ISOLATION AND IDENTIFICATION OF ECONOMICALLY IMPORTANT BACTERIA FROM THE BROILER FARMS AT DINAJPUR DISTRICT OF BANGLADESH WITH THEIR ANTIBIOTIC RESISTANCE

A THESIS

BY

MD. NURNOBI SHARKAR

REGISTRATION NO. 1605121

SEMESTER: JANUARY–JUNE, 2017

SESION: 2016

MASTER OF SCIENCE (M.S.)

IN

MICROBIOLOGY

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JULY, 2017

Dedicated

To

My Beloved Parents and Teachers

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The author is ever grateful and indebted to the Almighty Allah without whose grace she would have ever been able to pursue her higher studies in this field of science and to complete her research work successfully for the degree of Master of Science (MS) in Microbiology.

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The Author *July 2017*

ABSTRACT

The present research work was carried out for the isolation and identification of economically important bacteria from the broiler farms at Dinajpur district of Bangladesh with their antibiotic resistance during the period from January 2017 to June 2017. A total 100 samples (liver,

lungs, esophagus, duodenum and tracheal swab) were collected from broiler farms (n=10) in Dinajpur district. The samples were shifted aseptically to the Bacteriology laboratory of the department of Microbiology, Hajee Mohammed Danesh Science and Technology University following standard bacteriological techniques. Out of 100 samples *Escherichia coli* was isolated from 50 samples. Similarly, *Salmonella* spp*., Staphylococcus* spp., and *Pasteurella* spp. were isolated from 28, 20, and 8 samples, respectively. Antibiogram studies revealed that Ciprofloxacin was highly sensitive against all the isolated bacteria. *Escherichia coli* resistant to ampicillin 100 and amoxicillin 100. *Salmonella* spp*.,* resistance to Amoxycilin 89, Erythromycin 89. *Pasteurella* spp. resistance to amoxicillin 88. Diversified bacterial species are prevalent in broiler in Dinajpur district However, *E. coli* and *Salmonella* spp. infection might make the bird vulnerable for easy access of infection. Proper vaccination and use of selective antibiotics are crucial in protecting broilers from these pathogens.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	:	Negative
#	:	Identifying number
%	:	Percentage
@	:	At the rate of
+	:	Positive
µg	:	Microgram
µl	:	Microlitre
^{0}C	:	Degree of celcius
Ag	:	Antigen
Assist	:	Assistant
BA	:	BloodAgar
BD	:	Bangladesh
BGA	:	Brilliant Green Agar
EMB	:	Eosin Methylene Blue
ER	:	Erythromycin
et al.	:	Associated
etc	:	Etcetera
FAO	:	Food and Agricultural Organization
Gm	:	Gram
H.S	:	Haemorrhagic septicemia
H_2O_2	:	Hydrogen peroxide
H_2S	:	Hydrogen sulphide

HSTU	:	Hajee Mohammad Danesh Science and Technology University
i.e.	:	That is
Ltd	:	Limited
M.S	:	Master of Science
MC	:	MacConkey Agar
MI	:	Milliliter
MIU	:	Motility Indole Urease
MR	:	Methyl Red
NA	:	Nutrient Agar
NB	:	Nutrient Broth

LIST OF ABBREVIATIONS AND SYMBOLS (Contd.)

No.	:	Number
PBS	:	Phosphate Buffer Saline
PM	:	Post Mortem
Prof.	:	Professor
PSS	:	Physiological Saline Solution
RPM	:	Rotation Per Minute
SC	:	Subcutaneous
SE	:	Standard Error
SL	:	Serial number
Sp	:	Species
SSA	:	Salmonella Shigella Agar

v/v : Volume by volume

VP : Voges-Proskauer

w/v : Weight by volume

CHAPTER 1

INTRODUCTION

Bangladesh is a developing country where poultry industry is one of the emerging, promising, beneficial and important sectors that has been contributing progressively to economy of Bangladesh. Poultry industry started practicallyduring 1980s in our country (Huque, 2001). There has been a tremendous development ofhis sector since the last decades (1996-2006) in the country (Rahman, 2004). There are several constraints for the expansion of this industry in our country. Some infectious diseases are considered as the most leading causes of economic loss and discouraging poultry rearing in this country (Das *et al.,* 2005). *Escherichia coli* is a common pathogen for commercial poultry causing Colibacillosis all over the world. It is a major cause of respiratory and septicemic diseases in broiler chicken causing mortality less than 5% with morbidity over 50% but in layer it affects the reproductive tract resulting in failure of egg production and infertility (Barens and Gross., 1997). It may cause about 28% death in Sonali variety of birds in Bangladesh (Biswas *et al.,* 2006). The incidence rate of the disease has been reported to be about 22% in Bangladesh (Kamaruddin *et al*., 2007). *E. coli* infections cause many clinical manifestations such as airsacculitis, pericarditis, septicemia, and death of the birds (Hofstad *et al*., 1984). The infection has also been extended to various parts and organs such as skin, joints, eyes, head, blood, heart, yolk sac, peritoneum, etc. (Stebbins *et al*., 1992).In India the disease was first isolated and recorded from yolk sac infection (Pathak *et al*., 1960). At the present time there is a silent killer called "Salmonellosis" which causes constant losses to poultry industry economically and thus makes difficult in the establishing this industry (Khan *et al.,* 1998). Pullorum disease and Fowl typhoid have become wide spread in Bangladesh like other area of the world (Sarker, 1976 and Rahman *et al.,* 1979). Mortality may very from negligible to (10% to 80%) or higher in severe outbreaks (Kumar and Khaushi, 1988; Kaura *et al,*. 1990; Kleven and Yoder. 1998). The diseases are caused by Gram-negative bacteria, *Salmonella*

gallinarum and *Salmonella pullorum* respectively. Pullorum disease, formerly referred to as fatal septicaemia of chicks, white diarrhoea or white bacillary diarrhea. Pullorum disease is usually confined to the first 2-3 weeks of age occasionally occurs in adults. Fowl typhoid is frequently referred to as a disease of adult birds; there are also reports of high mortality in young chicks (Christensen *et al.,* 1992). *Salmonella gallinarum* is the most devastating disease in Bangladesh (Begum *et al.,* 1993 and Hoque *et al.,* 1992). Among the bacterial diseases, fowl cholera caused by *P. multocida* is a major threat for poultry industries in Bangladesh. Fowl cholera occurs sporadically or enzootically in most countries of the world (Heddleston and Rhoades, 1978) including Bangladesh. About 25% to 35% mortality in chickens of Bangladesh is due to fowl cholera (Choudhury *et al.,* 1985). *P. multocida* is a Gram-negative, nonmotile, penicillin-sensitive coccobacillus, capsulated, non spore forming rod shaped bacterium occurring singly, in pairs occasionally as chains or filaments belonging to the Pasteurellaceae family (OIE, 2008). *P. multocida* is a diverse organism; different genotype groups are implicated in different diseases, and genotype groups can also be paralleled with host preference (Amonsin *et al.,* 2002). The majority of respiratory tract infection in fowl species is caused by *P. multocida* (Bisgaard *et al.,* 2003). *P. multocida* can also be harbored in cloacal mucosa of asymptomatic birds and these strains can become sources of outbreaks (Muhairwa *et al.,* 2001).*Staphylococcus aureus* was discovered in Aberdeen, Scotland in 1880 by the Surgeon Sir Alexander Ogston in pus from surgical abscesses (Ogoston, 1984), abbreviated to *S. aureus* or *Staph aureus* in medical literature. According to McCaughey B (2007) it is found in United States, Denmark, Finland, and the Netherlands as a public health hazard. Giasuudin *et al.,* (2002) reported the disease to be found in Bangladesh as co-infection with other diseases. *S. aureus* are capable of producing pigment according to their strain called staphyloxanthin, a carotenoidpigment that acts as a virulence factor. It has an antioxidant action that helps the microbe to evade death by reactive oxygen species used by the host immune system. Staphyloxanthin is responsible for its characteristic golden colour in agar (Claudit *et al*., 2006). This pigment helps to survive incubation with an oxidizing chemical, such as hydrogen peroxide. For this reason, drugs designed to inhibit the bacterium's production of the staphyloxanthin may weaken it and renew its susceptibility to antibiotics (Liu *et al.,* 2005).Antibiotic resistance has been recognized as a global health problem for many decades. The problem has now been recognized as one of the top health challenges that the world will be facing in the 21st century (FDA, 2000 and CDC 2010). Some of its causes are widely accepted, for example, the overuse and inappropriate use of antibiotics for nonbacterial infections such as colds and

other viral infections and inadequate antibiotic stewardship in the clinical arena (Levy, 2002). Microbial resistance to antibiotic is also a decade old problem in veterinary medicine (Smith *et al.*, 1957; Howells *et al*., 1975 and Gorbach, 2001). There also exists a relationship of drug-resistant bacteria in people to antibiotic use in food animals (Witte,2000 ;Sorensen *et al*., 2001; Donabedin *et al*., and Lee, 2003; Perreten *et al.*, 2005; De Boer *et al., (*2009) and some of it is probably due to the use of antibiotics as growth promoters in animal feed (Johnson *et al*, 2006). Bangladesh is also not out of this problem. Antibiotics are extensively used as growth promoters in poultry production or to control infectious disease. Anti-microbial abuse is considered to be the most vital selecting force to antimicrobial resistance of bacteria (Okeke *et al.*, 1999 and Moreno *et al.*, 2000). Moreover, antibiotic treatment is considered the most important issue that promotes the emergence, selection and spreading of antibiotic-resistant microorganisms in both veterinary and human medicine (Neu, 1992 and Witte, 1998). A good number of scientists from Bangladesh worked on the antibiotic resistant organisms in food animals (Khan *et al.*, 2005; Akter *et al*., 2007; Purkayssttha *et al.*, 2010; Hashem *et al.*, 2012). They have only studied whether the organisms isolated were sensitive or resistant to some selected antibiotics but other factors that might or might not have any effect were not studied. Considering the above facts that there is a lack of information on the prevalence of bacterial diseases of poultry with their risk factors from the poultry zones of Dinajpur and surrounding areas, there is a need to develop fast and reliable diagnostic techniques for the diagnosis of poultry diseases and whether the antibiotic resistance of the causal agents of the diseases have any relation with other environmental factors or not the present study was designed to fulfill the following objectives:

- To isolate and identify the causative bacteria and **t**o determine the prevalence of bacterial diseases of poultry in the Broiler farms of Dinajpur in Bangladesh.
- To determine the antibiotic sensitivity of the organisms to help suggesting effective treatment.

CHAPTER II

REVIEW OF LITARATURE

The purpose of this chapter is to provide a selective review of the past researches conducted in relation to the present research work. The reviews are briefly presented under the following heading and subheading.

2.1Isolation, identification and characterization of *Escherichia coli* from chicken

AI-Ghamdi *et al*. (2001) studied the identification of common bacterial organisms in locally produced healthy chickens. Among the isolated 28 types of bacterial organisms, *E. coli* was the most common (16.2%) cases.

Chau *et al*. (2002) isolated *E. coli* from chickens and eggs. Of 47 *E. coli* isolates, 36 were from chickens and 11 were from eggs. The isolates were identified as *E. coli* belonging to the 01:K1, 02: K l and 078:K80 serotypes.

Derakhshantar and Ghanbarpour (2002) studied avian cellulites in broiler chickens. The authors identified 91.8% of *E. coli* infection from cellulites of broiler by bacteriological investigation

Dho-Moulin *et al.* (1999) stated that avian pathogenic *E. coli* (APEC) cause airsacculitis, polyserositis, septicemia and other mainly extra intestinal diseases in chickens, turkeys and other avian species. APEC are found in the intestinal microflora of healthy birds and most of the diseases associated with them are secondary to environmental and host predisposing factors. APEC isolates commonly belonged to certain serogroups, O1, O2 and O78.

Ewers *et al*. (2004) investigated molecular biology and epidemiology of 150 avian pathogenic *Escherichia coli* strains (APEC) isolated from septicemic poultry in Germany by

serotyping, pulsed field gel electrophoresis (PFGE), and polymerase chain reaction (PCR). Only 49.6% of the isolates were grouped to serogroups O1, O2, and O78.

Guan *et al.* (2002) identified *E. coli* by the analysis of 16S rRNA gene by PCR.

Hossain *et al.* (2008) isolated *E. coli* from apparently healthy broilers and layers from different poultry farms adjacent to the Bangladesh Agricultural University. A total of 110 fecal samples were collected from broiler (n=55) and layer (n=55) chickens. *E. coli* were isolated and identified by cultural, biochemical, motility test and the heat-stable toxins were determined by Infant Mouse Assay (IMA). In case of broilers, 35 (63.6%) samples were found positive while 31 (56.4%) from layers. The overall prevalence of *E. coli* was 60%.

Kalin *et al.* (2012) investigated the presence of *E. coli* O157 and its virulence genes in various samples collected from broiler chickens and humans in Eastern Turkey by culture, immunomagnetic separation (IMS), and polymerase chain reaction. In the PCR *E. coli* O157 was identified in 0.1% (1/1000) and 0.4% (4/1000) of the liver and cecum samples of broiler chickens, respectively. On the other hand, none of the carcass samples were determined to be positive for *E. coli* O157. Overall, the results indicated that 12% (3/25) of the flocks were

Pandian (2006) isolated 25 avian pathogenie *E. coli* (APEC) from 105 heart blood samples from birds in Tamil Nadu, India. They identified that the isolates were as belonging to the 01, 02, 015, 018, 024, 053, 060, 091, 0103, 016 and 0169 serotypes, respectively.

Positive for *E. coli* O 157.

Roy *et al.* (2006) isolated *E. coli* from Japanese quail and their environment. Of 31 *E. coli* isolates, 11 were from heart blood of dead Japanese quail and 20 were from dead in shell embryos, fluff sample, foot bath and drinking water.

Ali *et al.* (1998) described the colony characters of *E coli*. They found metallic sheen on the EMB agar, rose pink colony on the MacConkey agar and pinkish colony on the SS agar media.

Hossain *et al.* (2008) observed that *E. coli* shows characteristic yellow green metalic sheen on EMB agar, whereas on MacConkey agar and SS agar, bright pink or red colonies and pinkish colonies were seen respectively.

Sharada *et al*. (1999) reported that the *E. coli* colonies on nutrient agar are smooth, moist and low convex. The colonies on EMB agar appear dark with characteristics metallic sheen.

Dey *et al.* (2013) stated that pink colored, rod shaped, short chain, single or paired Gram negative bacilli were observed in *E. coli* isolates from pigeon after Gram's staining.

Hossain *et al*. (2008) observed the staining characters of *E. coli* as a gramnegative, short rod, single, pair or in short chain.

Merchant and Packer (1967) described the staining characters of *E. coli* as a gram-negative, short rod, varying from coccoid bipolar shape to long filamentous form.

Mishra *et al*. (2002) stained *E. coli* from domestic poultry and found short rod to coccoid shape bacteria.

Adeyanju and Ishola (2014) conducted that catalase test, sugar fermentation using TSI (LabM, Uk), Kovac's test for identification of *E. coli* isolates.

Ahmad *et al*. (2009) identified 26 isolates of *E. coli* on the basis of indole test and biochemical reaction.

Ali *et al*. (1998) reported the fermentation tests of *E. coli*. The fecal isolate fermented four sugars except maltose whereas urinary isolate fermented all the four sugars (Dextrose, lactose, sucrose and mannitol).

Collins and Lyne (1976) described the indole formation by *E. coli* with kovac's reagent which was a rose pink color.

Edward *et al.* (1968) conducted some tests with *E. coli* and concluded that *E. coli* produced indole, gave a negative Voges-Proskauer reaction and did not utilize citrate as the sole carbon source. Isolates of these genera usually did not produce extracellular DNase, H2S, phenylalanine deaminase, urease, and did not grow in inositol and KCN containing media. Typical gas production from glucose, indole, lysine, arabinose, mannitol, ortho-nitrophenyl galactocide (ONPG), trehalose and xylose was found in this genus.

Joshi *et al*. (2012) performed indole, methyl red, Voges-Proskauer, Simon's citrate test (IMViC), catalase, oxidase, urease, H2S production in TSI and sugar fermentation test for biochemical characterization of *E. coli.*

Khaton *et al.* (2008) stated that isolates of *E. coli* fermented dextrose, lactose, maltose and mannitol with the production of acid and gas but did not ferment inositol. Acid production was indicated by the change from reddish to yellow and gas production by the accumulation of gas bubbles in the inverted Durham's tube.

Thomas (1988) performed some biochemical tests for *E. coli* and observed that most *E. coli* fermented lactose, reduced nitrates and were methyl red positive. Approximately 10% of the species were late lactose fermenters; and some of them were non lactose fermenters.

2.2 Antibiogram profiles of *E. coli*

Adeyanju and Ishola (2014) conducted antibiotic sensitivity test of *E. coli* isolates of poultry by using different Gram-negative antibiotics. Nitrofurantion and augmentin showed a decrease in their sensitivity to isolates than they normally should. *E. coli* showed 100% resistance to augmentin and amoxicillin.

AI-Ghamdi *et al.* (2001) evaluated the overall resistance rates of isolated organisms to 28 types of antimicrobial agents. They found that 98.5% *E. coli* was resistant to erythromycin and 84.7% was resistant to gentamycin, ampicillin, streptomycin, tetracycline, sulphamethoxazole/trimethoprim, and kanamycin (42.51%).

Blanco *et al.* (1997) stated that antimicrobial therapy is an important tool in reducing the enormous losses in the poultry industry caused by *E. coli* infections. However, resistance to existing antimicrobials was widespread and of concern to poultry veterinarians. Antimicrobial resistance testing of 468 avian *E. coli* strains isolated in Spain showed very high levels of resistance to trimethoprim-sulfamethoxazole (67%) and the new fluoroquinolones (13 to 24%). chloramphenical and gentamicin ($p<0.05$). Multiple drug resistance was found in isolates from both sources and was usually associated with tetracycline resistance.

Chowdhury *et al.* (1994) studied the prevalence and patterns of drug resistance of Enterobacteriaceae, isolated from the cases of urinary tract infection. Out of 90 Enterobacteriaceae isolates, 95.5% were resistant to the different antimicrobials tested. The most common resistance pattern was observed against ampicillin, trimethoprim, sulphamethoxazole, tetracycline and chloramphenicol in all four genera of Enterobacteriaceae. Transferable drug resistance R (+) factor was detected in 68.5% *E. coli.*

Fluckey *et al.* (2007) determined antibiotic susceptibility of *E. coli* commonly associated with cattle. Samples were cultured for non-type-specific *E. coli*. Isolates were tested for antimicrobial drug susceptibility. For nonspecific *E. coli*, 80.3% (n = 270) of the isolates were resistant to at least one antimicrobial drug. The most common resistance was to sulfamethoxazole.

Hasina (2006) studied antibiotic resistance pattern of SPEC and ETEC and found that all the isolates were highly sensitive to ciprofloxacin (100%) and chloramphemnicol (100%). All the isolates of ETEC and EPEC were fully resistant to erythromycin (100%) and cloxacillin (100%). Multiple drug resistance was also observed to kanamycin and nalidixic acid.

Hossain *et al*. (2008) observed that *E. coli* isolates obtained from broilers were 100% resistant to nalidixic acid, 97.14% to cloxacillin, 91.42% to erythromycin and 62.85% to ampicillin. About 91.43% broiler isolates were moderately sensitive to cephalexin, 77.74% to ciprofloxacin and 85.71% to kanamycin while 54.28% isolates were highly sensitive to chloramphenicol and 45.71% were moderately sensitive to the same antibiotic. On the other hand, 14.29% and 22.86% isolates were highly sensitive to kanamycin and ciprofloxacin respectively. The *E. coli* isolates obtained from layers were 100% resistant to cloxacillin and nalidixic acid and 93.55% isolates were resistant to erythromycin. A total of 32.26% layer isolates were found resistant to Ciprofloxacin and 25.81% to Ampicillin.

Jones (1987) carried out an experiment on antibiotic sensitivity pattern of *E. coli* and they claimed that resistance to cephaloxium and cephaloridine was frequently seen in *E. coli.*

Joshi *et al*. (2012) observed that *E. coli* isolates were 100% sensitive to chloramphenicol followed by sulphamethizole, co-trimoxazole (84.21% each) and amikacin (78.95%). A high degree of resistance was found to be against cephalexin (73.68%) followed by neomycin and enrofloxacin (31.58% each).

Kikuvi *et al.* (2006) investigated 235 *E. coli* isolates from cattle, pigs and chickens for their resistance to seven antimicrobials by the disc diffusion method. Minimum inhibitory concentrations were determined for 154 isolates showing resistance to at least one of the antimicrobials tested. Resistance was found in 65.5% and mufti-resistance (resistance to=2 antibiotics) in 37.9% of the isolates. Resistance was highest in the isolates from chickens (74.0%), followed by pigs (64.8%) and cattle (61.3%).

Miles *et al*. (2006) investigated the prevalence and genetic basis of tetracycline resistance in faecal *E. coli* isolated from healthy broiler chickens and compare these data with isolates obtained from hospitalized patients in Jamaica. Eightytwo *E. coli* strains isolated from faecal samples of broiler chickens and urine and wound specimens of hospitalized patients were analyzed by agar disc diffusion to determine their susceptibility patterns to 11 antimicrobial agents. Plasmid profiling, transformations, and amplification of Plasmid-borne resistance genes investigated tetracycline resistance were determinants. Tetracycline resistance occurred at a frequency of 82.4% in avian isolates compared to 43.8% in human isolates. In addition, among avian isolates there was a trend towards higher resistance frequencies to kanamycin and nalidixic acid ($p<0.05$).

Nazir *et al.* (2004) studied the antibiotic sensitivity pattern of *E. coli* isolated from broilers. The authors found that the organisms were 100% resistant to cloxacillin and nalidixic acid, 92.30% to erythromycin and 61.53% to ampicillin. Broilers isolates were 76.92% sensitive to both kanamycin and ciprofloxacin. The authors also observed that the organisms were highly

Olarinmoye *et al*. (2013) found that *E. coli* were highly resistant to amoxicillin (99.2%), chloramphenicol (96.5%), tetracycline (91.4%), and ofloxacin (86.4%); moderately resistant to gentamicin (67.2%), augmentin (63.5%), nitrofurantoin (51.7%) and fosfotrim (37.9%); and least resistant to streptomycin (14.3%).

Sayah *et al.* (2005) reported that *E. coli* isolated from domestic species showed resistance to the largest number of antimicrobial agents compared to isolates from human, wildlife and surface water. The agents to which resistance demonstrated most frequently were tetracycline, cephalothin, sulfisoxazole and streptomycin. Sensitive to chloramphenicol (53.83%) and moderately sensitive to the same antibiotic 46.16%) and cephalexin (92%).

Shuchismita *et al.* (2007) conducted a study to discriminate *E. coli* strains based on their susceptibility or resistance to antibiotics. 100 strains of *E. coli* isolated from, cattle, camels, sheep and poultry were used in this study. Most of the isolates from camels, cattle, sheep and poultry were sensitive to gentamicin, chloramphenicol and streptomycin but resistant to ampicillin and trimethoprim.

***Zhao et al.* (2005)** recovered ninety-five avian pathogenic *E. coli (*APEC.) isolates from diagnosed cases of avian colibacillosis. Multiple anti microbial12 resistant phenotypes were observed in 92% of *E. coli* isolates with the majority of isolates displaying resistance to

sulfamethoxazole (93%), tetracycline (87%), streptomycin (86%), gentamicin (69%), and nalidixic acid (59%). Fifty-six *E.coli* isolates displaying resistance to nalidixic acid were coresistant to difloxacin (57%), enrofloxacin (16%), gatifloxacin (2%), and levofloxacin (2%).

2.3 Isolation, identification and characterization of *Salmonella spp.* from chicken

Ellerbroek *et al.*, (2010) isolated *Salmonella* from 400 imported chicken carcasses in Bhutan and from 178 pig carcasses in Vietnam for antibiotic resistance analyzed on a random basis against 14 antimicrobial agents. Among the poultry samples tested, 13% were positive for *Salmonella*.

Ahmed *et al.*, (2009) isolated 69 *Escherichia coli* and 10 *Salmonella*, from retail chicken meat in Hiroshima prefecture, Japan, the samples were assayed for antimicrobial susceptibility, the presence of integrons and antimicrobial resistance genes.

Akter *et al.*, (2007) determined the seroprevalence of Salmonellosis in layer flocks and antibiogram study following isolation of *Salmonellae*. This study was conducted during the period from January to May 2006 at Gobindapur of Dinajpur district. A total of 225 Star cross 579 brown chickens were studied with rapid serum plate agglutination test. Liver of 200 dead birds was studied for isolation and identification of *Salmonellae*. In vitro antibiotic sensitivity test of isolated *Salmonellae* was performed with commercial sensitivity discs. The overall seroprevalence was recorded 23.11%. The prevalence was varied from age to age. The highest rate was 28% in above 20 weeks of age. The antibiogram study revealed that the isolates were sensitive to ciprofloxacin (80%), nitrofurantoin (100%), sulphamethoxazole/trimeoprim and amoxycillin (50%), tetracycline (60%) but resistant to penicillin-G and erythromycin. The author suggest for further studies on serotyping of the isolated *Salmonellae*, isolation and identification of *Salmonellae* from different feed and environmental samples.

Roy *et al.*, (2002) isolated five hundred sixty-nine *Salmonella* out of 4745 samples from poultry products, poultry, and poultry environment in 1999 and 2000 from the Pacific

Northwest. These *Salmonella* were identified to their exact source, and some were serogrouped, serotyped, phage typed, and tested for antibiotic sensitivity.

Muktaruzzaman *et al.,* (2010) mentioned that *Salmonella* organisms showed different cultural characteristics in different media. These were turbidity in Tetra Thionate broth, pink white color colonies in Brilliant Green agar, gray white colony in Nutrient agar, slightly grayish color colonies in *Salmonella*-Shigella agar, black color colony in Tripple Suger Iron agar, pale color colonies in MaConkey's agar, well defined glistening colonies in Blood agar and pinkish colonies in EMB agar.

