Study of Microbial Load and Their Antibiotic Resistance in Different Samples of Ostrich

A THESIS

BY

ISRAT JAHAN

REGISTRATION NO. 1605132

SEMESTER: JANUARY–JUNE, 2017

SESSION: 2016

MASTER OF SCIENCE (MS)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY,DINAJPUR-5200

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SESSION: 2016

Submitted to the
Department of Microbiology
Hajee Mohammad Danesh Science and Technology University, Dinajpur
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE (MS)
IN
MICROBIOLOGY

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JULY, 2017

Abstract

The study was conducted to monitor the microbial assessment in oropharyngeal swab , cloacal swab and fecal samples of ostrich and also update knowledge on antimicrobial sensitivity of isolated organisms .The study was performed at HSTU ostrich farm from 16th January to 15th June, 2016. A total 75 samples were collected from 8 ostriches at different age of which 25 oropharyngeal, 25 cloacal swabs sample and 25 were feces samples. The organisms were isolated by using standard microbiological method. The result revealed that the average microbial load in plate count agar was highest in feces ($1.48 \times 10^9 \pm 0.27$CFU) than oropharyngeal swab($1.36\times10^9 \pm 0.38$ CFU) and cloacal swab ($1.28\times10^9 \pm 0.34$ CFU) .Among seventy five sampls, 29 (38.66%) *Escherichia coli*, 21 (28%) *Salmonella spp*, *20 (26.67%) Staphylococcus spp*. 5 (6.6%) *Bacillus spp*. were identified. In twenty five oropharyngeal swab sampls, *Staphylococcus spp* was found higher 15 (60%) than other organisms but *Escherichia coli* were found higher both in cloacal 11(44%) and faecal 12(48%) samples.

On antibiogram study *Escherichia coli*, *Salmonella spp*, *Staphylococcus spp*. and *Bacillus spp* were more sensitive to Levovidine, Azithromycin, Nalidixic Acid and Amoxicillin respectively while more resistant to Azithromycin, Penicillin ,Chloramphenicol and Erythromycin respectively . The obtained results indicated that ostrich excreta is one of the most important sources of *Escherichia coli*, *Staphylococcus spp, Salmonella spp, Bacillus spp.*in ostrich farms. Finally, it may be concludes that the logical use of antibiotics must be adopted in ostrich farms reared in Bangladesh for prevention the appearance of multi drug resistance bacteria. Moreover proper measures should be taken to ward off zoonotic diseases in peoples who are related to ostriche farming.

ACKNOWLEDGEMENT

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.

The author sincerely desires to express her heart felt respect, deepest sense of gratitude, sincere appreciation and profound indebtedness to her respected teacher and Research Supervisor, ***Dr. Nazmia Ara Rumi*** *Lecturer, Department of Microbiology, Faculty of Veterinary and Animal Science (FVAS), Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur or her constant supervision, scholastic guidance, uncompromising principles, helpful advice, constant inspiration, affectionate feeling, radical investigation and constructive criticism in all phases of this study and preparing the manuscript.*

The author finds it a great pleasure in expressing her heartfelt gratitude and immense indebtedness to her honorable teacher and respected Research Co-Supervisor, ***Dr. Md. Khaled Hossain*** *Associate Professor and Chairman Department of Microbiology, Faculty of Veterinary and Animal Science (FVAS), Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, for his sympathetic supervision, kind co-operation, inspiration and valuable suggestions, constructive criticism and valuable advice for the completion of the research work and preparation of the thesis.*

The author wishes to express her cordial respect and profound gratitude to her teachers Dr. Mir Rowshan Akter, Associate Professor, Dr. Md. Fakhruzzaman, Associate Professor, Dr. Farzana Afroz, Assistant Professor, Dr. Atiqul Haque, Dr.Most.Deloara Begum, Department of Microbiology, FVAS, HSTU, Dinajpur, Dr. Shajedur Rahman, Assistant Professor, Department of Medicine, Surgery & Obstetrics, Faculty of Veterinary & Animal Science for their valuable advice and constant inspiration throughout the entire period of study.

The author wishes to express her cordial respect and profound gratitude to her teachers Dr. Abdul Gaffar Miah, Professor and Chairman Department of Genetics and Animal Breeding, Faculty of Veterinary and Animal Science (FVAS), Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, for his ideas and suggestion to do this research work .

The author expresses her cordial appreciation to all the office staff of the Department of Microbiology FVAS, HSTU, Dinajpur.

The author expresses heartfelt gratitude and gratefulness to her beloved parents, friend Samina Akter and Md. Gausur Alal well wishers for their blessing, inspiration, sacrifice and moral support which opened the gate and paved the way to her higher studies and also provided her with the best of everything in her life.

Finally the author is extending her cordial thanks to all of her friends, relatives and well wishers.

The Author *July 2017*

Chapter-1

Introduction

The ostrich (*Struthio camelus*) is the largest and heaviest living bird. As its species name, *camelus*, suggests, the ostrich was once known as the "camel bird" because of its long neck, prominent eyes, and sweeping eyelashes, as well as its jolting walk. Also, like camels, the ostrich can tolerate high temperatures and go without water for long periods of time. As it is so heavy, this flightless bird that can never take to the skies; instead, it's built to run. Its long, thick, and powerful legs can cover great distances without much effort, and its feet have only two toes for greater speed. Ostriches are omnivores, and they eat whatever is available in their habitat at that time of the year. They mostly eat plants, especially roots, leaves, and seeds, but they also munch on insects, snakes, lizards, or rodents that come within reach. When an ostrich eats, food is collected in the crop at the top of the throat until there is a large enough lump to slide down the throat.

