a mycotoxin producing fungus that predominately infects food grains. Preharvest contamination of food commodities by toxigenic fungi results in production of mycotoxins under favorable conditions and these organisms can be carried over into and persist during storage of the commodity.

There are two important groups of fungi: field fungi and storage fungi. First one are those that invade the seeds while the seccond is still in the field and require high moisture conditions (20-21%) (CAST, 2003).

Field fungi may affect the appearance and quality of seed or grain. Usually damage caused by field fungi occurs before harvest, can be detected by routine inspection and does not continue to increase in storage if grain is stored at the proper moisture content and temperature. Most field fungi are more prevalent when rainfall is above normal during grain fill and harvest. Invasion by field fungi may be more severe is the crop has been damaged by insects, birds or hail. With corn, ears well covered by husks and maturing in a downwards position usually have less rot than ears with open husks or ears maturing in an upright position.

Storage fungi (also called storage molds) are fungi which invade grains or seeds during storage. Storage fungi are usually not present to any serious extent before harvest. Small quantities of spores of storage fungi may be present on grain going into storage or may be present on spilled grain present in harvest, handling and storage equipment or structures. Under improper storage conditions this small amount of inoculum can increase rapidly leading to significant problems. The development of storage fungi in stored grain is influenced by the moisture content of the stored grain, the temperature of the stored grain, the condition of the grain going into storage, the length of time the is grain stored and the amount of insect and mite activity in the grain. The most common storage fungi are species of *Aspergillus* and *Penicillium.* These fungi are widely distributed and almost always present.

Fungi produce mycotoxins under stressful conditions such as temperature, moisture or aeration. There are some conditions which influence the fungal growth such as: species, a high temperature and moisture. Fungi grow at temperatures between 20–30oC. Normally, fungi grow in storage conditions at a13–18% moisture (Novošinskas H. *et al.,* 2005).

Amongst the fungal genera, Fusarium and Alternaria are considered most important because of their toxigenic ability to produce mycotoxins and they are classified by some authors as field fungi, while, Aspergillus and Penicillium species are often considered storage fungi (Roige M. et al, 2009).

Fumonisin, aflatoxin, ochratoxin, zearalenone and trichothecenes such as deoxynivalenol, T-2 toxin and nivalenol are appreciate as most important mycotoxins (Shepard G. S., 2008).

A variety species of microorganisms often contaminate the wheat grains before harvesting or after harvesting and other species may develop when grains are in silo conditions. In all cases they decrease quality of wheat (Magan, N., *et al.,* 2003).

There are a lot of factors that affects wheat grains health, but from all of them, the most important are fungi. Some of their effects on wheat grains consists in fact that they reduces seed germination and vigor and cause decrease quality during storage (Doohan, F.M., *et al.,* 2003).

Maize (*Zea mays* L.) is one main cereal crop that has been broadly cultivated worldwide for human consumption and animal feed. However, maize is susceptible to be infected with toxigenic fungi, such as Fusarium spp. (*F. verticillioides, Gibberella moniliformis, F. graminearum*) and Aspergillus spp. (*A. Niger, A. flavus, A. parasiticus*), in the field and/or post-harvest conditions. Different toxigenic fungi can produce relevant toxic metabolites, for example, aflatoxins by *A. flavus and A. parasiticus, ochratoxins by A. Niger, fumonisins by Fusarium spp.* Among the various toxins, aflatoxin is highly toxic and carcinogenic to animals and humans.

Therefore, there has been a mass of studies focusing on detection of aflatoxins and fungi on maize to prevent health threats to people resulting from consumption of infected kernels. Traditional methods to detect fungal contamination on cereals are microbiological methods and diagnostic media for toxigenic fungi identification, and immunological methods for toxin detection.
So earlier detection of aflatoxigenic fungi can be made by simple traditional identifications using macro and micro morphological fungal features rather than adopting the time and cost consuming molecular identification techniques.

Bearing in mind the above facts the present study was undertaken with following specific objectives:

i. Isolation and identification of potential bacteria of aflatoxigenic fungus from grain sample.
ii. Isolation and identification of aflatoxigenic fungus from grain sample.
iii. Determination of antibiotic resistant pattern of isolated bacteria.

CHAPTER II

REVIEW OF LITERATURES

CHAPTER II

REVIEW OF LITERATURES

Maize is the third cereal crop in the world as well as Bangladesh. A good number of literatures are available on seed storage of many other cereal but very few literatures are found in case of maize. Literature related to the effect of temperature, humidity and moisture content on germination, vigor and insect infestation, role of insect infestation on seed germination, their interaction and storage condition have been reviewed below.

Maryam *et al* (2017) reported A total of 10 species of fungi belonging to 5 genera were isolated and identified from four cereal grains; wheat, barley, rice and maize collected from three Libyan cities known to grain producers (Al-Zawia, Subratah and Tripoli) on 1% dextrose-Czapkes agar medium at 28 ± 2°C for 7-15 days using seed-plate method. Two species of *Alternaria* (*A. raphani* and *A.tenusinae*); two species of *Aspergillus* (*A. flavus* and *A. Niger*); three *Fusarium* (*F. graminearum*, *F. moniliforme* and *F. solani*); one *Rhizopus* species (*Rhizopus stolonifer*) and two species of *Penicillium* (*P. digitatum* and *P. notatum*) were isolated from the grains. The densities of these fungi and their frequencies of occurrence have been investigated. It can be concluded that for human public health, cereal grains of production chain must be subjected to quality control and microbiological examinations.

Xin *et al.* (2017) Fungi infection in maize kernels is a major concern worldwide due to its toxic metabolites such as mycotoxins, thus it is necessary to develop appropriate techniques for early detection of fungi infection in maize kernels. Thirty-six sterilised maize kernels were inoculated each day with Aspergillus parasiticus from one to seven days, and then seven groups (D1, D2, D3, D4, D5, D6 and D7) were determined based on the incubated time. Another 36 sterilised kernels without inoculation with fungi were taken as control (DC). Hyperspectral images of all kernels were acquired within spectral range of 921–2529 nm. Background, labels and bad pixels were removed using principal component analysis (PCA) and masking. Separability computation for discrimination of fungal contamination levels indicated that the model based on the data of the germ region of individual kernels performed more effectively than on that of the whole kernels.

Fatih *et al.* (2016) conducted a study aimed to review fungal mycotoxins in foods, their roles and significance in human nutrition and health. This paper provided comprehensive information on the mycological quality and mycotoxin safety of foods. The review showed that moulds are multicellular fungi that form thin thread like structures called hyphae. They are widely distributed and found wherever moisture is present with adequate nutrients that can sustain their growth. Fungi are major spoilage of foods and feedstuffs.

Yongna *et al.* (2015) stated that the Probiotic" refers to viable microorganisms that promote or support a beneficial balance of the autochthonous microbial population of the gut. In the present study, we isolated and identified one potential probiotic of *Lactococcus lactis* WH-C1 from Tibetan Kefir grain. We assessed its probiotic and fermentation properties. WH-C1 showed good tolerance in the condition of high bile salt concentration and acidity. Moreover, WH-C1 could remove cholesterol from the culture medium at a percentage of 31.23% during 24 h and it had high capability of exopolysaccharide production. As part of starter culture it gave the better quality yogurt with good mouth feeling and a great viscosity. The solid-phase microextraction-gas chromatography-mass spectrometry analysis showed that fermented milk had 25 kinds of flavor compounds including acids, esters, alcohols, carbonyls, diacetyl, etc. In conclusion, our results showed that WH-C1 is a very promising strain which can be used in various fields.

Hossain *et al.* (2015) described one of the most important ubiquitous fungal species in tropical environments is *Aspergillus flavus* that can be found in soil and other substrates (Powell *et al*. 1994). *Aspergillus flavus* is reported to be associated with many diseases of human, most severe of which is invasive aspergillosis. It can also cause diseases in insects (Campbell, 1994) as well as in crops (such as maize, rice, peanuts etc.). Agricultural products including cereals e.g. maize, wheat, sorghum and by products thereof and variety of oilseeds are major constituents of poultry feed (Okoli *et al*. 2006).

Pruthviraj *et al.* (2015) In India, Maharashtra state is popular for the fermented wheat product especially kurdai and popdum. The aim of this study to optimize the biochemical activity of wheat fermentation to achieve improved the flavor, texture and shelf- life and to determine how the bacteria are responsible for fermentation of wheat and to check the prevalence of different microflora from the procedure of steeping and fermentation,

attempts were made to isolate fermenting microorganisms. The wheat grains were soaked in water in 1:2 proportions and its slurry allowed fermenting. From this, the bacteria especially lactic acid bacteria were tried for its isolation morphological and biochemical characterization. The total 37 lactic acid bacterial species from fermentation batter were isolated. Out of 37 isolates, 28 were identified. Among these isolates 24.57% of isolates found to be P. pentosaceus, 24.57% L. plantarum, 21.42% L. brevis, 14.28% L. lactis, 7.14% L. fermentum. At this preliminary study, it can be at least seen clearly that P. pentosaceous prevails dominantly during fermentation of steeped wheat grains

Peter *et al*. (2014) described Aflatoxins are toxic carcinogenic secondary metabolites produced predominantly by two fungal species: Aspergillus flavus and Aspergillus parasiticus. These fungal species are contaminants of foodstuff as well as feeds and are responsible for aflatoxin contamination of these agro products. The toxicity and potency of aflatoxins make them the primary health hazard as well as responsible for losses associated with contaminations of processed foods and feeds. Determination of aflatoxins concentration in food stuff and feeds is thus very important. However, due to their low concentration in foods and feedstuff, analytical methods for detection and quantification of aflatoxins have to be specific, sensitive, and simple to carry out. Several methods including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), mass spectroscopy, enzyme-linked immune-sorbent assay (ELISA), and electrochemical immunosensor, among others, have been described for detecting and quantifying aflatoxins in foods.

Emanuela *et al.* (2014) *Zea mays* is one of the most cultivated grain crop. The yield, quality and safety of its kernels are continuously challenged by pathogens. Amongst these, the mycotoxigenic fungi are actually the most health hazardous. *A. flavus* under suitable conditions infect maize kernels both during development in the field as well as during the storage (Sheidegger and Payne, 2003). In most cases *A. flavus* produces the harmful and carcinogenic aflatoxins, among which B1 is considered by IARC (International Agency for Research on Cancer) as belonging to group 1, i.e., carcinogenic to humans and animals. Seed composition, notably lipid composition, may affect susceptibility to fungal infection and mycotoxins production (Reddy *et al.,* 1992; Dall'Asta *et al.,* 2012).

Renter *et al.* (2014) stated that distiller's grains, a coproduct of ethanol production from cereal grains, are composed principally of the bran, protein, and germ fractions and are commonly supplemented in ruminant diets. The objective of this study was to assess the effect of feeding wet distillers grains with solubles (WDGS) and monensin and tylosin on the prevalence and antimicrobial susceptibilities of fecal foodborne and commensal bacteria in feedlot cattle. Cattle were fed 0 or 25% WDGS in steam-flaked corn-based diets with the addition of no antimicrobials, monensin, or monensin and tylosin. Fecal samples were collected from each animal (n = 370) on d 122 and 136 of the 150-d finishing period and cultured for *Escherichia coli* O157.

Yuyan *et al.* (2014) found this study is to investigate the possibility of zinc (Zn) biofortification in the grains of rice (*Oryza sativa* L.) by inoculation of endophytic strains isolated from a Zn hyper accumulator, *Sedum alfredii* Hance. Five endophytic strains, *Burkholderia sp.* SaZR4, *Burkholderia sp.* SaMR10, *Sphingomonas sp.* SaMR12, *Variovorax sp*. SaNR1, and *Enterobacter sp*. SaCS20, isolated from *S. alfredii*, were inoculated in the roots of Japonica rice Nipponbare under hydroponic condition. Fluorescence images showed that endophytic strains successfully colonized rice roots after 72 h. Improved root morphology and plant growth of rice was observed after inoculation with endophytic strains especially SaMR12 and SaCS20. Under hydroponic conditions, endophytic inoculation with SaMR12 and SaCS20 increased Zn concentration by 44.4% and 51.1% in shoots, and by 73.6% and 83.4% in roots, respectively. Under soil conditions, endophytic inoculation with SaMR12 and SaCS20 resulted in an increase of grain yields and elevated Zn concentrations by 20.3% and 21.9% in brown rice and by 13.7% and 11.2% in polished rice, respectively. After inoculation of SaMR12 and SaCS20, rhizosphere soils of rice plants contained higher concentration of DTPA-Zn by 10.4% and 20.6%, respectively. *In situ* micro-X-ray fluorescence mapping of Zn confirmed the elevated Zn content in the rhizosphere zone of rice treated with SaMR12 as compared with the control.

Camelia *et al.* (2014) found the purpose of this study was to identify mycobiota and mycotoxins content of wheat grains. For this purpose, 15 samples were collected from wheat grains depositing in several storages from Bihor County, during period October - March. Analyses were done using the conventional methods, on fungal species isolated of Aspergillus and Fusarium, in order to determine their mycotoxigenic potential, while mycotoxins analysis for aflatoxin B1, ochratoxin A and zearalenone were assessed using

thin layer chromatography and high performance liquid chromatography. Two different genera including Fusarium and Aspergillus were isolated from wheat samples. Of the fungal spp. isolated, Aspergillus was the most predominant and was succeeded by species of Fusarium. Two dominant species from Aspergillus genus: Aspergillus ochraceus and Aspergillus flavus were isolated from the wheat samples. Mycotoxins were identified and quantified with HPLC and TLC techiques.

Schwarz *et al.* (2013) stated that cereal crops grown in the biosolids-amended soil can potentially become contaminated with pathogenic micro-organisms during growth and at the time of harvesting. There is small but unquantified potential risk of transfer of enteric pathogens to humans and animals from contaminated plants and grains. This study examined decay of *Escherichia coli, Salmonella enterica serovar Typhimurium* and bacteriophage MS2 on the wheat phyllosphere and on stored grains. This was done to assess the health implications of cereal crops contaminated from land application of biosolids. *E. coli*, S. enterica and MS2 were inoculated onto the leaves, spikelets and grains of wheat. The change in the numbers of inoculated micro-organisms was determined over time to calculate the respective 90% reduction time (T90) in each of these environments. A rapid inactivation (T90 <1–3 days) of *E. coli* and *S. enterica* and MS2 from the plant phyllosphere was observed, particularly from the spikelets. The decay rates were influenced by micro-organism type and location on the plant phyllosphere. Decay times on the stored grains were longer (T90 9–71 days), with some observed influence of grain variety on pathogen decay times.

Detlev *et al.* (2011) fungal infestation on wheat is an increasingly grave nutritional problem in many countries worldwide. *Fusarium* species are especially harmful pathogens due to their toxic metabolites. In this work we studied volatile compounds released by *F. cerealis, F. graminearum, F. culmorum*and *F. redolens* using SPME-GC/MS. By using an electronic nose we were able to differentiate between infected and non-infected wheat grains in the post-harvest chain. Our electronic nose was capable of distinguishing between four wheat Fusaria species with accuracy higher than 80%.

Qinghaihu *et al.* (2011) stated that the three pathogens, *Riemerella anatipestifer*, *Escherichia coli*, and *Salmonella enterica*, are leading causes of bacterial fibrinous pericarditis and perihepatitis in ducks in China and worldwide. It is difficult to differentiate these pathogens when obtaining a diagnosis on clinical signs and

pathological changes. The aim of this research was to develop a multiplex polymerase chain reaction (m-PCR) that could discriminate *R. anatipestifer*, *E. coli*, and *S. enterica* rapidly in field isolates, or detect the three bacteria in clinical samples from diseased ducks. We selected the DnaB helicase (*dnaB*) gene of *R. anatipestifer*, alkaline phosphatase (*phoA*) gene of *E. coli* and invasion protein (*invA*) gene of *S. enterica* as target genes. In optimized conditions, the limitation of detection was approximately 10^3 colony forming units (CFU) of each of these three bacterial pathogens per PCR reaction tube.

Beccari *et al.* (2011) Surveys were carried out in 2006 and 2007 in Umbria (central Italy) to evaluate the presence of mycotoxigenic fungi and mycotoxins in maize grain sampled at harvest. *Fusarium* spp., were the most abundant species detected in maize kernels, followed by *Aspergillus* species of sections *Flavi* and *Nigri* and by *Penicillium* spp. Among *Fusarium* species, *F. verticillioides* was the most prevalent species, as detected by PCR directly on the kernels and on the fungi isolated from the kernels, followed by *F. proliferatum* and *F. subglutinans*. Fumonisins were the predominant mycotoxins with values, on average, of 4.3 and 5.7 mg kg^{-1}, in 2006 and 2007, respectively, with a maximum of 76.3 mg kg^{-1} in the second year. Deoxynivalenol ranged from 0.2 to 3.98 mg kg^{-1} in 2006 (average 1.04 mg kg^{-1}) and from undetectable levels to 14 mg kg^{-1} in 2007 (average 0.86 mg kg^{-1}). Aflatoxins, analyzed only in 2007, averaged 26.3 μg kg^{-1}, with a maximum of 820 μg kg^{-1}. Zearalenone content was always very low. Results indicate that EU legal limits for these mycotoxins were rarely exceeded with low levels across most of the examined area, suggesting that this region could be considered suitable for the production of healthy maize.

Correa *et al.* (2010) stated that the effect of storage on mycoflora profile was monitored bimonthly in 36 corn (*Zea mays* L.) samples, dividing the same sample into groups dried to 11 and 14% moisture content (1008 analysis). These groups were further subdivided based on the initial total count (moulds and yeasts) up to 10^4 CFU g^{-1} (12 samples, range 1.6×10^4 to 9.0×10^4, mean 3.8×10^4 CFU g^{-1}) and up to 10^5 CFU g^{-1} (24 samples, range 1.0×10^5 to 5.0×10^5, mean 2.7×10^5 CFU g^{-1}). In the corn group dried to 11%, the fumonisin content was analysed at the initial stage (freshly harvested) and at the end of 12-month storage. *Fusarium* spp. and Penicillium spp.

Matilda *et al.* (2010) described Preservation of moist crimped cereal grain is made feasible through fermentation by lactic acid bacteria. Climatic variations make it difficult to harvest at moisture contents (0.30–0.45 g/g) to support optimal fermentation under practical conditions. Therefore, the yeast, *Pichia anomala* J121, previously found to prevent mould spoilage and improve preservation of moist grain in malfunctioning airtight silos, was added to moist crimped cereal grain stored in large plastic tubes.

Amadi *et al.* (2009) found a study was conducted to determine the fungi associated with maize (Zea mays), rice (Oryza sativa) and millet (Pennisetum typhoiodes) in storage. Mycotoxin production by isolated fungi was subsequently evaluated using the thin layer chromatography technique. Eight different fungi were isolated altogether namely Aspergillus terreus, Aspergillus flavus, Aspergillus Niger, Aspergillus oryzae, Penicillium italicum, Penicillium spinulosum, Rhizopus stolonifer and Fusarium sp. The results of this study show that all the fungi produced one toxin or the other as detected in the culture filtrates of isolated fungi.