Hossain (2002) isolated *Streptococcus aureus, Escherichia coli* and *Salmonella* from diarrhoeic calves. The author stated that the *Salmonella spp.* produced small round and smooth colonies on nutrient agar and opaque, translucent and colorless colonies on SS agar. The organisms produced colourless, pale, transparent colonies on MacConkey agar and small, round, low convex, translucent, pale red colour colony on BGA against pinkish background which was initially green in colour.

Freeman (1985) stated that the optimum growth temperature of *Salmonella* is 37 _C, but good growth is observed at room temperature.

Freeman (1985) stated that *Salmonella typhi* produce "maple leaf" like irregular margin and slightly roughened glistening surface colony on nutrient agar.

Cheesbrough (1985) noted that on SS and MC agar *Salmonella* produce lactose nonfermenting colony. Most strains shows blackening of the colony due to H2S

Production.

Gene O (2002) reported the rod and short to long chain forming *Salmonella* organisms, which were isolated and identified from liver, spleen, intestinal contents of animals and birds.

Cheesbrough and Freeman (1985) reported that *Salmonellae* are gram negative bacilli whose cellular morphology closely resembles and indistinguishable from other enterobacteria.

Freeman (1985) showed Giemsa stain from a pure culture of *Salmonella* reveals that the organisms are rod shaped, single of paired in arrangement.

Sujatha *et al.*, (2003) isolated *S. gallinarum* from poultry in and around Hyderabad and Secunderabad cities in India and characterized. All isolates showed positive reaction to MR, citrate and H2S. Sugar fermentation tests revealed acid production occur without gas from glucose, maltose, dulcitol, galactose, trehalose, xylose and rhamnose.

Lee *et al.*, (2003) reported that Fowl typhoid (FT) in Korea is caused by *Salmonella gallinarum*. The authors investigated the biochemical characteristics and antimicrobials susceptibility of field isolates of *S. gallinarum* isolates in Korea. A total of 258 isolates of *S. gallinarum* from 1995 to 2001 showed the same pattern in the majority of biochemical test such as IMViC (indole, methyl red, Voges-Proskauer and citrate utilization), carbohydrate fermentation and amino acid decarboxylation, and these results were almost in accordance with the traditional biochemical characteristics of *S. gallinarum* strain. Therefore, results indicate that sorbitol fermentation and arginine decarboxylation showed the diversity by isolates and the vast majority of isolates from 2001 showed the reduced susceptibility to antimicrobials tested.

Hossain (2002) conducted an experiment that the *Salmonella* when allowed to ferment the five sugars were found to ferment dextrose, maltose and mannitol with the production of acid and gas but no fermentation was observed in lactose and sucrose.

Buxton and Fraser (1977) stated that the *Salmonellae* did not ferment lactose, sucrose or salicin but did ferment glucose, maltose, mannitol, dulcitol and dextrin with production of acid and gas. These were also characterized by their ability to reduce nitrates to nitrites and to produces H2S. The authors could not decompose urea, liquefy gelatin or produce indole.

Dhruba *et al*., (2000) isolated the *Salmonella* organism using five basic sugar fermentation reactions. Nearly all isolates were positive for mannitol, maltose and dextrose with the production of acid while lactose and sucrose are not fermented.

2.4Antibiotic Susceptibility test

Seyyedeh *et al.*, (2013) described that the stereotyping results showed that 34 of 44 isolates of *Salmonella* belonged to S*almonella infantis* (79.5 %), one strain (2.3%) of group C and 8 strain (18.2%) of group D. However, all these strains were sensitive to Cefotaxime and Ciprofloxacin, and 100% were resistant to Nalidixic acid, Tetracyclin and Sterptomycin. The

most common resistance pattern (34.1%) was towards six antibiotics, and 6.8% of strains were resistant to at least three antibiotics.

Ramya *et al* (2013) described that the sensitivity of *S. Enteritidis* was 100% for ciprofloxacin followed by chloramphenicol and amikacin (96%), gentamycin (90%), amoxicillin (82%), streptomycin (80%), tetracycline (76%), nalidixic acid (68%), ampicillin (58%) and sulfonamide (10%). The resistance was highest for sulfonamide (76%) followed by ampicillin (32%), nalidixic acid (30%) and 6- 20% for gentamycin, amoxicillin and tetracycline.

Bae *et al.*, (2013) reported that regarding the characteristics of their antibiotic resistance, 8 of the 11 ampicillin resistant isolates carried blaTEM only, two carried blaTEM and blaCTX-M-14 and one carried blaCTX-M-3 and only one AmR isolate with the blaCTX-M-3 β-lactamase gene Salmonella strain. Twenty seven Salmonella isolates showed nalidixic acid resistance with a mutation at amino acid codon Asp87 in gyrA and no mutation in the parC gene.

Gallati *et al.*, (2013) reported that **o**ver the years 2007-2011, the reports of salmonellosis caused by *Salmonella enterica* serovar 4,[5],12:i:- significantly increased. A high prevalence of multidrug-resistant isolates, mainly showing an ampicillin-streptomycin-sulfonamide-tetracycline resistance pattern (ASSuT), was observed. In addition, four extended spectrum beta lactamase (ESBL) (CTX-M- 55)-producing isolates were found.

Li *et al.*, (2013) identified a total of 165 *Salmonella enterica* isolates from 1382 samples taken from conventional farms, abattoirs and retail markets from 2010 to 2011 in Sichuan, China. Among these isolates, *S. enterica* serotypes *derby* (76 isolates, 46%) and *typhimurium* (16 isolates, 10%) were the most prevalent, and high antimicrobial resistance observed for tetracycline (77%), nalidixic acid (41%) and spectinomycin (41%).

Imad *et al.*, (2012) stated that antimicrobial susceptibility test of the 98 isolates of *Salmonella* revealed that 32.7% were resistant to one or more of the 24 antimicrobials tested. Generally, resistance for 13 different antimicrobial drugs was recognized. The most common resistance was to streptomycin (24/32, 75%), ampicillin (19/32, 59.4%), tetracycline (15/32, 46.9%).

De *et al.*, (2012) stated that the antimicrobial susceptibility of *E. coli,* S*almonella spp.* in chicken. For *E. coli* and *Salmonella spp.* clinical resistance to newer compounds (Cefepime,

cetotaxime and ciprofloxacin) was absent. Colistin sulphate resistance was absent for *E. coli* but apparent for *Salmonella spp*

Iwabuchi *et al.*, (2011) described that among 452 *Salmonella* isolates, 443 (98.0%) were resistant to one or more antibiotics, and 221 (48.9%) showed multipleantibiotic resistance, thereby implying that multiple-antibiotic resistant Salmonella organisms are widespread in chicken meat in Japan. Resistance to oxytetracycline was most common (72.6%), followed by dihydrostreptomycin (69.2%) and bicozamycin (49.1%).

Hyeon *et al.*, (2012) observed that in *salmonella* the highest antibiotic resistance was to erythromycin (100%) followed by streptomycin (22.2%) and tetracycline and chloramphenicol (16.7%). Of the 18 isolates, 5 (27.8%) were resistant to two or more antibiotics, and 1 isolate from chicken meat was resistant to eight antibiotics, including cephalosporins.

Lu *et al.*, (2011) evaluated the antimicrobial resistance of *Salmonella* isolated in 2008 from a chicken hatchery, chicken farms, and chicken slaughterhouses in China. More than 80% of the *S. indiana* isolates were highly resistant to ampicillin (97.7%), amoxicillin/clavulanic acid (87.9%), cephalothin (87.9%), ceftiofur (85.7%), chloramphenicol (84.9%), florfenicol (90.9%), tetracycline (97.7%), doxycycline (98.5%), kanamycin (90.2%), and gentamicin (92.5%). About 60% of the S. *indiana* isolates were resistant to enrofloxacin (65.4%), norfloxacin (78.9%), and ciprofloxacin (59.4%). Of the *S. indiana* isolates, 4.5% were susceptible to amikacin and 5.3% to colistin. Of the *S. enteritidi*s isolates, 73% were resistant to ampicillin, 33.1% to amoxicillin/clavulanic acid, 66.3% to tetracycline, and 65.3% to doxycycline, whereas all of these isolates were susceptible to the other drugs used in the study.

Petrovic *et al*., (2011) worked on 480 samples of chicken liver and carcasses swabs were taken from 7 abattoirs. The presence of *Salmonella spp*. was found in 69 samples (14.37%). Intermediate susceptibility or resistances to one or more antimicrobial drugs were found in 28(40.58%) isolates of *Salmonella spp*. It was concluded that the common zoonotic pathogens transferred by chicken meat. The application of prophylactic and sanitary measures in facilities, abattoirs and during meat processing may considerably reduce the incidence of resistance zoonotic bacteria.

Kang *et al.*, (2010) determined the antimicrobial susceptibility of *Salmonella enterica* serovar *gallinarum* isolates (n=105) from chickens in South Korea between 2002 and 2007 by minimum inhibitory concentrations of 16 antimicrobials, and their predominant resistance profiles were genetically characterized. Most isolates (99/105; 94.3%) were resistant to nalidixic acid and resistant/intermediately resistant to fluoroquinolones, and 63.8% (67/105) of the isolates were resistant to three or more antimicrobials.

2.5 Isolation, identification and characterization of *Staphylococcus spp.*from chicken

B. Y. Rashed. (2011) studied that sixty chickens 30-55days of age with arthritis symptoms, were collected from different broiler chickens farm, all sample were examined clinically, post mortem and bacterial isolation were done. The result revealed isolation of 26(50.98%) of *staphylococcus aureus*, which were found highly sensitive to amoxicillin. The experimental infection of 10 chickens was carried out on 35 days old by intravenous inoculated with 10cfu/ml of isolated *staphylococcus aureus*. Arthritis occurred in 8(80%) chickens. Clinical sign and postmortem findings confined to depression, swollen joints and inability to stand.

Wladyka B*et al.* (2011) reported that *Staphylococcus aureus* strain CH-91 isolated from chicken arthritic lesions produces large quantities of thiol protease implicated in disease formation. Observed overproduction requires efficient activation of the protease precursor which mechanism is studied here in detail. Wild type and mutant precursor forms are expressed in E. coli to test different hypotheses on the activation process. It is demonstrated that wild type precursor undergoes rapid autocatalytic processing whereas proteolytically inactive catalytic triad cysteine mutant (C(249)A) of the precursor is stable, but can be processed by minute quantities of active protease. It is concluded that limited intramolecular proteolysis is mainly responsible for efficient activation but, a positive feedback loop also contributes to the process. Both activation pathways allow efficient production of mature extracellular thiol protease, a putative virulence factor specific for avian strains of *S. aureus*.

Mamza SA *et al.* (2010) reported that the occurrence of beta-lactamase-producing *Escherichia coli* and *Staphylococcus aureus* in chickens was investigated. Specimens (n = 1,300) were collected from 400 chickens and were streaked on MacConkey agar plates. From each plate, presumptive growths of organisms were picked and streaked on eosin methylene

blue and Baird-Parker agars, respectively. Typical colonies of *E. coli* and *S. aureus* with similar morphologies were identified by biochemical tests. Isolates were tested for beta-lactamase production and antimicrobial susceptibilities. Results indicated that 805 *E. coli* isolates from which 89 (11%) were beta-lactamase-positive and 660 S. aureus from which 58 (8.8%) were beta-lactamase-positive. The implications of this for humans when handling and/or consuming chickens and chicken products contaminated with strains of such isolates, is a risk of transferrable multi-drug resistance and a failure of treatment. The results of our study indicated that beta-lactamase-producing *E. coli* and *S. aureus* are prevalent in chickens in Nigeria.

Nemati M *et al.* (2009) *Staphylococcus aureus* isolates recovered from poultry between 1970 and 1972 (90 old isolates) and in 2006 (81 recent isolates) were screened for the presence of bap, icaA and icaD genes associated with biofilm formation, and for bbp, cna, ebpS, eno, fib, fnbA, fnbB, clfA and clfB genes that encode microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Most of the old isolates were collected from broiler breeders affected by staphylococcal tenosynovitis and arthritis, whilst nearly all recent isolates were derived from the nose and cloaca of healthy broilers**.** From this study, there is no indication that the presence of these genes has changed over time and no specific association could be found between the presence of certain MSCRAMM or biofilm genes in poultry *S. aureus* isolates and the isolate's capacity to cause disease.

Zhong Z*et al.* (2009) reported that *staphylococcus aureus* was used as an indicator to study the origin and spread of microbial aerosol in and around chicken houses. Air samples indoor, upwind (10 and 50 m), and downwind (10, 50, 100, 200, and 400 m) of four chicken houses were collected using Andersen-6 stages sampler. The genetic relationship among the isolates was determined by profiles of PCR-amplified repetitive extragenic palindromic (REP-PCR) elements. The results showed that the concentrations of *S. aureus* indoor of four chicken houses were higher than those upwind and downwind sites ($P < 0.05$ or $P < 0.01$), but there were no significant concentration differences among downwind sites ($P > 0.05$). The fingerprints and the phylogenetic tree indicated that a part of the S. aureus (55.6%, 10/18) isolates from indoor air had the same REP-PCR fingerprints as feces isolates. Thus, the use of *S. aureus* as an indicator to study the origin and spread of airborne pathogens from chicken houses is potentially useful for enhancing public health and understanding the airborne epidemiology of this pathogen. Meanwhile it can provide evidence for studying the spreading model of airborne pathogens.

Lowder BV*et al.* (2009) reported that the impact of globalization on the emergence and spread of pathogens is an important veterinary and public health issue. *Staphylococcus aureus* is a notorious human pathogen associated with serious nosocomial and community-acquired infections.In addition, *S. aureus* is a major cause of animal diseases including skeletal infections of poultry, which are a large economic burden on the global broiler chicken industry**.** Here, we provide evidence that the majority of *S. aureus* isolates from broiler chickens are the descendants of a single human-to-poultry host jump that occurred approximately 38 years ago (range, 30 to 63 years ago) by a subtype of the worldwide human ST5 clonal lineage unique to Poland. These genetic events have resulted in enhanced resistance to killing by chicken heterophils, reflecting avian host-adaptive evolution. Taken together, we have determined the evolutionary history of a major new animal pathogen that has undergone rapid avian host adaptation and intercontinental dissemination. These data provide a new paradigm for the impact of human activities on the emergence of animal pathogens.

Lin J*et al.* (2009) studied that *Staphylococcus aureus* is a cause of many diseases in both humans and animals. This pathogen is also a major target in the screening of slaughterhouse carcasses to monitor hygienic conditions during slaughter. During 2004 to 2006, *S. aureus* was recovered from 8.8% (38 of 430), 11.3% (77 of 680), and 4.3% (13 of 300) of pork carcass samples, respectively, collected at 53 slaughterhouses in Taiwan. During 2003 to 2005, it was recovered from 0.3% (1 of 305), 0.4% (1 of 260), and 7.8% (31 of 395) of rinse fluids from chicken carcasses, respectively, collected at 17 meat processing plants. The percentages resistant to methicillin (oxacillin), chloramphenicol, erythromycin, and tylosin were 19.4% (40 of 207), 18.8% (39 of 207), 23.2% (48 of 207), and 20.8% (43 of 207) with MIC90s of 8, 64, > or = 64, and > or = 128 microg/ml, respectively. The methicillin-resistant *S. aureus* (MRSA) strains exhibited resistance to more antibiotics than did the methicillin-susceptible strains, and 87.5% (35 of 40) of the MRSA strains carried the mecA gene sequence. Since MRSA infections have become a public health concern in both communities and hospitals, testing for the presence of MRSA in animal carcasses during slaughtering operations are warranted.

Zhou Q*et al.* (2007) reported that evaluate the role of interleukin-6 (IL-6) in arthritis induced by *Staphylococcus aureus*, a chicken model was developed for study. A total of 120 healthy broilers (8 wk old) were randomly divided into 4 groups. Two groups were injected with 0.35 mL of *Staph aureus* (7.1x10(9) cfu/mL) into the right hock joints and the other 2 were

injected with 0.35 mL of sterile saline into the same joints. One group of each of the 2 treatment groups was fed levofloxacin at a dose of 5 mg/kg of BW on the third day postinoculation for 4 successive days. There was a strong correlation (r=0.91) between serum chIL-6 activities by the B9 bioassay and serum IL-6 concentrations determined by the human IL-6 ELISA. We concluded that chIL-6 is involved in the progression of chicken arthritis induced by *Staph aureus*, and that it contributes to disease incidence and mortality.

Pepe O*et al.* (2006) studied that we examined the presence of *Staphylococcus aureus* and staphylococcal enterotoxin A (SEA) in 20 industrial breaded chicken products obtained from different retail butchers and supermarket stores in Italy**.** The levels of contamination in the products analyzed were quite low, although the pH values and water activities a (w) in the samples considered were in ranges favorable for *S. aureus* growth. As demonstrated by phenotypic and molecular characterization, in spite of the high percentage of coagulase-positive Staphylococcus strains, only three strains could be referred to the species *S. aureus*. Moreover, all the strains were negative in PCR assays targeting staphylococcal enterotoxin genes (seA to seE, seG to seJ, and seM to seO), as well as the toxic shock syndrome toxin 1 gene, and no SEA was detected in the retail breaded chicken samples analyzed by a reversed passive latex agglutination assay or by Western blotting. Hence, we evaluated the thermal resistance of two strains of SEA-producing *S. aureus* in a laboratory-scale preparation of precooked breaded chicken he thermal process used during their manufacture can limit staphylococcal contamination but cannot eliminate preformed toxins.

Rodgers JD *et al.* (2006) this study aimed to develop an enzyme-linked immunosorbent assay to detect antibody associated with *Staphylococcus aureus* that is produced during the chicken immune response to this organism. The protein nuclease was tested for suitability as an antigen to detect antibody in sera from broiler chickens that had been exposed to aerosolized *S. aureus* on day 1 post hatch during experiments to reproduce bacterial chondronecrosis and osteomyelitis**.** The influence of CAV and IBDV on the number of chickens with positive levels was most prominent in chickens aged up to 42 days. The study showed that nuclease-specific antibodies form part of the humoral immune response in broiler chickens that have been exposed to *S. aureus*. Co-infection with CAV and IBDV appeared to promote development of antibody in birds younger than 42 days; however, the presence of antibody did not necessarily prevent systemic infection.

Lowder BV*et al.* (2006) studied that*Staphylococcus aureus* is a major human pathogen associated with nosocomial and community-acquired infections, and is also responsible for several economically important infections of livestock. However, the evolutionary origin of animal strains and the potential for cross-species transmission has not been well examined. We recently traced the origin of a common *S. aureus* clone which is a significant cause of morbidity in the global broiler poultry industry. We provided evidence that it evolved from a single human to poultry host jump which was followed by extensive genetic diversification including acquisition of novel mobile genetic elements and loss of virulence gene function. The clone has since been disseminated widely to several different continents presumably through globalization of the poultry industry. In the current article, we summarise the findings of the paper, discuss their implications and speculate on the potential for other *S. aureus* cross-species transfer events.

Vadari Y*et al.* (2006) reported that Poultry litter extracts were plated onto brain-heart infusion medium (BHI) containing an additional 0.75 mol l (-1) phosphate (BHI-P). Colonies were screened for the presence of inclusion granules with five being selected for further study. All strains displayed identical biochemical characteristics consistent with *Staphylococcus spp*. and grouped with Staphylococcus spp. by comparative 16S rDNA analysis. Thus all five strains were identified as such. All strains displayed elevated intracellular phosphate levels when cultured in BHI-P broth (0.417-0.600 microg phosphate mg(-1) protein) vs BHI broth (0.075-0.093 microg phosphate mg(-1) protein). Poultry litter contains novel *Staphylococcus spp*. capable of robust growth when exposed to phosphate levels comparable with that typically found in poultry litter. Data suggest intracellular

phosphate levels in these strains increase in response to increasing phosphate in the medium or austere medium conditions. Intracellular phosphate did not reach levels comparable with known hyper-accumulating micro-organisms.

El-Sayed A*et al.* (2005) reported that the present study was designed to comparatively investigate 19 *Staphylococcus aureus* strains isolated from specimens of 19 different birds during routine microbiological diagnostics. The S. aureus strains were characterized genotypically by polymerase chain reaction (PCR) amplification using 62 different oligonucleotide primers amplifying genes encoding staphylococcal cell surface proteins, exoproteins and two classes of the accessory gene regulator agr. In addition, all tested strains were positive for the genes hla and fnbA and negative for the genes seb, sec, sed, see, sej, tst, eta and etb. The remaining genes, including sbi, hlb, fnbB, ebpS, cna (domains A and B), cap5, cap8, set1, agr class I, agr class II, sea, seg, seh and sei were detected in a variable number of isolates. The presented data give an overview on the distribution of virulence determinants of *S. aureus* strains isolated from birds. This might be useful to understand the role of these virulence determinants in bird infections.

Kitai S *et al.* (2005) reported that a total of 444 samples of raw chicken meat (thighs, breasts, wings, livers, gizzards, hearts and ovaries) that retailed at 145 different supermarkets in 47 prefectures in Japan were examined for contamination with *Staphylococcus aureus* in association with its enterotoxigenicity. *S. aureus* was isolated from 292 (65.8%) of the samples, and from 131 of the 145 supermarkets Staphylococcal enterotoxins (SEs) produced were SEB (50 isolates), SEA (14), SEC (8), SED (2), SEA+SEB (2), and SEA+SEC (2). Most of the enterotoxigenic isolates belonged to the human and poultry biotypes, coagulase type VII, VIII or IV, and were lysed by phages of group III. The three patterns were found in isolates of samples that retailed at 17 supermarkets in 11 prefectures, indicating that they may be disseminated among raw chicken meat in Japan.

Capita R*et al.* (2002) studied that ninety-six *Staphylococcus aureus* isolates from retail chicken carcasses in Spain were characterized using cultural and biochemical tests. The strains were phage typed with the international bacteriophage set for typing *S. aureus* of human origin. Eighty-eight (91.7%) strains were of the poultry ecovar. Strains of human ecovar were not found. These facts are congruent with findings of other authors. Ninety (93.7%) strains were phage type able. Lyses by phages of Group III were the most frequent with 66 (68.7%) sensitive strains. More than one *S. aureus* phage type was detected in 14

(35%) carcasses, which emphasizes the convenience of subtyping several S. aureus isolates from the same food sample in epidemiological studiesThe S. aureus isolates were closely related, as 78 strains showed the most common or indistinguishable (<2 phage reaction differences) phage patterns.

Eremin VI*et al.* (2002) studied that the data on the biological properties of the culture fluid of Streptococcus strain sp. TOM-1606 are presented. The native preparation has been shown to possess the capacity for stimulating the rate of the clearance of the peritoneal cavity of mice from *Staphylococcus aureus* cells, strain MT-1, rif. r., found to be insensitive to the action of the above-mentioned preparation in vitro. The crude preparation produces a transitory bacteriostatic effect on the streptococcal and staphylococcal strains under study. The preparation produces a prolonged bacteriostatic effect only on *Micrococcus luteus* test strain. All these data suggest that the crude preparation contains at least two active principles.

Takeuchi S*et al.* (2002) reported that recently whole genome sequencing of *Staphylococcus aureus* has revealed the genes encoding cysteine proteases such as staphopain and SspB. In this study, we cloned and sequenced the structural gene (ScpA) encoding a cysteine (thiol) protease of *S. aureus* strain CH-91 from a chicken with dermatitis using polymerase chain reaction (PCR) and inverse PCR methods. Which is not found in the published whole genome sequences of *S. aureus*? In immunoblot, PCR, and Southern blot assays, the ScpA or its gene was detected in high protease-producing strains from chickens, but was not recognized in bovine and porcine strains or low protease-producing avian strains. These results indicate that the ScpA of CH-91 type may be specific to the high protease-producing strains of *S. aureus* from chickens, namely, there is a strain specificity of the ScpA.

Hazariwala A*et al.* (2002) studied that food poisoning by *Staphylococcus aureus* affects hundreds of thousands of people each year. *Staphylococcus aureus* also causes invasive diseases such as arthritis (in poultry) and septicemia (in poultry and humans). Food borne disease is caused by the ingestion of a staphylococcal enterotoxin (SE). Enterotoxin has also been associated with other *S. aureus* illnesses in humans and domestic animals. In this study, polymerase chain reaction was used to detect the staphylococcal enterotoxin genes, SEA, SEB, SEC, SED, and SEE, in *S. aureus* isolates associated with invasive disease in poultry and humans. In the 34 poultry isolates, only one isolate was found to contain a SE gene, sec. In the 41 human isolates, over 51% tested positive for an SE gene with 12.2% positive for the gene for SEA, 2.4% for SEB, 22% for SEC, 24.4% for SED, and 0 for SEE. The disparity

between the rates for SE gene(s) in poultry and human isolates suggests a lesser role for the enterotoxins in invasive poultry disease than in human disease.

Stedman NL *et al.* (2001) Avian leukosis virus subgroup J has a high tropism for myeloid lineage cells and frequently induces neoplastic transformation of myelocytes. The impact of congenital avian leukosis virus subgroup J infection on the function of circulating heterophils and susceptibility to staphylococcal infection was investigated. Six-week-old broiler chickens negative for exogenous avian leukosis viruses or congenitally infected with avian leukosis virus subgroup J were inoculated intravenously with 10(6) colony-forming units of *Staphylococcus aureus*, and pre- and postinoculation heterophil function was assessed. Osteomyelitis in the tibiotarsus or tarsometatarsus developed in 5/10 (50%) of the chickens in each group. *S. aureus* was recovered from the hock joint of 6/10 (60%) of the chickens in each groupHeterophils isolated from broiler chickens congenitally infected with avian leukosis virus subgroup J exhibit no significant functional deficits, and infected and uninfected chickens exhibit similar susceptibility to staphylococcal infection.