The natural home of the ostrich is Africa. Keeping ostriches has a long history dating back to the Egyptian, Babylonian, Greek and Roman empires. The Sahara desert contained many ostriches and was used as a hunting ground. Ostriches also inhabited Palestine, Persia and the Arabian desert. Large numbers of ostriches were exported from Africa in the latter half of 19th century to Australia, New Zealand, Europe, North and South America (Osterhoff 1979, Bertram 1992).Ostrich farming in South Africa today plays a minor role in agriculture, but in earlier years it played a major part in the economy of certain regions of the country. At the peak of ostrich farming in South Africa (1913), there must have been at least one million birds being farmed. Ostrich feather was ranked fourth in value after gold, diamonds and wool, on the list of exports from the then Union of South Africa. The feather market collapsed at the onset of World War I (Smit 1963, Osterhoff 1979, Bertram 1992).

Farm ostriches are called *Struthio camelus var. domesticus*. Their body weight is 30 - 40 kg less than the weight of wild ostriches (which can weigh up to 150 kg at an adult age) and their legs are shorter, but the feather quality is much better. Ostriches have a life span of 30 - 70 years (Hallam 1992, Hildebrandt & Raucher 1999).Wild ostriches are very unmanageable. The first

ostriches were only tamed by about 1863. It seems that a few farmers in the Karoo and Eastern Cape started this branch of agriculture at approximately the same time (Osterhoff 1979).

Ostrich eggs are famous for their size, averaging 6 inches (15 centimeters) in length, 5 inches (13 centimeters) across, and weighing about 3 pounds (1,500 grams). Yet an ostrich egg is small in relation to the size of the adult. An ostrich hen lays 7 to 10 eggs at a time, but her large body can easily cover dozens more. Communal laying has advantages for an ostrich flock: more eggs successfully hatch overall in a communal nest than if each female ostrich had her own nest to incubate and protect. Usually the drab-colored main hen takes incubation duty during the day; the black-plumed male takes over and incubates at night .The life span of ostrich is 30-40 years. One ostrich egg is equivalent to the weight of about 24 chicken eggs. Ostrich farming has been rapidly expanded in Worldwide to produce usable products such as meat, hides, feathers, and eggs. Ostrich (*Struthio camelus var. domesticus)* raising needs experience and information from farmers and the successful ostrich farming is largely dependent on the ability of farmers to rear sufficient numbers of viable and healthy chicks (Christensen JW, Nielsen BL 2004).

The main products obtained from ostriches are plumes (feathers), ostrich skin and a variety of meat products, for example, the liver, the heart and fresh meat (steaks and roasts), processed meats (sausage, ham-type products, salami and biltong) and health care products (ostrich fat) (Jones *et al*. 1997).

Feathers are used in the household and motorcar industries as feather dusters. The leather of ostriches is the most valuable product. Leather is imported by countries that are orientated towards the fashion industry. Approximately 1.3 m^2 of leather is produced by a 12 - 14 month old bird (Hastings 1991).

Ostriches are being explored for medical and medicinal purposes. The tendons of the ostrich leg are used to replace torn tendons in humans, as they are long and strong enough for the human leg. Recent research in ophthalmology points to the possible use of ostrich eyes in corneal transplants. Furthermore, the ostrich brain produces a substance that is being studied for the treatment of Alzheimer's disease and other types of dementia (Odendaal 2000).

Ostrich environment and its microbial load play a significant role in influencing the growth performance of ostrich and thus affect the quality of ostrich product. Ostrich meat and other products can be sources for human infections and may get contaminated through handling, processing, cooking, packaging and storage. Such contamination with pathogenic microorganisms not only renders ostrich products unfit for human consumption but also increase human risk (R.A. Elbassouny and E.S. Shabana, 2009).Meat quality is dependent on the entire meat production chain from the farm where animals are conceived to the consumer (Monin, G. Ouali, A. 1991).

Housing design also contribute to the level of microbes in ostrich bodies as ostriches penned on cement or tiles are restless and defecate readily when compared to those penned on sand. Cement or tiled flooring becomes wet and soiled and when ostriches lie down, expensive body feathers are soiled with faeces and urine. On the other hand, ostriches penned on sand are less restless and defecate less. Another advantage of sand is that the urine drains away in the sand, keeping the surface dry, so that when ostriches lie down their feathers are less soiled (Burger W.P., Peyrot B., Bekker A.et.al. 1995). The environment of a farm as heavy soil and poor drainage often result in animals arriving at the abattoir with muddy feet and abdomens. Dirty skins provide major sources of microbial contamination for the carcase (Edwards D. *et al;*. 1997).

Ostrich are susceptible to a number of infectious agents which are common to other avian species. They have no infectious or contagious species specific diseases **(**Huchzermeyer, F.W. 1998**)**.The bacterial pathogens most frequently involved in infectious enteritis of ostriches are: *Escherichia coli*, *Salmonella spp* **(**Doneley, B. 2006**).** *E.coil* Contaminate environmental sources (vegetation, soil and water) contribute to exposure, soon after birth (S. Fanning, and E. FitzPatrick. 2011). Some *E. coli* strains are pathogenic and have been associated with specific diseases in humans and animals: gastroenteritis, urogenital disease, septicemia, and pleural infections (A. Caprioli. 2000). Salmonella was isolated from ratites birds 5 days to 4 years of age (Vanhooser, S. L. and Welsh, R. D. 1995). The affected birds were from flocks that had fence-to fence contact with other animal species, such as pigs, goats, free-roaming guinea fowl or domestic turkeys. Different Salmonella serotypes cause enteritis in ostriches especially chicks.