Germini *et al.* (2009) stated that the wide application of nucleic acid amplification techniques and the increasing industrial interest toward rapid methods has led to the development and application of PCR based methods for the detection of microbial pathogens in food. In the present paper we describe the development of a multiplex PCR method for simultaneous detection of *Salmonella enterica* serovar *Typhimurium*, *Listeria monocytogenes* and *Escherichia coli* O157:H7 in a complex food matrix (liquid whole egg).

Four different DNA extraction procedures were evaluated for their application on food and, among these, Chelex resin combined with a DNA purification step were found to better perform on the food system considered.

Chiachen *et al.* (2008) reported Lactic acid bacteria (LAB) in different original kefir grains were first assessed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) by a culture-dependent way, and were further confirmed by DNA sequencing techniques. Results indicated that a combined method of cultivation with PCR-DGGE and subsequent DNA sequencing could successfully identify four LAB strains from three kefir grains from Taiwan (named Hsinchu, Mongolia and Ilan). *Lactobacillus kefiri* accounted, in the three kefir grains, for at least half of the isolated colonies while *Lb. kefiranofaciens* was the second most frequently isolated

species. *Leuconostoc mesenteroides* was less frequently found but still in the three kefir grains conversely to *Lactococcus lactis* which based on culture-dependent isolation was only found in two of the kefir grains.

Karina *et al.* (2008) reported novel qualitative as well as Semi quantitative rapid strip tests for screening of T-2 mycotoxin in agricultural commodities were developed. Colloidal gold particles were coated with monoclonal anti-T-2 antibodies and used as detector reagent, indicating the strip test results by formation of up to two colored lines in a competitive assay format. The test line comprises a protein conjugate of the T-2 mycotoxin and the control line an antispecies-specific antibody to confirm the correct test development. To perform the test, 5 g of sample was extracted in a ratio of 1:5 with methanol/water (70:30) by shaking for 3 min and the extract directly used without further cleanup steps. The T-2 toxin lateral flow device (LFD) presented has a cutoff level around 100 µg/kg for naturally contaminated wheat and oat.

ZhuBoLou *et al.* (2008) stated that the survey on isolation and detection of the casual organism of bacterial grain rot of rice was conducted during 1997–2006. In 2006, six pathogenic bacterial strains were isolated from two symptomless seed samples of rice (*Oryza sativa* L.) originally produced in Hainan Province and then planted in Zhejiang Province, China. They were identified as *Burkholderia glumae* which is the causal organism of bacterial grain rot of rice by physiological characteristics, colony morphology, pathogenicity test, Biolog, fatty acid methyl ester (FAME) analysis and RAPD-PCR compared with the four standard reference strains. It is confirmed that there is the infection of *B. glumae* in so-called 'health looking seeds.

Neethirajan *et al.* (2007) stated that the cereal grains are the major source of food for humans and most domesticated animals. In many developing countries, overall post-harvest losses of cereals and legumes of about 10–15% are fairly common. Consumption of cereals and legumes by pests such as insects during storage and microbial spoilage or contamination may make these totally inedible. On farms, manual samples, traps, and probes have been used to determine the presence of insects. Manual inspection, sieving, cracking-floatation and Berlese funnels are being used at present to detect insects in grain handling facilities. These methods are not efficient and are time consuming.

David *et al.* (2007) described contamination of cereal commodities by moulds and mycotoxins results in dry matter, quality, and nutritional losses and represents a

significant hazard to the food chain. Most grain is harvested, dried and then stored on farm or in silos for medium/long term storage. Cereal quality is influenced by a range of interacting abiotic and biotic factors. In the so-called stored grain ecosystem, factors include grain and contaminant mould respiration, insect pests, rodents and the key environmental factors of temperature, water availability and intergranular gas composition, and preservatives which are added to conserve moist grain for animal feed.

Maciorowski *et al.* (2007) reported animal feed may serve as a carrier for a wide variety of microorganisms. The primary mode of inoculation of feed materials is the transference of soil by wind, rain, mechanical agitation, or insects to standing crops. Some of the microorganisms are adapted to the desiccated and relatively nutrient-poor conditions in soil and survive in similar niches on growing crops. Gastrointestinal pathogens can also introduce into the food chain by animals defecating in the farm environment or by fertilization of crops with manures. Other microorganisms are introduced during storage. In general, the amount of available water in the feed matrix determines whether a microorganism will grow or survive. Some microorganisms, primarily moulds, are adapted to the low amount of available moisture and grow actively within stored seeds and grains.

Jongsookim *et al.* (2006) suggested multiplex PCR procedure was established to detect *Escherichia coli, Listeria monocytogenes and Salmonella typhimurium* in artificially inoculated wheat grain. The PCR protocol with an enrichment step successfully detected all three organisms inoculated together in non-autoclaved wheat grain. After a one day enrichment, *E. coli*, *L. monocytogenes* and *S. typhimurium* were detected at levels of 56, 1800 and < 54 CFU/mL, respectively, in the initial sample. For *L. monocytogenes*, an improved detection limit of < 62 CFU/mL was achieved using single plex PCR. For autoclaved wheat grain inoculated with the three bacterial strains individually, a detection limit of 3 CFU/mL was achieved after an enrichment step. The ability to test for the three bacteria simultaneously will save time and increase the ability to assure grain quality.

Adriano *et al.* (2006) stated that fungal growth on cereal grains decreases their nutritional value and constitutes health hazards, probably, because of the production of toxic metabolites (mycotoxins). Therefore, attempts are coming out to detect and quantify the degree of fungal infection at the early stage of mold infection. One of the

most promising techniques is the analysis of volatile compounds in the headspace gas surrounding the samples.

The aim of this work was to study the possibility of the application of electronic nose for an early detection of volatile compounds in infected samples and to discriminate between non-infected and infected samples with two different species of fungi (Penicillium chrysogenum and Fusarium verticillioides).

Zuoxinliu et al.(2006) stated that Aflatoxin contamination and its relationship to storage length in stored maize and rice in Liaoning Province, northeastern China, was investigated. Aflatoxins in 110 samples collected from an area of 14.68 million km^2 covering storage length from 1 yr to over 10 yr were determined by high-performance liquid chromatography with fluorescence detection. The results showed that almost all samples collected contained aflatoxins. The average contents in maize, whole grain rice and brown rice were found to be 0.99, 3.87 and 0.88 $\mu g\ kg^{-1}$, respectively. Three-fourths of the total aflatoxins in whole grain rice (3.87 $\mu g\ kg^{-1}$) could be removed by dehusking to as low as 0.88 $\mu g\ kg^{-1}$ in brown

Nayak *et al.* (2004) stated that rice harvested 18-30 days after full stigmatic stage. The moisture content of the physiologically mature grains was arounded 26% and the physiologically maturity was accompanied by the black region appearance arounded 24 days after full stigmatic stage, which served as a good morphological criterion for harvesting. Germination percentage of freshly harvested grains increased with delay in harvest from 1% at the first harvesting date to 28% at the last date. The low levels were due to dormancy. Germination after 8 months storage was highest (83-85%) with harvest 24, 27 or 30 days after full stigmatic stage.

Christopher *et al.* (2003) this is the first report of filamentous actinobacteria isolated from surface-sterilized root tissues of healthy wheat plants (*Triticum aestivum* L.). Wheat roots from a range of sites across South Australia were used as the source material for the isolation of the endophytic actinobacteria. Roots were surface-sterilized by using ethanol and sodium hypochlorite prior to the isolation of the actinobacteria. Forty-nine of these isolates were identified by using 16S ribosomal DNA (rDNA) sequencing and found to belong to a small group of actinobacterial genera including *Streptomyces*, *Microbispora*, *Micromonospora*, and *Nocardiodes* spp. Many of the *Streptomyces* spp.

was found to be similar, on the basis of their 16S rDNA gene sequence, to *Streptomyces* spp.

David *et al.* (2003) showed that the grain quality after harvest is influenced by a wide variety of abiotic and biotic factors and has been studied as a stored grain ecosystem. Important factors include grain and contaminant mould respiration, insects and mites, and the key environmental factors of water availability and temperature. Interactions between these factors influence the dominance of fungi, particularly mycotoxigenic species. Studies have shown that growth, mycotoxin production, competitiveness and niche occupation by mycotoxigenic species are influenced by the presence of other contaminant moulds and environmental factors.

Abraham *et al.* (2001) reported chemical and microbiological composition of four Argentinean kefir grains from different sources as well as characteristics of the corresponding fermented milk was studied. Kefir grains CIDCA AGK1, AGK2 and AGK4 did not show significant differences in their chemical and microbiological composition. In contrast, protein and yeast content of AGK3 was higher than in the other grains. Although grain microflora comprised lactobacilli, lactococcus, acetic acid bacteria and yeast, we found an important difference regarding species. *Lactococcus lactis* subsp. *lactis*, *Lactobacillus kefir*, *Lactobacillus plantarum*, *Acetobacter* and *Saccharomyces* were present in all types of kefir grain.

Magan *et al.* (2000) stated that there is significant interest in methods for the early detection of quality changes in cereal grains. The development of electronic nose technology in recent years has stimulated interest in the use of characteristic volatiles and odours as a rapid, early indication of deterioration in grain quality. This review details the current status of this area of research. The range of volatiles produced by spoilage fungi in vitro and on grain are described, and the key volatile groups indicative of spoilage are identified. The relationship between current grain quality descriptors and the general classes of off-odours as defined in the literature, e.g. sour, musty, are not very accurate and the possible correlation between these for wheat, maize and other cereals, and volatiles are detailed.

Petersson *et al.* (1999) stated that *Pichia anomala* is antagonistic against a range of spoilage molds in vitro as well as against *Penicillium roqueforti* in high-moisture wheat during malfunctioning airtight storage in laboratory experiments. The use of *Pichia*

anomala to improve the postharvest control of *Penicillium roqueforti* during airtight storage of feed grain was evaluated in outdoor silos. Inoculated and control winter wheat (cultivar Kosack) in 160-kg portions were stored at a water activity of 0.93 for 12 months in silos that were opened twice a week. During the first 2 months, inoculated *Pichia anomala* increased to about 10^7 colony-forming units (CFU)/g, while naturally occurring *Pichia anomala* in the treatments without inoculated yeast increased from 10^4 to 10^6 CFU/g.

Arpadbata et al. (1999) Different physical and chemical methods have been recommended for detoxification of mycotoxin-contaminated food and feed. Nevertheless, only a few of them (e.g. destruction of aflatoxin by ammonia treatment) have been accepted for practical use. Many specialists are of the opinion that the best approach for decontamination should be degradation by selected microorganisms, giving a possibility of removal of mycotoxins under mild conditions, without using harmful chemicals and without significant losses in nutritive value and the palatibility of detoxified food or feed. The present state of research in this field and the perspectives of such procedures are reviewed.

Francisco *et al.* (1998) described that the gastric stomach of human is a barrier to food-born pathogen, but *Escherichia coli* but can survive at pH 2.0 if it is grown under mildly acidic conditions. Cattle are a natural reservoir for pathogenic *E. coli*, and cattle fed mostly grain had lower colonic pH and more acid-resistant *E. coli* than cattle fed only hay. On the basis of numbers and survival after acid shock, cattle that were fed grain had 10^6-fold more acid-resistant *E. coli* than cattle fed hay, but a brief period of hay feeding decreased the acid-resistant count substantially.

Ansari et al (1996) stated that the highest germination of Rice, Mustard, and Jute seed was found when stored in tin pot. Percentage of germination for Jute, Mustard, and Wheat stored in different container were below the recommended germination standard. Polythene bag was good for retaining higher seed germination in wheat.

Kurdikeri *et al.* (1996) reported that seeds of 5 maize hybrids were stored at 9-10% moisture in cloth or polythene bags for 3-4 months. Viability interims of certification standard was maintained for 11,3,7,13 and 3 months in cloth bags in cv. Deccan, Ddeccan -103 Gonga-5, Ganga safed-2 and MMh-6 respectively. Corresponding value

for polythene bags were 13, 13, 13, 17 and 5 months respectively. Seed vigor index was higher in seeds stored in polythene bags than cloth bag.

Angela *et al.* (1995) Sixty nine samples of maize were collected from pre-harvest standing crops and on-farm storage facilities from 52 smallholder farms located within 4 regions of Honduras during October 1992 and November 1993. Samples were visually assessed for insect damage and fungal spoilage, and the mycoflora quantified on artificial media. The major components of the ear rot complex were *Fusarium moniliforme, F. moniliforme* var. *subglutinans, Penicillium* species, *Stenocarpella maydis, S. macrospora* and *Acremonium* spp.

Kurdikeri *et al.* (1994) reported revealed that percentage seed damage and loss in weight increased with the increase in storage period in all the maize hybrids, while the viability of seeds decreased.

Wang *et al.* (1990) stated that worked on summer storage of maize seed. They stored the seeds maize hybrid Danya (12.1-17% moisture content) at 20-30^{o}C to determine the optimum storage condition. The regration equation between seed moisture contents, storage temp. and storage days to achieve an 85% germination rate is presented. Max, and ideal moisture contents or seed storage in summer were 14and <13%, resp. Sealed storage at natural low temperature in spring slowed down the aging rate of maize seeds and offset the effect of high temperature. Hybird and initial germination rates were also factors affecting summer storage of maize.

Narkiewicz *et al.* (1990) observed effect of storage condition on sowing value and health of winter triticale seed. They harvested the seeds in 1981-82 and stored for 3years in (a) linen bag sat 40,60 and 70% RH and variable temperatures of 11.0-30.0^{o}C and (b) in air tight containers at the variable temperatures with seed moisture content of 11.0-11.7%. Germination percentage detracted in (a) at 70% RH and fell to 30-80% depending on seed lots at 60% RH but remained close to the initial levels of 88-97% for 3 years in (b) and in (a) at 40% RH.

Wallbridge *et al.* (1988) Post-harvest yellowing of rice was monitored during a research storage trial in Indonesia in which bulk rough rice (2283 tonnes) was dried by aeration with ambient air and stored over a period of a year. Yellowing and moisture content were measured in rice samples from different depths at intervals throughout the year. A test

for determining the yellowing potential of rice was applied, following observation of the effect of heat on certain mould contaminated grains. Yellowing increased from 0–0.5% up to 4.5–5.5% when the grain was dry, at moisture contents of less than 14%. This was related to earlier mould growth before and during the drying period, particularly in the upper layers of grain which had taken longer to dry.

Loschiavo *et al.* (1985) Post-harvest grain temperature and moisture were consistently higher at the center than at the edge roof openings of steel granaries in Manitoba in 1982 and 1983. In 1982, temperatures at the center were significantly higher in infested than in uninfected granaries and declined only slowly during a 2-week period. Several kinds of insects were found in grain samples or in beetle-detection traps inserted into grain at the center location but none was found in grain or in traps at the edge. The heaviest infestations coincided with the temperatures and moistures at the center. In 1983, initial temperatures at the center and edge roof openings were high when the granaries were filled but declined rapidly, particularly at the edge, during the next 2 months. Between late August when the granaries were filled and early November, moisture content increased at the center and edge roof openings. It was significantly greater at the surface than at a depth of 20–130 cm. Insects were found in traps inserted 1 m into the grain at the center location. The results indicate that the most likely place to find insects in filled granaries is at the center near the surface and the most opportune time to begin checking the grain is 2 months after the granaries have been filled.

Xzim *et al.* (1961) observed stored seeds of rice in sealed cans at four different moisture content-low, medium, high medium and high, which were, respectively, 15-17.9, 18-20.9, 21-33.9 and 24 percent at temperatures of 100, 90, 80, 60 and 50^{O}F. He found that over a 100 day storage period the rice at medium and low moisture contents stored at the lower temperature decline only in germination during the storage period, whereas, rice with high medium and high moisture content at the three highest temperatures showed some decline in viability oven on the third day of storage. A similar study conducted by Houston et al. (1959) with rice at 12.0, 13.1, 14.1 and 15.9 precent moisture in friction top cans for three years at $30^{0,}$ 20^{0} and 20^{0}F. Showed that storage was satisfactory at moisture levels at -20^{o}F. Appreciable losses on viability occurred in the three wettest samples at 30^{O}F. And in the very highest moisture sample at 20^{o}F.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The present research work was conducted during the period from July to December 2017, in the Bacteriology Laboratory of Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur.

3.1 Materials

3.1.1 Study Area

Grain samples were collected from 3 different local markets of the Dinajpur District of Bangaldesh.

3.1.2 Study population

5 types of grains (Maize, Rice, wheat, khessari dal, anchor dal), comprising a total of 25 grain samples were collected and brought to the Department of microbiology, Hajee Mohammad Danesh Science and Technology University for laboratory analysis.

3.1.3 Media for culture

The media and reagent that have been used for the isolation and identification of the bacteria and mentioned bellow

3.1.3.1 Liquid media:-

- Nutrient broth (NB)

3.1.3.2 Solid culture media for bacteria

- Nutrient agar (NA)
- MacConkey agar (MAC)
- Eosin methyl blue agar (EMB)
- Salmonella-Shigella agar (SSA)
- Blood agar (BA)
- Brilliant green agar (BGA)
- Manitol salt agar (MSA)

3.1.4 Media Used for Biochemical Test

In order to identify bacteria the following media were used for biochemical tests:

- Triple sugar iron (TSI) agar slant.
- MTU agar base media.
- Bufferd peptone water.
- Methy Red- Voges Proskauer medium base (MR-VP medium base).
- Simmon's Citrate Agar.
- Sugar media

3.1.5 Chemicals, Reagents and Solutions

The following reagents were used for conducting the various bacteriological tests:

- Crystal violet
- Gram's iodine
- Acetone alcohol
- Safranin
- Methyl-Red solution
- Alpha-naphthol
- Potassium hydroxide solution
- Kovac's reagent
- Phenol red solution
- Phosphate buffered saline solution
- Distilled water
- H_2O_2
- Lactophenol cotton blue stain.

3.1.6 Glass Wares, Instruments and Appliances

The different kinds of glass wares and appliances used during the course of the experiment were Test tubes (with or without Durham's fermentation tube and stopper),

- Conical flask, Petri dishes,
- Pipette, Slides,
- Cover slips,
- Clean free grease slide,

- Stop Watch,
- Test tube stand,
- Inoculating loop (Straight and coiled),
- Water bath, Detergent power,
- Sealed poly bags
- Electronic compact balance
- Aluminum foil roll
- Sterile cotton,
- Bacteriological incubator,
- Autoclave,
- Refrigerator,
- Hof air oven,
- Compound microscope

3.1.7 Materials required for antibiogram study

3.1.7.1 Muller Hinton Agar (MBA) Muller Hinton Agar plates were specially used for the antibiotic sensitivity test (Hi media, India).

3.1.7.2 Antibacterial Sensitivity Discs

To determine the drug Sensitivity and resistance patterns of isolated organisms with different types of antimicrobial discs, commercially available antimicrobial discs (Oxoid Ltd., UK) were used (Table 1). The method allowed for the raped determination of the efficacy of the dmgs by measuring the diameter to the zone of inhibition that resulted from different diffusion of the agent into medium surrounding the disc. The list of commercially available antimicrobial disc used in this study with their concentration is given in.