Mutalib A*et al.*(2001) studied that groups of 6-week-old broiler chickens were exposed to *Staphylococcus aureus* by aerosol, intratracheal or intravenous (control) inoculation. No lesions were produced by aerosol. A single dose of up to 5 X 10(10) organisms given intratracheally failed to produce any lesions. Single or repeated doses of 5 X 10(11) organisms given intratracheally resulted in osteomyelitis in a low proportion of chickens.

X. Y. Zhu *et al.* (2001) conducted an experiment with broilers to determine if prior exposure to *Staphylococcus aureus* would facilitate the systemic infiltration of this pathogen following intradermal footpad challenge with live *S.aureus*. Litter-raised broilers were sensitized at 3 and 4 wk of age with s.c. injections in the neck with heat-killed *S. aureus* diluted in polyethylene glycol (PEG). Equal numbers of control birds were injected at the same times with PEG. At 7 wk of age, chicks previously sensitized to killed *S. aureus* or injected with PEG were injected intradermally in the right footpad with PBS or live *S.aureus.* The left footpads of all birds were injected with PBS. The difference in thickness between the right and left footpads was determined at 0, 24, and 48 h postchallenge. Blood, liver, spleen, lung, and synovial fluid were collected six times between 1 and 48 h postchallenge to *Staphylococcus aureus*, systemic infiltration, footpads, broilers, chickens.

Butterworth A*et al.* (2001) reported that a study was conducted to investigate the relationship between the presence of localized bacterial infection and lameness in broiler fowl

(Gallus gallus domesticus). Isolation of bacteria from the proximal femur, proximal tibia, and tibiotarsus from broilers with lameness revealed a probable association between lameness and the presence of *Staphlylococcus aureus.* Other potential pathogens, including Escherichia coli and DNase-negative staphylococci, were also isolated from sound and lame birds, and their association with pathologies causing lameness was less well defined. After trials with a set of twenty 10-base oligonucleotide primers, a pair of primers giving optimal performance was selected. The *S. aureus* isolates were typed by random amplification of polymorphic DNA (RAPD) by using the pair of 10-mer primers, and groupings were defined by banding patterns after agarose gel electrophoresis. The putative RAPD groupings may provide a basis for epidemiological studies of *S. aureus* in broiler production systems.

2.6 Antibiotic sensitivity and resistance pattern.

Graham JP*et al.*(2009) stated thatthe use of antimicrobials in commercial broiler poultry production results in the presence of drug-resistant bacteria shed in the excreta of these birds. Because these wastes are largely land-disposed these pathogens can affect the surrounding environment and population. In this analysis, we characterized the survival of antimicrobial-resistant enterococci and staphylococci and resistance genes in poultry litter. . persisted throughout the 120-day study period. Resistance genes identified in the study include: erm(A), erm(B), erm (C), msr(A/B), msr(C), and vat(E). This study indicates that typical storage practices of poultry litter are insufficient for eliminating drug-resistant enterococci and staphylococci, which may then be released into the environment through land disposal.

Graham JP*et al.* (2009) studied that use of antibiotics as feed additives in poultry production has been linked to the presence of antibiotic resistant bacteria in farm workers, consumer poultry products and the environs of confined poultry operations. There are concerns that these resistant bacteria may be transferred to communities near these operations; however, environmental pathways of exposure are not well documented. We assessed the prevalence of antibiotic resistant enterococci and staphylococci in stored poultry litter and flies collected near broiler chicken houseswere found in isolates recovered from both poultry litter and flies. Erm(B) was the most common resistance gene in enterococci, while erm(A) was the most common in staphylococci. We report that flies collected near broiler poultry operations may be involved in the spread of drug resistant bacteria from these operations and may increase the potential for human exposure to drug resistant bacteria.

Persoons D*et al.* (2009) Methicillin-resistant *Staphylococcus aureus* (MRSA) has been detected in several species and animal-derived products. To determine whether MRSA is present in poultry, we sampled 50 laying hens and 75 broiler chickens. MRSA was found in some broiler chickens but no laying hens. In all samples, spa type t1456 was found.

Miranda JM*et al.* (2008) the presence of Escherichia coli, *Staphylococcus aureus*, and Listeria monocytogenes was determined in 55 samples of organic poultry meat and in 61 samples of conventional poultry meat. A total of 220 E. coli, 192 *S. aureus*, and 71 L. monocytogenes strains were analyzed by an agar disk diffusion assay for their resistance to ampicillin, cephalothin, chloramphenicol, ciprofloxacin, doxycycline, fosfomycin, gentamicin, nitrofurantoin, streptomycin, and sulfisoxazole (*E. coli*); chloramphenicol, ciprofloxacin, clindamycin, doxycycline, erythromycin, gentamicin, nitrofurantoin, oxacillin, and sulfisoxazole (*S. aureus*); and chloramphenicol, doxycycline, erythromycin, gentamicin, sulfisoxazole, and vancomycin (L. monocytogenes). The results indicated a significantly higher ($P < 0.0001$) prevalence of *E. coli* but not of *S. aureus* and L. monocytogenes in organic poultry meat as compared with conventional poultry meat In the case of E. coli, the presence of multiresistant strains was significantly higher ($P < 0.0001$) in conventional poultry meat as compared with organic poultry meat. Organically farmed poultry samples showed significantly lower development of antimicrobial resistance in intestinal bacteria such as *E. coli*.

Nemati M, *et al.* (2008) reported that the susceptibilities of 12 antimicrobial agents for two collections of *Staphylococcus aureus*, isolated in the 1970s and in 2006 from poultry, were determined. For eight antibiotics, the percentage of resistance was significantly higher in the recent isolates. Ten recent isolates were methicillin resistant and had spa types t011 and t567, belonging to multilocus sequence type 398. This is the first report of "livestock-associated" methicillin resistant *S. aureus* from healthy poultry.

Lee JH *et al.* (2006) studied from 2001 to 2005, various specimens from cattle, pigs, and chickens were collected and examined for the presence of methicillin (oxacillin)-resistant *Staphylococcus aureus* (MRSA). The isolates from 19 specimens were tested for the presence of the mecA gene. Methicillin resistance was confirmed by determining the MICs for these isolates. Among these 19 mecA-positive isolates, 16 were consistently found to be resistant to methicillin. The mecR1 gene was found in all 19 mecA-positive *S. aureus*, and mecI was also detected in 15 of the mecA-positive *S. aureus*. In all 15 mecI-positive MRSA, the sequence

of the mec promoter/operator region was identical to the reference sequence. This suggests other mechanisms for overcoming the repression of resistance caused by mecI, beyond the simple product interaction between the mecA, mecRI, and mecI genes.

White DG *et al.* (2003) stated that *Staphylococcus aureus* is an important opportunist that can cause superficial to life-threatening illnesses in a variety of animal species. In poultry, this organism has been implicated in osteomyelitis, synovitis, and cellulitis. Whereas most infections can be treated with antibiotics, because of the organism's propensity to acquire antimicrobial resistance, it is important to continually monitor antibiotic susceptibilities of clinical isolates. We surveyed 77 clinical poultry *S. aureus* isolates, collected from 1998 to 2000, for susceptibilities to a panel of 18 antimicrobial agents *Staphylococcus aureus* isolates were commonly resistant to tetracycline (40%; minimal inhibitory concentration [MIC]90 > 32 microg/ml), lincomycin (19%; MIC90 > 32 microg/ml), erythromycin (12%; MIC90 > 8 microg/ml), and kanamycin (8%; MIC90 < 128 microg/ml). A periodic assessment of antimicrobial susceptibilities of important avian pathogens like *S. aureus* will be important in helping the clinician's choice of antibiotic to control infection.

Khan SAWhite DG*et al.* (2003) reported that the transfer of erm A and ermC genes, the two most common resistance determinants of erythromycin resistance, was studied with Luria-Bertani broth in the absence of additional Ca(2+) or Mg(2+) ions. Since both the donors (Amp(s)-Tet(r)) and recipients (Amp(r)-Tet(s)) were resistant to erythromycin, the transconjugants were initially picked up as ampicillin- and tetracycline-resistant colonies. A high frequency of transfer (4.5 x 10(-3)) was observed in all of the 23 transconjugants obtained, and the direction of tetracycline and erythromycin resistance marker transfer was determined to be from poultry to clinical isolates. The transfers of the ermA and ermC genes were via transposition and transformation, respectively.

Zhu XY*et al.* (2001) conducted an experiment with broilers to determine if prior exposure to *Staphylococcus aureus* would facilitate the systemic infiltration of this pathogen following intradermal footpad challenge with live *S. aureus*. Litter-raised broilers were sensitized at 3 and 4 wk of age with s.c. injections in the neck with heat-killed *S. aureus* diluted in polyethylene glycol (PEG). Equal numbers of control birds were injected at the same times with PEG. At 7 wk of age, chicks previously sensitized to killed *S. aureus* or injected with PEG were injected intradermally in the right footpad with PBS or live *S. aureus*. The left footpads of all birds were injected with PBS. The difference in thickness between the right

and left footpads was determined at 0, 24, and 48 h postchallenge. It was concluded that the intradermal challenge of the footpad with *S. aureus* resulted in systemic infiltration of *S. aureus* into the spleen, liver, and blood. Prior exposures to killed *S. aureus* as compared to PEG controls did not affect the systemic distribution of *S aureus*.

2.7 Isolation, identification and characterization of fowl cholera organism from chicken

Christensen et al. (**2000)** found that P. multocida subspecies multocida is the most common cause of fowl cholera, although P. multocida subspecies septica and gallicida may also cause fowl cholera-ike disease to some extent. However, the virulence properties of the different subspecies for various hosts have not been elucidated. In recent years, molecular typing methods have been applied to avian strains of P. multocida of different origin. The site of infection for P. multocida is generally believed to be the respiratory tract.

Olsen et al. (2001) found that a P. multocida species-specific oligonucleotide probe, pmhyb449, targeting 16S rRNA was designed and evaluated by whole-cell hybridization against 22 selected reference strains in animal tissues. It 6 differentiated P. multocida from other bacterial species of the families Pasteurellaceae and Enterobacteriaceae and also from divergent species of the order Cytophagales (except biovar 2 strains of P. avium and P. canis, which have high 16S rRNA similarity to P. multocida). In pig lung, P. multocida was detected in the alveoli. In poultry, infection with P. multocida may result in fowl cholera, a disease of economic importance in commercial production that may occur in different forms, such as peracute, acute, and chronic infections.

Leotta et al. (2006) reported the possible cause of outbreak of avian cholera occurred in Hope Bay area, located on the tip on the Antarctic peninsula, was that non breeder kelp gulls carried Pasteurella multocida gallicida to Hope Bay, and avian cholera was transmitted through water and to skuas and penguins. This study reports avian cholera in new bird species, their potential role in the transmission of the disease, and the different responses of these species to the disease.

Woo and Kim (2006) focused on whether the PM strains that originated in wild birds were transmitted in poultry farms. The possibility was tracked down by comparing phenotypic and genetic properties between the two types of PM strains. PM strains of chicken origin showed prominent differences from the PM strains of wild bird origin in both phenotypic and genetic

properties. The author conducted an examination of the origin of the wild bird bacteria but no evidence has been identified that PM strains from the wild bird were introduced into poultry farms.

Zhang et al. (2004) investigated the two outbreaks fowl cholera on a multiage free range egg farm. All 22 strains had the same phenotypic properties, all were confirmed as P. multocida by PCR, all were Heddleston serovar 4, and all had the same REA pattern. The results indicate that these 2 outbreaks were caused by the same clone of P. multocida despite the 8-year time period between the outbreaks.

Corney et al. (2007) designed a 5' Taq nuclease assay utilising minor groove binder technology and targeting the 16S rRNA gene to detect P. multocida in swabs collected from poultry. The assay correctly identified four P. multocida taxonomic type strains, 18 P. multocida serovar reference strains and 40 Australian field isolates (17 from poultry, 11 from pigs and 12 from cattle). Representatives of nine other Pasteurella species, 26 other bacterial species (18 being members of the family Pasteurellaceae) and four poultry virus isolates did not react in the assay. The assay detected 10 CFU of P. multocida per reaction. Of 79 poultry swabs 17 were positive in the 5' Taq nuclease assay, but only 10 were positive by culture. The other 62 swabs were negative for P. multocida by both 5' Taq nuclease assay and culture. The assay is suitable for use in diagnosing fowl cholera, is more rapid than bacteriological culture, and may also have application in diagnosing P. multocida infections in cattle and pigs.

Jabbari et al. (2003) described thephenotypic patterns of P. multocida strains by using different carbohydrates from hexoses, pentoses, disaccharides and polyhydric alcohols. The strains showed a high homogeneity. All strains were able to ferment sorbitol, mannitol, galactose, dextrose, fructose, glucose, mannose and sucrose. However none of them could produce acid from dulcitol, inositol, arabinose, salicine, raffinose and inoline. According to this pattern all of isolates belonged to subspecies multocida.

Kuczkowski et al. (2006) observed 123 cases of fowl cholera in south-west Poland over a 3-year period on geese and turkeys farms. A phenotypic analysis of 43 isolates of Pasteurella sp. was performed and all the tested isolates decomposed glucose, fructose, mannose and saccharose. Sorbitol, maltose and trehalose were fermented by 95.2%, 7.1% and 7.1% of the strains respectively. 83, 7% of the isolates tested were identified as P. multocida subs P. multocida, 2.3% as Avibacterium gallinarum and 14% could not be assigned to any of the

currently recognized subspecies or species of Pasteurella. The study revealed that capsular antigens belonging to group A and D occurred in 74.4% and 14.3% of the isolates, respectively. However, 16, 7% of the isolates did not reveal the presence of capsular antigens. Most of the isolates (76.2%) belonged to somatic serotype 1. The study indicated that the strains were most sensitive to amoxicillin (100%), amoxicillin with clavulanic acid (100%), colistin (93%) and gentamicin (88%).

Kwon and Kang (2004) diagnosed fowl cholera (FC) in waterfowl, Baikal teals (Anas formosa), at the National Veterinary Research and Quarantine in Korea. The total number of mortalities was 13,228 out of approximately 100,000 birds that wintered in Cheonsoo Bay, the most important habitat area of Baikal teals in the world. Clinical signs were detected in only a few birds because of sudden death. Grossly, the dead Baikal teals had lesions consistent with FC, including multifocal necrotic foci in the liver with enlargement, petechial or ecchymotic hemorrhages on the heart, and mucoid exudates in the duodenal mucosa. Microscopically, there were hepatocytic necrosis with bacterial colonization, hemorrhage and necrosis in the myocardium, and hemorrhagic enteritis. P. multocida was isolated from the liver and the heart of all birds examined by the agar gel immunodiffusion test. In order to estimate the virulence of P-627, 5-wkold commercial ducks were exposed intramuscularly or intracheally to the bacterium. On the basis of mortality rate, the isolate, P-627, was found to be highly virulent. This is the first report of an outbreak of FC in Baikal teals in Korea.

Mohamad et al. (2012) studied the prevalence of P. multocida strains among 275 backyard chickens from different regions of Upper Egypt. A total of 21 isolates P. multocida were recovered in 21 out of 275 chickens tested (7.6%) and were confirmed using phenotypic characterization. Somatic serotyping of the 21 isolates resulted in 12 isolates being classed are serotype A:1 (57.14%), 4 as serotype A:3(19.05%), 5could not be typed(23.8%). Capsular typing, using multiplex polymerase chain reaction (PCR), demonstrated that 18 strains were capsular type A (85.7%), and 3 were type D (14.3%).

Rutkowska and Borkowska (2000) described biochemical properties of P. multocida strains isolated from poultry. All isolates metabolized glucose, mannitol and saccharose. Sorbitol and arabinose were fermented by 82 and 43% of the strains, respectively. Xylose, trehalose and dulcitol were fermented by 30, 13 and 4% of isolates, respectively. All the tested strains produced catalase, ornithine decarboxylase, cytochrome-oxidase, indole and reduced nitrates

to nitrites. None isolate showed any motility or growth on MacConkey agar and could ferment maltose.

Shivachandra et al. (2006) suggested that a multiplex capsular PCR assay might be suitable for the rapid initial identification of the serotypes P. multocida during epidemiological studies of fowl cholera.

Tabatabai (2008) reported protective bacterial surface protein antigens such as: OMA87 hem P. multocidae-hemopexin receptor, HemR lactate permease, LctP and heptosyl transferase F and RfaF. Both the Oma87 and the HemR proteins would be of interest for subunit and modified live vaccine studies, respectively.

Varga et al. (2007) determined Pasteurella sp. by using biochemical characterization and PCR-based techniques and also determined subspecies, biovar, capsule type and presence of the toxA gene. Eighty-seven percent of the isolates belonged to P. multocida and 98% of these had biovar 3 or were trehaloseor lactose-fermenting or ornithine decarboxylase negative variants of that. Ten percent of the strains were P. multocida subsp. septica, and within this group 80% of the strains showed sorbitol-negative biovars (5, 6 and 7) but the rest (20%) were lactose positive. Only 3% of the porcine isolates were P. multocida ssp. gallicida and 3 out of the 4 strains belonged to the dulcitol-fermenting biovar 8. Using a capsule-specific multiplex PCR, 60% of the strains belonged to capsule type D, 38% to capsule type A, and only 1 isolate had capsule type F.

Wang et al. (2009) reported that 11 species of waterfowl (Anseriformes and Charadriiformes) were found dead in Hongjian Nur Lake in the Ordos wetland, Inner Mongolia of Northern China, in 2007. P. multocidawas isolated from tissue samples of dead and sick birds and identified as P. multocida subsp. muitocida, serotype A1, using serologic and molecular techniques. Eight bird species in this outbreak had never been previously reported with P. multocida infection. This was also the first report of fowl cholera in wild waterfowl in China.

Woo and Kim (2006) observed that a gram negative bi-polar staining bacillus was easily found in a direct smear. The biochemical properties of isolates were examined using a standard diagnostic method, proving that they were 99.7% similar to the P. multocida, a pathogenic and causative agent of fowl cholera.

Zahoor and Siddique (2006) characterized the 6 P. multocida isolates recovered from several farms in Faisalabad with outbreak of F. Cholera. Isolates were found resistant to streptomycin and cephradine and susceptible to ciprofloxacin and chloramphenicol. Analytical profile index (API) resulted in 86.1-98.6% identification. They concluded that API system and antimicrobial susceptibility testing were helpful for quick diagnosis and selection of appropriate antimicrobial agents.

Jabbari et al. (2003) described the phenotypic patterns of P. multocida strains by using different carbohydrates from hexoses, pentoses, disaccharides and polyhydric alcohols. The strains showed a high homogeneity. All strains were able to ferment sorbitol, mannitol, galactose, dextrose, fructose, glucose, mannose and sucrose. However none of them could produce acid from dulcitol, inositol, arabinose, salicine, raffinose and inoline. According to this pattern all of isolates belonged to subspecies multocida.

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Shah et al. (2008) carried-out a study on physico-chemical growth conditions of Pasteurella multocida at Vaccine Production Unit, Tandojam, Sindh, Pakistan. The organism was grown in brain heart infusion medium containing different physical and chemical agents. A suitable temperature for its growth was recorded in between 35°C and 40°C, whereas poor activities were observed at below 30°C and the organism did not grew well at 50°C and above. The influence of shaking in terms of different rotation per minute (rpm) was also determined during investigation. Under shaking conditions (500 rpm for 24 h) good growth was achieved. Positive correlation of shaking and growth at 500 rpm for 24 h was observed. The influence of pH was recorded during the experiment. No growth occurred at pH 0.5 and 10.0 but best growth was obtained at pH ranged from 7.0- 8.0. In addition to that, use of orange juice as supplement failed to support the growth, whereas apple juice (15%) showed positive influence on the growth of P. multocida. Moreover, different concentrations of succrose were used but only 1.2% supported the growth. As succrose was costly, it was replaced with table sugar at various concentrations, out of which only 0.6% well supported the growth.

Varga et al. (2007) determined Pasteurella sp. by using biochemical characterization and PCR-based techniques and also determined subspecies, biovar, capsule type and presence of the toxA gene. Eighty-seven percent of the isolates belonged to P. multocida and 98% of these had biovar 3 or were trehaloseor lactose-fermenting or ornithine decarboxylase negative variants of that. Ten percent of the strains were P. multocida subsp. septica, and within this group 80% of the strains showed sorbitol-negative biovars (5, 6 and 7) but the rest (20%) were lactose positive. Only 3% of the porcine isolates were P. multocida ssp.gallicida and 3 out of the 4 strains belonged to the dulcitol-fermenting biovar 8. Using a capsule-specific multiplex PCR, 60% of the strains belonged to capsule type D, 38% to capsule type A, and only 1 isolate had capsule type F.

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2.8 Antibiotic sensitivity and resistance pattern.

Reece RL*et al.* (1985) reported that the resistance to anti-microbial agents of bacteria isolated from pathological conditions of birds in Victoria, 1978 to 1983, was determined for

isolates of Escherichia coli, Salmonella species, *Staphylococcus aureus*, *Pasteurella multocida*, *P. anatipestifer*, *Yersinia pseudotuberculosis and Haemophilus paragallinarum.* The isolates of E. coli had a high prevalence of resistance to tetracycline and sulphonamides, and a lower prevalence of resistance to furazolidone and sulphamethoxazole-trimethoprim. The isolates of *Salmonella spp* commonly had resistance to tetracycline, sulphonamides, furazolidone and sulphamethoxazole-trimethoprim. Almost half the isolates of S. aureus showed resistance to lincomysin and many showed resistance to penicillin. Resistance to tetracycline was found in isolates of *P. multocida*, *P. anatepestifer and Y. pseudotuberculosis. Some isolates of H. paragallinarum* showed resistance to sulphonamides, streptomycin and sulphamethoxazole-trimethoprim.

Hill and Johnson (2005) examined susceptibility of *P. multocida* to antimicrobial agents. Of the 153 isolates, 6 were shown to be resistant to one or more antimicrobial agents. Of the six resistant isolates, five contained R plasmids. All but one of the R plasmids were small (6 to 7 megadaltons) and non conjugative, encoding resistance to tetracycline or kanamycin, streptomycin, and sulfonamides; the other was large (70 megadaltons) and conjugative, transferring resistance to kanamycin, streptomycin, sulfonamides, and tetracycline to *P. multocida* and Escherichia coli. The three plasmids encoding resistance to tetracycline alone appeared identical.

Mukkur (2004) reported that a *P. multocida* isolate of 12; A serotype from a rabbit caused typical pulmonary pasteurellosis and death in pasteurella-free rabbits by intranasal exposure. Rabbits stressed with hydrocortisone and inoculated with 12: A *P. multocida*, organisms developed a higher prevalence of pneumonia than rabbits not treated with hydrocortisone. Typical 12: A *P. multocida* was isolated from nasal cavity, trachea, and lung, and was, most prevalent in nasal cavities. Surviving rabbits developed serum agar gel precipitating antibody beginning 15 days post-inoculation. The data showed that the l2:A *P. multocida* isolate was pathogenic, caused mortality, colonized the respiratory tract, and stimulated systemic immune response by producing serum agar gel precipitating antibody.

P. Srinivasan *et al* . (2011)

In the *in vitro* drug sensitivity cotrimoxazolewas found to be highly effective (89 percent) followed by enrofloxacin, ciprofloxacin (78 percent) and oxytetracyclin (67 per cent) where as thenorfloxacin, cephotaxime and cephalexin were moderately sensitive and the remaining antibiotics(ampicillin, amoxicillin, chloremphenicol, colistin and gentamicin) were resistant .

The drug sensitivitypattern in the present study are in concurrence with those of Madhekar *et al*. (1982) and Ramasastry and Ramarao (1989), where as Shivachandra *et al*. (2004) observed a strong resistance against Sulfadiazine. These differences in antibiogram pattern may bedue the non acquaintance of antibiotic resistance, since broiler birds are not exposed to antibiotics very frequently as that of layer birds. Accordingly the birds were treated with Sulphamethaxazole and Trimethoprim at the dose rate of 25 mg/kg body weight for five days, administered in drinking water. Clinical signs and mortality associated with fowl cholera were reduced from second day after the commencement of the treatment and birds completely recovered on the 4thday of treatment.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Study area and period

The present research work was carried out on 10 different Broiler farms in Dinajpur district. The laboratory works were conducted in the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur, during the period from January toJune 2017.

3.1.2 Laboratory Preparations

All items of required glassware including test tubes, pipettes plate, slides, cylinder, flasks, conical flasks, glass and vials soaked in a household discwashing detergent solution (‘Trix’

Recket and Colman Bangladesh Ltd.) overnight. Contaminated glassware was disinfected with 2% sodium hypochloride solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly in running tape water, rinsed within distilled water and finally sterilized either by dry heat at 160˚C for 2 hours or by autoclaving for 15 minutes at 121˚C under 15 lbs pressure per sq inch. Autoclaved items were dried in a hot air oven over at 50˚C. Disposable plastic (items e.g. micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50˚C for future use.

3.1.3 Media for culture

The media and reagents that have been used for the isolation and identification of the bacteria are mentioned below.