Ostriches are susceptible to numerous diseases of bacterial, fungal or parasitic origin. (Cooper RG *et al* .2004). Enteric diseases are important concern in the poultry industry because of decreasing productivity, increased mortality and the associated hazard of poultry products for human food safety. Prebiotics and probiotics are two of several approaches with the potential to reduce enteric diseases and subsequent contamination of poultry products (Cooper RG.2005).

Bacterial infections are an important issue in intensive ostrich breeding. The most important thing is a high level of ostriches infection with *Escherichia coli, Salmonella sp, Pseudomonas spp*. (Wieliczko 2000). Bacteria isolated from respiratory disease in ostriches include *Staphylococcus spp, Pasteurella haemolytica, Pseudomonas aeruginosa, Bordetella spp, Haemophilus spp, Streptococcus viridans, Mycoplasma spp*.and*Chlamydia psittaci*(Huchzermeyer 1994)

There is little information about salmonellosis in ostriches. Most of the published reports come from research done with poultry from unknown healthy conditions or from sick birds that were sent to diagnostic centers where, *Salmonella spp* was reported to cause mortality in ostrich chicks (Shivaprasad, 1993; Higgins et al., 1997; Verwoerd, 2000). However, it was reported that immunosuppressed adult ostriches may shed *Salmonella spp.*, contributing to contamination of products during slaughtering process (Karama et al., 2003).

Now a days antimicrobial resistance that's occurs for microbes of animal origin, including food-producing animals, pet and companion animals, fish and other aquatic animals as well as wild animals, has gained particular attention (Schwarz et al., 2010). There are only some specific study was applied on the antimicrobial resistance for isolated microbes from ostriches in Bangladesh.

Due to the global expansion of ostrich farming and lack of information about the prevalence of microbes in commonly reared ostrich flock. The present study was designed with the following objectives:

1. To assess the microbial load in different samples of ostrich
2. Isolation and identification of organisms from oropharyngeal swabs, clocal swabs and feces of ostrich
3. Antibiogram study of the isolated organisms

Chapter-2

Review of literature

Yadav *et al.* (2017) conducted a cross sectional study to investigate the antibiogram of *Salmonellae* isolated from 23 Captive Ostrich reared in a park situated in Banskhali, Chittagong from 15th July to 15th October, 2015. Gene specific (InvA and SefA) PCR was also used to confirm the selective *Salmonella* serovar from isolated pure culture. Antibiogram was applied on Salmonella positive samples for 12 different antimicrobials.

Asmaa *et al.* (2016) monitored the microbial status of ostrich farms through isolation of bacteria causing disease especially family *Enterobacteriaceae (E. coli; Salmonella spp)* from chicks and adult yards. And Identification of isolated microorganism by serological tests and PCR.

Choboghlo *et al.* (2016) studied the prevalence and antimicrobial resistance characteristics of *Salmonella* spp from ostriches in the North-west of Iran. All 140 samples were collected from feces, feeds and different segments of gastrointestinal tract (GIT) of 5 healthy adult ostriches and isolated of sixteen *Salmonella* strains, belonging to different serotypes. The most frequent serotypes were *S. typhimurium* (37.5%) followed by *S. enteritidis* (31.25%).

Cerbova *et al.* (2016) conducted experiments to detect coliform bacteria in ostrich faeces and to test their antibiotic profile and sensitivity to enterocins. Pure colonies were identified using MALDI-TOF MS mass spectrometry and confirmed by phenotypization. Seventy-one strains were allotted to the species *E. coli*. Sixty-four of those 71 strains caused hemolysis. They were mostly poly resistant to antibiotics. Thirty-two poly resistant strains of *E. coli* were sensitive to enterocins. These strains were most sensitive to Ent 9296 (26 strains).

Nascimento *et al.* (2015) evaluated bacteriological characteristics and shelf life of three formulations of ostrich sausages (*linguiças*), only differing in lean meat percentage: Formula 1, 100% ostrich meat; Formula 2, 75% ostrich meat + 25% pork; and Formula 3, 50% ostrich meat + 25% pork + 25% chicken. Mesophilic and psychrotrophic bacteria, sulfite reducing Clostridia, coagulase-positive Staphylococci, and *Escherichia coli* were enumerated and *Salmonella* spp were isolated and identified and also showed that ostrich meat trimmings can be successfully used in the production of ostrich *linguiças*, and that the formula containing ostrich meat as the only source of lean meat presented the longest shelf life.

Keokilwe *et al.* (2015) investigated the infectious bacteria implicated in ostrich chick (1 day to 3 month) enteritis. Bacterial isolates were typed by PCR and serotyping. *Escherichia coli* (*E. coli*; 49%) was the most frequently isolated from the samples followed by *Clostridium perfringens* (*C. perfringens*; 20%), *Enterococcus* spp. (16%), and *Salmonella* spp. (7%). Of the *E. coli*, 39% were categorized as enteropathogenic *E. coli*, 4% enterotoxigenic *E.coli*, and no enterohaemorrhagic *E. coli* were found. The majority (93%) of *C. perfringens* was Type A and only 7% was Type E. *C. perfringens* Types B through D were not present. The *net B*-gene that encodes Net B toxin was identified from 16% of the *C. perfringens* isolated. Three *Salmonella* serotypes were identified: *Salmonella* Muenchen (*S.* muenchen; 80%), *S.* hayindongo (13%), and *S.* othmarschen (7%). The indication is that the cause of enteritis in ostrich chicks is bacterial-involving: enteropathogenic *E. coli* and enterotoxigenic *E. coli*; *C.perfringens* Types A and E (with the possible influence of *net B*-gene); and *S. muenchen, S. hayindongo, and S. othmarschen*.