Table-1: Antimicrobial agents with their disc concentration

Sl. No	Name of the antibiotics	Disc concentration (µg/disc)
1	Vancomycine (VA)	30 µg
2	Ciprofloxacin (CIP)	5 µg
3	Kanamycin (K)	30 µg
4	Neomycin (N)	30 µg
5	Gentamycin (GEN)	10 µg
6	Erythromycin (E)	15 µg
7	Amoxicillline (AMX)	30 µg
8	Gentamycine(GE)	(30) µg

Note: Sl. No. = Serial Number µg = Microgram.

3.1.8 Materials required for detection of Aflatoxigenic fungus

- Sabroud Dextrose Agar (SDA)
- Potato Dextrose Agar (PDA)
- ***Aspergillus flavus paraciticus*** **agar medium**

In the present study, *Aspergillus flavus parasiticus* agar medium (AFPA) was used to detect aflatoxin producing ability of the fungal isolates (R.Thilagam *et al.)*

3.1.9 Materials required for Rapid aflatoxin detection

Abraxis Aflatoxin Test Strip 20 ppb

Materials provided:

1. Aflatoxin Test Strips

2. Package insert sheet

Materials required but not supplied:

1. 25-50 ml screw cap vials for extracting specimens

2. Graduated cylinder for preparing Extraction Solution (70% Methanol/DI Water)

3. Methanol

4. 1.7 ml micro-centrifuge tubes

5. Pipets to deliver 250 ul

6. Timer

3.2 Methods

3.2.1 Experimental Design

The experimental work was divided into two (2) steps: the first step was performed for the isolation and identification of the organisms with detection of antibiotic resistance patterns of the isolated organisms. In the second step Aflatoxigenic fungus with aflatoxin was detected.

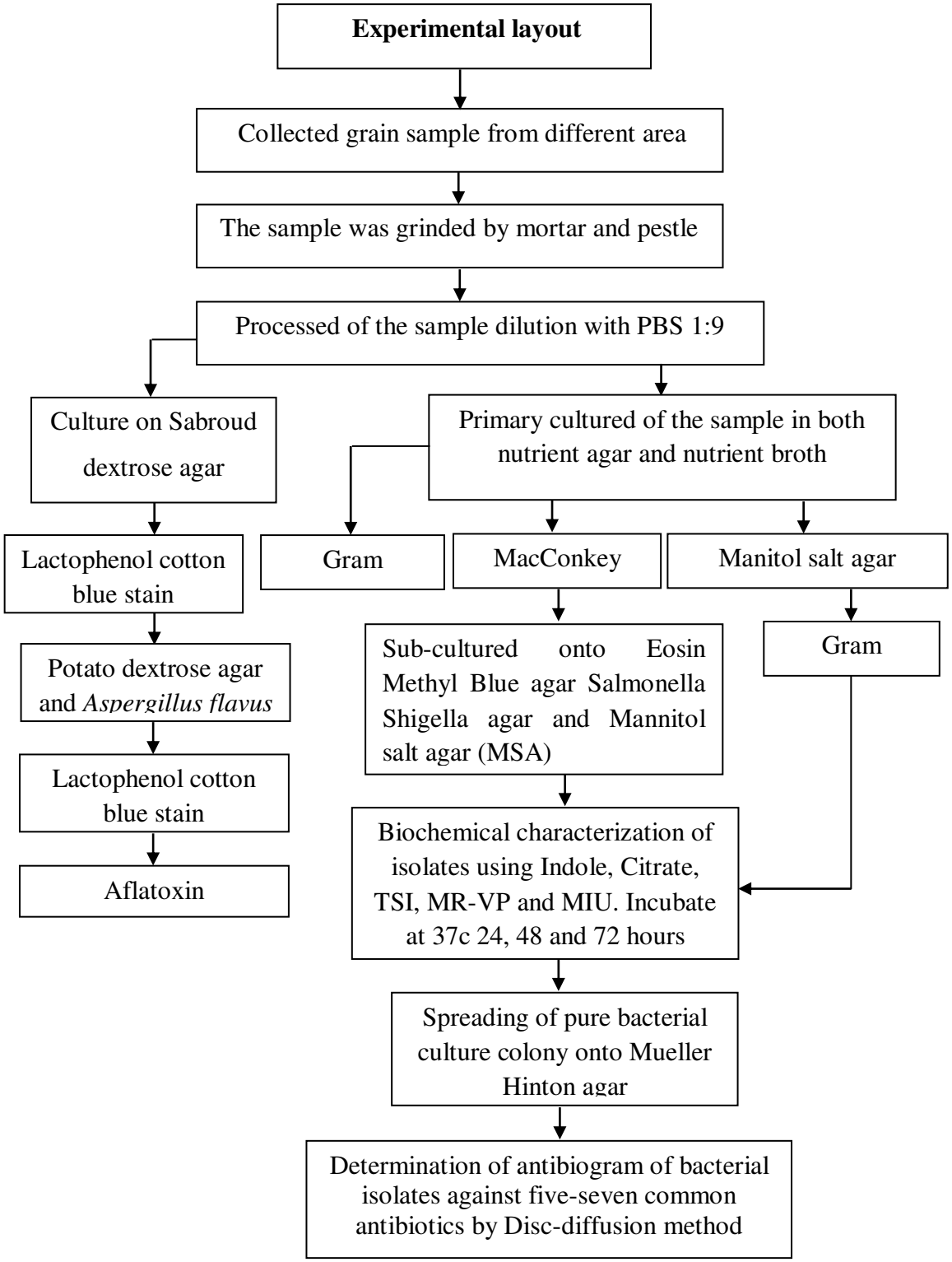

Figure 1: The schematic illustration of experimental layout

3.2.2 Samples Collection and transportation

A total of 25 samples were collected for bacteriological and fungal examination. The samples were collected from local market of Dinajpur district, Bangladesh and brought to the microbiology laboratory HSTU. Approximately 300 g of each grain sample were collected and kept in an ice-box during transportation to the laboratory and stored at 4°C until testing. They were analyzed within 24 hours of sampling.

Sample name	Number of sample	Location
Rice	5	Leili mall
Maize	5	Leili mall
Wheat	5	Doshmail
Khessari dal	5	Bahadur bazar
Anchor dal	5	Bahadur bazar

3.2.3 Processing of sample

300 gm of different grain (Rice, Maize, Wheat, Anchor and Khessari) samples were uniformly homogenized in mortar and pastel using a sterile diluent as per recommendation of ISO (1995). A homogenized suspension was made with the help of mortar and pastel. A quantity of 10 gm homogenate samples transferred carefully into a sterile pastle containing 90 ml of PBS. Thus 1:10 dilution of the samples was obtained.

3.2.4 Preparation of culture media and broth

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

3.2.4.1 Nutrient broth media

Thirteen grams of dehydrated nutrient broth (HI-MEDIA, India) was dissolved into 1 ml of distilled water and heated to boiling 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/cm2 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.

3.2.4.2 Nutrient agar medium

Twenty eight grams of nutrient agar powder (HI-MEDIA) 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 Petridish and allowed to solidify. After solidification of the medium in the petridishes, 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.

3.2.4.3 Blood agar medium

Forty grams of Blood agar base (HI-MEDIA, India) powder was suspended in 1000 ml of distilled water and boil to dissolve the medium completely. The medium was sterilized by autoclaving at 1.2 kg/cm2 pressure and 121° C for 15 minutes and 45° C. Then 5-10 % sterile defibrinated blood was added to the medium and distributed to sterile petridishes and allowed to solidify.

3.2.4.4 Eosin Methylene Blue agar

Thirty six grams of EMB agar base (HI-MEDIA, India) 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/cm2 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 Petridish sized and allowed to solidify. After solidification of the medium in the petridishs, these were incubated at 37° C for overnight to check their sterility and petridishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use.

3.2.4.5 MacConkey agar medium

51.5 grams MacConkey agar base (HI- MEDIA, India) 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 petridishes (medium sized) and in 15 ml quantities in sterile glass Petridishes (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 Petridishes 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 petridishes, 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.

3.2.4.6 Sugar media

The medium consists of peptone water of which fermentable sugar was added to the proportion of 1%. One gram of Bacto peptone (HI-MEDIA) and 0.5 gram of sodium chloride were added in 100 ml of distilled water. The medium was boiled for 5 adjusted to PH 7.0, cooled and then filtered through filter paper. Phenol red, indicator at the strength of 0.2% solution was added to peptone water and then in 5 ml into cotton plugged test tubes containing Durham's fermentation and placed invertedly. These were then sterilized by autoclaving at 1.2 kg /cm2 121° C for 15 minutes. The sugars used for fermentation were prepared separately 10% solution in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A mild heat was necessary to dissolve the sugar. The sugar solutions were sterilized in Arnold steam sterilizer at 100° C for 30 minutes for 3 consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture containing 4.5 ml sterile peptone water, indicator and Durham's fermentation tube. Before use, the sterility of the sugar media was examined by incubating it at for 24 hours.

3.2.4.7. Salmonella-Shigella agar media

The SS agar plates were prepared and stored following the procedure of Cowan, (1985). An amount of 63 gm. powder of SS agar base (Hi-media, India) was added to 1000 ml distilled water in a flask and heated to boil for dissolving the medium completely. The medium was then sterilized by autoclaving at 120C maintaining a pressure of 15 lb pressure/sq. inch for 15 minutes. After autoclaving, the medium was put into a water bath at 450C to cool down its temperature. Then 20 ml of medium was poured into each sterile petridishes and allowed to solidify. After solidification of the medium in the

petridishes, the petridishes were allowed for incubation at 37^0C for overnight to check their sterility and then stored at 40C in a refrigerator for future use.

3.3 Preparation of reagents

3.3.1 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 so 500 ml with the addition of distilled water.

3.3.2 Methyl red – Voges Proskauer broth

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

3.3.3 Voges - Proskauer solution

Alpha – napthol was prepared by dissolving 5gm of alpha- napthol in 100ml of 95% ethyl alcohol.

3.3.4 Potassium hydroxide solution

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

3.3.5 Phosphate Buffered saline solution

Eight grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate (Na_2HPO_4, $12H_2O$) 0.2 grams of potassium chloride (KCI) and o.2 grams of potassium hydrogen phosphate (KH_2PO_4) were suspended in 1000ml 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 1.2kg /cm^2 pressure and 121^oC for the 15 minutes and stored for future use.

3.3.6 Indole test

3.3.6.1 Kovac's reagent

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

3.3.7 Culture of samples

Homogenous for bacteriological examination cultured in to nutrient broth, NA, MAC, MSA, EMB, and BGA. For Fungal examination homogenous cultured on SDA, PDA, and YEA.

Each sample of sample earlier put into transport media was divided and inoculated separately in Nutrient agar (NA) and Blood agar (BA) To Promote growth of bacteria. Each group of these media was incubated 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 homogeneous colonies were obtained. Media such as Blood agar, Nutrient agar, MacConkey agar, Eosin methylene blue agar, were used for sub-cultures and were incubated 37^{o}C for 24 hours for growth.

3.3.8 Staining methods

Gram's staining method was followed to study the morphological and staining characteristics bacteria and to provide information about the presumptive bacterial identification as per recommendation of Merchant and packer (1967). The procedure was follows: A small colony was picked up from SS, MC, and BGA plates with the bacteriological loop, smeared on spread glass slide and fixed by gentle heating. Crystal violate was then applied on each smear to stain for two minutes and then washed with running water. Few drops of gram's iodine was then added to acts and then mordent for one minute and then again washed with running water. Acetone alcohol was then added (acts as decolorize) for few second. After washing with water, safranin was added as a counter stain and allowed to stain for few minutes. The slide were then washed with running water, blotted and dried in air and then examined under microscope with high power objectives (100 X) using immersion oil. For fungal the procedure was follows:

drop of ethanol on clean microscopic glass slide, immerse the specimen in the drop of alcohol then add one or most two drops of the lactophenol cotton blue stain (LPCB) before the alcohol dries out, holding the coverslip between the index finger and thumb, then preparation is now ready for examination, the initial examination using lower objective, switch to higher power (40X) Objectives for more detailed examination of spores and other structure.

3.3.9 Biochemical test

3.3.9.1 Catalase test

This test was performed by taking 2-3 drops of 3 percent H_2O_2 on clean grease-free glass slide and single colony was mixed with the help of wire loop. Immediate formation of gas bubbles was considered a positive test.

3.3.9.2 Methyl-red test

A colony of organism was inoculated in 0.5ml sterile glucose phosphate broth to perform this tube an incubated at 37^0C for overnight. After inoculation a drop of methyl red solution was added. A red color indicated an acid p^H resulting from the fermentation of glucose and was considered as positive. A yellow coloration indicated negative result (Cheesbrough, 1985).

3.3.9.3 Indole test

Two milliliter of peptone water was inoculated with the 5 ml of bacterial growth and incubated for 24 hours. Kovac's reagent (0.5ml) was added, shaked well and examined after 1 minute A red color in reagent layer indicated production of indole. The negative case there was no development of re colour (Cheesbrough, 1985).

3.3.9.4 Voges- Proskauer test

Two milliliter of sterile glucose phosphate peptone water was inoculated in 5ml of test organisms. It was incubated at 37^0C for 48 hours. A very small amount of creatine was added and mixed. Three milliliter of sodium hydroxide was added and shaked well. The bottle cap was removed and left at room temperature for an hour. It was observed closely for the slow the development of pink color in negative cases (Cheesbrough, 1985).

3.3.9.5 Haemolytic activity

To determine the haemolytic property of isolated bacteria, the colonies of bacteria were inoculated on to BA media and incubated at 37^0C for 24 hours. Various types of haemolysis were observed after development of bacteria colony on the BA. The haemolytic pattern of the bacteria was categorized according to the types of haemolysis produced on BA and this was made as per recommendation of Carter (1986) and was listed as mentioned bellow.

3.3.9.5.1 Alpha (α) hemolysis

A zone of greenish discoloration around the colony manifested by partial hemolysis.

3.3.9.5.2 Beta (β) hemolysis

Complete clear zone of hemolysis around the colony.

3.3.9.5.3 Gamma (ϒ) hemolysis

No detectable hemolysis.

3.3.10 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 suggested by Poindexter (1971)

Step1.A inoculum was picked up with sterile inoculating loop and was spread on a area of the medium in the petridish.

Step2. The loop was sterilized by being heated as red hot in a flame.

Step3. The inoculum was spread over the remainder of the plate by drawing the cooled, sterilized loop across the part of the inoculated area, then streaking in a single Erection in each 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.

3.3.11 Techniques for the Isolation and Identification of *Escherichia coli.*

3.3.11.1 Culture into different media

Sterilized platinum loop was used for streaking the lactose broth culture on EMB agar and MacConkey agar to get isolates in pure culture. All inoculated media were kept at 37^0C for overnight incubator.

3.3.11.2 MacConkey agar

Gram negative cultures were inoculated on MacConkey agar plates which after incubation, if positive *Escherichia coli* will show rose pink color colonies.

3.3.11.3 Eosin Methylene blue (EMB)

Materials from lactose fermentation tubes were inoculated into EMB agar plates which after incubation, will show smooth circular colonies with dark center and metallic sheen if *Escherichia coli.*

3.3.11.4 Microscopic study for identification of *Escherichia coli* suspected colonies by Gram's staining method.

Gram's staining was performed to determine the size, shape and arrangement of bacteria. Gram's staining reaction was performed according to the methods described by Cowan and Steel (1979). The organism if *Escherichia coli* will reveal Gram negative, pink color, large rod shape appearance, arranged in single or paired.

3.3.11.5 Identification of *Escherichia coli* isolated by biochemical test.

Sugar fermentation test was performed to identify *Escherichia coli.* For sugar fermentation on the tubes containing different sugar media such as sucrose, maltose, dextrose, lactose and Manitol were inoculated with loopful of broth culture of isolated and incubated at 37^0C for 18 hours. The isolate is positive, ferment five sugar viz. dextrose, maltose, lactose, sucrose and Manitol, the organism's acid and gas in all cases. Acid production was indicated by the change of the color reddish to yellowish in the medium and gas production was noted by the appearance of gas bubble in the inverted Durham's tube.

3.4 Isolation and identification of the *salmonella spp*.

3.4.1 Culture on nutrient agar

With the help of sterile inoculating loop the processed samples were inoculated into nutrient agar and incubated at 37^0C for 24 hours. The incubated media were then examined for the growth of bacteria. Smooth, glistening and opalescent colony were found on nutrient agar.

3.4.2 Culture into different media

The gram's negative organism were inoculated into MacConkey agar, EMB and incubated at 37^0C for 24 hours. The incubated media were then examined for the growth of bacteria.

3.4.3 Culture on selective media

In case of EMB agar non- metallic sheen colony was sub cultured. In case MC agar colorless, translucent colony was sub cultured on selective media (SS agar, BG agar, Selenite broth). In case of SS agar colorless, translucent, sometimes black colony was sub cultured. In case BG agar, light pink colony against a rose pink background was sub cultured. Thus single pure colony was obtained.

3.4.4 Morphological study (Gram's staining)

- A loop full of sterile distilled water was placed in the center of a clean sterile slide.
- A small colony was picked up with a bacteriological loop and mixed with a distal water of water slide.
- The colony was made to thin smear on slide.
- The smear were fixed by air drying
- 0.5% crystal violet solution was then applied on the smear for one minute.
- Lugols iodine was then added to act as mordant for one minutes.
- Acetone alcohol was then added to decolorize for 1-2 seconds.
- Then the slide was then washed with water.
- Safranin was added as counter stain and allowed for one minutes.
- Then the slide was then washed with water.

- Then the slide was blotted with blot paper and was allowed to dry. The slide was examined under microscope with high power.

Gram positive (violate colour) organisms are discarded and gram negative (pink coloured), small rod shaped, single or paired arranged organisms were selected.

3.4.5 Biochemical characterization

Isolated organisms with supporting growth characteristics of salmonella on various media were maintained on SS and BG agar and were subjected to biochemical tests (sugar fermentation test. TSI agar slant reaction, MR-VP and indole reaction, MIU test).

3.4.6 Sugar fermentation test

The carbohydrate fermentation was test by inoculation a loop of thick bacterial culture into the tubes containing five basic sugars (dextrose, maltose, lactose, sucrose and Manitol) and incubated at 37^0C for 24 hours. Acid production was indicated by the change media from pink yellow color and gas production was indicated by the appearance of gas bubble in the inverted Durham's fermentation tubes (Cheesbrough, 1985).

3.4.7 Methyl-red test

The test was performed by inoculating a colony of organism was inoculated in 0.5ml sterile glucose phosphate peptone broth. After overnight incubation at 37^0C, a drop of MR solution was added and mixed thoroughly. A red coloration was considered as a positive and indicated an acid p^H of 4.5 or less resulting from the fermentation of glucose while yellow coloration was considered negative result (Cheesbrough, 1985).

3.4.8 Indole test

Two milliliter of peptone water was inoculated with the 5 ml of bacterial growth and incubated for 48 hours. Kovac's reagent (0.5ml) was added, and mixed thoroughly. The tube was then allowed to stand for a while. The appearance of red color on the whole medium was considered as a positive test for the production of indole by the organisms (Cheesbrough, 1985).