3.1.3.1 Solid media:

- Nutrient Agar Medium, (HI-MEDIA, India)
- Cook Meat Media, (HI-MEDIA, India)
- Eosin Methylene Blue, (EMB) (HI-MEDIA, India)
- Agar Medium, (HI-MEDIA, India)
- Blood Agar Medium, (HI-MEDIA, India)
- Mac Conkey Agar medium, (HI-MEDIA, India)
- Brilliant Green Agar Medium, (HI-MEDIA, India)

3.1.3.2 Liquid media:

- Nutrient broth, (HI-MEDIA, India)
- Methyl Red-Voges Proskauer (MR-VP) broth, (HI-MEDIA, India)
- 1% Pepton Water, (HI-MEDIA, India)
- Tetrathionate broth, (HI-MEDIA, India)

3.1.4 Media for bichemical test

- Simmons Citrate Agar Medium, (HI-MEDIA, India)
- Lactose broth, (HI-MEDIA, India)

- Pepton broth, (HI-MEDIA, India)
- Methyl Red (MR) media
- Voges-Proskauer (VP) media

3.1.5 Reagents

The chemicals and reagents used during the study were-

- Gram's staining reagents (Crystal violet, Gram's iodine, Acetone alcohol, Safranin)
- Potassium- di-hydrogen phosphate (0.2M, KH_2PO_4 $2H_2O$)
- Dehydrated sodium citrate
- Phosphate Buffered Saline (PBS)
- Physiological Saline Solution (PSS)
- Methylene Blue stain
- Di-sodium hydrogen phosphate (0.2M, $Na_2HPO_4 12H_2O$)
- Voges-Proskauer (VP) Solution
- Sugar media (Dextrose, Maltose, Lactose, Sucrose, and Mannitol) and other chemicals and reagents as when required during the experiment.
- Indol Solution
- Methyl Red Solution

3.1.6 Preparation of MR-VP broth

A quantity of 17 gms of Bacto MR-VP medium (Hi Media, India) was dissolved in 1000 ml distilled water dispensed in 2 ml amount in each test tube and then the tubes were autoclaved at $l2l^0$ C maintaining a pressure of l5 pounds/sq. Inch for 15 minutes. After autoclaving, the tubes containing medium were incubated at 37^0 C for overnight to check their sterility and then stored in a refrigerator for future use (Cowan, 1985).

3.1.7 Preparation of physiological saline solution

For the preparation of this solution procedures suggested by Cowan (1985) were followed. A 0.85% PSS was prepared by dissolving 8.5 gms of chemically pure sodium chloride (NaCl) in 1000 ml of distilled water in a conical flask. The physiological saline solution was then sterilized by autoclaving at $l2l^0$ C under 15 lbs, for 15 minutes. Following sterilization, the saline was cooled and then kept at 4^0 C -8^0 C in the refrigerator until used.

3.1.8 Preparation of Phosphate buffered saline

PBS was prepared by dissolving the following ingredients according to Sambrook et al. (1989). For 1 liter of 1X PBS, prepare as follows:

1. Start with 800 ml of distilled water:
2. Add 8 g of NaCl.
3. Add 0.2 g of KCl.
4. Add 1.44 g of Na_2HPO_4.
5. Add 0.24 g of KH_2PO_4.
6. Adjust the pH to 7.4 with HCl.
7. Add distilled water to a total volume of 1 liter.

The solution was then sterilized by autoclaving at 121^0 C under 15 Ibs for 15 minutes and stored at 4^0 C -8^0 C in refrigerator until used.

3.1.9 Glassware's and appliances

The different kinds of glassware's and appliances used during the course of the experiment were as follows:

Test tubes (with or without Durham's fermentation tube and stopper).

- Conical flask
- Inoculating loop
- Petridishes
- Pipette
- Cover slips
- Hanging drop slide
- Glass rod spreader
- Test tube stand Water bath
- Ice box
- Autoclave
- Refrigerator
- Hot air oven
- Compound microscope
- Micropipette
- Centrifuged tube
- Spirit lamp
- Slide

3.1.10 Antimicrobial Sensitivity Discs

To determine the drug sensitivity pattern of different bacterial isolate with different types of antimicrobial. Commercially available antimicrobial discs (Oxoid Ltd., UK) were used. The

method allowed for the rapid determination of the efficacy of the drug by measuring the diameter of the zone of inhibition that result from different diffusion of the agent into the medium surrounding the disc. The followings are the antibiotics that were tested against, the selected organism with their disc concentration.

Table 1. Antimicrobial agents with their disc concentration.

S/N.	Name of antibiotics	Disc concentration (µg / disc)	S/N.	Name of antibiotics	Disc concentration (µg/disc)
1.	Amoxycillin (AMX)	10 µg	8.	Neomycin(NEO)	30 µg
2.	Ampicillin (AMP)	30 µg	9.	Colistin sulphate(CS)	25 µg
3.	Gentamicin (GN)	5 µg	10.	Erythromycin(E)	5µg
4.	Chloramphenicol (C)	30 µg	11.	Levofloxacin(LF)	5 µg
5.	Ciprofloxacin (CIP)	5 µg	12.	Doxicycline(DO)	10µg
6.	Enrofloxacin (ENR)	5 µg			
7.	Streptomycin (S)	30 µg			

Legend: µg = Microgram

3.2 Methods

The following methods were used for the isolation and identification of bacteria.

3.2.1 Experimental design

The experimental work was divided into two steps: The first step was performed for the isolation and identification of the organisms of the collected sample using cultural, staining and biochemical characteristics. The second step was conducted for the determination of antibiotic sensitivity and resistant pattern of isolated organisms of various samples by using different antibiotic discs available in the market. The layout of the diagrammatic illustration of the present study is shown in figure

Experimental design

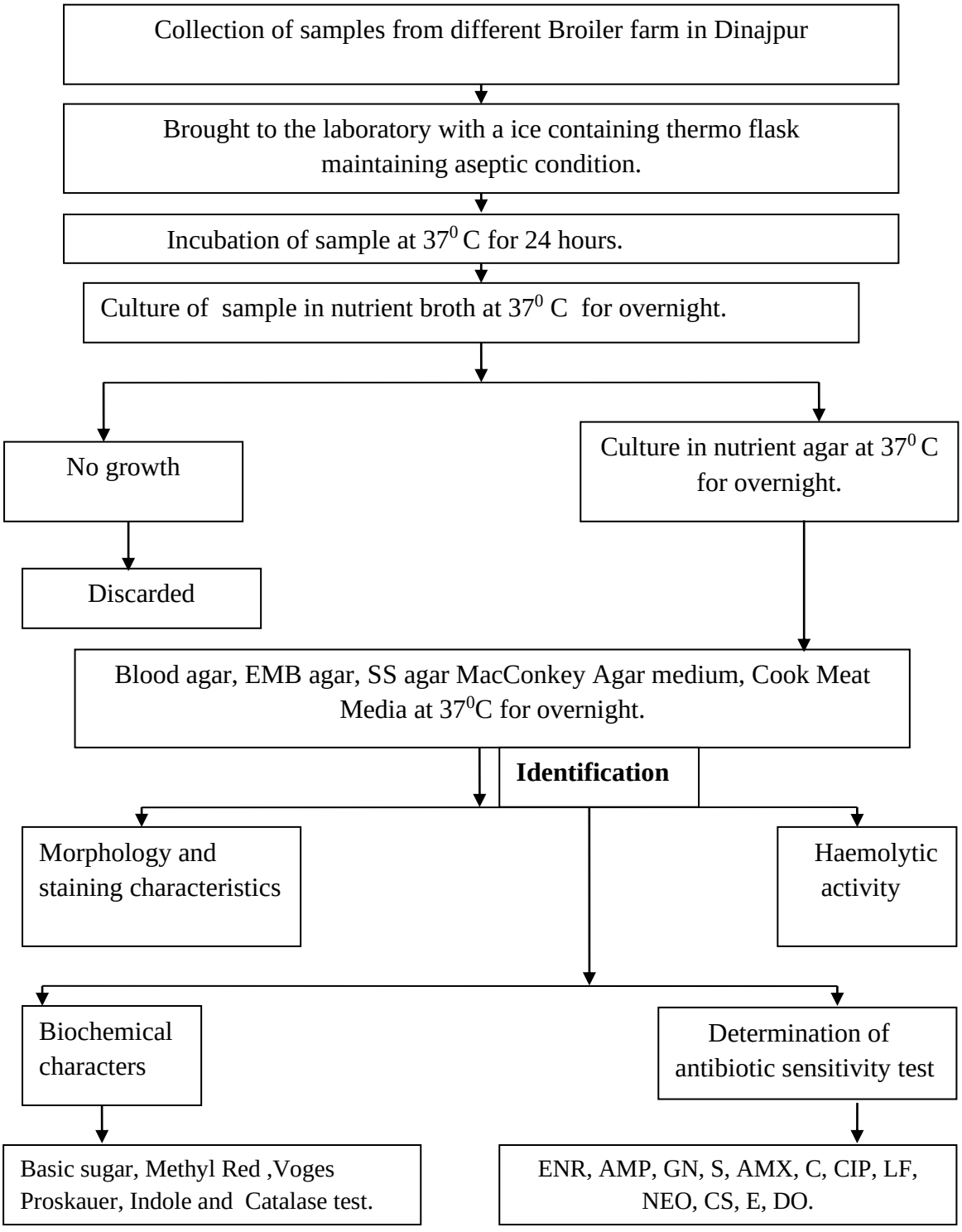


Fig. 1. The schematic illustration of the experiments.

3.2.2Collection and transportation of samples

A total of 100 samples were randomly collected directly from dead birds in Dinajpur district. The samples (Liver, Lungs, Esophagus, Duodenum, Tracheal swab) were carried to the bacteriological laboratory in the department of Microbiology, HSTU, Dinajpur, in an ice box contained ice and processed for the isolation and characterization of bacteria subsequently and kept in incubator at 37^0 C for 24 hours for the isolation and identification of bacteria by morphology, staining and cultural characteristics. Characterization of bacteria was done by biochemical reactions and antimicrobial sensitivitytest. The remaining samples were stored at 0° C for further use. Aseptic measures were followed during collection and transportation of samples.

Table 2: Collection area of sample

SL No.	Name of the Poultry farms and Locations
01	Nawab poultry farm, Birgong, Dinajpur.
02	Chan mia poultry farm, Birgong, Dinajpur.
03	Mofijul poultry farm, Birgong, Dinajpur.
04	Abul hossain poultry farm, Parbotipur, Dinajpur.
05	Nur poultry farm, Parbotipur, Dinajpur.
06	Kamu poultry farm, Sadar ,Dinajpur
07	Monir hossain poultry farm, Sadar, Dinajpur.
08	Saddam hossain poultry farm, Setabgong, Dinajpur.
09	Ria poultry farm, kaharol, Dinajpur.
10	Hasan Mia poultry farm, kaharol, Dinajpur.

3.2.3 Preparation of culture media and broth

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

3.2.3.1 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.

3.2.3.2 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.

3.2.3.3 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/cm^2 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.

3.2.3.4 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/cm^2 pressure and 121° C for 15 minutes and I to 50° C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile 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.

3.2.3.5 MacConkey agar medium

51.5 gramsMacConkey 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/cm^2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 45^0- 50^0C 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.

3.2.3.6 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 P^H 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 /cm^2 121° 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.

3.2.3.7. Salmonella-Shigella agar media

The SS agar plates were prepared and stored following the procedure of Cowan, (1985).An amount of 63 gms 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 12^0C maintaining a pressure of 15 lb pressure/sq. inch for 15 minutes. After autoclaving, the medium was put into a water bath at 45^0C 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 4^0C 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 to 500 ml with the addition of distilled water.

3.3.2 Methyl Red - Voges Proskauer broth

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

3.3.3 Voges – Proskauer solution

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

3.3.4 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of Potassium hydroxide crystals in100 ml 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 gram of potassium chloride (KC1) and 0.2 gram of potassium hydrogen phosphate (KH_2PO_4) were suspended in 1000 ml of distilled for the preparation of phosphate buffered saline solution. The solution was heated to dissolve completely. Then the solution was sterilized by autoclaving at 1.2 kg / cm^2 pressure and 121° C for 15 minutes and stored for future use.

3.3.6 Indole test

3.3.6.1 Kovac's reagent

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

3.3.7 Culture of samples

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 at 37°C for overnight. The colonies on primary cultures were repeatedly sub-cultured by streak plate method (Cheesbrough, 1985) until the pure culture with homogenous colonies were obtained. Media such as Blood agar, Nutient agar, MacConkey agar, Eosin Methylene Blue agar, were used for sub-cultures and were incubated at 37° 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 as follows: A small colony was picked up from SS, MC, and BGA plates with a bacteriological loop, smeared on separate 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 act as a mordent for one minute and then again washed with running water. Acetone alcohol was then added (acts as decolorizer) for few second. After washing with water, safranin was added as counter stain and allowed to stain for two minutes. The slides were then washed with running water, blotted and dried in air and then examined under microscope with high power objects (100 X) using immersion oil.

3.3.9 Biochemical Test

3.3.9.1 Catalase test

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

3.3.9.2 Methyl- Red test

A colony of test organism was inoculated in 0.5 ml sterile glucose phosphate broth to perform this tube an incubated at37^0 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 (0.5 ml) was added, shaked well and examined after 1 minute. A red color in the reagent layer indicated production of indole. In negative case there was no development of red colour (Cheesbrough, 1985).

3.3.9.4 Voges- Proskauer test

Two milliliter of sterile glucose phosphate peptone water was inoculated in 5 ml of test organisms. It was incubated at 37^o 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 positive cases. There was no 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^o for 24 hours. Various types of haemolysis were observed after development of bacteria colony on the BA. The hemolytic pattern of the bacteria was categorized according to the types of hemolysis produced on BA and this was made as per recommendation of Carter (1986) and was listed as mentioned below:

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 (γ) 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).

Step 1. A inoculum was picked up with a sterile inoculating loop and was spread on a area of the medium in the petridish.

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

Step 3. 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° C for overnight in an incubator.

3.3.11.2 MacConkey agar

Materials from lactose fermentation tubes were inoculated into Mac Conkey agar plates which after incubation, if positive for *Escherichia coli* will show rose pink color colonies.

3.3.11.3 Eosin Methylene Blue(EMB)agar

Materials from lactose fermentation tubes were inoculated into EMB agar plates which after incubation, will show smooth circular colonies with dark centers 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 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.

3.3.11.5 Identification of Escherichia coli isolates by biochemical test

Sugar fermentation test was performed to identify of *Escherichia coli*. For sugar fermentation on the tubes containing different sugar media such as sucrose, maltose, dextrose, lactose and mannitol were inoculated with a loopful of broth culture of the isolated and incubated at 37°C for 18 hours. The isolates if positive, ferment five sugar viz. dextrose, maltose, lactose, sucrose, and mannitol, the organisms 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.

3.4.1Culture on nutrient agar

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

3.4.2 Culture in to different media

The gram negative organisms were inoculated into MacConkey agar, EMB agar and incubated at 37º C for 24 hours. The incubated media were then examined for growth of bacteria.

3.4.3 Culture on selective media

In case of EMB agar non-metalic sheen colony was sub cultured. In case of 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 of BG agar, light pink colony against a rose pink background was sub cultured. Thus single pure colony was obtained. Thus single pure colony was obtained.

3.4.4 Morphological study (Gram's staining)

- A loopful of sterile distilled water was placedin the center of a clean sterile slide.
- A Small colony was picked up with a bacteriological loop and was mixed with distilled water of a slide.
- The colony was made to thin smear on a slide.
- The smears were fixed by air drving.
- 0.5% crystal violet solution was then applied on the smear for one minute.
- Lugol's iodine was then added to act as mordant for One minute.
- Acetone alcohol was then added to decolorize for 1-2 seconds.
- Then the slide was washed with water.
- Safranine was added as counter stain and allowed for one minute.
- 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 agar and BG agar and were subjected to biochemical tests (sugar fermentation test, TSI agar slant reaction, MR-VP reaction and indole reaction, **MIU test**).

3.4.6 Sugar fermentation test

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

3.4.7 Methyl Red (MR) test

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

3.4.8 Voges-Proskaure test

2ml of sterile glucose phosphate peptone water were inoculated with the 5ml of test organisms. It was incubated at 37°C aerobically for 48 hours. A very small amount (knifepoint) of creative was added and mixed. 3 ml of potassium hydroxide were added and snaked well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of pink color for positive cases. In negative cases, there was no development of pink color (Cheesbrough, 1985).

3.4.9 Indole test

Two ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours. Kovac's reagent (0.5 ml) 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).

3.4.10 Citrate utilization test

Suspected colony was inoculated on Simmons citrate agar. Then the medium was incubated at 37°C for 48 hours.In suspected case the colour of green medium turned to deep blue colour.

3.4.11 Triple sugar iron agar slant reaction

TSI agar was used to detect the nonlactose fermenters and the dextrose fermenters. The medium also helped to determine the ability of the organisms to produce hydrogen sulfide (H_2S). The organisms under study were heavily seeded with a platinum needle over the surface of the slant and stabbed into the butt 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 of acid and gas respectively in slants or in butt as the case may be. 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, Urea) medium

Suspected colony was inoculated into the tube containing MIU medium. Then the medium was incubated at 37°C for overnight. Absence of turbidity throughout the medium was indicated nonmotile *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 the 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 the concave depression of the hanging drop slide for better attachment of the cover-slip to prevent air current and evaporation of the fluid. The hanging drop slide was then 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 the Nutrient broth culture and streaked on Nutrient agar, Blood agar media to get isolates in pure culture. All inoculated media were kept at 37° C for overnight in an incubator.

3.5.2 Nutrient agar (NA)

Materials from Nutrient broth tubes were inoculated into Nutrient agar plates which incubation, showed grey-white to yellowish colony on nutrient agar in case of Staphylococcus spp.

3.5.3 Blood agar(BA)

Incubation of materials from Nutrient broth into Blood agar plates which after inoculation, if positive for 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 described by Cowan and Steel (1979). If the organism found as Gram positive, cocci, arranged in cluster indicating Staphylococcus spp.

3.5.5 Identification of Staphylococcus spp. isolates by biochemical test

Sugar fermentation test was performed to identify of *Staphylococcus spp*. For sugar fomentation tests the tubes containing different sugar media such as sucrose, maltose, dextrose, lactose, and mannitol were inoculated with a loopful of broth culture of the isolated organisms and incubated at 37° C for 18 hours. The isolates if positive, ferment five sugar viz. dextrose, maltose, lactose, sucrose, and mannitol, the organism produces acid in all cases and change color of media reddish to yellowish. Acid production was indicated by the change of the color reddish 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*. will reveal as positive reaction.

3.6 Isolation and identification of the *Pasteurella spp.*

The following steps were followed for isolation and identification of the *Pasteurella spp.*from collected samples, colony characteristics, morphology andstaining characteristics, motility and biochemical test.

3.6.1 Culture into different media

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

3.6.2 Nutrient agar (NA) media

Materials from Nutrient broth tubes were inoculated into Nutrient agar plates which incubation, if positive showed whitish, opaque, flat and translucent colonies in case of *Pasteurella spp.*

3.6.3 Blood agar (BA) media

Incubation of materials from Nutrient broth into Blood agar plates which after inoculation, if positive for *Pasteurella spp.*showed small colonies. The other characteristics of these colonies included whitish, opaque, circular and translucent appearance. No hemolysis was noticed on blood agar.

3.6.4MacConkey agar (MC) media

Incubation of materials from Nutrient broth into MC agar plates which after inoculation, if positive for *Pasteurella spp.*showed no colonies.

3.6.5 Salmonella-Shigella (SS) agar media

Incubation of materials from Nutrient broth into SS agar plates which after inoculation, if positive for *Pasteurella spp.*showed no colonies

3.6.6 Procedure of staining techniques

The smears prepared from isolated colonies of *Pasteurella spp.*were stained using the following staining method.

Gram's staining technique

This was conducted following the method described by Merchant and Packer (1967). Smears were fixed by gentle heat; Crystal violet solution was then applied on the smear to stain for two minutes and then washed with water. Lugol's iodine was then added to act as mordant for one minute and then washed with water. Acetone alcohol was used as decolourizer for few seconds. After washing with water, safranine was added as counter stain and allowed to stain for 1-2 minutes. The preparation was then washed with water, blotted and dried in air and then examined under microscope.

3.6.7 Procedure of Motility test

The motility test was performed according to the method described by Cowan, (1985) to differentiate motile bacteria from the non-motile one. Before performing the test, a pure culture of the organism was allowed to grow in NB. A drop of cultured broth was placed on the 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 concave depression of the hanging drop slide for better attachment of the cover slip to prevent air current and evaporation of the fluid. The hanging drop slide was then examined carefully under 100 power objective of a compound.

3.6.8 Bio-chemical test

3.6.9Procedure of MR test

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

3.6.10Procedure of VP test

2 ml of sterile glucose phosphate peptone water were inoculated with the 5 ml of test organisms. It was incubated at 37^0C for 48 hours. A very small amount (knife point) of creatine was added and mixed. 3 ml of 40% potassium hydroxide were added and shacked well. The bottle cap was removed and left for an hour at room temperature. It was observed

closely for the slow development of a pink colour for positive cases. In negative cases there was no development of pink colour (Cowan, 1985).

3.6.11Procedure of Indole test

2 ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours. 0.5 ml Kovac's reagent was added, shaked well and examined after 1 minute. A red colour ring at the top of the reagent indicated positive test. In negative case there is no development of red ring (Cowan, 1985).

3.6.12Procedure of Motility Indole Urease Test (MIU)

MIU was prepared in a test tube. Then the organism was inoculated into the media by stabbing method with the help of sterile straight wire. Then the test tube was incubated 37^0C overnight. Single stick that is no turbidity throughout the medium indicate gram negative organism (non motile) and turbidity throughout the medium indicate gram positive case (Cowan, 1985).

3.6.13Procedure of Triple Sugar Iron Test (TSI)

Triple sugar iron contain three sugar (Glucose, Sucrose, Lactose). At first TSI agar slant was prepared in a test tube. Then the organism was inoculated into the butt with a sterilized wire and on the slant with a wire loop producing zigzag streaking. The tube was incubated for 24 hours at 37^0C.Yellow colour of butt and slant of the test tube indicate fermentation of Glucose, Sucrose and Lactose fermentation and butt shows blacking indicate H_2S production (Cowan, 1985).

3.6.14 Maintenance of stock culture

During the experiment it was necessary to preserve the isolated organisms for longer periods. For this purpose the organisms from pure culture were inoculated into the tubes of nutrient agar slants and incubated at 37^0C for 24 hours. After the growth of organisms the tubes were sealed with paraffin wax and kept in the refrigerator at 4^0C following the procedures of (Choudhury *et al.*, 1985).

3.7 (20%) Sterile buffered glycerin

An amount of 20%o sterile buffered glycerin was made by mixing 20 parts pure glycerin and 80 parts PBS. Then a loopful of thick bacterial culture was mixed with 20%sterile buffered glycerin in small vials and was preserved at 20^0C. This method is more appropriate for preserving bacteria with no deviation of their original characters for several years (Buxton and Fraser, 1977).

3.8 In vitro antibiotic sensitivity test

The method allowed for the rapid determination of the efficacy of the drugs by measuring the diameter of the zone of inhibition that resulted from different diffusion of the agent into the medium surrounding the disc.

In vitro antibiotic sensitivity tests were done using disc diffusion test following the method Kirby- Bauer(Bauer *et al.,* 1966). 1-2 ml of freshly growing broth culture were poured on nutrient agar plate and spread uniformly. Antibiotic discs were placed apart on to the surface of the inoculated plates aseptically with the help of a sterile forceps and incubated at 37 ^{0}C for 24 hours.

After incubation the plates were examined and the diameter of the zone of inhibition was measured. The diameter of the zone for individual antibiotic was recorded as sensitive, intermediate and resistant. (According to EUCAST)

CHAPTER 4

RESULTS

Result of morphological, staining, cultural, biochemical, antibiotic sensitivity including percentage of incidence of isolated bacteria are presented in the table and described below under the following headings:

4.1Isolaton and identification of *Escherichia coli* by different acteriological methods

4.1.1 Results of cultural examination

4.1.2 Nutrients broth

Nutrients broth was inoculated with the milk sample and incubated at 37 for 24 hours. The growth of bacteria was indicated by the presence of turbidity.(**Plate-2).**

4.1.3 Nutrients agar

Nutrient broth plates streaked with E. coli broth then shown growth of bacteria after 24hrs of inoculation at 37^0c. The growth of E. coli on nutrient agar media was characterized by circular, smooth, opaque and colorless colonies. (**Plate-1).**

4.1.4. MacConkey (MC) agar

MacConkey 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 bright-pink colored smooth colonies. (**Plate-7).**

4.1.5. Eosin Methylene 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, circular , black color colonies with metallic sheen . (**Plate-4).**

4.1.6. Results of Gram's staining

The microscopic examination of Gram's stained smears from MC and EMB agar revealed Gram-negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain **.** (**Plate-3).**

4.1.7 Results of biochemical tests

4.1.8 Fermentation reaction with five basic sugars

The isolates fermented the five sugars (dextrose, maltose, lactose, sucrose, and manito) 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. (**Plate-14).**

4.1.9 Indole test

The isolates were indole positive. (**Plate-12).**

4.1.10 Methyl- red and Voges- proskauer test

The isolates were MR positive and VP negative . (**Plate-9, 10).**

Table 3 Cultural, morphological and biochemical properties of isolated Escherichia coli.