Tebyanian *et al.* (2014) showed that PCR method is time consuming, effective, and efficient method to detect *M. synoviae*infection in ostriches. PCR method could be recommended as an alternative for culturing; *M. synoviae*was isolated from ostriches for first time in Kerman Province, Iran.

Sohrabi *et al*. (2013) conducted of a total of 52 sample of various poultry meat products, including: chicken (n=30), turkey (n=10) and ostrich (n=12) obtained from retail stores in Isfahan, Iran and detected by using standard culture methods and biochemical tests. Out of 52 samples 12 (23.07%) were positive for *Listeria spp*. The occurrence of *Listeria spp*. in samples of chicken, turkey and ostrich meat was 20, 30 and 25% respectively.

Zakeri *et al*. (2012) reported of sudden death in one of the ostrich farms of Tabriz, the carcasses were necropsied. Symptoms that were seen include petechia and ecchymosis hemorrhagic in the end area of duodenum and the outset of jujenom along with gaseous gangren. For perfect study and certain diagnosis, sampling of the liver and intestine was done and *Clostridium Perfrigenes* was confirmed. Histological examination revealed multifocal necrosis of hepatocytes with infiltration of hetrophils and also apoptosis. Extensive superficial necrosis associated with fibrin and serocellular deposits was shown in intestine. Generally, sudden changes in diet, stress and nutrition from soil and sands that cause sudden death in young ostriches because of their high sensitivity. Application of effective antibiotics, adjustment and change of diet can be effective in controlling of this disease.

Rahimi (2012) isolated 4 serotypes identified, *S. enteritidis*was the predominant type (50.0%) following by *S. typhimurium* (27.3%) and *S. agona*(13.6%). All isolates were susceptible to amoxicillin, gentamicin, kanamycin, ceftizoxime and cefotaxine but 50.0% and 45.5% of isolates were resistant to nalidixic acid and tetracycline.

Bovera (2011) compared caecal and faecal fermentation characteristics, ostrich caecal content (CI) and faeces (FI) were used as *inocula* for an *in vitro* gas production trial in which four substrates (dehydrated alfalfa, alfalfa hay, maize and a commercial concentrate) were tested. The fermentation characteristics (degraded organic matter, OMd; potential gas production, *A*; acetate; branched chain proportion, BCP) were studied by *inoculum* and substrate. CI and FI showed significant differences for almost all the fermentation parameters, and CI had higher values than

FI for OMd (76.83 vs. 72.79%, p <0.01), A (250.3 vs. 229.3 ml/g, p<0.01), acetate (57.91 vs. 53.20 mmol/l, p<0.01) and BCP (0.031 vs. 0.027, p<0.05). CI and FI showed differences in carbohydrates and protein fermentation, but the interaction between the tested effects was not significant. The regression equations to estimate caecal fermentation characteristics from faeces suggest the possibility to use faeces as *inoculum* alternative to faeces.

Hoffmana *et al*. (2010) investigated the prevalent microbial growth on carcasses before and after overnight cooling in an ostrich abattoir and de-boning plant was. It was indicated that the cold trim (mainly of bruises) of carcasses was advantageous in terms of microbiological meat quality. Results indicated pooled water in the abattoir as the most hazardous vector for carcass contamination and that contaminants from this source are mostly Gram-negative pathogens. *Pseudomonas* and *Shigella*were frequently isolated from surface and air samples and indicated that the control of total plant hygiene is a requirement for producing ostrich meat that is safe to consume and has an acceptable shelf-life.

Alexandre *et al*. (2010) studied to estimate the occurrence of vancomycin-resistant enterococci (VRE) in fecal samples of ostriches from a farm of Southern Portugal, the mechanisms implicated, and the associated virulence factors, 13 years after the banning of the glycopeptide avoparcin as animal growth promoter in the European Union. Fifty-four fecal samples of ostriches were inoculated in Slanetz-Bartley supplemented with vancomycin (4 µg/mL) for VRE recovery. Susceptibility to 11 antibiotics was performed by disk-diffusion agar method in recovered VRE isolates. The mechanism of resistance to vancomycin and to other antibiotics and the presence of the *esp* and *hyl* virulence genes were determined by polymerase chain reaction and sequencing. VRE were detected in 7 of the 54 ostrich fecal samples (13%); *Enterococcus durans* isolates with the *vanA* genotype were found in 4 of the 54 fecal samples (7.4%), and *Enterococcus gallinarum* with the intrinsic *vanC1* genotype in the remaining three VRE-positive samples. All *vanA*-containing *E. durans* isolates showed resistance to tetracycline and erythromycin, and one of them also to ciprofloxacin; they harbored the *erm*(B) and *tet*(M) genes, as well as the specific sequences of Tn*916* and Tn*5397* transposons, but not the *esp* or *hyl*

virulence genes. Two of the three *vanC1* isolates showed resistance to tetracycline [with the *tet*(M) gene] and one to erythromycin [with the *erm*(B) gene], and all three contained the *hyl* gene. Fecal samples of ostriches represent a reservoir of *vanA*-containing enterococci that could be transmitted to humans through the food chain.

Jahantigh (2010) studied the bacterial flora of dead-in-shell ostrich chicks, twelve unhatched eggs which did not have external pipping during the hatching period were transferred to the laboratory of microbiology and *Salmonella* spp*; Bacillus* spp*; Staphylococcus* spp *were isolated*.