3.4.9 Voges- Proskauer test

Two milliliter of sterile glucose phosphate peptone water were inoculated with the 5ml of test organisms. It was incubated at 37^0C aerobically for 48 hours. A very small amount (knifepoint) of creative was added and mixed. Three milliliter of sodium hydroxide was added and shaked well. The bottle cap was removed and left at room temperature for an hour. It was observed closely for the slow the development of pink color for positive cases. In negative cases, there was no development of pink color (Cheesbrough, 1985).

3.4.10 Citrate utilization test

Suspected colony was inoculated on simmon citrate agar. Then the medium was incubatedat 37^0C for 48 hours. In positive case the colour of green medium turned on deep blue colour.

3.4.11 Triple sugar iron agar slant reaction

TSI agar was used to detect the non-lactose fermenters and the dextrose fermenters. The medium also helped to determine the ability of organism to produce hydrogen sulfide (H_2S).

The organism under study were heavily seeded with a platinum needle over the surface of the slant and stabbed in to the put of the tubes of TSI agar. After an incubation period of 24 hours at 37^0C, aerobically the tubes were examined for all changes in the slant or in the butt. In TSI agar slant the presence of yellow color and gas bubbles in the media were considered as production acid and gas respectively in slants or in butt as the case maybe. The red or dark pink coloration of the media in slant or in butt was considered as alkaline reaction. The black coloration in any part of media was considered as the production of H_2S (hydrogen sulfide).

3.4.12 MIU (Motility, Indole, Urease) medium

Suspected colony was inoculated in to the tube containing MIU medium. Then the medium was incubated at 37^0C overnight. Absence of turbidity throughout the medium was indicated no motile salmonella organisms.

3.4.13 Motility test

The motility test was performed to differentiate motile bacteria from non-motile one (Cheesbrough, 1985). Before performing the test, a pure culture of the organism was allowed to grow in NB. One drop of cultured broth was placed on clean cover slip and was placed invertedly over the concave depression of the hanging drop slide to make hanging drop preparation. Vaseline was used around the edge of concave depression of the hanging drop slide for better attachment of the cove slip to prevent air current and evaporation of the fluid. The hanging drop slide was than examined carefully under 100 power objective of a compound light microscope using immersion oil. The motile and non-motile organisms were identified by observing motility in contrasting with swinging movement of bacteria.

3.5 Techniques for the isolation and identification of *Staphylococcus spp.*

3.5.1 Culture into different media

Loopful aliquot was taken from nutrient broth culture and streaked on nutrient agar, Blood agar media to get isolates in pure culture. All inoculated media were kept at 37^0C for overnight incubator.

3.5.2 Nutrient agar (NA)

Materials from nutrient broth culture and streaked on nutrient agar, blood agar media to get isolate in pure culture. All inoculated media were kept at 37^0C overnight in an incubator.

3.5.3 Blood agar (BA)

Incubation of materials from Nutrient broth into Blood agar plates which after incubation, if positive staphylococcus showed white to golden yellow color on blood agar media. (β) hemolysis complete clear zone of hemolysis around the colony was occurred.

3.5.4 Microscopic study for identification of *staphylococcus spp.* Suspected colonies by Gram's staining method

Gram's staining was performed to determine the size, shape, and arrangement of bacteria. Gram staining reaction was performed according to the methods describe by

Cowan and Steel (1979). If the organism found as Gram positive, cocci, arranged in cluster indicating staphylococcus.

3.5.5 Identification of *staphylococcus spp*. Isolates by biochemical test

Sugar fermentation tests the tubes containing different sugar media such as dextrose, maltose, lactose, sucrose and Manitol were incubated with a loopful of broth culture of the isolated organisms and incubated 37^0C for 18 hours. The isolated if positive, ferment five sugar viz. dextrose, maltose, lactose, sucrose and Manitol, the organism produces acid in all cases and change color of media reddish to yellowish. Acid production was indicated was indicated by the change of the color to yellowish in the medium.

3.5.6 Catalase test

Catalase test was performed to differentiate catalase enzyme producing bacteria those of non-Catalase producing one if *staphylococcus spp.*

3.5.7 Methyl-red test

The test was performed by inoculating a colony of organism was inoculated in 0.5ml sterile glucose phosphate peptone broth. After overnight incubation at 37^0C, a drop of MR solution was added and mixed thoroughly. A red coloration was considered as a positive and indicated an acid p^H of 4.5 or less resulting from the fermentation of glucose while yellow coloration was considered negative result (Cheesbrough, 1985).

3.5.8 Indole test

Two milliliter of peptone water was inoculated with the 5 ml of bacterial growth and incubated for 48 hours. Kovac's reagent (0.5ml) was added, and mixed thoroughly. The tube was then allowed to stand for a while. The appearance of red color on the whole medium was considered as a positive test for the production of indole by the organisms (Cheesbrough, 1985).

3.5.9 Voges- Proskauer test

Two milliliter of sterile glucose phosphate peptone water were inoculated with the 5ml of test organisms. It was incubated at 37^0C aerobically for 48 hours. A very small amount (knifepoint) of creative was added and mixed. Three milliliter of sodium hydroxide was added and shaked well. The bottle cap was removed and left at room

temperature for an hour. It was observed closely for the slow the development of pink color for positive cases. In negative cases, there was no development of pink color (Cheesbrough, 1985).

3.5.10 Citrate utilization test

Suspected colony was inoculated on simmon citrate agar. Then the medium was incubatedat 37^0C for 48 hours. In suspected case the colour of green medium turned on deep blue colour.

3.5.11 Triple sugar iron agar slant reaction

TSI agar was used to detect the non-lactose fermenters and the dextrose fermenters. The medium also helped to determine the ability of organism to produce hydrogen sulfide (H_2S).

The organism under study were heavily seeded with a platinum needle over the surface of the slant and stabbed in to the put of the tubes of TSI agar. After an incubation period of 24 hours at 37^0C, aerobically the tubes were examined for all changes in the slant or in the butt. In TSI agar slant the presence of yellow color and gas bubbles in the media were considered as production acid and gas respectively in slants or in butt as the case maybe. The red or dark pink coloration of the media in slant or in butt was considered as alkaline reaction. The black coloration in any part of media was considered as the production of H_2S (hydrogen sulfide).

3.5.12 MIU (Motility, Indole, Urease) medium

Suspected colony was inoculated in to the tube containing MIU medium. Then the medium was incubated at 37^0C overnight. Absence of turbidity throughout the medium was indicated no motile salmonella organisms.

3.6 Techniques for the Isolation and Identification of *Klebsiella.*

3.6.1 Culture into different media

Sterilized platinum loop was used for streaking the lactose broth culture on EMB agar and MacConkey agar to get isolates in pure culture. All inoculated media were kept at 37^0C for overnight incubator.

3.6.2 MacConkey agar

Materials from lactose fermentation tubes were inoculated MacConkey agar plates which after incubation, if positive *Klebseilla.* pink color colonies.

3.6.3 Eosin Methylene blue (EMB)

Materials from lactose fermentation tubes were inoculated into EMB agar plates which after incubation will show smooth circular colonies with dark and pink color mucoid of plate.

3.6.4 Microscopic study for identification of *klebsiella Spp.* suspected colonies by Gram's staining method.

Gram's staining was performed to determine the size, shape and arrangement of bacteria. Gram's staining reaction was performed according to the methods described by Cowan and Steel (1979). The organism if *Escherichia coli* will revealed Gram negative, pink color, large rod shape appearance, arranged in single or paired.

3.6.5 Identification of *klebsiella spp.* isolated by biochemical test.

3.6.5.1 Methyl-red test

The test was performed by inoculating a colony of organism was inoculated in 0.5ml sterile glucose phosphate peptone broth. After overnight incubation at 37^0C, a drop of MR solution was added and mixed thoroughly. A red coloration was considered as a positive and indicated an acid p^H of 4.5 or less resulting from the fermentation of glucose while yellow coloration was considered negative result (Cheesbrough, 1985).

3.6.5.2 Indole test

Two milliliter of peptone water was inoculated with the 5 ml of bacterial growth and incubated for 48 hours. Kovac's reagent (0.5ml) was added, and mixed thoroughly. The tube was then allowed to stand for a while. The appearance of red color on the whole medium was considered as a positive test for the production of indole by the organisms (Cheesbrough, 1985).

3.6.5.3 Voges- Proskauer test

Two milliliter of sterile glucose phosphate peptone water were inoculated with the 5ml of test organisms. It was incubated at 37^0C aerobically for 48 hours. A very small amount (knifepoint) of creative was added and mixed. Three milliliter of sodium hydroxide was added and shaked well. The bottle cap was removed and left at room temperature for an hour. It was observed closely for the slow the development of pink color for positive cases. In negative cases, there was no development of pink color (Cheesbrough, 1985).

3.6.5.4 Citrate utilization test

Suspected colony was inoculated on simmon citrate agar. Then the medium was incubatedat 37^0C for 48 hours. In suspected case the colour of green medium turned on deep blue colour.

3.6.5.5 Triple sugar iron agar slant reaction

TSI agar was used to detect the non-lactose fermenters and the dextrose fermenters. The medium also helped to determine the ability of organism to produce hydrogen sulfide (H_2S).

The organism under study were heavily seeded with a platinum needle over the surface of the slant and stabbed in to the put of the tubes of TSI agar. After an incubation period of 24 hours at 37^0C, aerobically the tubes were examined for all changes in the slant or in the butt. In TSI agar slant the presence of yellow color and gas bubbles in the media were considered as production acid and gas respectively in slants or in butt as the case maybe. The red or dark pink coloration of the media in slant or in butt was considered as alkaline reaction. The black coloration in any part of media was considered as the production of H_2S (hydrogen sulfide).

3.6.5.6 MIU (Motility, Indole, Urease) medium

Suspected colony was inoculated in to the tube containing MIU medium. Then the medium was incubated at 37^0C overnight. Absence of turbidity throughout the medium was indicated no motile salmonella organisms.

3.6.5.7 Haemolytic activity

To determine the haemolytic property of isolated bacteria, the colonies of bacteria were inoculated on to BA media and incubated at 37^0C for 24 hours. Various types of haemolysis were observed after development of bacteria colony on the BA. The haemolytic pattern of the bacteria was categorized according to the types of haemolysis produced on BA and this was made as per recommendation of Carter (1986) and was listed as mentioned bellow.

3.6.5.7.1 Alpha (α) hemolysis

A zone of greenish discoloration around the colony manifested by partial hemolysis.

3.6.5.7.2 Beta (β) hemolysis

Complete clear zone of hemolysis around the colony.

3.6.5.7.3 Gamma (ϒ) hemolysis

No detectable hemolysis.

3.7 Maintenance of stock culture

Stock culture is a culture of a microorganism maintained solely for the purpose of keeping the microorganism in a viable condition by subculture, as necessary, into fresh medium.

3.7.1 Agar slant method

The stock culture was maintained following the procedures, Isolated and identified bacteria were inoculated into nutrient agar slants and incubated at 37°C for 24 hours and then examine for growth. One slant was used for an individual isolate. Then the sterile mineral oil was poured into the tube until the colonies were covered completely. The tube were sealed off with paraffin and kept at room temperature for future use as seed. By this method bacteria can be preserved with no deviation of their original characters for few months (Buxton and Fraser, 1977).

3.7.2 Sterile buffered glycerin method

Sterile buffered glycerin (20%) was prepared by mixing 20 parts of pure glycerin and 80 parts of PBS. Then a loopful of thick bacterial culture was mixed with 20%, sterile buffered glycerin in small vials and was preserved at -20°C This method is more appropriate for preserving bacteria with no deviation of their original characters for several years (Buxton and Fraser, 1977).

3.8 Antibiotic susceptibility test

Bacterial susceptibility to anti-microbial agent was determined in vitro by using the standardized agar disc-diffusion method known as the *Kirby Bauer* ,Labeled the covers of each of the agar plates with name of the test organisms was inoculated.

- Using sterile technique, inoculated all agar plates with their respective test organisms as follow:
    - Dipped a sterile cotton swab into a well-mixed saline test culture and removed excess inoculums by pressing the saturated swab against the inner wall of the culture tube.
    - Using the 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.
- Allowed all culture plates to dry for about 5 minutes.
- Distributed the individual antibiotic discs at equal distance with forceps dipped in alcohol and flamed.
- Gently pressed each disc down with the wooden end of the cotton swab or sterile forceps to ensure that the discs adhered to the surface of the agar.
- The plates were then inverted and incubated at 370 C for 24 hours.
- After incubation, the plates were examined and the diameter of the zones of complete inhibition was measured in mm.
- The zone diameter for individual anti-microbial agents was used to determine susceptible, intermediate, and resistant categories by referring to an interpreting (cappuccino and Sherman, 2005).

3.9 Techniques for the Isolation and Identification of Aspergillus *spp.*

3.9.1 Culture into different media

3.9.1.1 Sabroud Dextrose agar (SDA)

With the help of sterile inoculating loop the processed samples were inoculated into sabroud dextrose agar and incubated at 37^0C for 5-7 days. The incubated media were then examined for the growth of fungi. Colony, greenish colony were found Sabroud dextrose agar.

3.9.1.2 Potato Dextrose agar (PDA)

Colonies from Sabroud dextrose agar were subculture in potato dextrose agar.

3.9.2 Microscopic study for identification of *Aspergillus Spp.* suspected colonies by lactophenol cotton blue stain (LPCB) method.

Micro morphological characteristics of the pure culture colonies like conidia were observed as wet mount in Lactophenol cotton blue stain for identification by the conidiospore appearance and arrangement (R.Thilagam *et al*. 2016).

- Place a drop of 70% ethanol on a clean microscopic glass slide
- Immerse the colony from pure culture in the drop of alcohol
- Add one or at most two drops of the LPCB before the alcohol dries out
- Holding the coverslip between the index finger and thumb, touch one edge of the drop of mountant with a coverslip edge and lower gently avoiding air bubbles
- This preparation is now ready for examination
- Make the initial examination using lower power objectives. Switch to higher power (40X) objectives for more detailed examination of spores and other structures.

3.9.3 *Aspergillus flavus paraciticus* agar medium

Observed specific colonies from Sabroud dextrose agar and potato dextrose agar were subculture in *Aspergillus flavus parasiticus* agar medium. A bright orange colour on the reverse side of the plates of *Aspergillus flavus parasiticus* agar medium (AFPA) will indicate a positive result. (R.Thilagam *et al*. 2016).

3.9.4 Rapid Aflatoxin Determination by Abraxis Aflatoxin Rapid test strip 20 ppb

This Rapid Aflatoxin Test is designed solely for use in preliminary screening of grain samples. This test is a qualitative one-step competitive inhibition immunoassay for the detection of aflatoxin. It detects the presence of aflatoxin at 5 ppb or higher in grain samples by utilizing highly specific reactions between antibodies and aflatoxin in grain samples (Delmulle BS *et al*. 2005), (Xiulan S *et al*. 2005), and (Stubblefield RD *et al.*1991).

3.9.4.1 Test principle

The toxin conjugate competes for antibody binding sites with toxins that may be present in the grain sample. The test device consists of a membrane strip to which a conjugate of the toxin of interest is attached. A colloidal gold labeled antibody is located at one end of the membrane. A control line, produced by a different antibody/antigen reaction, is also present on the membrane strip. The control line is not influenced by the presence or absence of mycotoxins in the grain sample, and therefore, it should be present in all reactions. In the absence of toxin in the grain sample, the colloidal gold labeled antibody complex moves with the grain sample by capillary action to contact the immobilized aflatoxin conjugate. An antibody antigen reaction occurs forming a visible line in the 'test' area. The formation of two visible lines indicates a negative test result. This means the test did not detect the toxin at or below the cut-off point established for the toxin. If aflatoxin is present in the grain sample, it competes with the immobilized toxin conjugate in the test area for the antibody binding sites on the colloidal gold labeled antibody complex. If a sufficient amount of toxin analytic is present, it will fill all of the available binding sites, thus preventing attachment of the labeled antibody to the toxin conjugate. If a colored line is not visible in the Test Line region, or Test Line is lighter than negative controls, aflatoxin is present at levels of concern. Available Aflatoxin Controls may be used to approximate the quantity of toxin present in grain samples.

- **Sample collection and handling**

  1. A solution of 70% methanol by mixing 70 parts methanol and 30 parts deionized water was prepared
  2. 5 gm grain was added to 10 ml 70% methanol.
  3. Mixture was shaken for 3 minutes and allowed to settle for 2 minutes.
  4. Then it was filtered and the liquid portion is taken

- **Assay procedure**

  1. Test strip and grain extract was equilibrated to room temperature before conducting any testing.

  2. In a micro centrifuge tube 0.25 ml of extract was mixed with 0.25 ml deionized water.

  3. Test strip was removed from the desiccant vial and dipped into diluted extract with arrows pointing downward.

  4. The test was allowed to develop for 10 minutes and the result was read as explained below under Interpretation of Results.

- **Interpretation of results**

Control Line	Test Line	Interpretation
No control line present	No test line present	Invalid result
Control line present	Distinct test line present	0 ppb
Control line present	Moderate intensity test line present	Between 0 and 20 ppb
Control line present	No test line present	>20 ppb

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

4.1 Prevalence Study

4.1.1 Prevalence of Bacterial pathogens in Grain samples (25)

Total 20 no of grain samples comprising wheat (5), Khashari dal (5), Maize (5), Rice (5) and anchor dal (5) were analyzed. The total prevalence of *E. coli, Salmonella spp., Klebsiella spp and Staphylococcus spp* in the grain samples was 16%, 28%, 16% and 16% respectively. From the wheat samples *E.coli*, *Salmonella spp* was isolated from one samples and their prevalence was 20% separately. But no *Klebsiella spp* and *Staphylococcus spp* was found. From Khessari dal *E.coli* and *Staphylococcus spp* were isolated and their prevalence were 40% and 20% respectively. In Maize the prevalence of *Salmonella spp, Klebsiella spp* and *Staphylococcus spp* were 40%, 20% and 20% respectively. No *klebsiella spp* was found from maize. From rice no E.coli and Staphylococcus was isolated but *Salmonella spp* and *Klebsiella spp* was found and their prevalence was 20% and 40% respectively. From anchor dal *E.coli, Salmonella spp*, *klebsiella spp* and *Staphylococcus spp* was isolated and their prevalence was 20%, 60%, 20%and 40% respectively.

Table 2: Bacterial isolated from grain samples and prevalence (%)

Samples	**No. of samples**	**No. of isolated *E. coli***	**%**	**No. of isolated *Salmonella spp***	**%**	**No. of isolated *Klebsiella spp***	**%**	**No. of isolated *Staphylococcus spp***	**%**
Wheat	5	1	20%	1	20%				
Khesari dhal	5	2	40%					1	20%
Maize	5			2	40%	1	20%	1	20%
Rice	5			1	20%	2	40%		
Anchor dhal	5	1	20%	3	60%	1	20%	2	40%
Total	25	4	16%	7	28%	4	16%	4	16%

4.1.2 Prevalence of Aflatoxigenic *Aspergillus spp.* in grain samples (no=25)

Among the 25 grain samples *Aspergillus spp* was isolated from 4 grain samples with 16% prevalence. But Aflatoxigenic *Aspergillus spp* was isoled from 3 samples with 12% prevalence. From the wheat samples and maize the Aflatoxigenic fungus was isolated and their prevalence in maize and wheat was 20% and 40% respectively.