<table>
<tr><td colspan="2">Cultural characteristics</td><td colspan="2">Biochemical Characteristics</td><td>Staining and morphological Characteristics</td></tr>
<tr><td>EMB Agar</td><td>MC Agar</td><td>Tests</td><td>Results</td><td>Staining properties</td></tr>
<tr><td rowspan="12">Smooth, circular, black color colonies with metallic sheen were produced</td><td rowspan="12">Bright pink color, smooth colonies were produced smooth colonies were</td><td>Dextrose</td><td>AG</td><td rowspan="12">Gram-negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain</td></tr>
<tr><td>Maltose</td><td>AG</td></tr>
<tr><td>Lactose</td><td>AG</td></tr>
<tr><td>Sucrose</td><td>AG</td></tr>
<tr><td>Mannitole</td><td>AG</td></tr>
<tr><td>Catalase test</td><td>+</td></tr>
<tr><td>Indole</td><td>+</td></tr>
<tr><td>MR</td><td>+</td></tr>
<tr><td>MIU</td><td>-</td></tr>
<tr><td>TSI</td><td>+</td></tr>
<tr><td>VP</td><td>-</td></tr>
</table>

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 Isolation and identification of *Salmonellae spp.*

4.2.1 Cultural characteristics

4.2.2 On Nutrient agar (NA)

On Nutrient agar (NA) isolates produced translucent, opaque, smooth colonies. (**Plate-16).**

4.2.3 On Nutrient broth (NB)

Salmonellae isolates produced turbidity in nutrient broth. (**Plate-17).**

4.2.4. S-S agar

On S-S agar suspected isolates produced translucent, colorless, smooth, small round colonies (**Plate-21).**

4.2.5 Brilliant green agar

Samples inoculated onto BG agar plates produced pale pink color colonies against a pinkish background, which was rose-pink colour before growth.(**Plate-22).**

4.2.6 MacConkey agar

Samples inoculated onto MacConkey agar plates produced colorless, smooth, transparent and raised colonies. (**Plate-19).**

The overall Cultural characteristics in different culture media are summarized in Table 4

4.2.7 Simmons citrate agar

In the citrate utilization test the colour of green medium turned to deep blue colour indicating the isolated *salmonellae* were positive for Simmons citrate test. (**Plate-23).**

Table 4. Cultural characteristics of *Salmonella spp.* in different culture media

Media used	Colony characteristics	Isolated organism
Salmonella-Shigella agar	Translucent, Smooth, Small round black centered colonies	*Salmonella* spp.
MacConkey agar	Colorless, smooth, transparent, raised colonies	
Brilliant green agar	Pale pink color colonies against a yellowish background.	
TSI agar	Transparent, smooth round colonies	
Nutrient agar	Translucent, opaque, smooth colonies	
Nutrient broth	Turbidity in the broth	

4.2.8 Results of Gram's staining method

The thin smears prepared with the colony from SS agar, MC agar and BG agar for Gram's staining revealed Gram-negative, pink colored, small rod shaped appearance, arranged in single or paired under the microscopic examination . (**Plate-18).**

4.2.9. Results of motility test

Isolates was found to be non motile when examined using hanging drop slide under microscope. The Characteristics of chicken *Salmonellae* isolates by Gram's staining method and motility test are presented in Table 5.

4.2.10. Results of biochemical test

4.2.11. Fermentation reaction with five basic sugars

Organism isolated from chicken fermented dextrose, maltose and mannitol, 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 bubbles in the inverted Durham's tubes kept inside each of the test tubes containing sugar media.

4.2.12. Indole test

Salmonellae isolates from chicken was indole negative. They did not produce any red color. (**Plate-47).**

4.2.13 MIU test

Absence of turbidity trough out the medium was indicated non-motile *Salmonella* organisms (**Plate-25).**

4.2.14 Methyl red (MR) test

In the MR test the appearance of the red colour in the media after the addition of 3 ml methyl red with the cultural growth was observed and thus indicating the isolated *Salmonellae* were positive for MR test. (**Plate-26).**

4.2.15 Voges-Proskauer (V-P) test

In the Voges-Proskauer (V-P) test, no change of colour of the media was observed after the addition of 3 ml of 3% KOH to 3 ml V-P broth media with the cultural growth of the isolated *Salmonellae* and thus indicated that the isolated *Salmonellae* from chicken was negative for V-P test. (**Plate-27).**

4.2.16 Triple Sugar Iron (TSI) agar slant reaction

On TSI agar slant, *Salmonella* isolates from chicken 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-28).**

4.3. Identification of *Staphylococcus spp.* by different bacteriological methods

4.3.1 Results of cultural examination

4.3.2 Nutrients broth

Nutrients broth was inoculated with the milk sample and incubated at 37 for 24 hours. The growth of bacteria was indicated by the presence of turbidity. **(Plate-32).**

4.3.3 Nutrient agar

Nutrient agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37 aerobically and were indicated by the growth of circular, small smooth, convex and gray white or yellowish colonies. (**Plate-30).**

4.3.4 Staphylococcus agar no.110

Staphylococcus agar plates streaked separately with the organisms and incubated at 37^0C aerobically for 24 hours and observed golden yellowish colonies on staphylococcus agar no. 110. (**Plate-33).**

4.3.5 Blood agar

Blood agar plates streaked separately with the organism and incubated at 37 aerobically for 24 hours and hemolysis were produced. (**Plate-34).**

4.3.6 Results of Gram's staining

Gram's stained smears from NA and BA were examined agar revealed Gram-positive cocci arranged in grape like cluster. (**Plate-33).**

Table5.Cultural, morphological and biochemical properties of isolated *Staphylococcus spp.*

Cultural characteristics		Biochemical Characteristics		Staining and morphological Characteristics
Blood Agar	Nutrient Agar	Tests	Results	Staining properties
ß-Type of hemolysis were produced	Circular, small, smooth, convex and gray-white or yellowish colonies were produced were prod were	Dextrose	Acid	Gram positive cocci arranged' in grape like cluster
		Maltose	Acid	
		Lactose	Acid	
		Sucrose	Acid	
		Mannitole	Acid	
		Catalase test	+	
		MIU	-	
		VP	+	
		TSI	+	
		MR	+	
		Indole test	-	

Legends

AG = Acid and Gas, MR = Methyl-Red test, VP = Voges-Proskauer test,

+ = Positive reaction, - = Negative reaction, EMB = Eosin Methyle Blue, MC = MacConkey

4.3.7 Results of biochemical tests

4.3.8 Fermentation reaction with five basic sugars

The isolated bacteria fermented the five sugars (dextrose, maltose, lactose, sucrose, and mannitol) with the production of acid. The change of color from reddish to yellow indicated acid production. (**Plate-41).**

4.3.9 Catalase test

The isolates were catalase positive. (**Plate-42).**

4.3.10 Methyl- red and Voges- proskauer test

The isolates were MR positive and VP negative. (**Plate-37,38).**

4.3.11 Indole test

The isolates were indole positive. (**Plate-36).**

4.4 Results of cultural examination of the bacterial isolates *Pasteurella spp*.

4.4.1 Culture in nutrient broth (NB) media

The growth of *Pasteurella spp*. in NB was characterized by diffused turbidity and no pellicle was found to be formed (**Plate-45).**

4.4.2 Culture on Nutrient agar (NA) media

The culture of organism on NA produce whitish, opaque, flat and translucent colonies (**Plate-44).**

4.4.3Culture on blood agar (BA) media

The culture of organism on BA yielded small colonies. The other characteristics of these colonies included whitish, opaque, circular and translucent appearance. (**Plate-47).**

4.4.4Culture on MacConkey (MAC) agar media

Culture on MAC agar plates yielded no colonies . (**Plate-48).**

4.4.5Culture on Salmonella-Shigella (SS) agar media

Culture on SS agar plates yielded no colonies. (**Plate-49).**

4.4.6Results of staining characteristics of the bacterial isolates

Results of Gram's staining

Gram's staining was performed on smears of samples showed the presence of Gram negative, cocco-bacillary or bipolar shape organism and arranged singly or in paired. Similar characters were also observed with organisms grown in various artificial Media. (**Plate-46).**

Table 6. Results of cultural, morphological and motility characteristics of the isolated *Pasteurella spp.*

Colony characteristics						Staining characters	Motility
NA	BA	NB	MC	SS	NB		
Whitish opaque, circular, translucent	Whitish, opaque, circular, translucent Appearances and no hemolysis	Produce diffuse turbidity	No colony appears	Same as EMB agar	diffused turbidity and no pellicle	Gram negative, coccobacil-lary bipolar	-

Legends

BA: Blood agar, MC: MacConkey, SS: Salmonella Shigella, NA: Nutrient agar NB: Nutrient broth, -= Non motile

4.4.7 Results of biochemical tests

4.4.8 Results ofMethyl Red (MR) and Voges Proskauer (VP) tests

In MR test, persistence of red color indicated positive test while appearance of yellow color indicated negative test. In VP test, the appearance of pink color indicated the positive test. Both MR and VP tests were found to be negative for the isolates of *Pasteurella spp.* (**Plate-50, 51).**

4.4.9 Results of Indole test

In Indole test, the development of red color ring indicated the positive test. Indole test was found to be positive for the isolates of *Pasteurella spp.* (**Plate-52).**

4.4.10Results of TSI (Triple Sugar Iron) Test

In TSI test sugar fermentation occurred that means yellow color of the butt and slant and no H_2S gas production indicate the test is positive . (**Plate-54).**

4.4.11 Results of Motility Indolee Urease (MIU) Test

Single stick like appearance into the test tube was found that means there was no turbidity throughout the medium indicated that the organism was non motile and gram negative (Plate 53).

Table 7. Results of biochemical tests of the isolated *Pasteurella spp.*

Tests	Sugar	Results
TSI	Sugar fermentation	+
	H_2S gas production	-
Indole		+
MR		-
VP		-
MIU	Motility	-
	Indole	+
	Urease	+

TSI = Triple Sugar Iodine

+ = Fermentation occurred MR = Methyl Red

- = No Fermentation VP = Voges Proskauer

- = Non Motile MIU =Motility Indole Urease

Table 8: Bacterial flora isolated from internal organs (n=100) of broiler

No. of positive Isolated bacteria samples and prevalence (%)

Isolated bacteria	No. of positive Isolated bacteria samples and prevalence (%)					Total (%)
	Liver (n=20)	Lungs (n=20)	Esophagus (n=20)	Duodenum (n=20)	Tracheal swab (n=20)	
E coli	14(70)	8(40)	8(40)	14(70)	6(30)	50(50%)
Salmonella	6(30)	8(40)	8(40)	6(30)	NI	28(28%)
Staphylococcus	10(50)	6(30)	4(20)	NI	NI	20(20%)
Pasteurella	2(10)	4(20)	NI	2(10)	NI	8(8%)

Legend: NI- Not identified

4.5 Results of antibiotic sensitivity pattern of isolated bacteria

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

4.5.1 Antibiotic sensitivity pattern of *E. coli.*

The antibiotic study revealed that all of the isolates (50) were sensitive to ciprofloxacin (100%), followed by enrofloxacin (48%), chloramphenicol (90%), streptomycin (48%). The isolates were found resistance to ampicillin (100%) and amoxicillin (100%).

Table 9. Antibiotic sensitivity pattern of E. coli (n = 50)

Antibacterial agents	Disc concentration (µg /disc)	No. and Percentages (%) of isolates		
		Sensitive	Intermediate	Resistance
Ampicillin	10µg	(0) 0%	(0) 0%	(50)100%
Amoxicillin	10µg	(0) 0%	(0) 0%	(50)100%
Streptomycin	10µg	(24) 48%	(0) 0%	(26) 52%
Chloramphenical	30 µg	(45) 90%	(0) 0%	(5) 10%
Enrofloxacin	5 µg	(24) 48%	(18) 36%	(8) 16%
Ciprofloxacin	5 µg	(50)100%	(0) 0%	(0) 0%

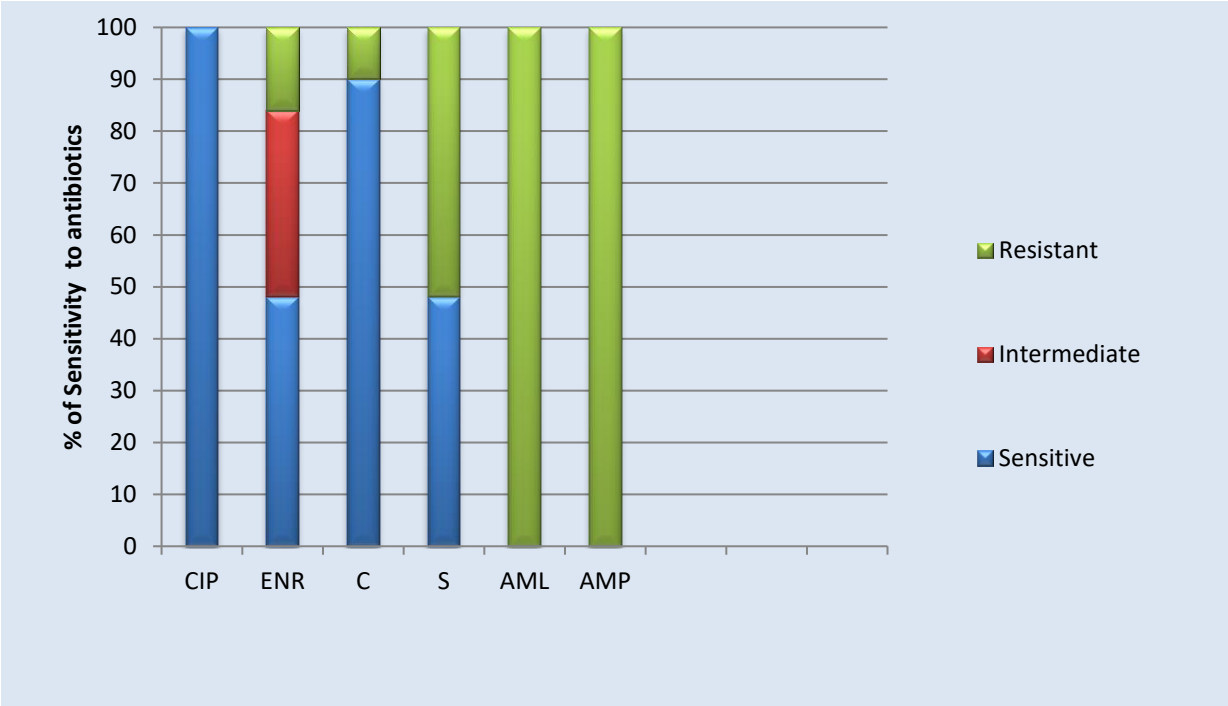


Fig. 2. Bar Diagram presenting antibiotic sensitivity pattern of isolated *E. coli*

Legends (ENR= Enrofloxacin, C = Chloramphenicol, AMP= Ampicillin, CIP= Ciprofloxacin, S = Streptomycin, AML=Amoxycillin).

4.5.2 Antibiotic sensitivity pattern of *Salmonella spp.*

The antibiotic study revealed that all of the isolates (28) were sensitive to ciprofloxacin (100%). The isolates were found resistance to Amoxycilin (89%), Neomycin (71%) Colistine sulphate (75%), Erythromycin (89%).

Table 10. Antibiotic sensitivity pattern of *Salmonella spp.* (n=28)

Antibacterial agents	Disc concentration (µg /disc)	No. and Percentages (%)of isolates		
		Sensitive	Intermediate	Resistance
Ciprofloxacin	5 µg	(28) 100%	(0) 0%	(0) 0%
Neomycin	10µg	(0) 0%	(8) 29%	(20) 71%
Colistine sulphate	5µg	(0) 0%	(7) 25%	(21) 75%
Amoxycilin	10 µg	(3) 11%	(0) 0%	(25) 89%
Erythromycin	10µg	(0) 0%	(3) 11%	(25) 89%

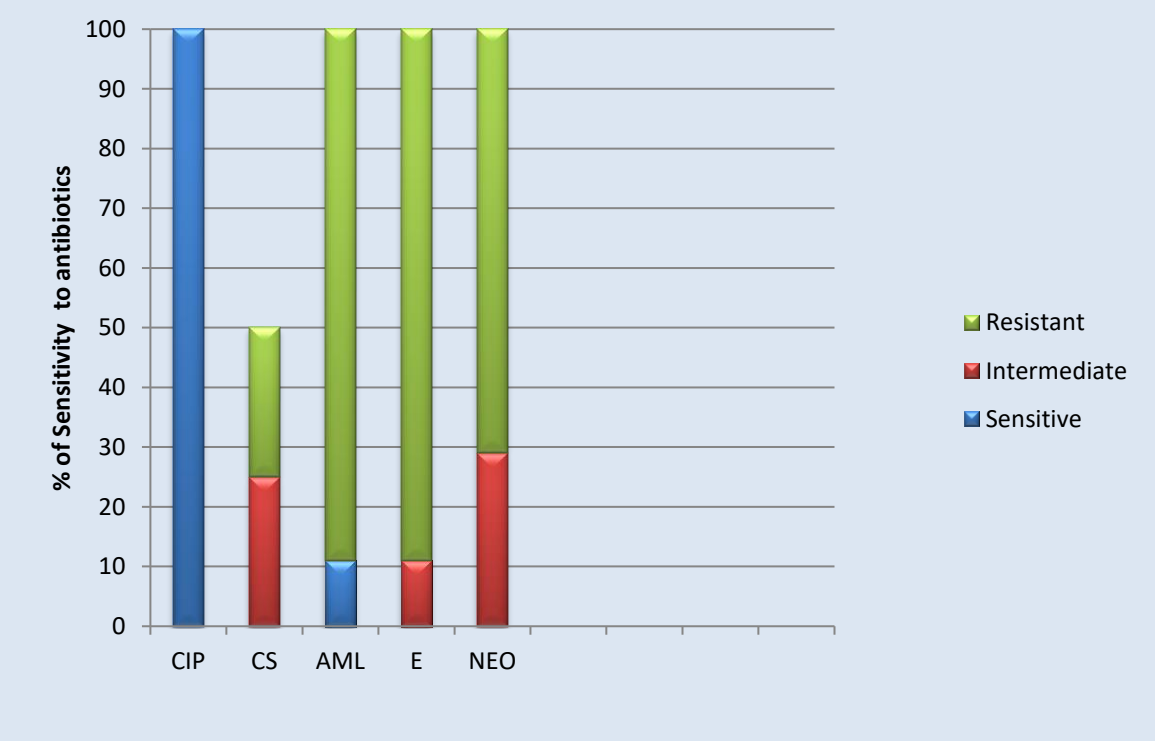


Fig. 3. Bar Diagram presentingantibiotic sensitivity pattern of isolated *Salmonella spp.*

Legends (NEO=Neomycin, CS=Colistine sulphate, E=Erythromycin, CIP= Ciprofloxacin, AML=Amoxycillin).

4.5.3 Antibiotic sensitivity pattern of *Staphylococcus spp.*

The antibiotic study revealed that all of the isolates (20) were sensitive to ciprofloxacin (100%), followed by gentamicin (95%), chloramphenicol (80%), enrofloxacin (85%), ampicillin (80%), amoxicillin (75%).

Table 11. Antibiotic sensitivity pattern of Staphylococcus spp.(n=20)

Antibacterial agents	Disc concentration (µg /disc)	No. and Percentages (%) of isolates		
		Sensitive	Intermediate	Resistance
Amoxicillin	10µg	(15) 75%	(4) 20%	(1) 5%
Ampicillin	10µg	(16) 80%	(3) 15%	(1) 5%
Enrofloxacin	5 µg	(17) 85%	(0) 0%	(3) 15%
Chloramphenical	30 µg	(16) 80%	(4) 20%	(0) 0%
Ciprofloxacin	5 µg	(20) 100%	(0) 0%	(0) 0%
Gentamicin	30 µg	(19) 95%	(1) 5%	(0) 0%

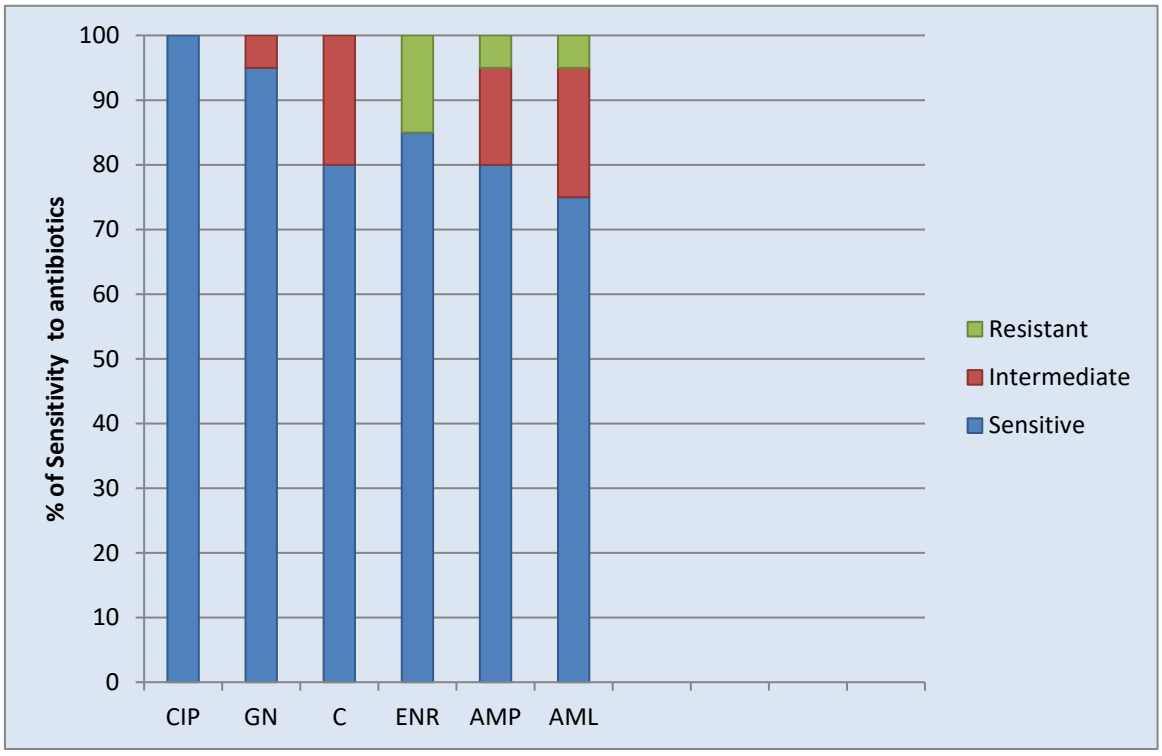


Fig: 4.Bar Diagram presentingantibiotic sensitivity pattern of isolated*staphylococcus spp.*

Legends: (ENR= Enrofloxacin, C = Chloramphenicol, AMP= Ampicillin, CIP= Ciprofloxacin, AML=Amoxycillin,GN=Gentamicin).

4.5.4 Antibiotic sensitivity pattern of *Pasteurella spp.(n=8)*

The antibiotic study revealed that all of the isolates (8) were sensitive to ciprofloxacin (100%), and Levofloxacin (100%), and resistance to amoxicillin (88%).

Table 12. Results of antibiotic sensitivity tests using various isolated *Pasteurella spp.* (n=8)

Antibacterial agents	Disc concentration (µg /disc)	No. and Percentages (%) of isolates		
		Sensitive	Intermediate	Resistance
Ciprofloxacin	5 µg	(8) 100%	(0) 0%	(0) 0%
Levofloxacin	5 µg	(8) 100%	(0) 0%	(0) 0%
Gentamicin	30 µg	(1) 12%	(5) 63%	(2) 25%
Doxicycline	15 µg	(2) 25%	(5) 63%	(1) 12%
Erythromycin	10µg	(0) 0%	(6) 75%	(2) 25%
Amoxicillin	10µg	(0) 0%	(1) 12%	(7) 88%

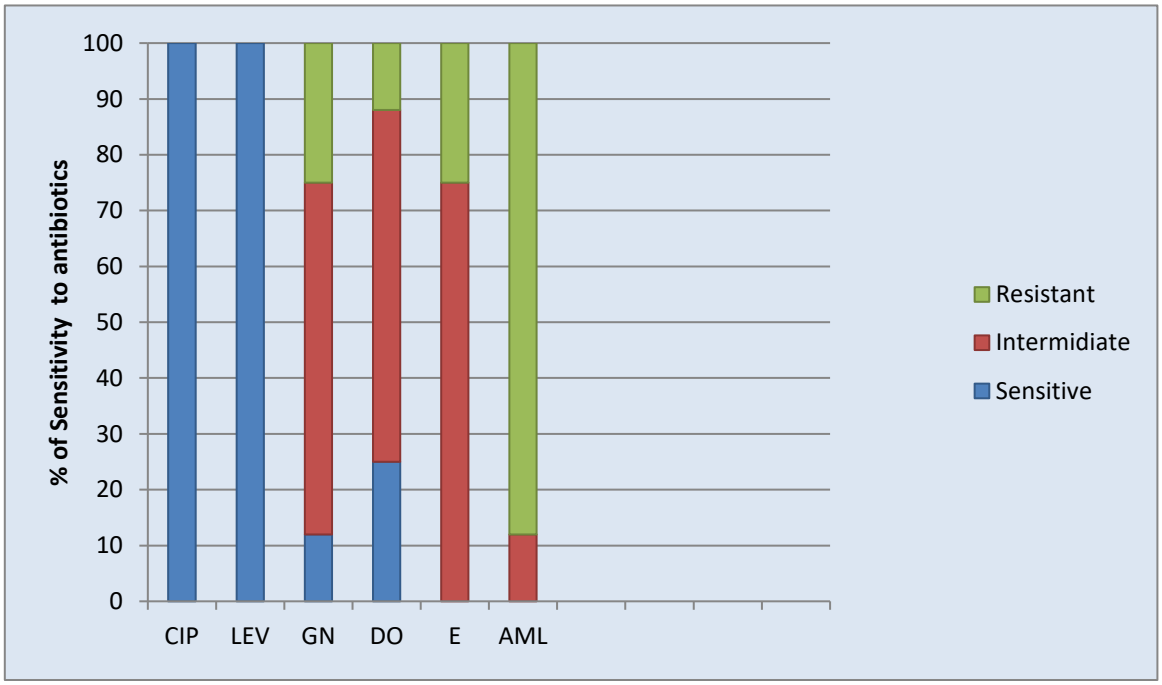


Fig.5. Bar Diagram presentingantibiotic sensitivity pattern of isolated *Pasteurella* spp.

Legends: (LEV=Levofloxacin, DO=Doxicycline, E=Erythromycin, CIP= Ciprofloxacin, AML=Amoxycillin,GN=Gentamicin).

Table 13. Antibiotic sensitivity test of the isolated bacteria obtained from chicken.

Name of the isolates	***E. coli***		***Salmonella spp.***		***Staphylococcus spp.***		***Pasteurella spp.***	
	zone size(mm)	***out come***	**zone size(mm)**	***out come***	**zone size(mm)**	***cout come***	**zone size(mm)**	***out come***
CIP	**21**	S	31	S	20	S	27	S
GN	-	-	-	-	18	S	13-14	I
ENR	**15**	I	12	R	25	S		
C	**18**	S	-	-	18	S		
AML	**13**	R	13	R	20	S	15	R
AMP	**13**	R	-	-	29	S	-	-
S	**11**	R	-	-	-	-	-	-
NEO	-	-	18	I	-	-	-	-
CS	-	-	13	I	-	-	-	-
E					-	-	18	I
LF	-	-	-	-	-	-	27	S
DO	-	-	-	-	-	-	22	I

Legends: (ENR= Enrofloxacin, C = Chloramphenicol, AMP= Ampicillin, CIP= Ciprofloxacin, GN= Gentamicin, S = Streptomycin, AML=Amoxycillin, NEO= Neomycin, CS= Colistin sulphate, E=Erythromycin, LF =Levofloxacin, DO=Doxicycline, S= sensitive, I= intermediate, R= resistant.