Matsui *et al*. (2010) detected three fibrolytic bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*, in the cecaldigesta of the ostrich (*Struthio camelus*) by PCR using a species-specific primer set for each 16S ribosomal RNA gene (16S rDNA). Although amplified DNA fragments obtained from each primer set had the expected size, the clone library derived from the amplimer contained non-specific sequences. The *F. succinogenes*-specific primer set recovered a partial 16S rDNA sequence of an uncultivated *Fibrobacter* with low similarity (<95%) and distantly related phylogenetic positioning to *Fibrobacter* sequences deposited in the databases, indicating a novel species of *Fibrobacter*. The sequence was considered to be identical to a clone detected in our previous experiment. Thus, we confirm that the gastrointestinal tract of the ostrich is one of the habitats of *Fibrobacter* species. The clone library derived from the *R. flavefaciens*-specific primer set contained a 16S rDNA sequence with 97% similarity to *R. flavefaciens*, indicating it could be one of a major fibrolytic bacterium in the ostrich ceca. No *R. albus* 16S rDNA sequence was found in the clone library of the *R. albus*-specific primer set.

Harry *et al*. (2007) reported that a fecal sample from a 42-year-old goat with a 2-month history of poor weight gain and diarrhea yielded a moderate growth of an organism resembling *Salmonella spp*. on MacConkey agar. The organism was identified as *Escherichia fergusonii*.

The animal was euthanized. Samples of intestine, lung, liver, and kidney yielded the same organism, *E. fergusonii*.

Cuomo (2007) investigated to during period January 2004 to December 2005 four different ostrich farms were evaluate the presence of thermotolerant *Campylobacter*. A total of 150 ostriches were examined and all were found to be clinically healthy. *Campylobacter* spp. were isolated from 60/150 cloacal swabs. Among the isolates, 48 were identified as *Campylobacter jejuni* whereas the remainder were identified as *Campylobacter coli*. *Campylobacter lari* was not isolated. The ostriches, although apparently in a healthy body condition, can be considered, theoretically, as potential *Campylobacter* carriers.

Karama (2005) evaluated the microbial quality of ostrich carcases produced in a South African export-approved ostrich abattoir. Ninety surface samples were collected on 30 ostrich carcases at three processing points in the abattoir: post-flaying, post-evisceration and post-chilling. *Pseudomonas* spp., Enterobacteriaceae, *Staphylococcus aureus*and for the presence of *Escherichia coli* and presumptive *Salmonella* spp isolates were identified.

Sahinduran (2004) observed 49 (10-12 months old, 30 males and 19 females) ostriches were kept in the small pen with high levels of ammonia and insufficient ventilation for two months in winter. After two months in sixteen ostriches (12 females and 4 males) respiratory problems. Clinical signs included loss of appetite, ocular and nasal discharge, conjunctivitis and dyspnea. *Staphylococcus aureus* and *Escherichia coli* were isolated from the nasal cavity and conjunctiva. In according to antibiogram results, isolated microbial agents were the most susceptible to amoxycillin and clavulanic acid combinations.

Cooper (2001) reported that ostrich production is highly management intensive. Losses to producers commonly arise from infertile eggs, poor egg handling, and incorrect storage and

incubator settings (temperature, relative humidity, and air flow). Early chick mortality is also a significant factor influencing successful ostrich management. Microbial infection of ostrich eggs, caused by contaminated nests, inadequate egg cleaning, and poor incubator and hatcher sanitation, results in low hatchability. Adequate breeder nutrition is vital for ensuring fertility, increasing the number of eggs laid, and ensuring good survival rates of hatched chicks. The producer must work closely with veterinary extension officers, health laboratories, ostrich producer associations, researchers, and other farmers so that ostrich egg production is molded into a process of excellence.

Liu *et al*. (2001) monitored a disease occurred from August, 1999 in an ostrich farm with more than 450 African ostriches in the south of Hunan Province. Some birds showed various clinical signs, including depression, progressive emacipation, enteritis etc. Two strains of Salmonella typhimurium were isolated and identified for two 3 ~ 4 month old ostriches died from the disease. Their antigen pattern was 4, 5, 12: i: 1, 2.They had hemolytic activities on blood agar Plate. The disease was put under control by using sensitive drugs selected by ding sensitive tests.

Odendaal (2000) studied that ostriches were being explored for medical and medicinal purposes. The tendons of the ostrich leg are used to replace torn tendons in humans, as they are long and strong enough for the human leg. Recent research in ophthalmology points to the possible use of ostrich eyes in corneal transplants. Furthermore, the ostrich brain produces a substance that is being studied for the treatment of Alzheimer's disease and other types of dementia.

Elizabeth *et al*. (2000) conducted research on the prevalence of food borne pathogens such as *Escherichia coli* 0157:H7, *Salmonella*, and *Campylobacter* on ostrich carcasses. Antimicrobial susceptibility testing on 93 carcass *E. coli* isolates showed resistance to erythromycin (99%), neomycin (65%), netilmicin (2%), oxy- tetracycline (22%), streptomycin (2%), and trimethoprim (3%). All isolates were resistant to bacitracin, lincomycin, penicillin, and vancomycin. No Salmonella colony was detected. Antimicrobial susceptibility testing on 131 intestinal *E. coli*

isolates showed resistance to erythromycin (98%), neomycin (66%), netilmicin (34%), oxytetracycline (34%), streptomycin (40%), and trimethoprim (13%). All isolates were resistant to bacitracin, lincomycin, penicillin, and vancomycin.

Gopo *et al*. (1997) conducted to determine the status of *Salmonella spp* from a total number of 1429 samples during the processing of ostriches to ostrich meat and product.

Jones *et al*. (1997) obtained from ostriches are plumes (feathers), ostrich skin and a variety of meat products, for example, the liver, the heart and fresh meat (steaks and roasts), processed meats (sausage, ham-type products, salami and biltong) and health care products (ostrich fat). Feathers are used in the household and motorcar industries as feather dusters. They are also used in the fashion industry as feather fans and capes, artificial flowers, feather-trimmed hats and frocks. Emptied, cleaned and carved, unhatched eggs are commercialised for the tourist industry.