Table 3: Prevalence of Aflatoxigenic *Aspergillus spp.* in grain samples (no=20)

Samples	**No.**	**No. of isolated Aspergillus spp (fungus)**	**%**	**No. of isolated Aflatoxigenic *Aspergilus spp***	**%**
Wheat	5	2	40%	2	40%
Khesari dhal	5	1	20%		
Maize	5	1	20%	1	20%
Rice	5				
Anchor dhal	5				
Total	25	4	16%	3	12%

4.2 Isolation and identification of *Escherichia coli* by different bacteriological methods

4.2.1 Results of cultural examination

4.2.1.1 Nutrient broth

Nutrient broth was inoculated with the grain samples and incubated at 37 ^{o}C for 24 hours. The growth of bacteria was indicated by the presence of turbidity.

4.2.1.2 Nutrient agar

Nutrient broth plates streaked with E.coli broth then shown growth of bacteria after 24 hours of inoculation at 37^{o}C.The growth of E.coli on nutrient agar media was characterized by circular, smooth, opaque and colorless colonies. (Plate 2)

4.1.1.3 MacConkey (MC) agar

MacConkey agar plates streaked separately with the organism revealed the growth of bacteria after 24 hours of incubation at 37 ^{O}C aerobically and were indicated by the growth of bright-pink colored smooth colonies. (Plate 3)

4.2.1.4 Eosin Methyl Blue (EMB) agar

EMB agar plates streaked separately with the organism revealed the growth of bacteria after 24 hours of incubation at 37 aerobically and were indicated by the growth of smooth metallic sheen color colonies. (Plate 4)

4.2.2 Results of Grams staining

The microscopic examination of Grams stained smears from MC and EMB agar revealed

Gram-negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain. (Plate 16)

Table 4: Cultural, Morphological and biochemical properties of isolated of isolated *Escherichia coli*

Cultural characteristics	Biochemical characteristics		Staining and morphological Characteristics	
EMB Agar	MC Agar	Tests	Results	Staining properties
Smooth Metallic Sheen color colonies Were Produced	Bright Pink color Smooth Colonies Were Produced	Indole	+	Gram-negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain.
		MR	+	
		MIU	+	
		TSI	+	
		VP	_	
		Citrate utilization test	_	

Legends: AG= Acid and Gas, MR= Methyl-Red test, VP= Voges-Proskauer test, += Positive reaction,- = Negative reaction, EMB = Eosin Methyle Blue, MC = MacConkey.

4.2.3 Result of biochemical tests

4.2.3.1 Fermentation reaction with five basic sugars

The isolated organisms fermented the five sugars (dextrose, maltose, lactose, sucrose, and Manitol) with the production of acid and gas. The change of color from reddish to yellowish indicated acid production. The presence of gas bubble in the inverted Durham's tube indicated gas production.

4.2.3.2 Indole test

The isolates were indole positive (Plate 9).

4.2.3.3 Methyl-red and Vegos-proskauer test

The isolates were MR positive (plate 10) and VP negative (Plate 14).

4.2.3.4 MIU test

Presence of turbidity trough out the medium indicated motile *E. coli* organisms (Plate 11).

4.2.3.5 Triple Sugar Iron (TSI) agar slant reaction

On TSI agar slant, *salmonella* isolated from grain produced acid (yellow) and gas in the butt, hydrogen sulfide gas in both butt and slant and the alkaline reaction in the slant which revealed red color (Plate 12).

4.3 Isolation and identification of *salmonella spp.* different bacteriological methods

4.3.1 Cultural characteristics

4.3.1.1 Nutrient broth

Salmonellae isolate produced turbidity in nutrient broth.

4.3.1.2 On Nutrient agar

On Nutrient agar (NA) isolated produced translucent, opaque, smooth colonies (Plate 2).

4.3.1.3 MacConkey (MC) agar

Samples inoculated onto MacConkey agar plates produced colorless, smooth, transparent and raised colonies.

4.3.1.4 S-S agar

On S-S agar suspected isolated produced Translucent, smooth, small round black centered colonies (Plate 5).

4.3.2 Results of Grams staining method

The thin smears prepared with the colony from SS agar and MC agar for Grams staining revealed Gram-negative, pink colored, very small plump rod shaped appearance, arranged in single, paired under the microscope examination (Plate 17).

4.3.3 Results of motility

Isolated was found to be motile when examined using hanging drop slide under microscope.

4.3.4 Result of biochemical tests

4.3.4.1 Fermentation reaction with five basic sugars

Organism isolated from chicken fermented dextrose, maltose, and Manitol, glucose and produced acid and gas but did not ferment lactose and sucrose. Acid production was marked by the color change from reddish to yellow and the gas production was noted by the presence of gas bubble in the inverted Durham's tube kept inside each of the test tube containing sugar media.

4.3.4.2 Indole test

Salmonellae isolate from grain was indole negative. They did not produce any red color (Plate 9).

4.3.4.3 MIU test

Presence of turbidity trough out the medium indicated motile Salmonella organisms (Plate 11).

4.3.4.4 Methyl red (MR) test

In the MR test the appearance of the red colour in the media after the addition of 3ml methyl red with cultural growth was observed and thus indicating the isolated *Salmonellae* were positive for MR test (Plate 10).

4.3.4.5 Voges-Proskauer (V-P) test

In the Vegos-proskauer (V-P) test, no change of colour of the media was observed after the addition of 3ml of 3% KOH to 3ml V-P broth media with the cultural growth of isolated *salmonellae* and thus indicated that the isolated *salmonellae* were negative for V-P test (Plate 14).

4.3.4.6 Triple Sugar Iron (TSI) agar slant reaction

On TSI agar slant, *salmonella* isolated from grain produced acid (yellow) and gas in the butt, hydrogen sulfide gas in both butt and slant and the alkaline reaction in the slant which revealed red color (Plate 12).

4.3.4.7 Citrate utilization test

In the citrate utilization test the colours of green medium turned to deep blue colour indicating the isolated salmonella were positive for simmon citrate test (Plate 13).

4.4 Identification of *Staphylococcus spp*. By different bacteriological methods

4.4.1 Results of Cultural examination

4.4.1.1 Nutrient broth

Nutrient broth was inoculated with the grain samples and incubated at 37 ^{o}C for 24 hours. The growth of bacteria was indicated by the presence of turbidity.

4.4.1.2 Nutrient agar

On Nutrient agar (NA) plates streaked separately with the organism revealed the growth of bacteria after 24 hours of incubation at 37 ^{o}C aerobically and were indicated by the growth of circular, small smooth, convex and grey white or yellowish colonies (Plate 2).

4.4.1.3 Manitol Salt Agar (MSA)

On Mannitol salt agar plates streaked separately with the organism from nutrient agar revealed the growth of bacteria after 24 hours of incubation at 37 aerobically and were indicated by the growth of circular, small, yellowish colonies (Plate 6).

4.4.1.4 Blood agar

Blood agar plates streaked separately with the organism and incubated at 37^{o}C aerobically for 24 hours and hemolysis were produced. (Plate 7)

4.4.2 Results of gram's staining

Gram's stained smear from NA and BA were examined agar revealed Gram-positive cocci arranged in grape like cluster. (plate 15)

Table 5: Cultural, Morphological and biochemical properties of isolated of isolated *Staphylococcus* spp.

Cultural characteristics	Biochemical characteristics		Staining and morphological Characteristics	
Blood Agar	Nutrient Agar	Tests	Results	Staining properties
β-type of hemolysis Were Produced	Circular, small, smooth, convex and gray-white or yellowish colonies produced were prod	Catalase Test	+	Gram-positive Cocci arranged in grape like cluster
		Indole	+	
		MR	+	
		MIU	+	
		TSI	–	
		VP	+	
		SC	+	

Legends: AG= Acid and Gas, MR= Methyl-Red test, VP= Voges-Proskauer test, += Positive reaction,- = Negative reaction, EMB = Eosin Methyle Blue, MC = MacConkey.

4.4.3 Results of biochemical tests

4.4.3.1 Fermentation reaction with five basic sugars

The isolated bacteria fermented the five sugar (dextrose, maltose, lactose, sucrose, and Manitol) with the produced of acid. The change of color from reddish to yellow indicated acid production.

4.4.3.2 Indole test

Staphylococcus isolates from grain was indole negative. They did not produce any red color.

4.4.3.3 MIU test

Presence of turbidity trough out the medium indicated motile Staphylococcus organisms.

4.4.3.4 Methyl red (MR) test

In the MR test the appearance of the red colour in the media after the addition of 3ml methyl red with cultural growth was observed and thus indicating the isolates were positive for MR test.

4.4.3.5 Voges-Proskauer (V-P) test

In the Voges-Proskauer (V-P) test, change of colour of the media was observed after the addition of 3ml of 3% KOH to 3ml V-P broth media with the cultural growth of isolated *Staphylococcus spp.* and thus indicated that the isolated *Staphylococcus spp.* were positive for V-P test.

4.4.3.6 Triple Sugar Iron (TSI) agar slant reaction

The isolates were TSI negative (plate 12)

4.4.3.7 Citrate utilization test

In the citrate utilization test the colours of green medium turned to deep blue colour indicating the isolated *Staphylococcus spp* were positive for simmon citrate test (Plate 13).

4.4.3.8 Catalase test

The isolates were catalase positive (Plate 5).

4.5 Isolation and identification of *Klebsiella spp.* by different bacteriological methods

4.5.1 Results of cultural examination

4.5.1.1 Nutrient broth

Nutrient broth was inoculated with the grain samples and incubated at 37 ^{o}C for 24 hours. The growth of bacteria was indicated by the presence of turbidity.

4.5.1.2 Nutrient agar

Nutrient broth plates streaked with organisms from nutrient broth then shown growth of bacteria after 24 hours of inoculation at 37^{o}C.The growth of *Klebsiella spp*. on nutrient agar media was characterized by circular, smooth, opaque and colorless colonies. (Plate 2)

4.5.1.3 MacConkey (MC) agar

MacConkey agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37 ^{O}C aerobically and were indicated by the growth of bright-pink colored smooth mucous colonies.

4.5.1.4 Eosin Methyl Blue (EMB) agar

EMB agar plates streaked separately with the organisms from MacConkey agar revealed the growth of bacteria after 24 hours of incubation at 37 aerobically and were indicated by the growth of smooth mucoid pink color colonies (Plate 4).

4.5.2 Results of Grams staining

The microscopic examination of Grams stained smears from MC and EMB agar revealed Gram-negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain (Plate 14).

Table 6: Cultural, Morphological and Biochemical Properties of Isolated *Klebsiella spp.*

Culture characteristics		**Biochemical Characteristics**		**Staining and morphological**
MC Agar	**EMB Agar**	**Test**	**Result**	**Staining properties**
Pink colored colonies	Pink mucose large colonies.	Indole	+	gram-negative, pink colored, rod shaped organism
		MIU	+	
		MR	+	
		VP	+	
		TSI	–	
		SC	+	

Legends: MC= MacConkey, EMB=Eosin Methyl Blue, MR=Methyl-red test, VP=Voges-Proskauer test, TSI=Triple Sugar Iron, MIU=motility Indole urease test, SC=Simon citrate, + positive,- negative

4.5.3 Result of biochemical test

4.5.3.1 Indole test

The isolated were Indole positive (plate9)

4.5.3.2 Methyl-red and Vegos-proskauer test

The isolated were MR positive (plate 10) and VP negative (plate 14)

4.5.3.3 Motility Indole urease test

The isolated were MIU positive (plate11).

4.5.3.4 Triple sugar iron test

The isolated were TSI negative (plate12).

4.5.3.5 Simon's citrate test

The isolated were SC positive (plate13).

4.6 Isolation and Identification of Aflatoxigenic Aspergillus *spp.*

4.6.1 Culture into different media

4.6.1.1 Sabroud Dextrose agar (SDA)

Greenish colonies were found on Sabroud dextrose agar (Plate 18).

4.6.1.2 Potato Dextrose agar (PDA)

On PDA the colonies were yellow green with white to cream mycelia and yellow green edges and also in some plates greenish colony were found (Plate 19).

4.6.2 Microscopic study for identification of *Aspergillus Spp.* suspected colonies by lactophenol cotton blue stain (LPCB) method.

Characteristics conidia were found under microscope by lactophenol cotton blue stain (LPCB) method (Plate 21).

4.6.3 Aflatoxin production on Aspergillus flavus parasiticus agar medium (AFPA)

In the present study, *Aspergillus flavus parasiticus* agar medium (AFPA) was used to detect aflatoxin producing ability of the fungal isolates. A bright orange colour on the reverse side of the plates of *Aspergillus flavus parasiticus* agar medium (AFPA) indicated a positive result (Plate 20).

4.6.4 Screening of Aflatoxin by Abraxis Aflatoxin Rapid Test Strip 20ppb

Aflatoxigenic *Aspergillus spp* was isolated from 3 samples with 20 ppb aflatoxin with no test line.

4.7 Result of antibiotic sensitivity pattern of isolated bacteria

The isolated bacterial pathogens were tested for the antibiotic sensitivity and resistance pattern against commonly used antibiotic. The results of sensitivity against antibiotic discs (zone of inhibition) were categorized as resistance, intermediate, sensitive. The results of antibiotic sensitivity are given in table.

4.7.1 Antibiotic sensitivity pattern of *E. coli*

The antibiotic study revealed that isolated *E. coli were* sensitive of Ciprofloxacin (100%) followed by Gentamycin (66.7%), the isolates were found resistant to Penciling (100%), Vancomycin (100%) and Erythromycin (100%).

Table 7: Antibiotic sensitivity pattern *E. coli* (no. 4)

Antibacterial agents	Disc concentration (µg/disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistance	Sensitive	Resistance
Erythromycin	5 µg	0	3	0	100
Pencilline	10µg	0	3	0	100
Vancomycine	30 µg	0	3	0	100
Gentamycin	10 µg	2	1	66.7	33.3
Ciprofloxacin	10µg	3	0	100	0

4.7.2 Antibiotic sensitivity pattern of *Salmonella spp.*

The antibiotic study revealed that isolated *Salmonella spp*. were sensitive of Ciprofloxacin (100%) and the isolates were found resistant to Penciline (100%), Erythromycin (100%), followed by Gentamycin (60%), Neomycin (60%).

Table 8. Antibiotic sensitivity pattern *Salmonella spp.* (no.7)

Antibacterial agents	Disc concentration (µg/disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistance	Sensitive	Resistance
Erythromycin	15 µg	0	5	0	100
Pencilline	10µg	0	5	0	100
Gentamycine	10µg	2	3	40	60
Neomycin	30 µg	2	3	40	60
Ciprofloxacin	5 µg	5	0	100	0

4.7.3 Antibiotic sensitivity pattern of *Staphylococcus spp.*

The antibiotic study revealed that isolated *Staphylococcus spp*. were sensitive to Ciprofloxacin (100%), Followed by Gentamycine (66.7%) and the isolates were found resistant to Pencilline (100%), Amoxycilline (100%) Vancomycine (100%).

Table 9. Antibiotic sensitivity pattern *Staphylococcus spp.* (no.4)

Antibacterial agents	Disc concentration (µg/disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistance	Sensitive	Resistance
Amoxycilline	30 µg	0	3	0	100
Pencilline	10µg	0	3	0	100
Ciprofloxacin	5 µg	3	0	100	0
Gentamycine	10µg	2	1	66.7	33.3
Vancomycine	30 µg	0	3	0	100

4.7.4 Antibiotic sensitivity pattern of *Klebsiella spp*

The antibiotic study revealed isolated *Klebsiella spp.* were sensitive of Ciprofloxacin (100%) and the isolates were found resistant to kanamycin (100%) followed by neomycin (50%), Gentamycine (50%) and chloramphenicol (50%).

Table 10. Antibiotic sensitivity pattern *Klebsiella spp.* (no. 4)

Antibacterial agents	Disc concentration (µg/disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistance	Sensitive	Resistance
Kanamycin	30 µg	0	4	0	100
Neomycin	30 µg	2	2	50	50
Gentamycine	10 µg	2	2	50	50
Chloramphenicol	10µg	2	2	50	50
Ciprofloxacin	5 µg	4	0	100	0

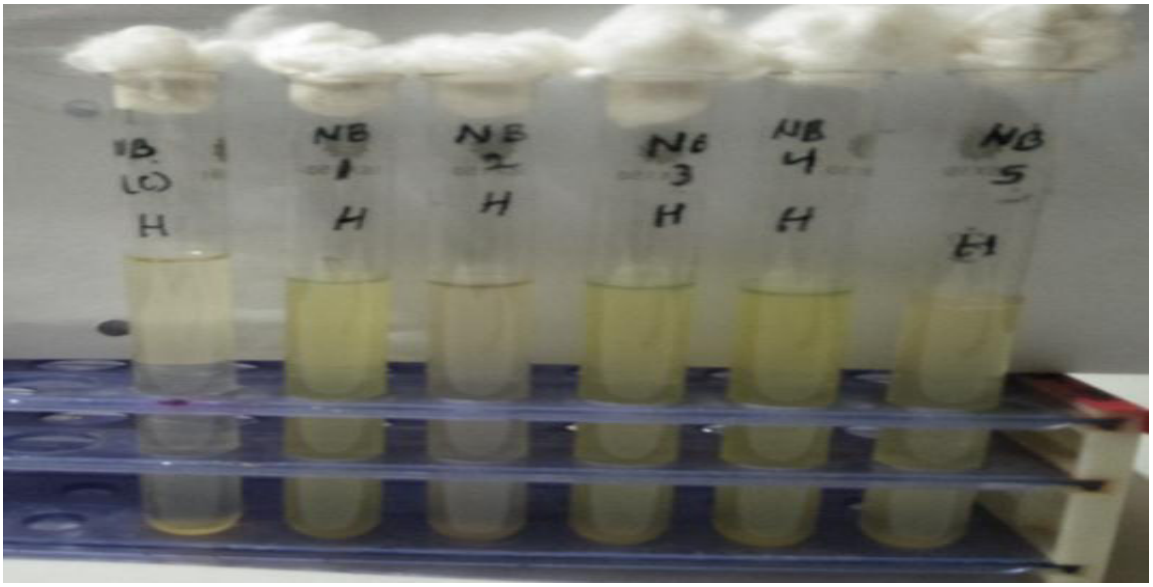

Plate 1: (A) Control (B) Wheat (C) khessari (D) Maize (E) Rice (F) Anchora. Growth of microorganism culture on nutrient broth compare with control test tube left