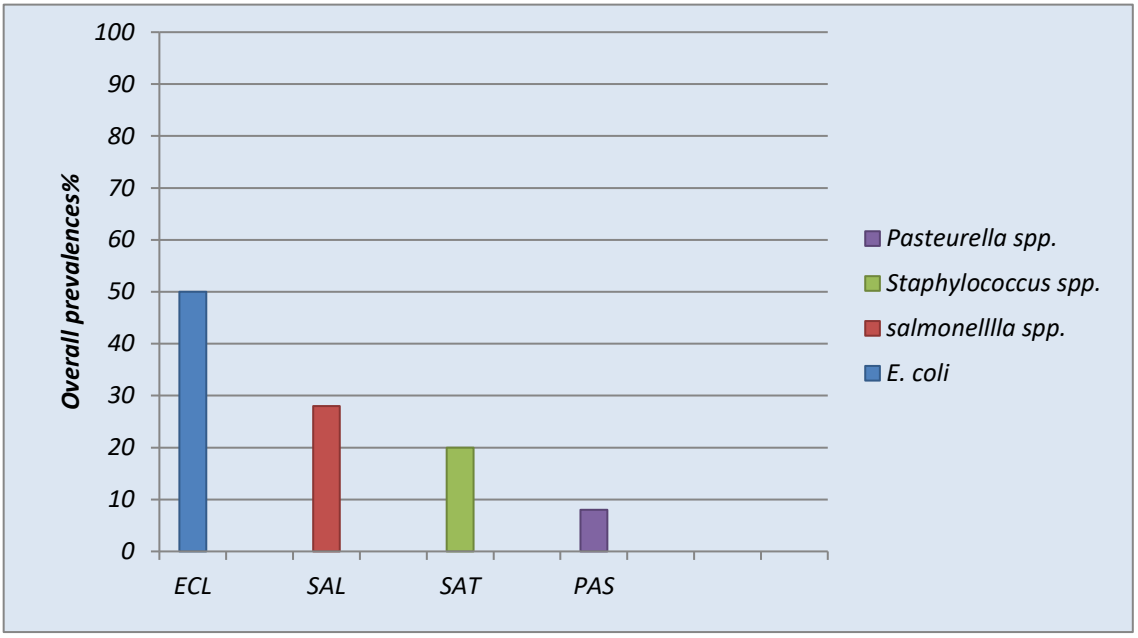


Fig: 6. Overall prevalence of bacteria found in internal organs of broiler. The value indicated for each bar is the overall prevalence of each bacterium.

Legend:

ECL= *Escherichia coli. sp.*PAS= *Pasteurella spp.*

SAL= *Salmonella spp.*SAT= *Staphylococcus spp.*

Plate: 1. Thick grayish white colony of E. coli on **Nutrient agar**.

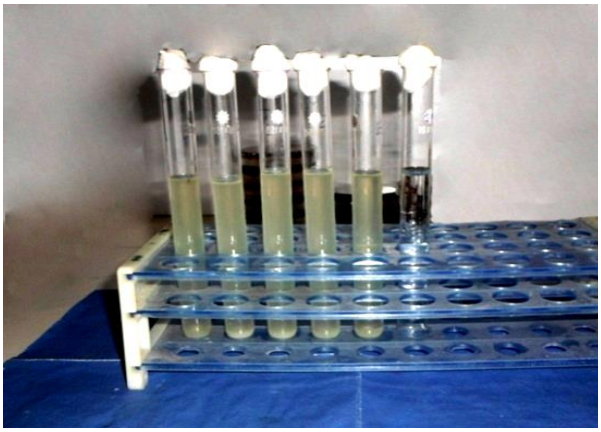


Plate: 2. Culture of E. coli in nutrient broth showing turbidity (left) and uninoculated contro (right).

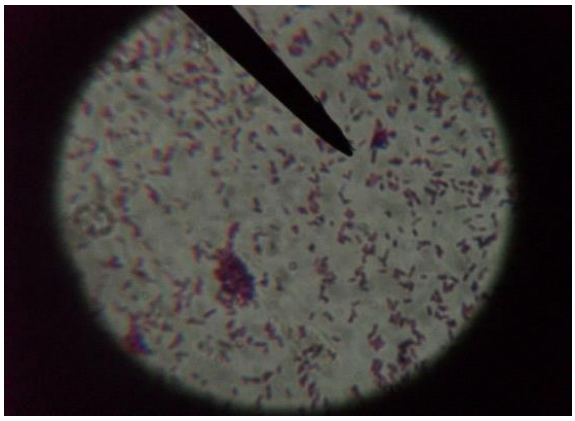


Plate 3: Gram-negative single or paired short plump rods of *E. coli*

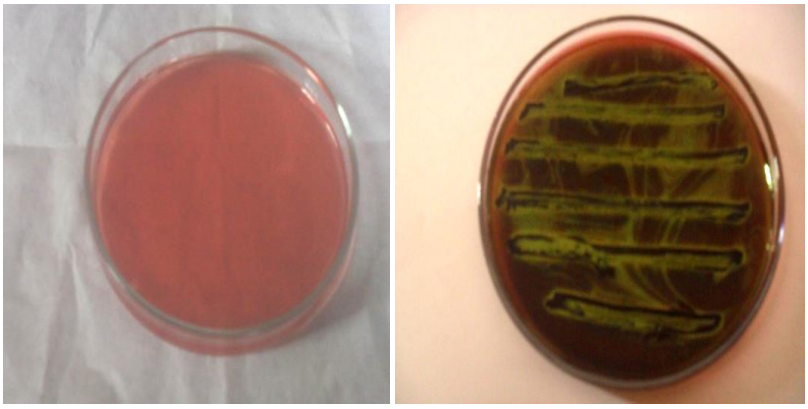


Plate: 4.. Metallic sheen colony of E. coli on **EMB** agar(right)and uninoculated control (left)

Plate: 5. Yellowish green colour colony produced of *E. coli* on **Brilliant Agar media** (right) and uninoculated control (left).

Plate: 6. *E. coli* are grown on Simmons citrate agar media (left) , the medium is changed to blue colour

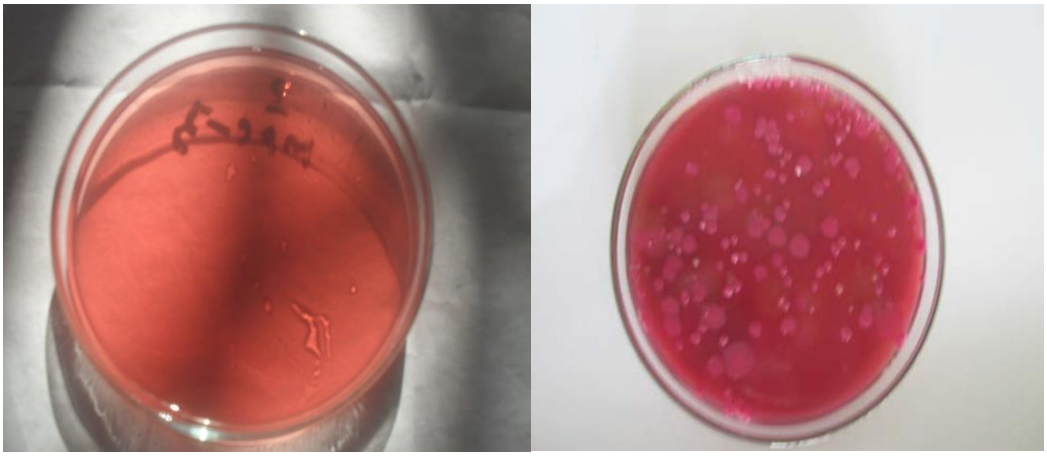


Plate 7: *E. coli* produces dark pink color colony in Mac Conkey agar (right) and uninoculated control (left)

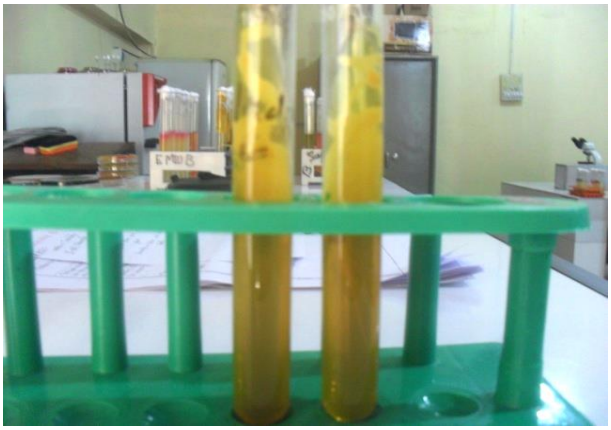


Plate:8. Culture in **Triple Sugar Iron (TSI)** agar slant reaction showing yellow slant and yellow butt (left) and production of gas by *E. coli*.

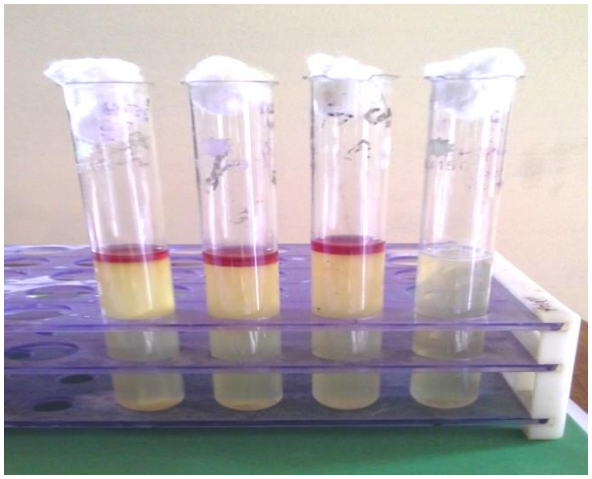


Plate: 9. **Methyl-Red test** for *E. Coli* showing bright red color (right) and uninoculated control (left)

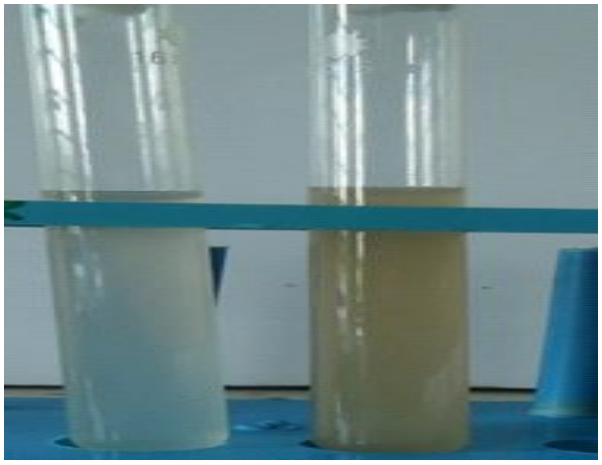


Plate: 10. **Voges-proskauer test** for **E. coli.** Showing no change of the medium (right) and uninoculated control. (night).

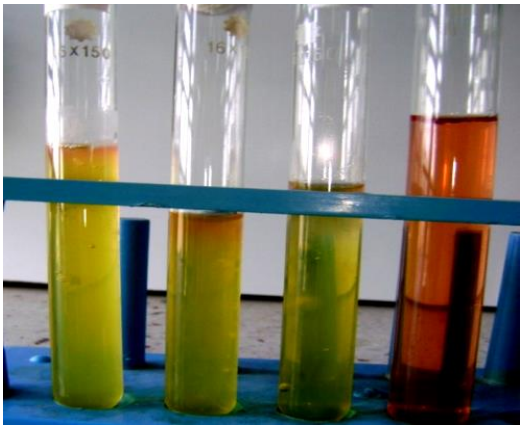


Plate: 11.**MotilityIndole Urease test** causing **turbidity** Urease production with indole positive by *E. Coli* (left) and uninoculated control (right).

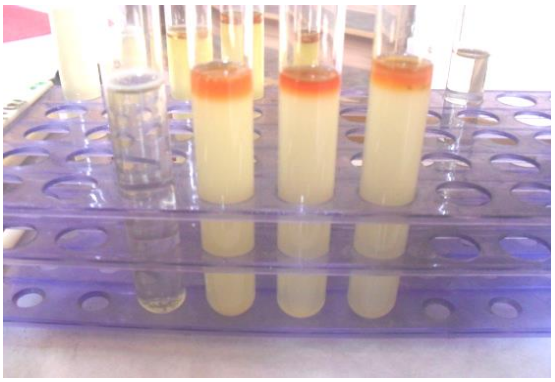


Plate: 12.**Indole test** showing positive results with a red color in the reagent layer indicating indole production with reaction of *E. coli* (right) and uninoculated control (left).

Plate: 13. **Catalase(positive)** for *E. coli* with gas bubble formation.

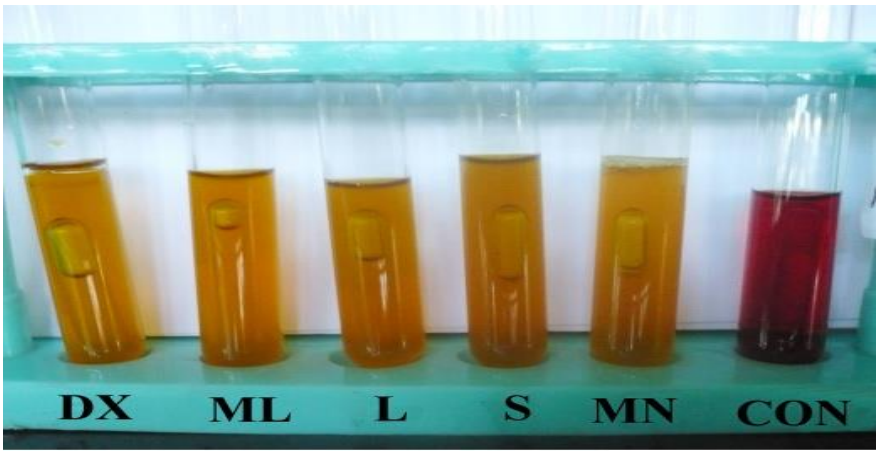


Plate: 14. Fermentative activity of *Escherichia coli.***with five basic sugars** (DX=Dextrose, ML= Maltose, L= lactose, S= Sucrose, M= Mannitol, C= Control) with production of acid and gas. Change of medium color reddish to yellowish and gas bubble formation.

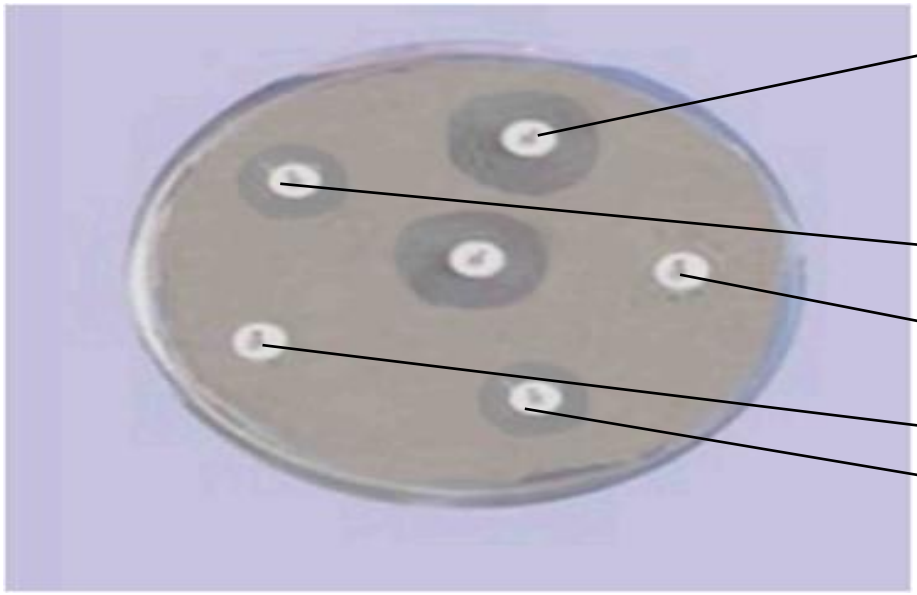


Plate: 15. Antibiotic sensitivity test for *Escherichia coli* on **nutrient agar**. (ENR= Enrofloxacin, C = Chloramphenicol, AMP= Ampicillin, CIP= Ciprofloxacin, S =

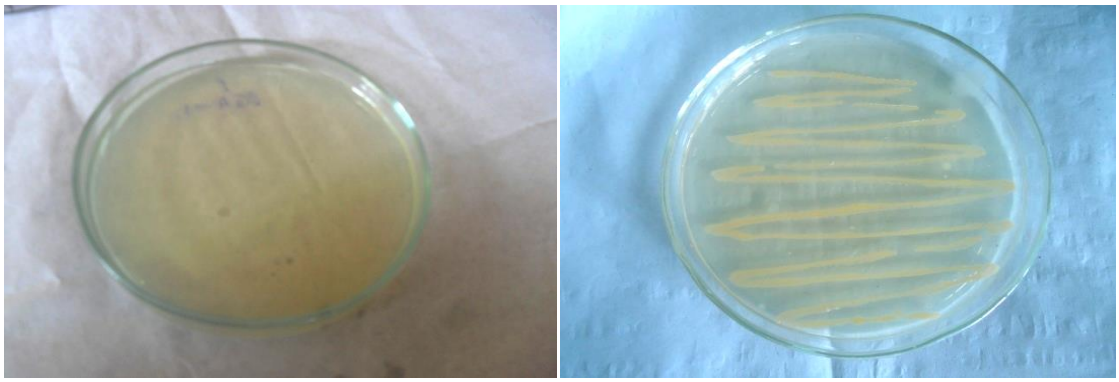


Plate 16: Growth of *Salmonella* in Nutrient agar showing circular, smooth, opaque, translucent colonies(right)and uninoculated control (left)

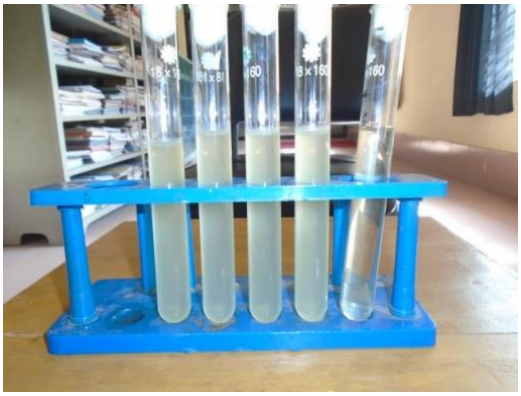


Plate 17: Growth of *Salmonella* in nutrient broth showing turbidity (left) and control(right)

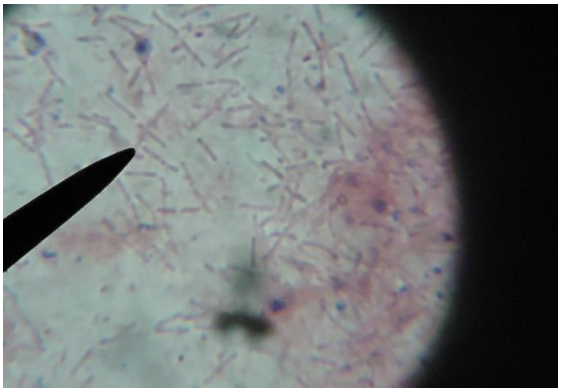


Plate 18: Gram negative rod *Salmonella* spp

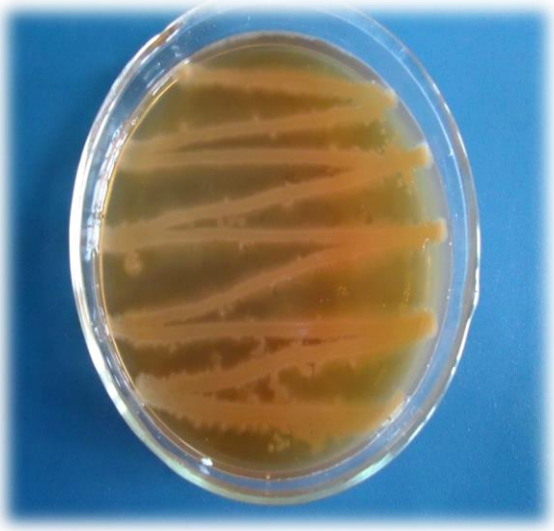


Plate. 19: Growth of *Salmonella in* MacConkey agar showing colorless, smooth, pale and transparent raised colonies (left) and control (right).

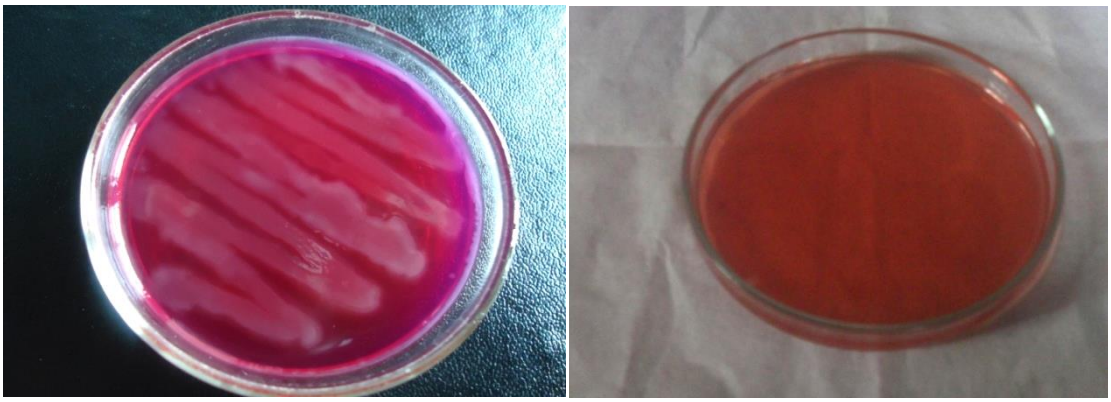


Plate 20: Growth of *Salmonella* in EMB agar showing pink color colonies (left) and control (right).

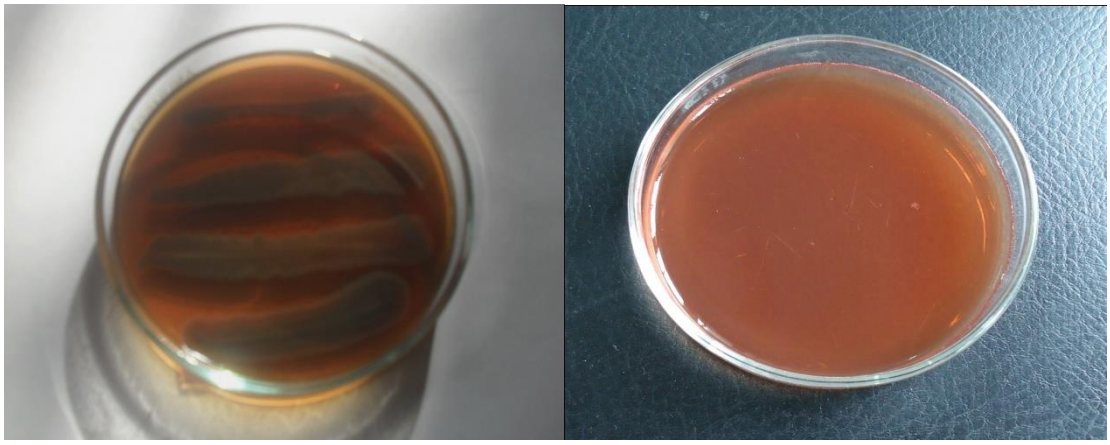


Plate 21: Black center colonies produced by *Salmonella* on SS agar(left) and un-inoculated control (right).

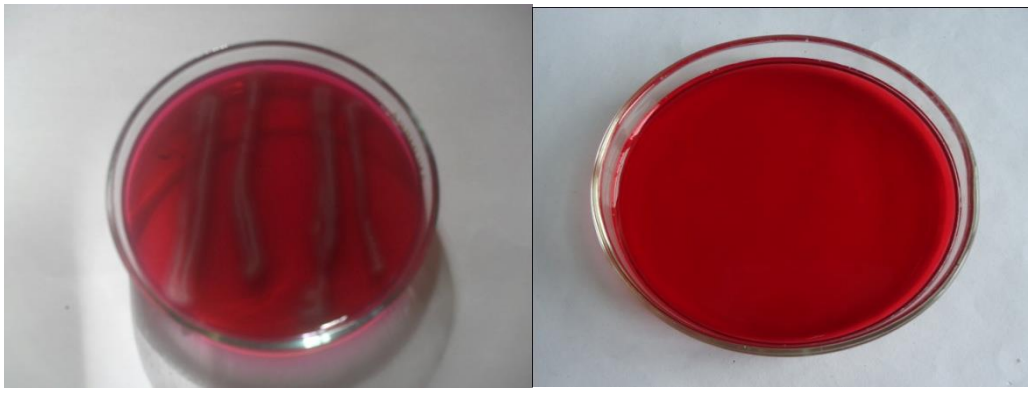


Plate 22: Growth of *Salmonella* in Brilliant green agar showing pale pink color colonies against a pinkish background (left) and control (right).