Anonymous (1997) conducted experiment ostrich meat, once only served locally in the production area in South Africa (fresh and biltong), has long been served in gourmet restaurants in Europe. Demand is growing in the Pacific Rim countries and in the United States.

Rahkio *et al*. (1997) documented the source of meat contamination is airborne contamination. It appears that airborne bacteria contribute to carcase contamination. studied microbiological contamination of abattoirs. They found out that there was an association between microbiological contamination of air and carcase contamination, and the movement of personnel between the clean and dirty areas, appeared to be associated with higher carcase contamination level. Airborne contamination originates from skins of animals and lair ages. Separation of the clean and unclean areas of the abattoir decreases the level of contamination.

Hudson *et al*. (1996) monitored to the slaughter process inevitably involves some degree of meat contamination, whether from the animals themselves, the abattoir environment or through contact with personnel and equipment as carcases move through the process. At the end of the slaughter process, beef carcasses are likely to have an aerobic count/cm^2 of 10^3 - 10^5 on the meat surface, mostly less than 10^2 psychotrophs/cm^2 and 10^1 -10^2 coliforms/cm^2 of meat surface. Sheep carcasses usually have a slightly higher level of contamination than beef with 10^3 - 10^6 aerobes/cm^2, about 20% of samples have up to 10^3 or more psychotrophs/cm^2 of meat surface (International Commission on Microbiological Specifications for Food (ICMSF) 1980).

Anonymous (1996) observed the ostriches are poultry, the pH of their flesh is similar to that of beef. Therefore, some classify ostriches as "red meat". In ostriches, there is no breast meat (no white meat). The bulk of the meat is obtained from the leg and thigh.

Van Zyl (1996) observed that in South Africa, ostriches are slaughtered mainly for the export market. In 1993, income generated from ostrich meat was 31.4 million rands. The total income from all ostrich products combined (leather, feathers and meat) was 189.9 million rands in the same year. In 1995, about 170 000 ostriches were slaughtered in South Africa at six European Union approved abattoirs. Calculations were that the rest of the world was slaughtering approximately 15 000 - 20 000 ostriches.

Burger *et al*. (1995) studied the microbial assessment of two methods of ostrich lair age, on sand and cement, found that penning ostriches on clean river sand had to be well-managed by adhering to strict management procedures. The physical condition of the sand had to be efficiently monitored by keeping it well drained, raked at least once a day and kept dry at all times to prevent soil age of birds while lying down.

Burger *et al*. (1995) concerned the design of ostrich lair ages, it has been observed that ostriches penned on cement or tiles are restless and defecate readily when compared to those penned on sand. Cement or tiled flooring become wet and soiled and when ostriches lie down, expensive body feathers are soiled with faeces and urine. On the other hand, ostriches penned on sand are less restless and defecate less. Another advantage of sand is that the urine drains away in the sand, keeping the surface dry, so that when ostriches lie down their feathers are less soiled .

Nortje *et al*. (1990a) showed that the contamination of meat during storage in chillers. Organisms like *Pseudomonas* spp. were found on structural surfaces in the chillers. It was demonstrated that contamination during chilling was also airborne. The presence of spoilage flora in chillers indicated that the disinfection and cleaning routines were inadequate with regard to removal of spoilage micro-organisms.

Monin *et al*. (1991) showed that meat quality is dependent on the entire meat production chain from the farm where animals are conceived to the consumer. It covers sensory and microbiological properties (colour, tenderness, smell, taste, microbial load and shelf-life).

Hudson *et al*. (1991, 1996) observed that post mortem meat inspection has been designed to ensure that meat and meat products entering the human food chain are safe, sound and wholesome. However, it is clear that post mortem meat inspection does not deal adequately with the problem of microbial contamination of meat during the slaughter process, and its consequences for human food-borne diseases.

Hastings (1991) showed that the leather of ostriches is the most valuable product. Leather is imported by countries that are orientated towards the fashion industry. These countries buy tanned skins from South Africa and process them into handbags, purses, briefcases, footwear,

belts, upholstery, and jackets. Approximately 1.3 m^2 of leather is produced by a 12 - 14 month old bird.

Simonsen (1989) observed that some laboratories prefer to use coliform counts instead of Enterobacteriaceae. A European Economic Community (EEC) study carried out to compare the coliform count (on the Violet Red Bile Agar medium) and the Enterobacteriaceae count (on the Violet Red Bile Glucose Agar), demonstrated a high correlation between these two types of counts on samples of poultry carcases taken at different stages of processing. Enterobacteriaceae counts were generally higher, and the coliforms constituted 80 – 90% of the total Enterobacteriaceae count. From this correlation, it was established that either group of organisms could be used for hygiene control checks.

Mead *et al*. (1989) examined care must be exercised when interpreting Enterobacteriaceae count on carcases as indicators of intestinal tract content contamination. (cited by Grau 1986), found that most of the Enterobacteriaceae on poultry carcases were psychrotrophic and originated from the equipment used for slaughter. Because of the presence of psychrotrophic bacteria in the Enterobacteriaceae group, they were found to be less reliable as indicators of contamination with mesophilic organisms when used for chilled meat.

Simonsen (1989) conducted that Enterobacteriaceae species able to grow at low temperatures include members of the genus *Kluyvera, Citrobacterfeundii, Enterobacter cloacae, Erwinia herbicola, Serratia liquefaciens, Klebsiella aerogenes* and *Enterobacter hafniae* (Kleeburger*et al.* 1980). The mesophile Enterobacteriaceae are the pathogenic ones**:** *E. coli*, *Salmonella* spp., *Yersinia* spp., *Shigella* spp. and *Edwardsiella* spp.