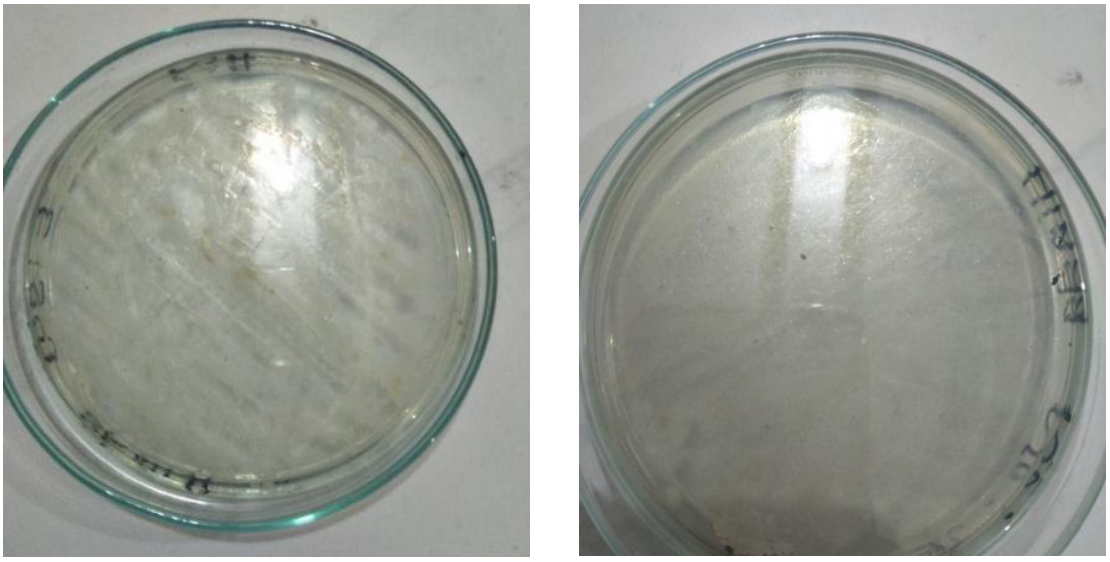

Plate 2: Growth of microorganism culture on nutrient agar compare with control test (right)

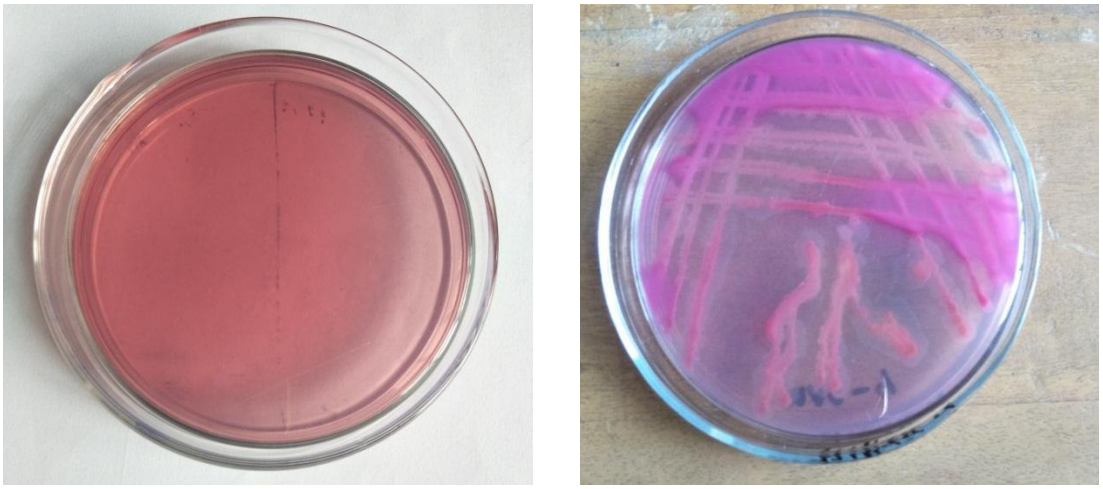

Plate 3: Microorganism Grows in MacConkey agar Plate culture (right) compare with control Petridis (left)

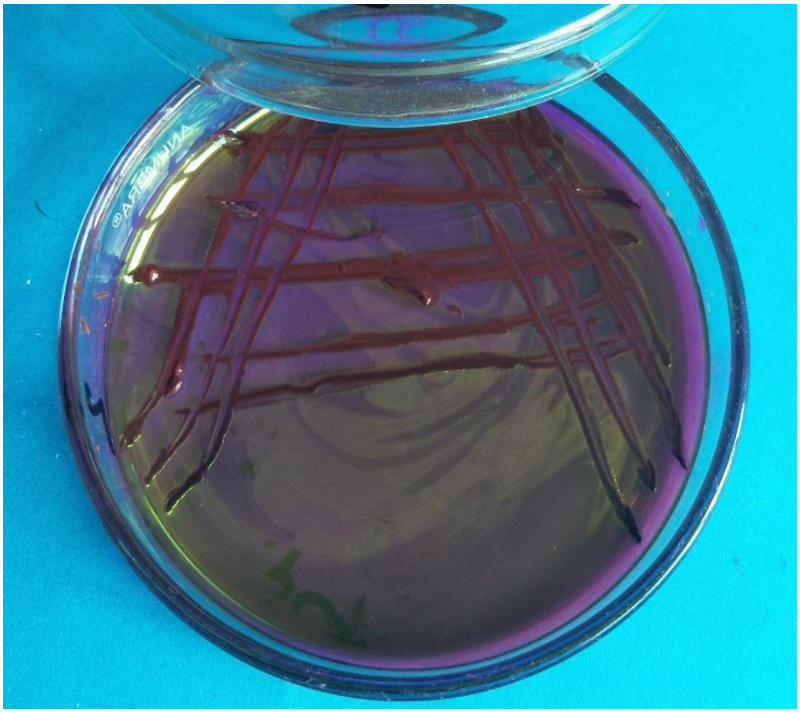

Plate 4: *E. coli* grows in Eosin Methylene Blue (EMB) in changed metallic sheen colored colonies formed

Plate 5: ***Salmonella spp.*** **grows in Salmonella-Shigella (SS) agar formed black color colony**

Plate 6: ***Staphylococcus spp*** **on Mannitol salt agar**

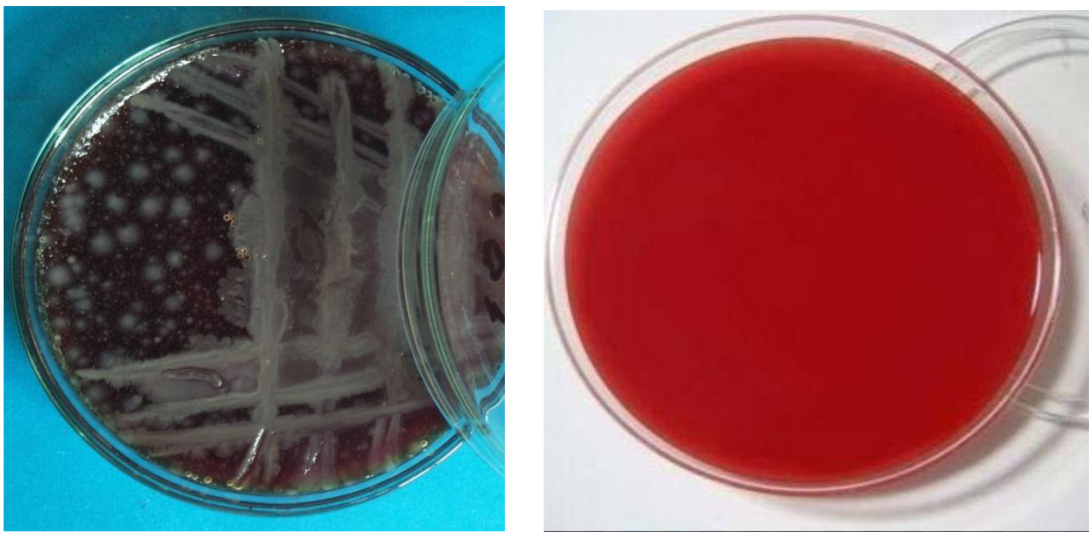

Plate 7: ***Staphylococcus spp.*** **grows in blood agar changed to β- hemolysis colony**

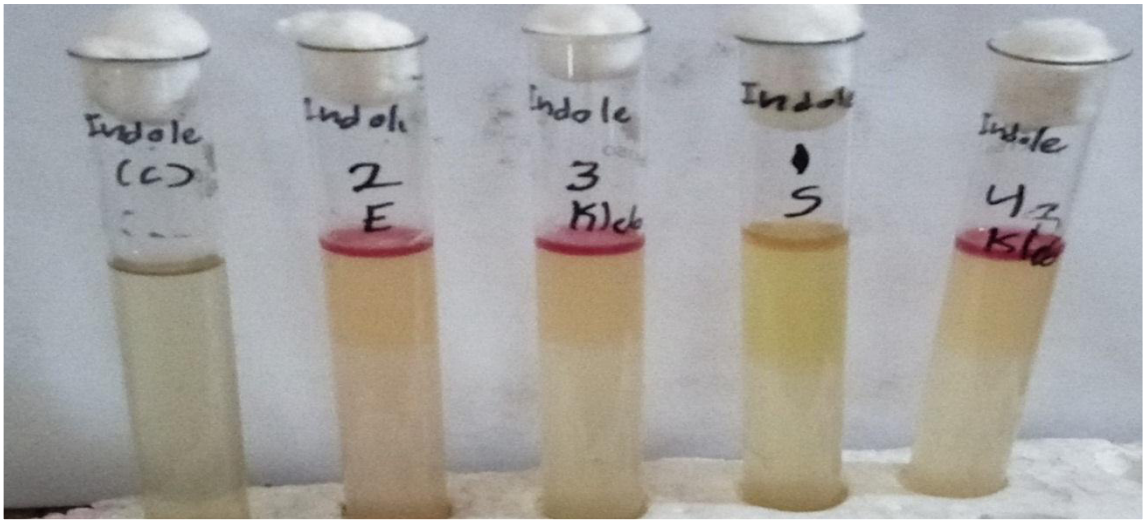

Plate 8: (A) Control (B) ***E.coli,*** **C)** ***Klebsiella Spp*** **(D)** ***salmonella Spp*** **(E)** ***Staphylococcus Spp.*** **Biochemical test showed buffered peptone water (indole) negative reaction and positive reaction**

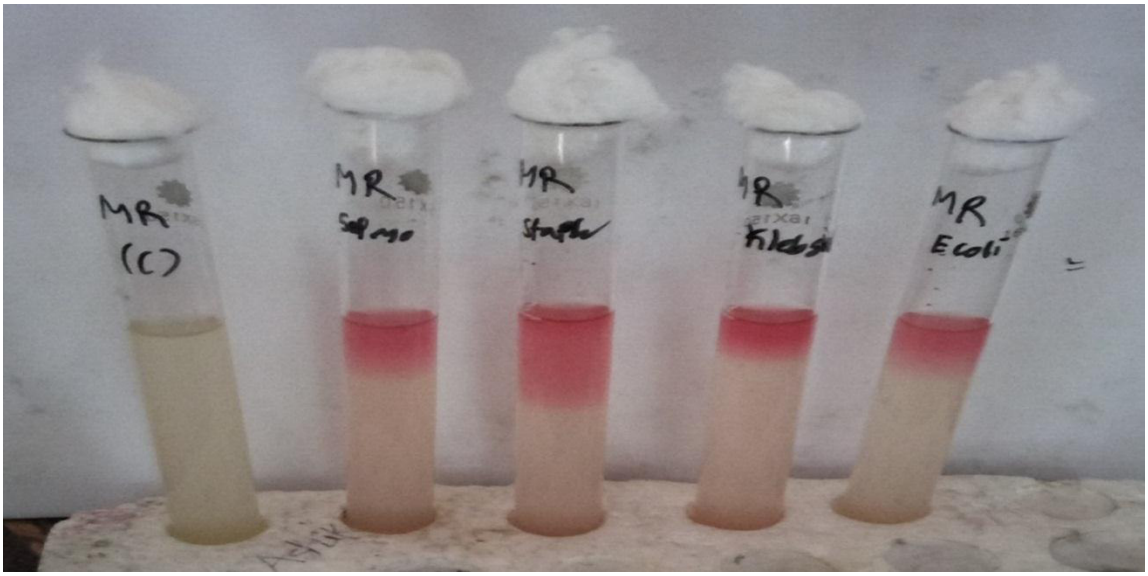

Plate 9: (A) Control (B) *salmonella Spp,* C) *Staphylococcus Spp.* (D) *Klebsiella Spp* (E) *E. coli*. Biochemical test showed buffered Methyl-red (MR) negative reaction and positive reaction biochemical test showed

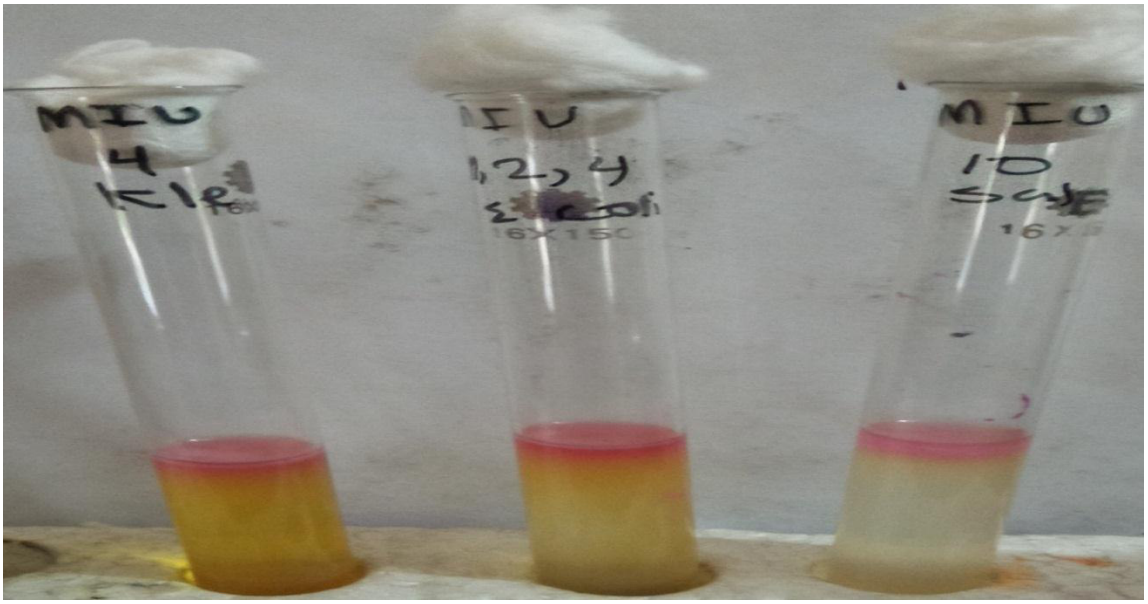

Plate 10: (A) *Klebsiella Spp* (B) *E. coli* (C) *Salmonella Spp.* biochemical test showed buffered Motility Indole urease test (MIU) negative reaction and positive reaction

Plate 11: (A) *E. coli* (B) *Klebsiella Spp* (C) *Staphylococcus spp.* (D) *Salmonella Spp* (E) Control. Biochemical test showed buffered Triple sugar iron (TSI) positive reaction and negative reaction

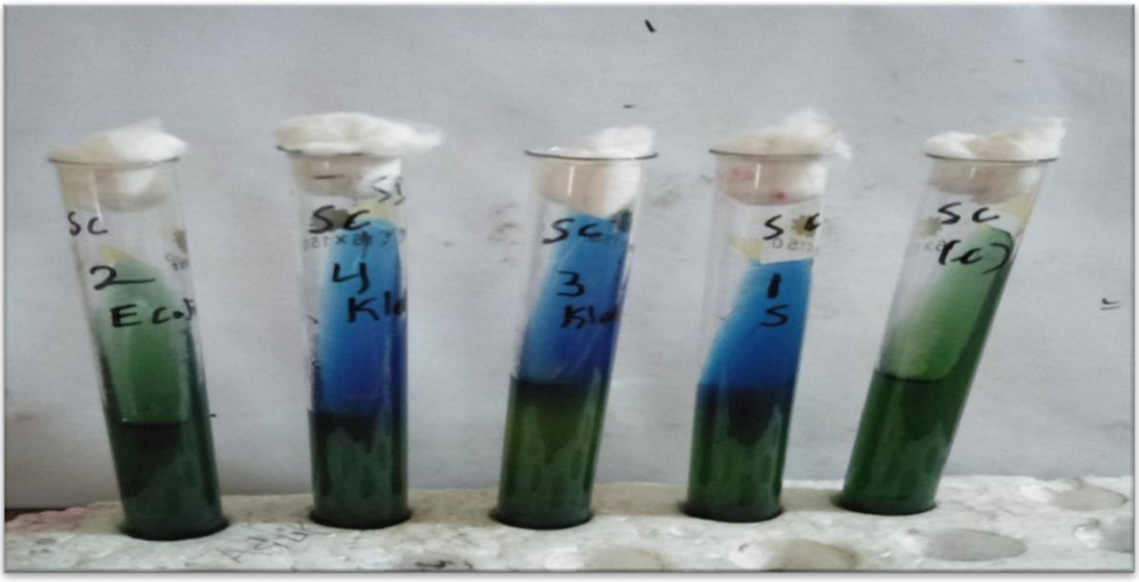

Plate 12: (A) *E. coli* (B) *Klebsiella Spp* (C) *Staphylococcus Spp* (D) *Salmonella Spp* biochemical test showed buffered Simmon citrate (SC) positive reaction and negative reaction

Plate 13: (A) control (B) *Salmonella Spp* (C) *Klebsiella Spp* (D) *E. coli* (E) *Staphylococcus spp* biochemical test showed buffered Vegos-proskauer test (VP) positive reaction and negative reaction