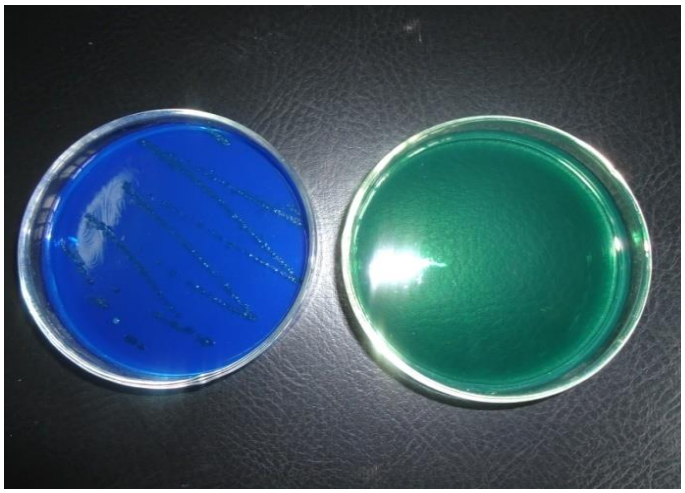
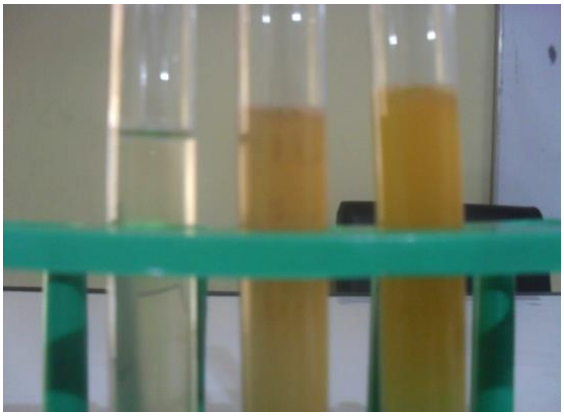


Plate 23: Culture of *Salmonella* on Simmons citrate agar showing deep blue coloration of the medium(left) and Control(right).



Indole test showing no indole ring in the medium (right) and control (left)
Plate 24: Indole test (*Salmonella).*

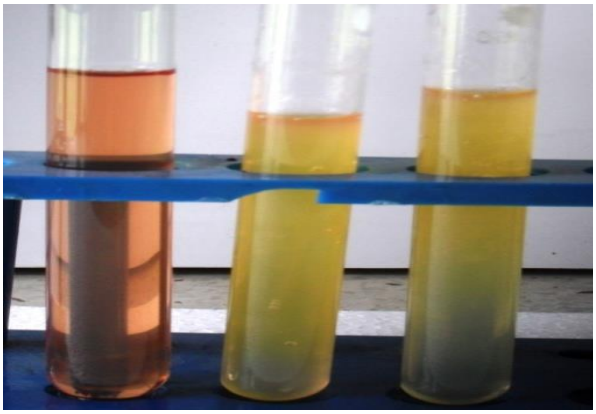
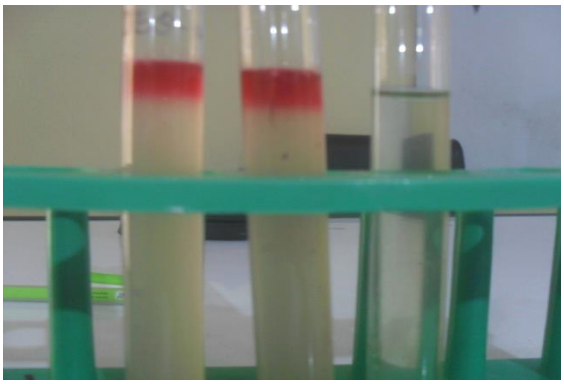
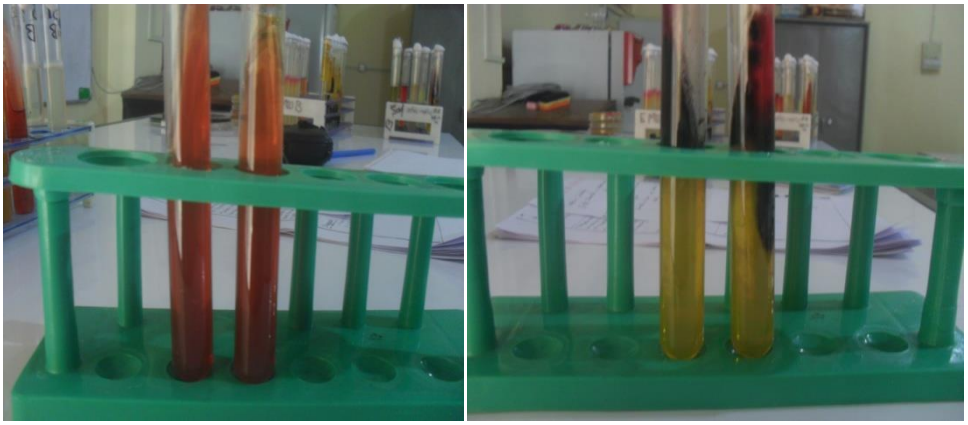


Plate 25: MIU test showing absence of turbidity indicating non motile *Salmonella spp*(Right) and control(left)



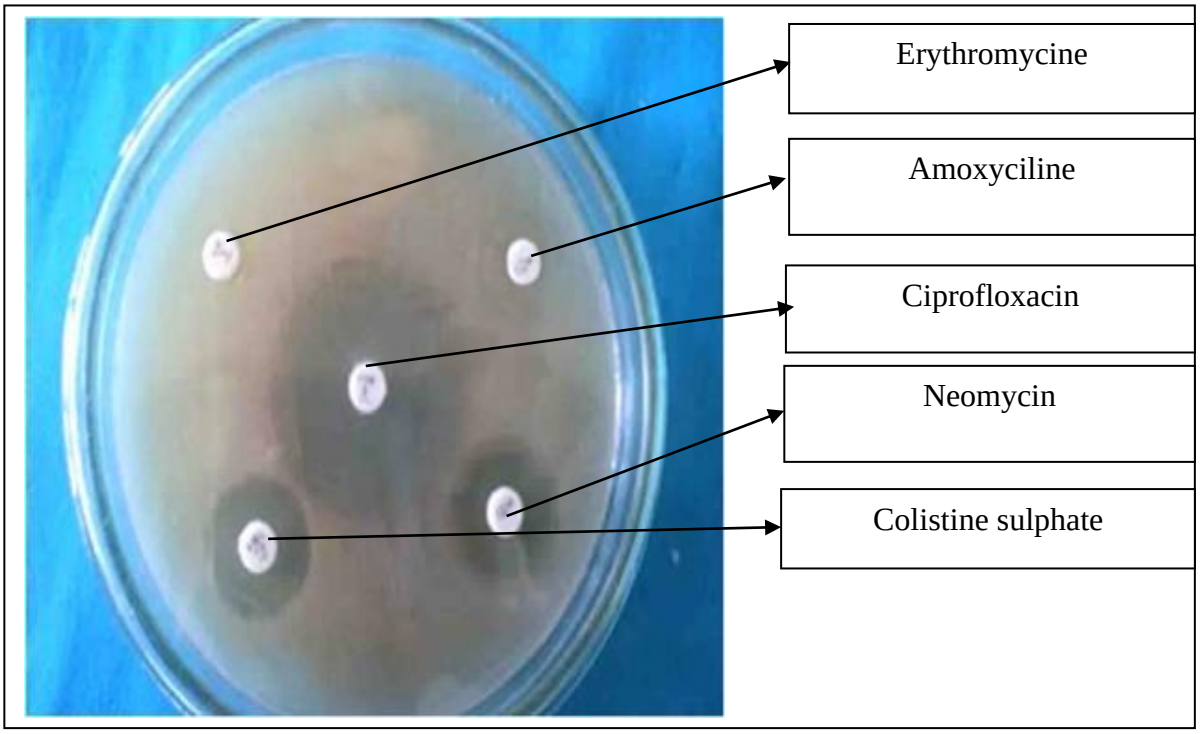
Methyl -Red test showing bright red colour of the medium(left) and control(right)
Plate 26: Methyl -Red test(*Salmonella).*

Voges-Proskauer test showing no change of the color of the medium(left) and control(right)
Plate 27: Voges-Proskauer test(*Salmonella).*



Triple Sugar Iron (TSI) agar slant test showing yellow butt , red slunt and black colour in the slunt due to hydrogen sulfide production(right) and control(left).

Plate 28: Triple Sugar Iron (TSI) agar slant test(*Salmonella).*



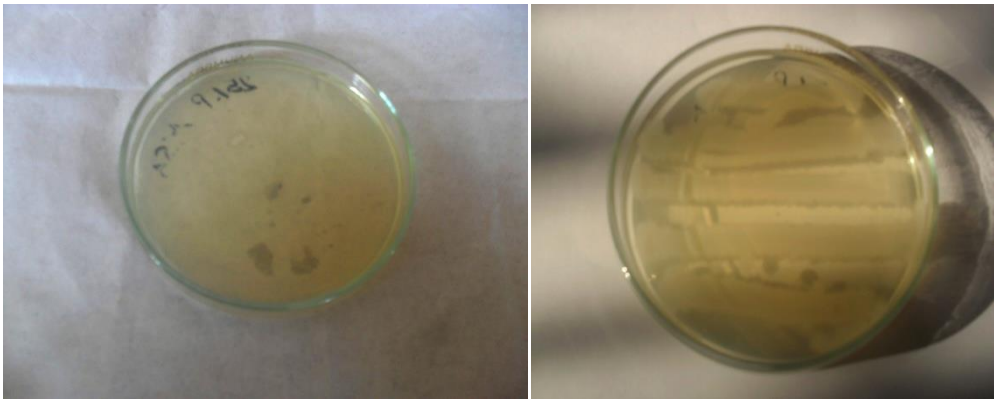


Plate: 30. Yellowish colony of *Staphylococcus spp.* on **Nutrient agar**(right)and uninoculated control (left) .

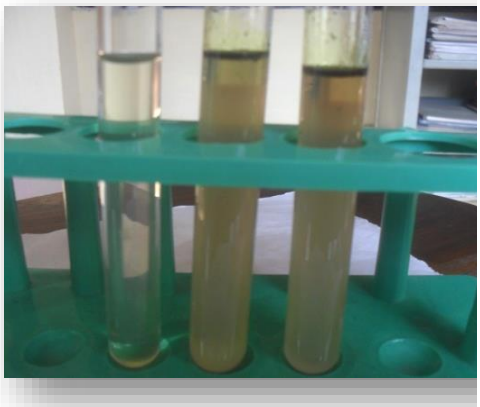


Plate: 31. Culture of Staphylococcus spp.in **nutrient broth** showing turbidity (right) and uninoculated control (left).

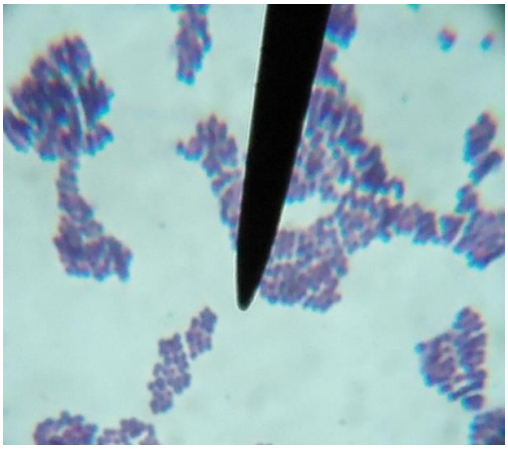


Plate: 32. Gram's staining of *Staphylococcus spp.* Showing Gram-positive cluster form of organisms.

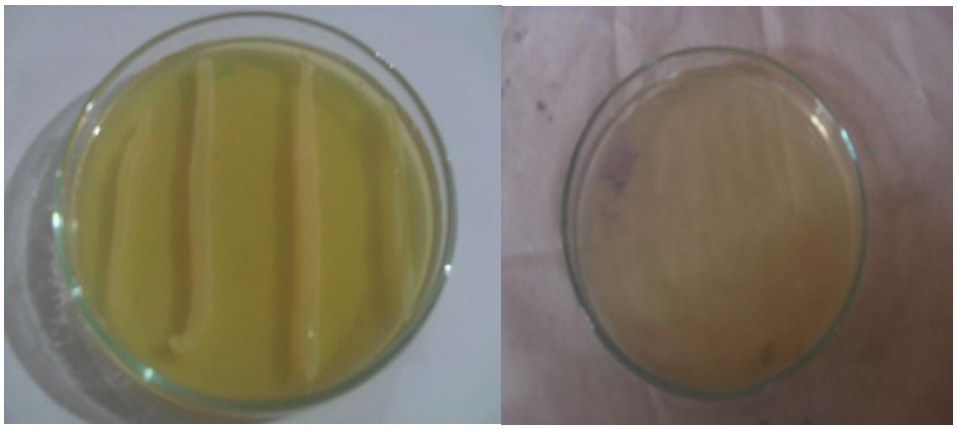


Plate: 33. Culture of Staphylococcus spp. **on Staphylococcus agar no. 110.** (left) showing yellowish colonies and uninoculated control (right)

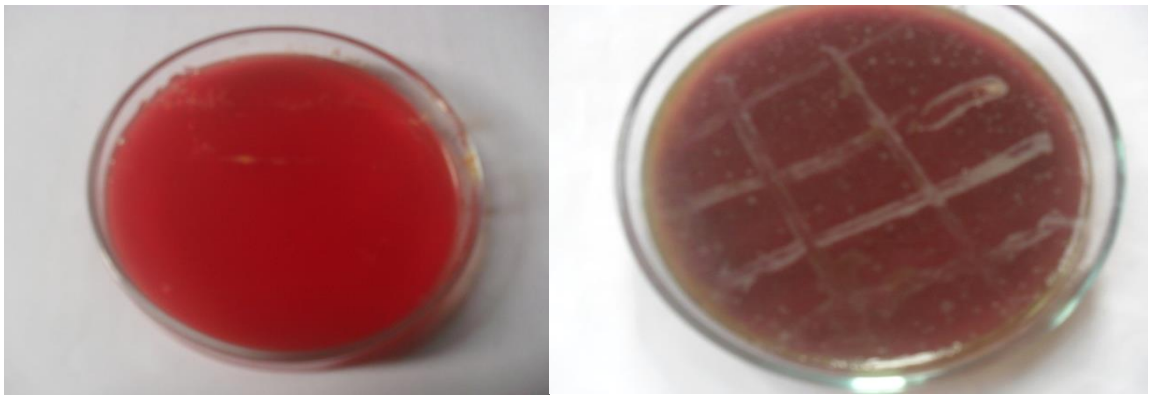


Plate: 34. Staphylococcus spp**.** produces (β) hemolysis **on blood agar medium** with clear zone of hemolysis around the colony (right) and uninoculated control (left)

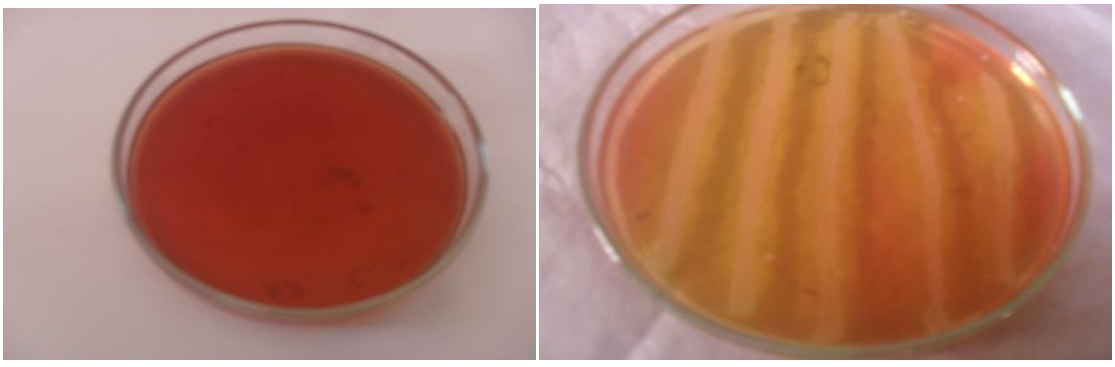


Plate: 35. In Manitol salt base agar Staphylococcus produces non fermenting white colony with pink color media(right)and uninoculated control (left).

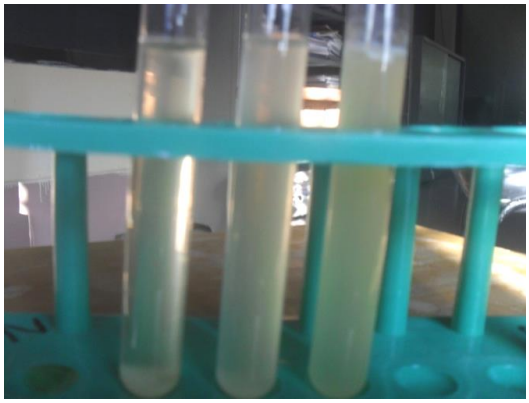


Plate: 36.**Indole test** showing no change of the medium with the reaction of the Staphylococcus spp. (right) and uninoculated control (left)

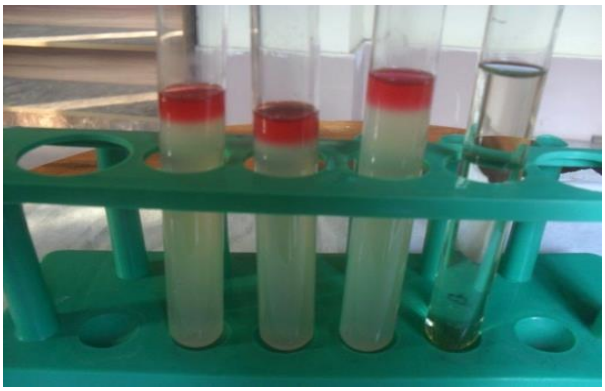


Plate: 37. **Methyl-Red test** for staphylococcus spp. showing the medium was changed to bright red colour (left) and uninoculated control (right).

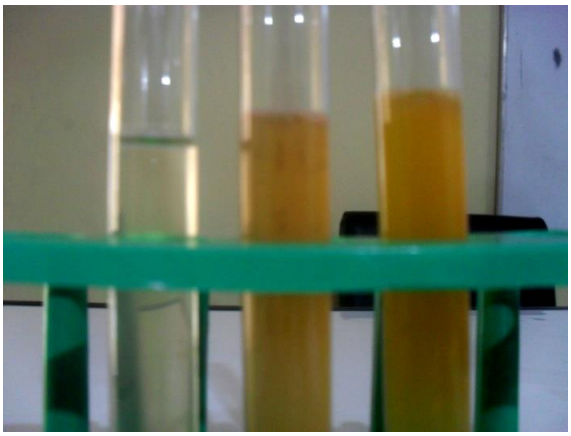


Plate:38. VP test negative (no color change after adding reagent) **by** *staphylococcus spp.*

Plate: 39. **Motility Indole Urease test** causing no **turbidity** and Urease production with indole positive by staphylococcus spp. (left) and uninoculated control (right)

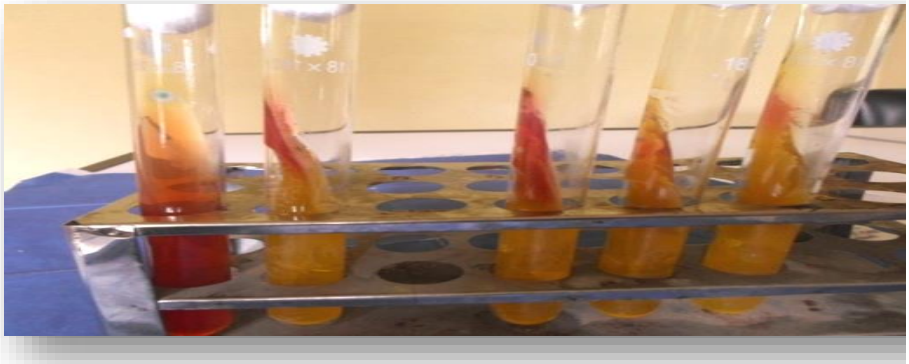


Plate: 40. Culture in **Triple Sugar Iron (TSI)** agar slant reaction showing yellow slant and yellow butt (right)and production of gas by *Staphylocccus spp.* and uninoculated control (left).

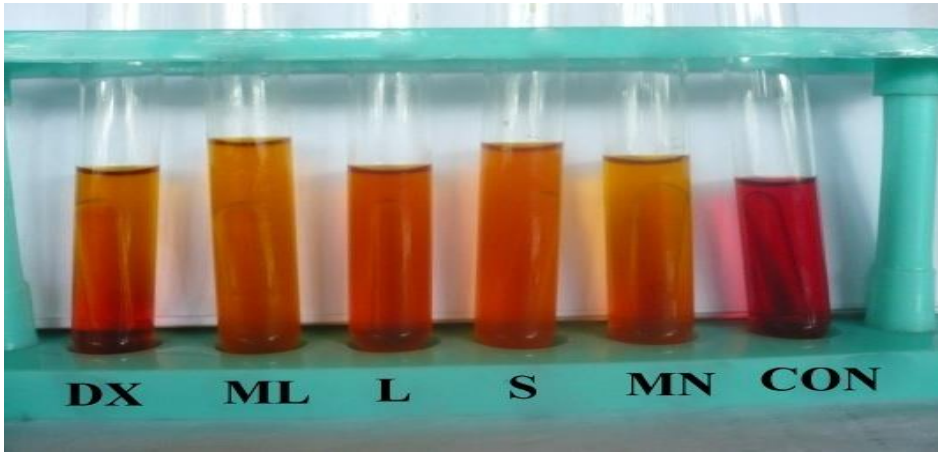


Plate: 41. Fermentative activity of Staphylococcus spp**. with five basic sugars** (DX= Dextrose, ML= Maltose, L= lactose, S= Sucrose, M=Mannitol, C = Control) with production of gas. Change of medium color reddish to yellowish.

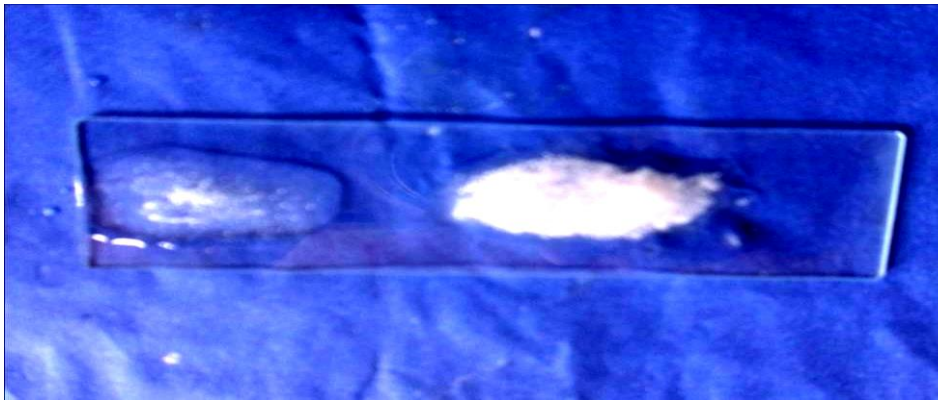


Plate: 42.**Catalase (positive)** for *Staphylococcus spp.* with gas bubble formation.