Ternstrom *et al*. (1987) investigated a number of infectious micro-organisms associated with food have been identified. These include *Aeromonas hydrophylia*, *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*, *E. coli* 0157:H7, *Klebsiella pneumonia*, *Listeria monocytogenes*, Norwalk virus, *Plesiomonas shigelloides*, *Serratiam arcescens*, *Toxoplasma gondii*, *Vibrio vulnificus*, *Vibrio parahaemolyticus* and *Yersinia enterocolitica*. Of particular importance are *Salmonella* spp., such as *Salmonella enteriditis*PT4 in poultry.

Grau (1986) evaluated the presence of *E. coli* on meat does not necessarily mean that a pathogen could be present, it only implies that there may be a risk of pathogens of faecal origin like *Salmonella* spp., *Campylobacter* spp. and *E. coli* 0157:H7 being present. *Salmonella* spp. have been isolated from samples taken from carcases in which the *E. coli* count ranged from 0.1 to 1 800 per cm^2 of meat surface and the count of Enterobacteriaceae ranged from less than 20 to more than 1 000 per cm^2 of meat surface .Nevertheless, with these observations, *E. coli a*nd Enterobacteriaceae can be useful in the definition of the stages of slaughtering and dressing responsible for contamination, and the sites on carcases most likely to be contaminated with *Salmonella* spp.

Grau *et al*. (1986) counted of Enterobacteriaceae and *E. coli* have been used as indicators of direct contamination of carcases with organisms associated with faecal material. The detection of such organisms on carcases could also indicate indirect contamination from the intestinal tract during slaughter, since these organisms, along with *Salmonella* spp. are frequently found on the outside surface of animals. There is usually not a very large difference between counts of Enterobacteriaceae and *E. coli* obtained from intestinal tract contents. Enterobacteriaceae, on the outside surfaces of animals, are often 100 to 1 000-fold more numerous than *E. coli.*

Grau (1986) monitored the flaying process, when an incorrect technique is used, most of the carcase bacterial contamination is acquired on the first incision, when the knife being used for

slaughter penetrates a heavily contaminated skin and comes into contact with the underlying tissue. Further contamination occurs, if the skin or workers' hands come into contact with the carcase.

Gill *et al*. (1986) stated that pre-slaughter handling of animals influences to a large extent the rate of pH decline in the muscles after slaughter. According to Sales & Mellet (1996), the mean ultimate pH of ostrich muscles suggest that ostrich meat may be classified as an intermediate type between normal (pH <5,8) and extreme Dark Firm Dry meat (pH >6.2). Dark Firm Dry is a condition normally associated with pre-slaughter stress. It occurs mostly in beef, if muscle glycogen reserves are depleted before slaughter, with subsequent production of meat with a low shelf-life.

Grau (1986) differentiated in slaughter and dressing techniques used for different meat animal species, the significance of the APC will not be the same for all meats. For example, in the production of pig and poultry carcases, the skin is not removed so that the number of organisms on the skin is a reflection of the destruction of organisms by scalding (and singeing) and of recontamination in the abattoir. On ostriches, sheep and cattle, the number of APC is a consequence of contamination of a surface, which was sterile before removal of skin or viscera.

Grau *et al*. (1986) conducted contamination during the slaughter process is inevitable, the first aim of the abattoir is to harvest the edible tissue (meat) with as little contamination as possible, by ensuring that the contamination of dressed carcases and edible offal from sources within the abattoir itself is kept to a minimum. This can only be achieved by the use of good manufacturing practices. This entails specific measures to prevent meat contamination at all stages of meat production resulting in prevention of microbial contamination of meat during chilling, freezing, deboning and cutting, packaging and distribution to the consumer.

Dainty *et al*. (1985) observed microbial contaminants that are associated with meat will also include some species of the following genera: *Bacillus* spp., *Aeromonas* spp., *Corynebacterium* spp., *Staphylococcus* spp., *Alcaligenes* spp., *Proteus* spp., *Alteromonas* spp., *Psychrobacter* spp., the *Moraxella/ Acinetobacter* group, *Kingella* spp., Micrococcaceae and lactic acid bacteria. studied the events taking place and their influence on meat quality when P*seudomonas* spp. and *Brochothrix* spp. contaminate meat.

Nortje *et al*. (1985) evaluated meat from animals which have undergone prolonged muscular activity or stress before slaughter, with consequent depletion of glycogen reserves in muscles, undergoes spoilage at low cell (bacterial) densities (10^{6}/cm²). This meat contains little or no glycogen and, therefore, spoilage bacteria growing on such meat, immediately attack amino acids, so that spoilage odours and ammonia are detected.

Nortje *et al*. (1990b) observed the level of contamination of the carcase depends on the cleanliness of the animal before slaughter, the number of bacteria introduced during slaughter and processing, as well as the temperature, the time and the conditions of storage and distribution.

Roberts (1982) conducted many of the procedures involved in stages of breeding and fattening meat animals to processing them into meat for the table, serve to spread the microorganisms from animal to animal and from carcase to carcase. The spread of contamination can be divided into several stages: on the farm, during transport and holding prior to slaughter, during slaughter and post-slaughter.

Tompkin *et al*. (1983) studied a high APC on carcases usually indicates the degree of care taken during slaughter and unsuitable time or temperature conditions during the production and storage

of meat. It can also indicate heavy post-slaughter or post-processing contamination. The presence of a high APC may also mean that the plant used has been poorly cleaned or is contaminated with raw product. In addition, high counts can predict the likelihood of product spoilage (ICMSF 1973).