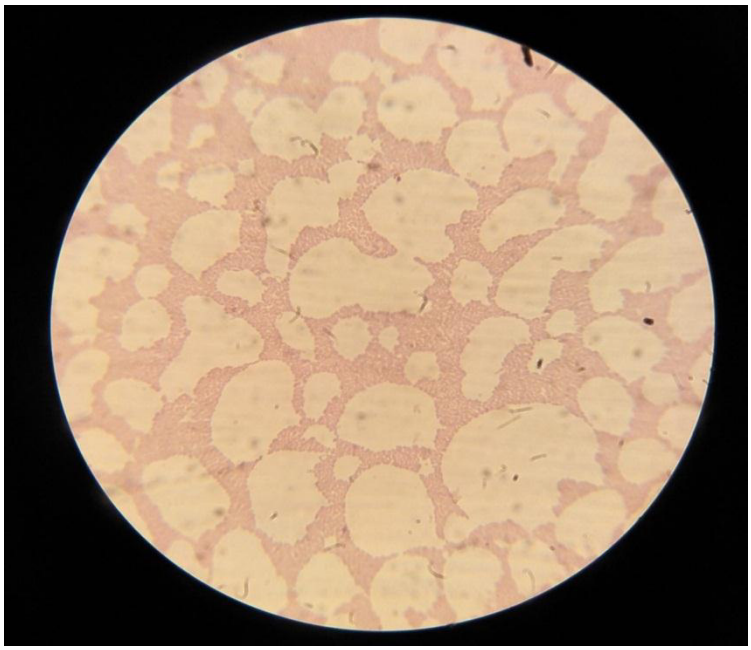

Plate 14: Gram negative klebsiella with the short rods, single of paired chain

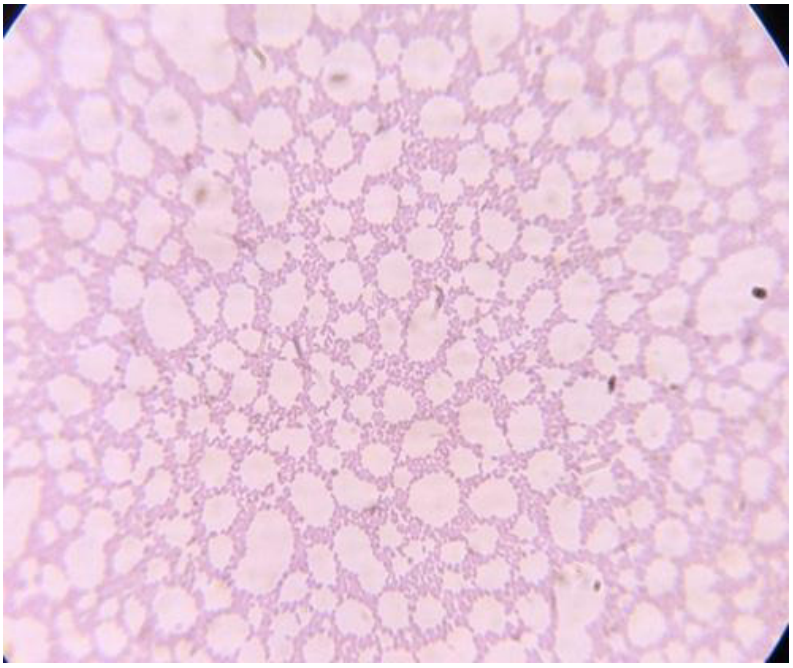

Plate 15: Gram's staining of *Staphylococcus spp.* Showed gram-positive cluster form of organism

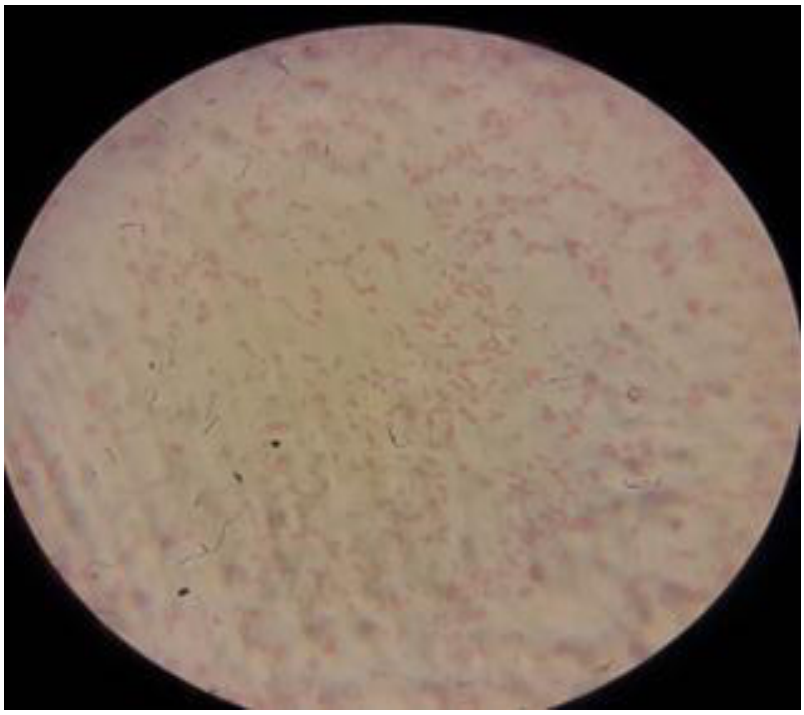

Plate 16: Gram's staining of *E. coli*. gram-negative single or paired short plumb rod

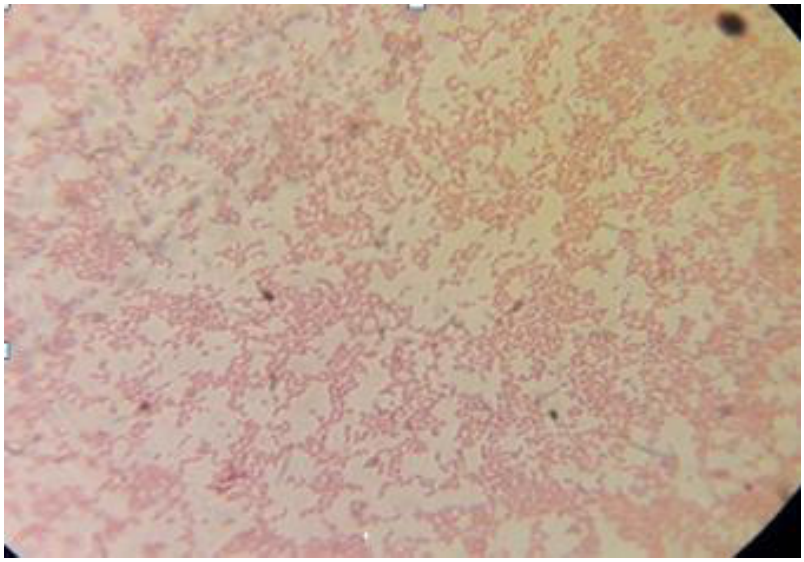

Plate 17: Gram negative *Salmonella spp.*

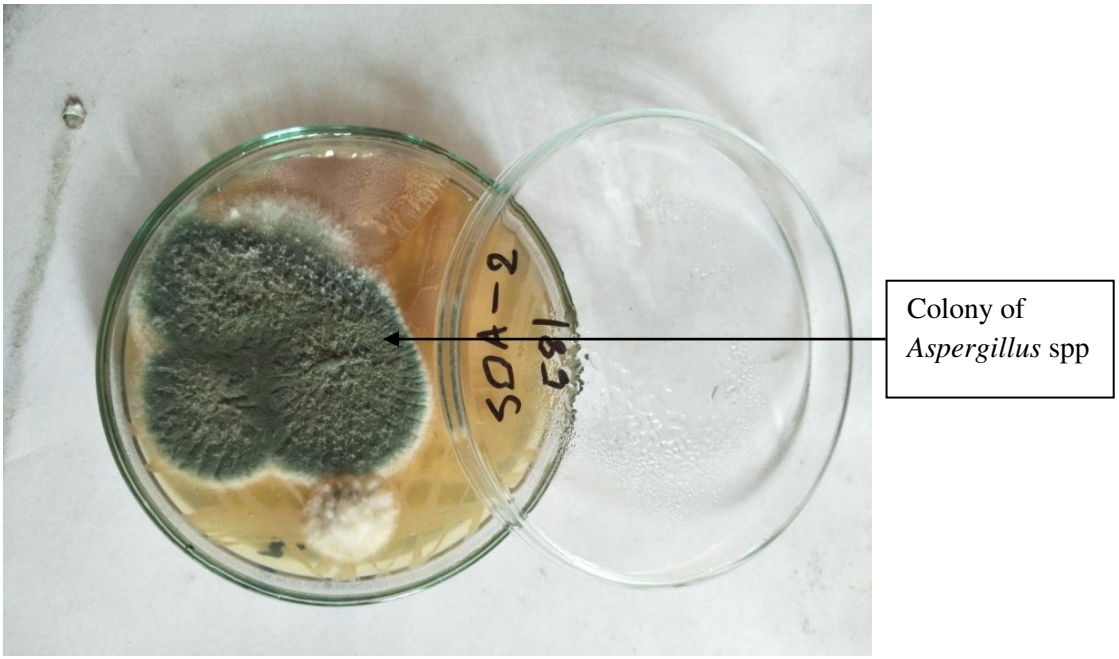

Plate 18: *Aspergillus spp* on SDA agar

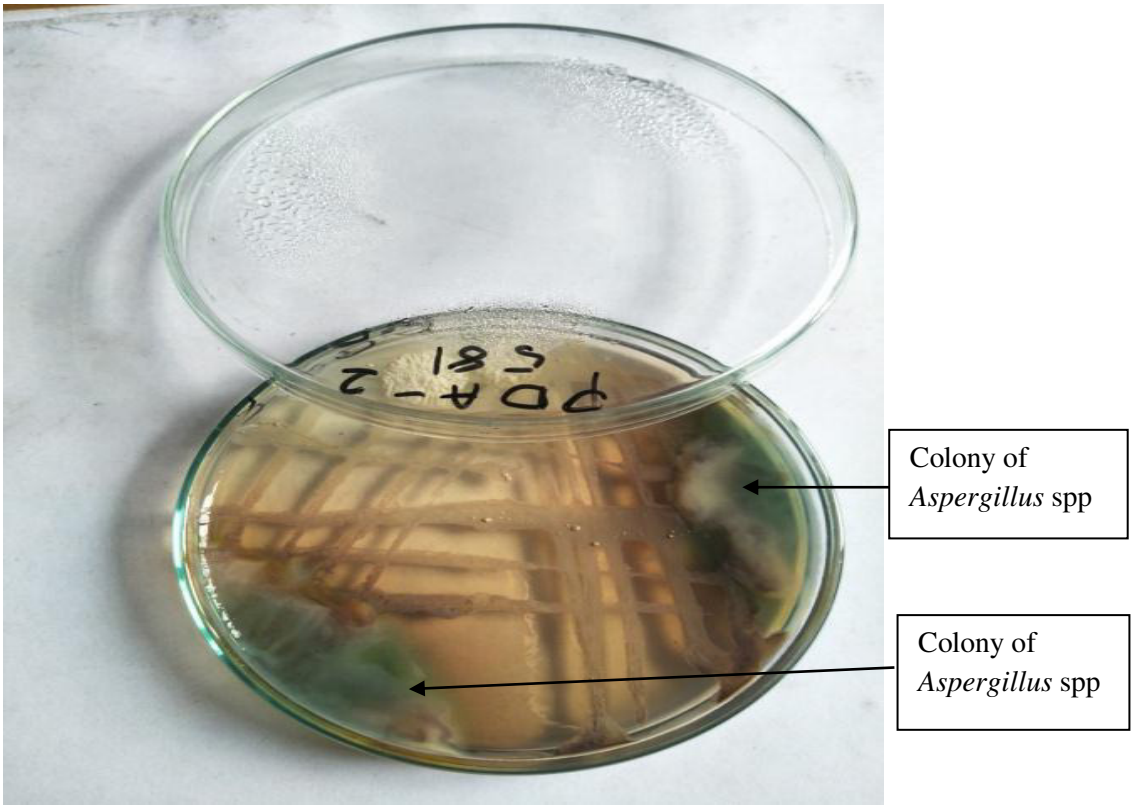

Plate 19: ***Aspergillus spp*** **on PDA agar**

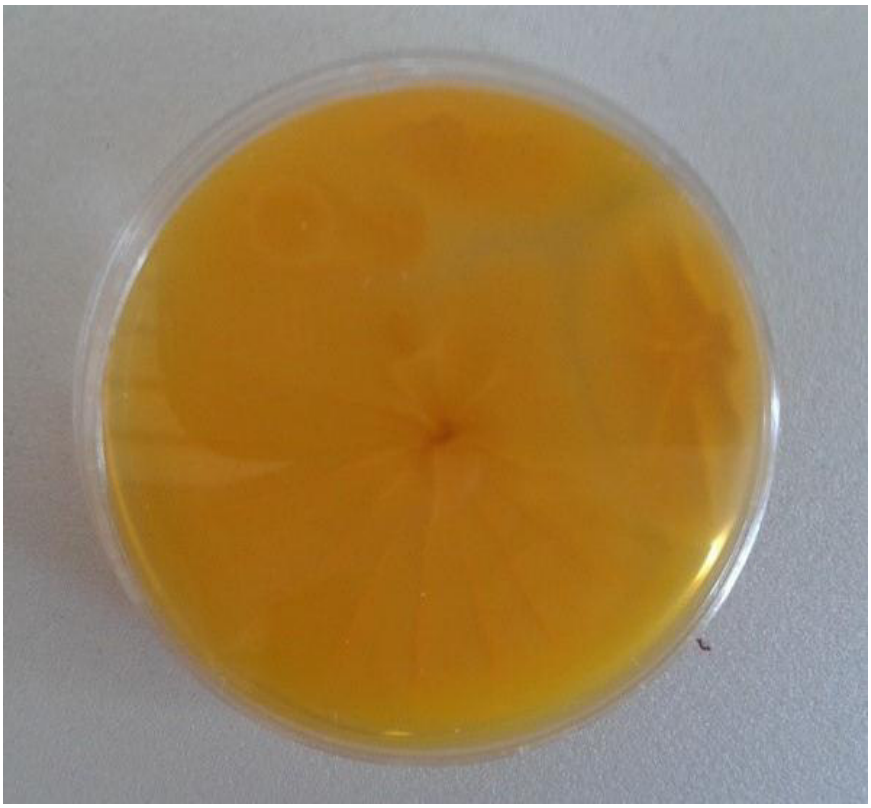

Plate 20: ***Aspergillus spp*** **on Aspergillus flavus paraciticus agar medium**