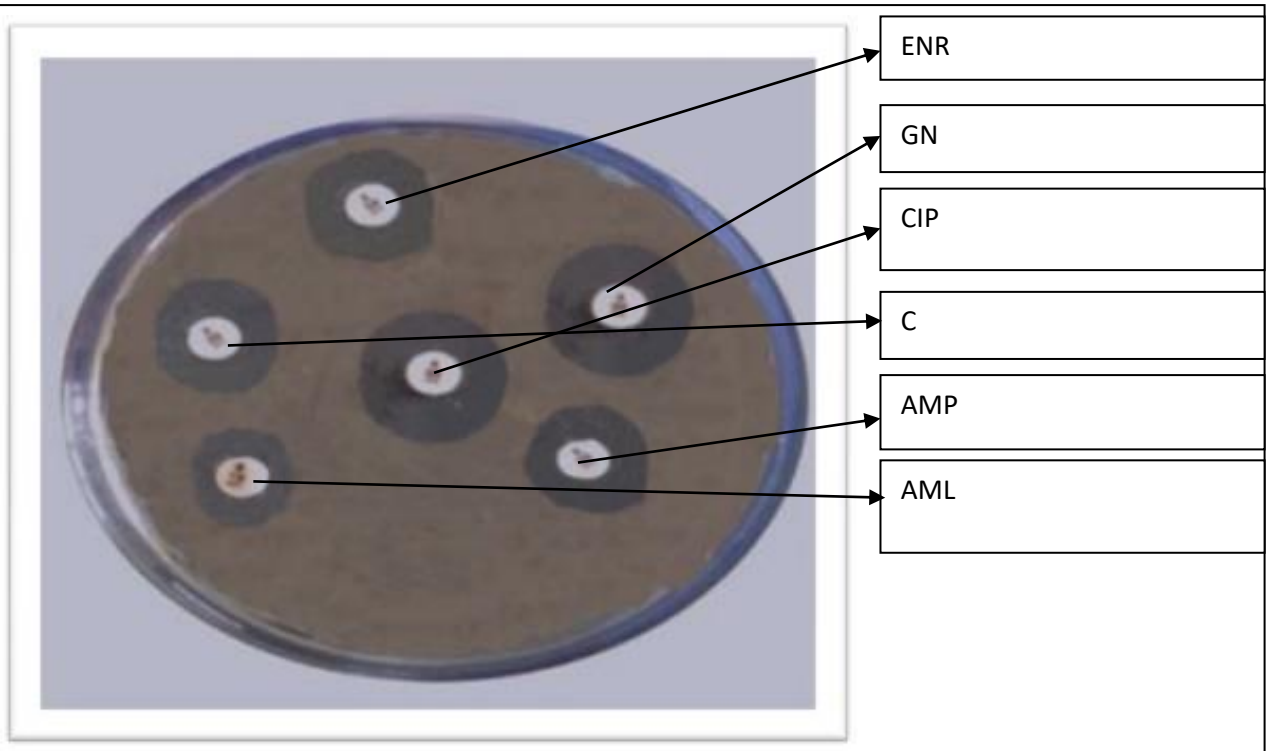


Plate: 43 Antibiotic sensitivity test for isolated *Staphylococcus spp.* on **nutrient agar**. (ENR = Enrofloxacin, C = Chloramphenicol, AMP = Ampicillin, CIP = Ciprofloxacin, GN = Gentamicin, AML=Amoxycillin)

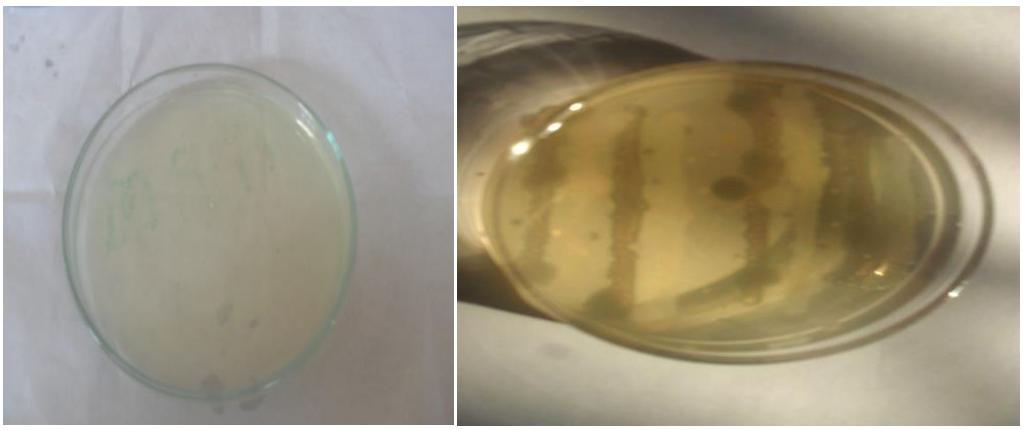


Plate-44: Whitish, opaque, translucent colony of *P. multocida* on **Nutrient agar** (right)and uninoculated control (left)

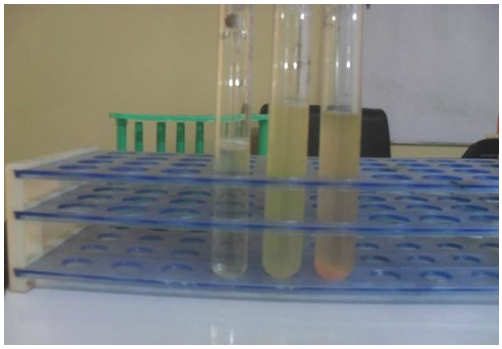


Plate 45: Growth of *Pasteurella spp.*in nutrient broth (right)and uninoculated control (left)

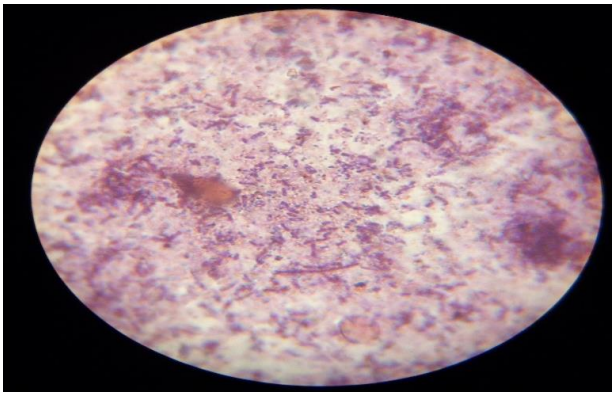


Plate: 46. Gram's staining of *Pasteurella spp.* Showing Gram-negative cluster form of organisms.

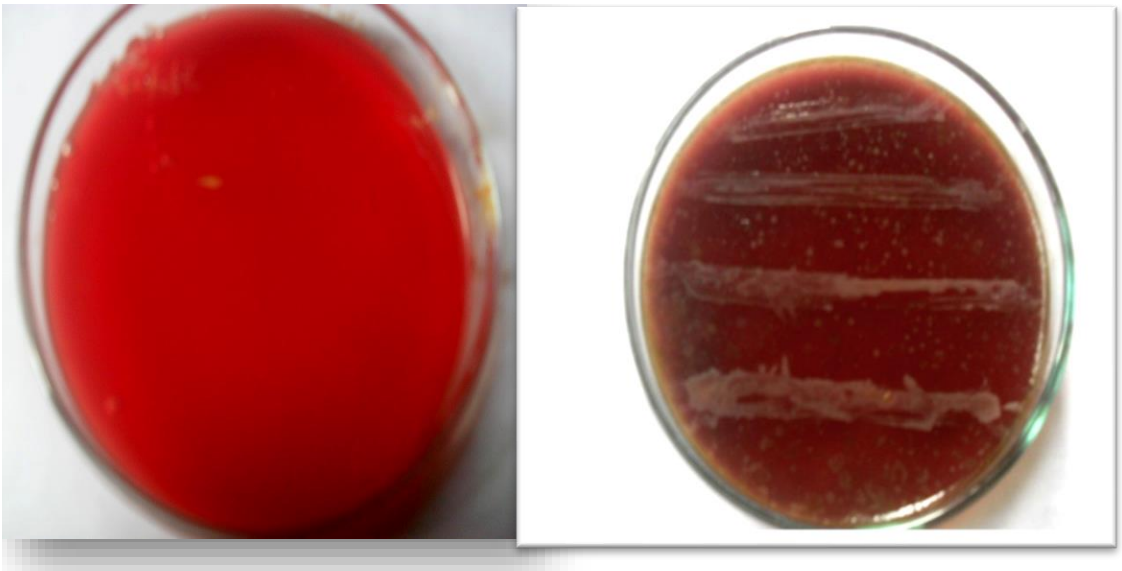


Plate-47: Whitish, opaque, translucent colony without hemolysisof *P. multocida* on blood agar(right)and uninoculated control (left).

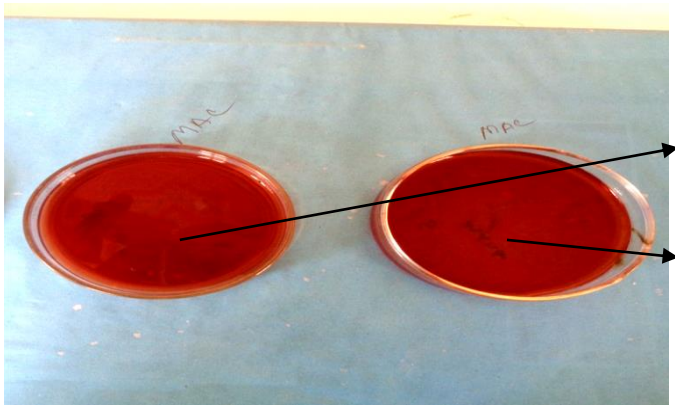


Plate 48- Culture on **MAC**agar plates yielded no colonies (Plate 4).

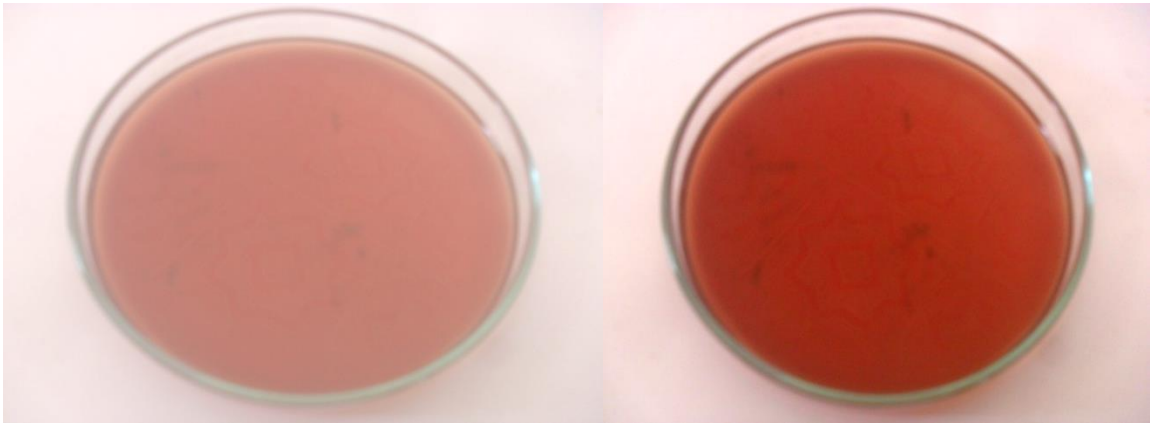


Plate 49: Culture on **SS agar** plates yielded no colonies (right)and uninoculated control (left)

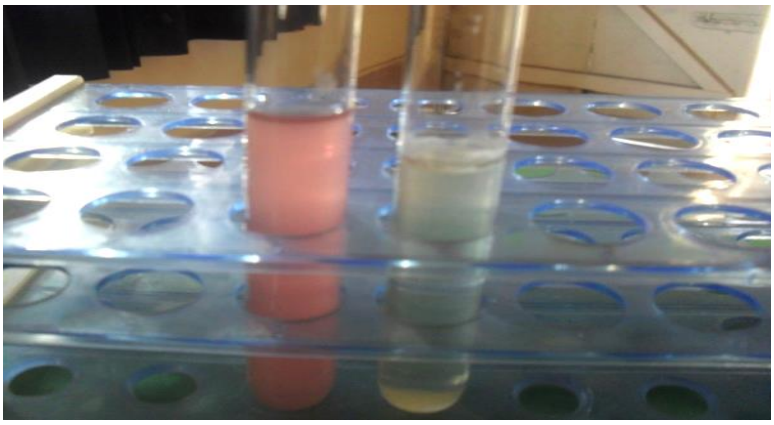


Plate 50: MR test (-ve) (No color change after adding reagent) **by** *Pasteurella spp.*

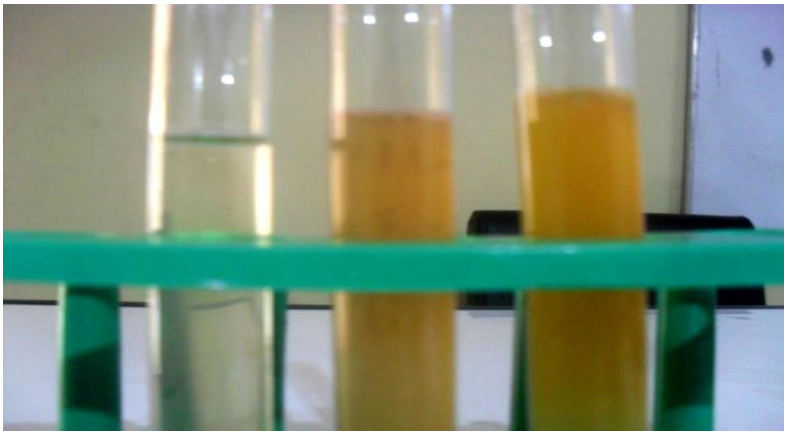


Plate 51: VP test (-ve) (no color change after adding reagent) **by** *Pasteurella spp.*

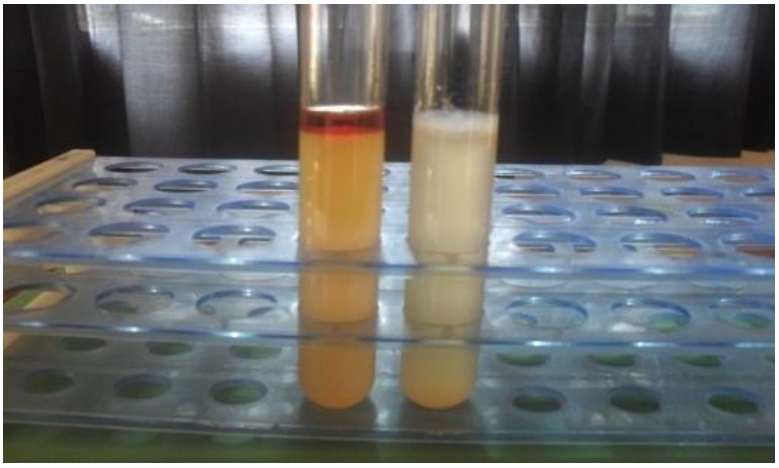


Plate 52: Indole test (+ve) (change after adding reagent) **by** *Pasteurella spp*

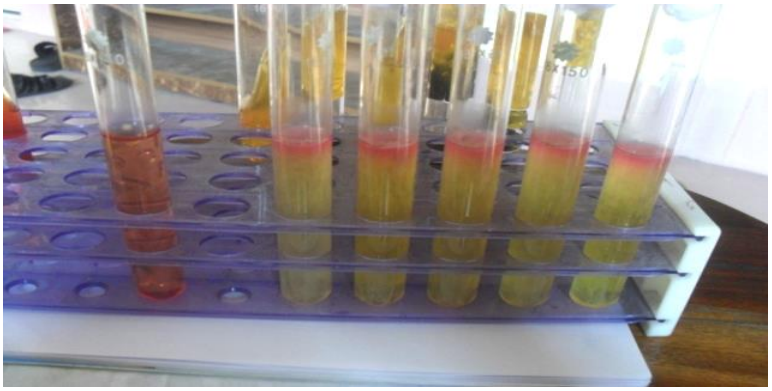


Plate:53: MIU negative (No color change after adding reagent) **by** *Pasteurella spp*

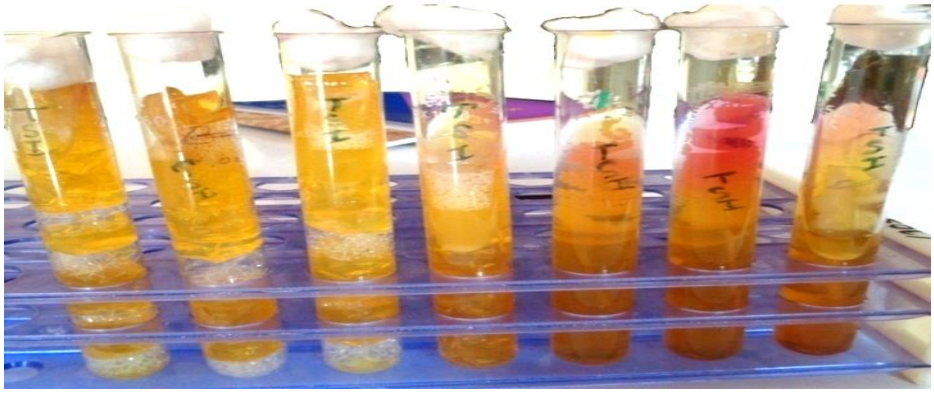


Plate 54: In TSI test sugar fermentation occurred that means yellow color of the butt and slant and no H_2S gas production indicate the test is positive.

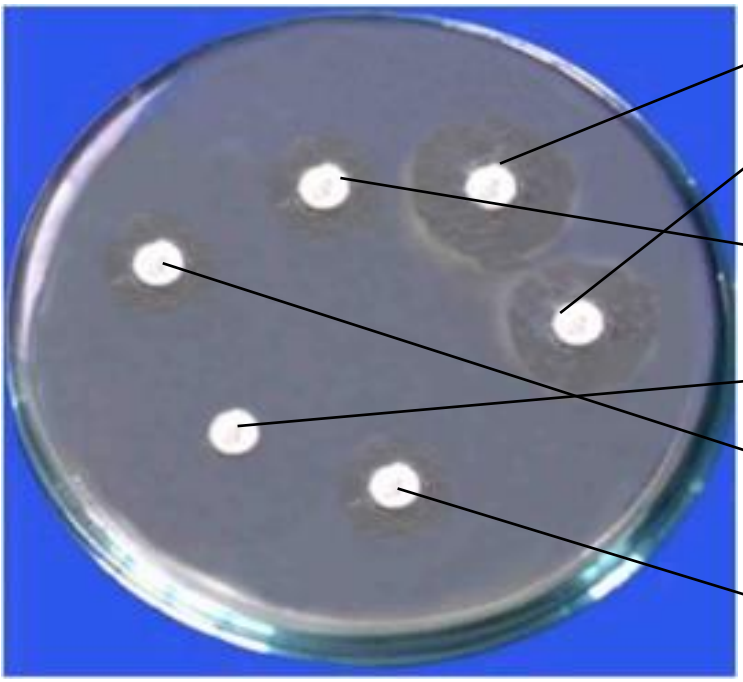


Plate55- Results of representative samples of antibiogram study of *Pasteurella spp*

CHAPTER 5

DISCUSSION

In the present study, four different bacteria (*E. coli*, *Salmonella* spp., *Pasteurella* spp. and *Staphylococcus* spp.) were isolated from the internal organs of broiler. This is in line of findings by Malmuthuge *et al.* (2012) and Voidarou *et al.* (2011). Considering all the 100 samples, *E. coli* was isolated from 50 (50%) samples. This finding is consistent with that of Awad- Alla *et al*. (2010) and Aguirre *et al*. (1992) who described a prevalence of 51% in broiler and 52% in black-billed ducks, respectively. The prevalence of *Salmonella spp.* was 28% in broiler, which is supported by Cardinale *et al*. (2003), Temelli *et al.* (2012) and Alcaine *et al.* (2007). However, a significant variation regarding prevalence of *Salmonella* spp. was described in other findings, such as 17.9% by Tibaijula *et al*. (2003), 14.37% by Petrovic *et al.* (2011) and 13% by Ellerbroek *et al.* (2010). Similarly, Afroz et al. (2012) reported a 26.02% prevalence of *Salmonella* spp. in internal organs of layer. These variations might be due to difference in sample size, geographical location and type of bird. In this study, the prevalence of *Pasteurella* spp. was 8%. Similar observation was also recorded by Spadafora *et al.* (2011) and Tatum *et al.* (2005). Among the Gram positive bacteria, overall prevalence of *Staphylococcus spp.* was 20%, which is similar to the findings described by Hanning *et al.* (2012) and Alfonso and Barnes (2006).

The antibiotic sensitivity was indicated by diameter of the Zone of growth inhibition by specific antibiotic supported by EUCAST. From the antibiogram study, it was observed that among the isolates of *Pasteurella spp*. isolated from broiler showed different level of sensitivity against different antibiotics used in the study. From the antibiogram study, it was observed that the isolates *Pasteurella spp*. showed the sensitivity to ciprofloxacin and levofloxacin, intermediate to erythromycin, doxycycline, gentamycin but resistant to amoxycillin.

The antibiotic sensitivity test for all positive isolates *Salmonella* using five drugs revealed the sensitivity to ciprofloxacin, resistant to Amoxycilin, and Erythromycin, while neomycin and Colistinesulphate,were found intermediate . Ramya *et al* (2013) described that the sensitivity of *Salmonella spp*. was 100% for ciprofloxacin followed by amoxicillin (82%). Hyeon *et al.,* (2012) stated that in *salmonella* the highest antibiotic resistance observed was to erythromycin (100%) followed by streptomycin (22.2%) and tetracycline and

chloramphenicol (16.7%). All isolated *Salmonella* in our study were multidrug resistant because, the farmers use several types of antibiotics in sick as well as healthy birds also without maintaining proper dose.

In the present study, *E. coli* isolated from broiler were found to be sensitive to Ciprofloxin, Chloramphenicol. The results strengthen the earlier observations of Jeyasanta *et al.* (2012), Akond *et al.* (2009) and Nazir *et al*. (2005). The *E. coli* was resistant against Amoxicillin, Ampicillin. Similar findings were reported by Jeyasanta *et al.* (2012) and Ako nd *et al* (2009).

Among the Gram positive bacteria, *Staphylococcus* spp. was found to be sensitive to all tested antibiotics. Landman and Cornelissen (2006) reported that more than one predisposing factors such as environmental and managemental factors (housing, climate etc), imbalance nutrition and immune status of the poultry might play roles in developing diseases while harboring the potential pathogenic bacteria. Additional research is required for further characterization of the bacterial isolates described in this study.

CHAPTER 6

SUMMARY AND CONCLUSION

The research work was conducted for the identification of important bacteria causing economic losses in the chicken farms at Dinajpur District of Bangladesh and study of their antibiotic resistance. Samples from chicken farm were collected during the period fromJanuary to June 2017. The laboratory works were conducted in the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU). A total of 100 samples from poultry farm of Dinajpur district were collected for this study. A series of bacteriological methods were used for the isolation and identification of different types of bacteria and to determine the antibiotic sensitivity of those isolates to different antibiotics. Different types of ordinary, enriched and selective media such as Nutrient broth, Nutrient agar, Mannitol Salt agar, Blood agar, Brilliant Agar, Eosine Methylene agar, Cook Meat Media test, MacConkey Agar, were used for the determination of the cultural characteristics of the different types of isolated bacteria. Biochemical properties of the isolated bacteria were studied by sugar fermentation, indole test, catalase TSI test, and MR-VP test. On the basis of morphology, staining, cultural and biochemical characteristics, the isolated organisms were identified as, *Escherichia coli, Salmonella spp., Staphylococcus aureus, and Pasteurella spp.*Bacteriological examination of total samples 100. Out of 100 samples, 50 were *Escherichia coli (50%),* 28 were *Salmonella spp* (28%), 20 were staphylococcus aureus (20%), and 8 were *Pasteurella spp.* (8%).

The antibiotic study revealed that all of the isolates (50) *Escherichia coli,* were sensitive to ciprofloxacin (100%), followed by enrofloxacin (48%), chloramphenicol (90%), streptomycin (48%). The isolates were found resistance to ampicillin (100%) and amoxicillin (100%). The isolates of*Salmonella spp* (28), were sensitive to ciprofloxacin (100%) andthe isolates were found resistance to Amoxycilin (89%), Neomycin (71%) Colistine sulphate (75%), Erythromycin (89%). The all of the isolates (20) *staphylococcus aureus* were sensitive to ciprofloxacin (100%), followed by gentamicin (95%), chloramphenicol (80%), enrofloxacin (85%), ampicillin (80%), amoxicillin (75%). The all of the isolates (8) *Pasteurella spp.* were sensitive to ciprofloxacin (100%) and resistace toAmoxacillin (88%).

Prudent use of antibiotics should be considered in broiler production (where permissible) since many strains are resistant to common antibiotics as described in this study. Potential

drug resistant pathogens in otherwise normal broilers may be a serious concern for public health. Current findings warrants further studies with the isolated strains of bacteria.

From the results of the present study, it may be concluded that:

1. *Escherichia coli, Salmonella spp, Staphylococcus aureus, and Pasteurella spp.* were the major disease forming causal agents found in of the broiler chicken.
2. Antibiogram studies revealed that Ciprofloxacin in optimum doses would be the drug of choice to treat the all the isolated bacteria.

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APPENDICES

APPENDIX 1

Composition of Media

1. Selenite broth

Ingredients	**g/L**
Casein enzymic hydrolysate	5.0
Lactose	4.0

	Sodium phosphate	10.0
	Final pH(at 25°C)	7.0±0.2

2. Nutrient broth

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

3. Nutrient agar

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

4. Salmonella Shigella agar

Ingredients	5.00 gm
Peptic digest of animal tissue	
Beef extract	5.00 gm
lactose	10.00 gm
Bile salts mixture	8.50 gm
Sodium citrate	10.00 gm
Sodium thiosulphate	8.50 gm
Ferric citrate	1.00 gm
Brilliant green	0.00033 gm
Neutral red	0.025 gm
Agar	15.00 gm
Distilled water	1000 ml
Final pH(at25°C)	7.0±0.2 gm

5. Brilliant green agar

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

6. MacConkey Agar

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

7. Eosine methylene blue agar

Ingredients	**g/L**
Peptone	100
Lactose	10.0
K2HP04	2.0
Eosin	0.4
Methylene blue	0.065

	Agar	20.0
	Final pH	6.8±0.2
8.	**Blood agar**	
	Ingredients	**g/L**
	Agar	15.0
	Beef extract	10.0
	Peptone	10.5
	Sodium chloride	5.0
	Final pH	7.3±0.2
9.	**MR VP medium (Himedium, India)**	
	Composition	7.0
	Buffered peptone	
	Dextrose	5.0
	Dipotassium phosphate	5.0
	Final pH(at 25°CO	6.9±0.2
10.	**Sugar media**	
	a. Peptone water	10.0 gm
	Bacto-peptone	
	Sodium chloride	5.0 gm
	0.5% phenol red	0. 1 ml
	Distilled water	1000 ml
	b. Sugar solutions	5 gm
	Individul sugar	100 ml
	Distilled water	
	c. Sugar mediua preparation	4.5 ml
	Pepton water	
	Sugar solution	0.5 ml
11.	**Simmons citrare agar**	
	Ingredients	**g/L**
	Magnessium sulphate	0.20
	Ammunium dihydrogen phosphate	1.0

	Dipotassium phosphate	1.0
	Sodium citrate	2.0
	Sodium chloride	5.0
	Bromothvmol blue	0.08
	Agar	15.0
12.	**TSI Agar slant**	
	Ingredients Lab Lamco Powder	3.00 gm
	Yeast extract	3.00 gm
	Peptone	20.00 gm
	Sodium chloride	5.00 gm
	Lactose	10.00 gm
	Sucrose	10.00 gm
	Glucose	1.00 gm
	Ferric citrate	0.3 gm
	Sodium thiosulphate	0.3 gm
	Phenol red	0.3 gm
	Agar	12.00 gm
	Distilled water	1000 ml

APPENDIX 2

Preparation of reagents

1.	**Peptone water** peptone	I gm
	Distilled water	1000 ml
2.	**Kovacs reagent for indole preparation** P- dimethyl aminobenzal dehyde	5 gm
	Amyl alcohol	75 gm

Conc. HCL	25 ml

3. V-P reagent-1

5% alpha- naphthanol in absolute ethyl alcohol

4. V-P reagent-2

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

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

5. Methyl red Solution

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

6. Phenol red solution

0.2^{0}o aqueous solution of phenol red

7. Gram stain solutions

a. **Stock crystal violet**

crystal violet	10 gm
Ethy1 alcohol	1000m1

b. Stock oxalate

Ammonium oxalate	1 gm
Distilled water	1000 ml

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

c. Lugol's Iodine solution

Iodine crystal	I gm
Potassium iodide	2gm

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

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

Safranine working solution:

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