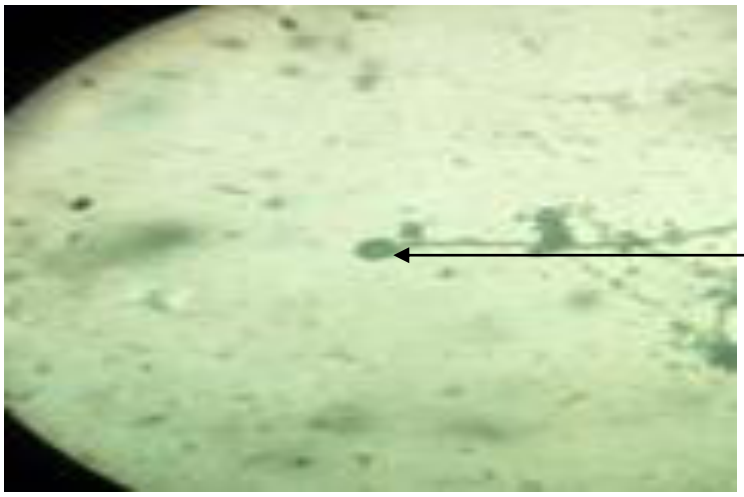

Plate 21: Conidia of *Aspergillus spp* by lactophenol cotton blue stain

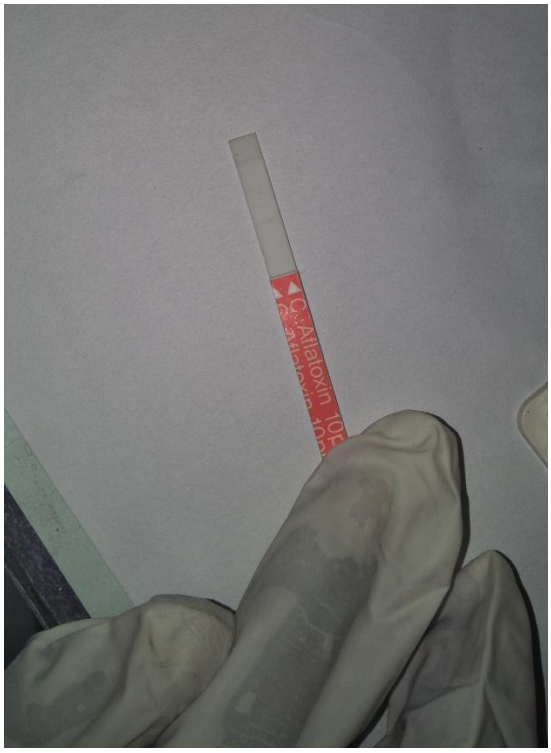

Plate 22: Aflatoxine test stripe

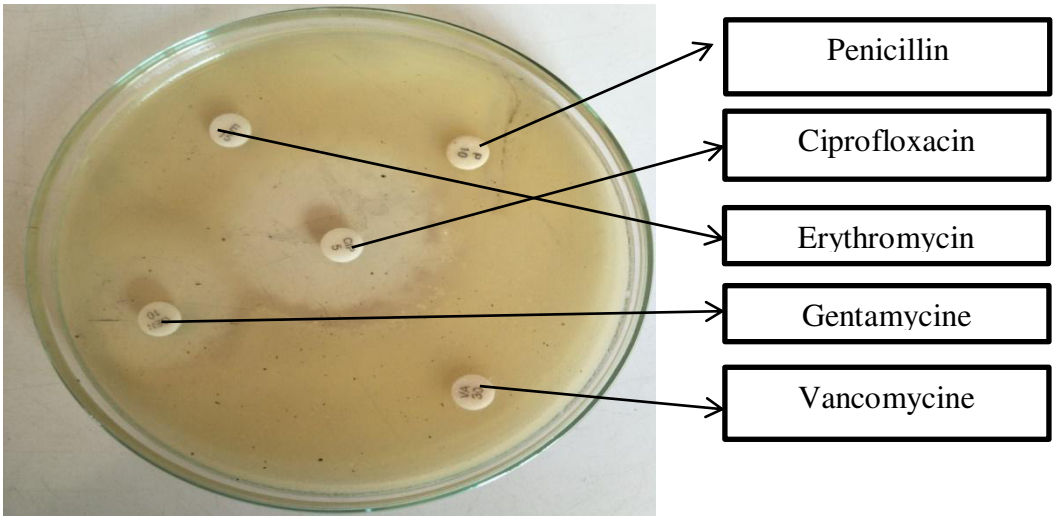

Plate 23: Antibiogram of *E. coli.*

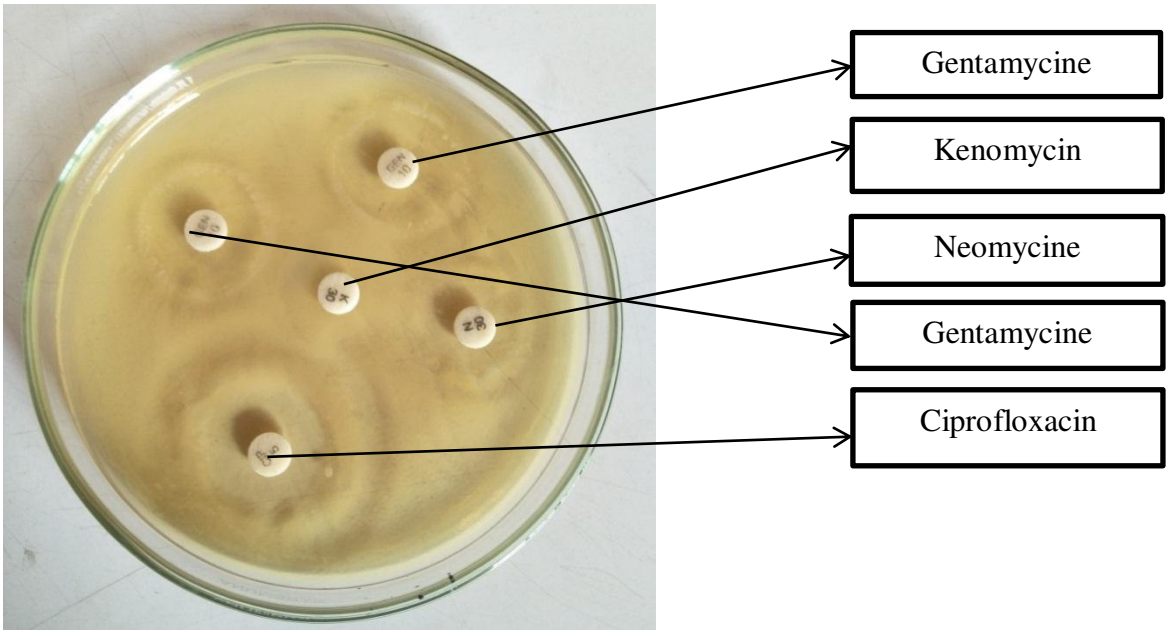

Plate 24: Antibiogram of *Klebsiella spp*

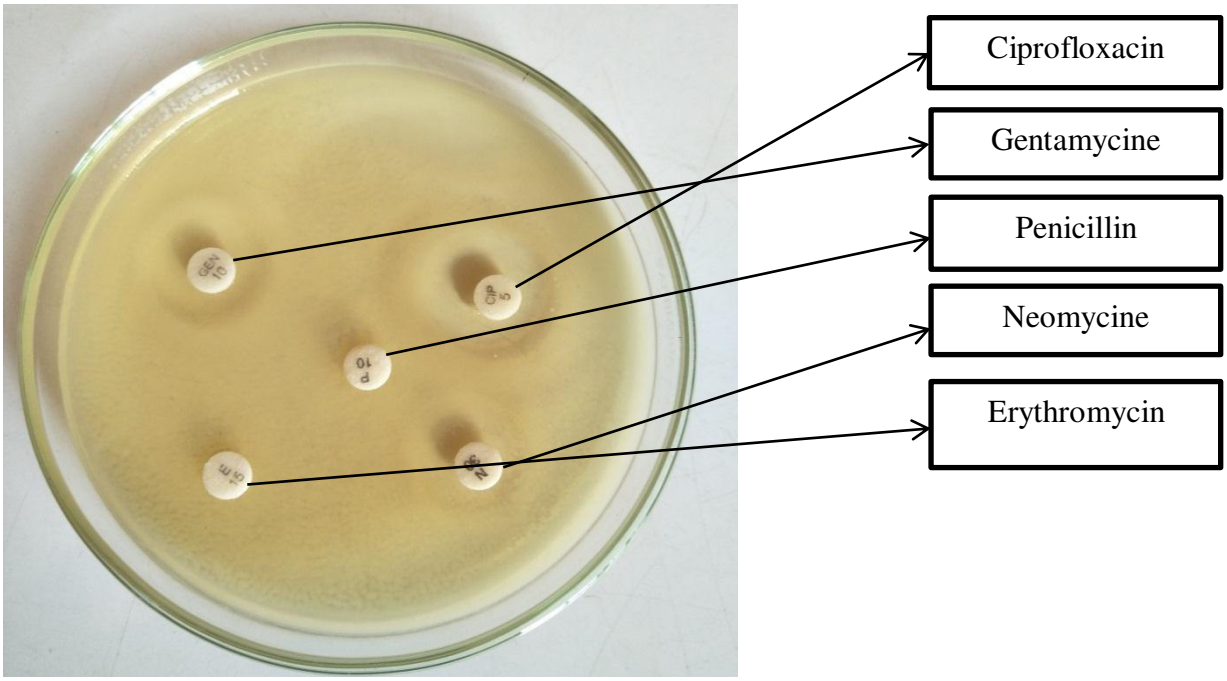

Plate 25: Antibiogram of *Salmonella spp.*

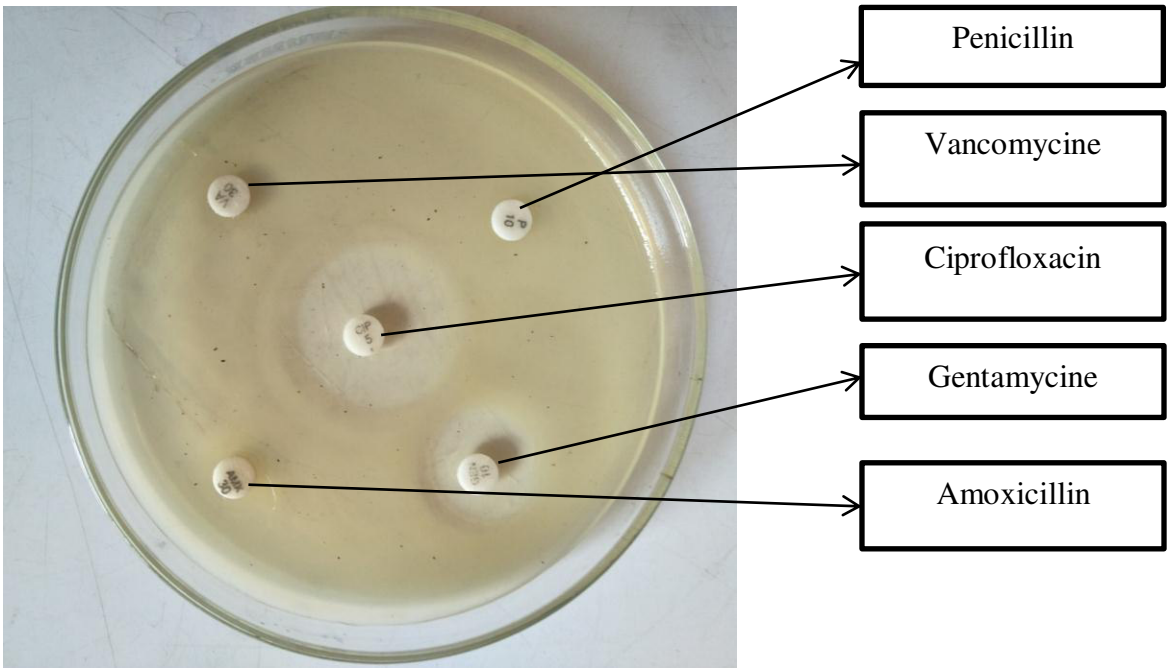

Plate 26: Antibiogram of *Staphylococcus spp.*

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

The experiment was carried out for detection of potential bacterial and aflatoxigenic fungi pathogens from grain samples. The three locations Leili mall, Doshmail, and Bahadur bazar were selected for sample collection. For this study a total of 25 grain samples were collected. A series of test were conducted for detection, frequency distribution and drug sensitivity of various kinds of bacteria in grain and for detection of aflatoxigenic fungus from grain samples.

In this study the 15 bacterial isolates comprising 4 genera of bacteria were found from a total of 25 samples. The Isolated bacteria were *Staphylococcus spp., Escherichia coli, Klebsiella spp and Salmonella spp*. Among the 25 grain samples, 4 *Staphylococcus spp.,* 7 *Salmonella spp.,* 4 *Escherichia coli* and 4 *Klebsiella spp*. were isolated with 16%, 28%, 16% and 16% prevalence respectively. Similar study was made by Md. Mazedul Haque *et al* (2017) in which they reported the *Escherichia coli* and *Staphylococcus spp.* from the positive grain samples with prevalence of 100%, 50%, and 80% respectively. So Above report was less similar with the findings of this study.

Among the 25 grain samples *Aspergillus spp* was isolated from 4 grain samples with 16% prevalence. But Aflatoxigenic *Aspergillus spp* was isolated from 3 samples with 12% prevalence. From the wheat samples and maize the Aflatoxigenic fungus was isolated and their prevalence in maize and wheat was 20% and 40% respectively which was more or less similar to the study of *Bakr. M. A (1992).*

Many infectious agents might be present in grains but *Staphylococcus spp, E.coli, Klebsiella spp*, *Salmonella spp* were isolated from grain samples in this study. The frequency distribution of different bacterial isolates in different grain samples was found variable. Result of the present study indicates that all the four different genera of bacteria were not present in the same grain sample collected from the different market. From the wheat samples, *E.coli* and *Salmonella spp* were isolated from one samples and their prevalence was 20% separately. But, no *Klebsiella spp* and *Staphylococcus spp* was found. From Khessari dal, *E.coli* and *Staphylococcus spp* were isolated and their prevalence were 40% and 20% respectively. In Maize, the prevalence of *Salmonella spp, Klebsiella spp* and *Staphylococcus spp* were 40%, 20% and 20% respectively. No

klebsiella spp was found from maize. From rice no E.coli and Staphylococcus was isolated but *Salmonella spp* and *Klebsiella spp* was found and their prevalence was 20% and 40% respectively. From anchor dal *E.coli, Salmonella spp*, *Klebsiella spp* and *Staphylococcus spp* was isolated and their prevalence was 20%, 60%, 20%and 40% respectively.

The isolated *Staphylococcus spp. E.coli, Klebsiella spp, Salmonella spp,* showed identical result in different biochemical tests of including catalase test, indole test, methyl-red, Vegos-proskauer test, motility indole urease test, triple sugar iron test, and citrate utilization test.

The in vitro antibiotic sensitivity test of isolated bacteria was done with 8 different antibiotics, such as Gentamycin, Ciprofloxacin, Kanamycin, Neomycin, Erythromycin, Vancomycin, and Amoxicillline, chloramphenicol.

The antibiotic study revealed that isolated *Staphylococcus spp*. were sensitive to Ciprofloxacin (100%), Followed by Gentamycin (66.7%) and the isolates were found resistant to Pencilline (100%), Amoxycilline (100%) and Vancomycine (100%) These findings were more or less similar to the findings of Md. Mazedul Haque *et al.* (2017).

The antibiotic study revealed that isolated *Salmonella spp*. were sensitive of Ciprofloxacin (100%) and the isolates were found resistant to Pencilline (100%), Erythromycin (100%), followed by Gentamycin (60%), Neomycin (60%). These findings were more or less similar to the findings of Md. Mazedul Haque *et al.* (2017).

The antibiotic study revealed that isolated *E. coli were* sensitive of Ciprofloxacin (100%) followed by Gentamycin (66.7%), the isolates were found resistant to Pencilline (100%), Vancomycin (100%) and Erythromycin (100%).These findings were more or less similar to the findings of Md. Mazedul Haque *et al.* (2017).

The antibiotic study revealed isolated *Klebsiella spp.* were sensitive of Ciprofloxacin (100%) and the isolates were found resistant to kanamycin (100%) followed by neomycin (50%), Gentamycin (50%) and chloramphenicol (50%).These findings were more or less similar to the findings of Md. Mazedul Haque *et al.* (2017).

Overall effective drugs against isolated bacteria were ciprofloxacin followed by Gentamycine. But resistant drugs were Penicillin, Vancomycin, Erythromycin,

Kanamycin and Amoxicilline. The variation in the sensitivity of common antibiotic could be result of extensive and indiscriminate use of these antibiotics.

So, for effective treatment of infection caused by consumption of such grain food, medicinal formulation should preferably contain antibiotics that have good spectrum of inhibition against all species of these bacteria. In this context, it is interesting to note ciprofloxacin and Gentamycin should be the antibiotic of choice and these antibiotics appear to be promising for the treatment of the infection caused by grain food in Bangladesh.

Grain samples were also found to be contaminated with *Aspergillus spp*. Not only *Aspergillus spp* but also Aflatoxigenic *Aspergillus spp.* The Food and Drug Administration (FDA) has established action levels of 20 parts per billion (ppb) for grain and feed products, and 0.5 ppb for milk. Mixing aflatoxin contaminated grains with sound grains for sale is illegal. In the U.S. corn and other grain with less than 20 ppb aflatoxin can be sold as normal grain.

But according to this study in our maize samples and in two of our wheat samples the aflatoxins are found to be as 20ppb. So it was a great concern for human health because Aflatoxin is a potent liver toxin known to cause cancer in animals. Their early detection can help to take preventive measures to combat economic and health losses. The study showed that earlier detections can be made by simple traditional identifications using macro and micro morphological fungal features rather than adopting the time and cost consuming molecular identification techniques.

CHAPTER VI

SUMMARY AND CONCLUSION

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SUMMARY AND CONCLUTION

The recent study was conducted for detection of potential bacterial and aflatoxigenic fungi pathogens from grain samples. Properties and antibiotic sensitivity pattern of the bacterial and fungal isolated from infected grain food. A total of 25 grain food samples were collected from different areas of Dinajpur district in Bangladesh for this study.

Staphylococcus spp, Salmonella spp, E. coli spp, Klebsiella spp, were the major bacterial etiological agent isolated from grain food sample with 16%, 28%, 16% and 16% prevalence respectively.

Among the 25 grain samples, Aspergillus spp was isolated from 4 grain samples with 16% prevalence. But Aflatoxigenic Aspergillus spp was isolated from 3 samples with 12% prevalence.

From the wheat samples and maize the Aflatoxigenic fungus was isolated and their prevalence in maize and wheat was 40% and 20% respectively.

Their early detection can help to take preventive measures to combat economic and health losses. The study showed that earlier detections can be made by simple traditional identifications using macro and micro morphological fungal features rather than adopting the time and cost consuming molecular identification techniques.

Antibiogram result indicated the ciprofloxacin and Gentamycin, in optimum doses would be the drug of choice to treat the most cases of infection caused by consumption of contaminated grain foods.

Antibiotic sensitivity test revealed that kanamycin, Vancomycine, Neomycine and Erythromycin would not be recommended because isolated bacteria were resistant to these drugs.

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APPENDICES

APPENDICES

APPENDIX-I

Composition of the media used:

Nutrient Agar	**Grams/Liter**
Peptone	5.0
Bacto beef extract	3.0
NaC1	5.0
Agar	15.0
Distilled water	1000 ml
pH	7.2

Sterilized at 121°C under 15 lb/in2 pressure for 15 minutes.

Nutrient Broth	**Grams/Liter**
Peptone	5.0
NaCI	5.0
Beef Extract	1.5
Yeast Extract	1.5
pH	7.4

Distilled water 1000 ml Sterilized al 121°C under 15 lb/in2 pressure for 15 minutes.

MacConkey agar	**Grams/Liter**
Bctoeptone	17.0
Proteas Peptone	3.0
Lactose	10.0
Bile Salt	1.5
Agar	15.0
Neutral red	0.03
Crystal violet	0.100
Distilled water	1000 ml
pH	71

Sterilized at 121°C under 15 lb/in2 pressure for 15 minutes.

Eosine methylene blue(EMB) agar	**Gram/Liter**
Peptone	10.0
Lactose	10.0
K_2HpO_4	2.0
Eosin	0.4
Methylene blue	0.065
Agar	20.0
Distilled water	1000 ml
pH	6.8

Sterilized at 121°C under 1 Sib/in^2 pressure for 15 minutes.

Mueller Hinton Agar	**Gram/Liter**
Beef infusion	2.0
Bacto casamino acid (technical)	17.5
Starch	1.5
Bacto agar	17.5
Distilled water	1000 ml
pH	73

Sterilized at 121°C under 151 b/in^2pressure for 15 minutes.

Mannitol Salt Agar	**Gram/Liter**
Proteas peptone	10.0
Beef extract	1.0
D-Mannitol	10.0
NaCl	75.0
Phenol red	0.025
Agar	20
Distilled water	1000ml

Sterilized at 121°C under 15 lb/in^2pressure for 15 minutes.

***Salmonella Shigella* Agar**	**Gram/Liter**
Lactose	10.0
Bile salt No.3	8.5
Sodium citrate	8.5
Sodium Thiosulfate	8.5
Beef extract	5.0
Proteose peptone	5.0
Ferric citrate	1.0
Brilliant Green	0.33
Nuetral Red	0.025
Agar	13.5
Distilled water	1000ml

Sterilized at 121°C under 15 lb/in^2 pressure for 15 minutes

Normal Saline	**Gram/Liter**
NaCl	0.85
Distilled water	1000ml

Autoclaved at 121°C for 15 minutes

Sabourad Dextrose Agar (SDA)	**Gram/Liter**
Ingredients	In gm/L
Dextrose (Glucose)	40 gm
Peptone	10 gm
Agar	15 gm
Distilled Water	1000 ml

Potato Dextrose Agar (PDA)	**Gram/Liter**
Ingredients	In gm/L
Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled Water	1 L

APPENDIX-Il

Composition of the media used in biochemical test

MR-VP broth	**Gram/Liter**
Peptone	7.0
Dextrose	50
Dipotassiuni phosphate	5.0
Distilled water	1000ml
pH	6.9

Sterilized at 121°C under 151 b/in^{2}pressure for 15 minutes.

Triple Sugar Iron TSI Agar	**Gram Liter**
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Lactose	10.0
Saccharose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosuiphate	0.3
Phenol Red	0.024
Agar	12.0
pH	7.4

Sterilized at 121°C under 151 b/in^{2}pressure for 15 minutes.

Simmons citrate Agar	**Gram/Liter**
Magnesium sulphate	0.2
Manoammonium phosphate	1.0
Dipotassium phosphate	1.0

Sodium citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Brom-Thymol Blue	0.08
pH	6.8

Sterilized at 121°C under 15 lb/in^{2}pressure for 15 minutes.

Indole tryptopon broth medium	**Gram/Liter**
Tryptone	10.0
Distilled water	1000ml

Sterilized at 121°C under 15 lb/in^{2}pressure for 15 minutes.

Nitrate broth	**Gram/Liter**
Peptone	5.0
Beef extract	3.0
NaCI	5.0
Potassium nitrate	1.0
Agar	1.0
Distilled water	1000 ml
pH	7.2

Sterilized at 121°C under 151 b/in^{2}pressure for 15 minutes.

APPENDIX III

Composition of chemicals and reagents

Crystal violet

Solution-A

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol (95%)	20.0 ml

Solution-B

Ammonium oxalate	0.8
Distilled water	80.0 ml

Note-Mix the solution A and B

Gram's iodine

Iodine	l.0g
Potassium iodide	2.0 g
Distilled water	300.0 ml
Ethyl alcohol	(95%)
Ethyl alcohol (100%)	95.5 ml
Distilled water	5.0 ml

Safranin

Safranin O	0.25 ml
Ethyl alcohol (95%)	10.0 ml
Distilled water	100. 0 ml

Kovac's reagent (for detection of indole)

P-Dim ethylaminobenzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Hydrochloric acid (concentrated)	25.0 ml

Concentrated P-Dimethylaminobenzaldehyde was dissolved in the amyl alcoholadded slowly.

Methyl red solution

Methyl red	0.04g
Ethanol	40.0g
Distilled water	100.0 ml

Methyl red dissolved in ethanol and diluted water.

Barrit's reagent

Solution-A

α- naptho	15.0 g
Ethanol (Absolut)	95.0 g

α- naptho was dissolved in ethanol with constant stirring.

Solution-B

KOH	40.0g
Creatirie	0.3 g
Distilled water	100.0 ml

Hydrogen peroxide

3% aqueous solution of H_2O_2 was prepared from the H_20_2 absolute solution.