Roberts (1982) conducted experiments symptomless carriers of pathogenic infections are also of particular significance in meat contamination. In symptomless carriers, the pathogens are generally found in the gastrointestinal tract, but they may also be confined to the mesenteric lymph nodes and the gallbladder (Brown & Baird-Parker 1982, Samuel *et al*. 1979). It has been recognized for decades that pigs and poultry are major reservoirs of *Salmonella* spp.

Nottingham (1982) suggested that generally *E. coli* comprises a greater proportion of the total aerobic flora of the intestine than that of the hide or fleece. The ratio of *E. coli* to total aerobic count can be used as an indicator of whether the major source of carcase contamination is the intestinal tract or skin.

Kilsby (1982) evaluated the microbes on carcases and primal cuts will usually be most numerous on the surfaces. Exceptions to this do occur from time to time as in the case of bone taint, but it is rare. Routine sampling of whole joints is usually confined to the surface of the meat.

Mead (1982) this study was conducted the stress condition before slaughter also contributes to meat contamination in the live animal. Transport stress may lead to increased frequency of defecation and discharge of caecal contents resulting in shedding of bacteria in the faeces, with increased risk of contamination of hides and subsequently of carcase meat.

Notermans *et al*. (1982) evaluated before slaughter, meat and other edible organs without contact with the exterior of healthy and physiologically normal animals, may be regarded as sterile with the exception of the gastrointestinal tract and the tongue. Usually, meat contamination occurs during the slaughter process due to contact with the skin, hair, wool or feathers and the gastrointestinal tract contents. Contamination of carcases during the slaughter process depends on care taken during flaying and evisceration. The skin and viscera are both reservoirs of human pathogens and spoilage micro-organisms.

ICMSF (1980) showed that aerobic organisms as detected with APC on carcases varies with the incubation temperature used for their culture. The approach of the Meat Industry Centre laboratory of the Agricultural Research Council Animal Nutrition and Animal Products Institute (ARC-ANPI), and many other laboratories in the world, is to use an incubation temperature from 20°C - 30°C. The rationale behind the use of this incubation temperature (20°C - 30°C) is that many bacteria present on meat are unable to grow above 30°C. Another reason is that, since the APC is done with the intention of enumerating bacteria which may spoil the product and to check the level of hygiene during slaughter, a temperature from 20°C to 30°C would be suitable for the recovery of the combined flora on meat which is psychrotrophilic (spoilage) and mesophilic because they both grow in this range.

Simmons *et al*. (1978) examined that other sources of meat contamination during the slaughter process include clothing of workers, processing equipment such as saws, boning tables, conveyors and mincers, and the water used to wash carcases, hands and equipment. It has been demonstrated that there is a significant decrease in the degree of contamination of meat, if the hands and tools of operators are thoroughly cleaned. Although water at 82°C is provided for decontamination of equipment used during the slaughter process, the time of immersion is usually not enough (must be at least 10 seconds) to kill bacteria .

Simmons (1978) investigated the evisceration process, contamination occurs if there is puncture or spillage of intestinal or bile content on the carcase. The operations involved in the freeing of the anal sphincter and the rectal end of the intestine constitute an important source of contamination for the carcase. The perianal region of the carcases is often heavily contaminated with *E. coli* and *Salmonella* spp. The incision of the gallbladder, lymph nodes and bile ducts may contribute to contamination of the carcase with *Salmonella* spp. and *Campylobacter* spp.

Kuhne *et al*. (1977) regarded to the nutrient profile of cooked lean meat from ostrich carcases, ostrich meat is low in fat (0.5%). The cholesterol content of raw ostrich meat is 62 mg/100 g, which compares favourably with that of chicken (with skin) at 98 mg/100 g. In view of the trend towards the consumption of lean meat, this should make ostrich meat suitable for the health-conscious consumer. The iron content of ostrich meat is closer to that found in beef rather than that found in cooked lean meat from chickens. This is one reason why ostrich meat is more red in appearance than conventional poultry meat

Ingram *et al*. (1976) conducted to get a reliable indication on the hygienic quality of meat and meat products, micro-organisms on the meat surfaces must be enumerated. One would want to know the identity and numbers of all the micro-organisms on the carcasses, but this is impractical. The best way is to make separate estimates of a few organisms or groups of particular significance for hygiene.

Patterson *et al*. (1969) investigated on the farm, heavy soil and poor drainage often result in animals arriving at the abattoir with muddy feet and abdomens, thus the state of the animal at slaughter is important. Dirty skins provide major sources of microbial contamination for the carcasses. Soiling can be influenced by many factors including the prevalence of diarrhea in animals, climatic conditions on the farm and the length of time spent in the lair age. The design of transport trucks and abattoir lair ages can also make a significant contribution to the level of soiling.

Smit *et al.* (1963) stated that ostrich farming in South Africa today plays a minor role in agriculture, but in earlier years it played a major part in the economy of certain regions of the country. At the peak of ostrich farming in South Africa (1913), there must have been at least one million birds being farmed. Ostrich feather was ranked fourth in value after gold, diamonds and wool, on the list of exports from the then Union of South Africa.

Ayres *et al.* (1955) conducted examined that the animals from feedlots frequently carry variable amounts of manure, bedding and soil on their skins when they enter the abattoir. Mud, bedding and manure adheres to the skin of the animal and may contribute to microbiological contamination of carcasses during skin removal. Microbial contamination from the skin normally includes staphylococci, micrococci, pseudomonas, yeasts and moulds. Skins may also carry as many as log 9 bacteria of soil or faecal origin per cm^2 of skin. Mud and faeces may contain food-borne pathogens like *E. coli*, *Clostridium perfringens* and *Salmonella* spp.

Donkersgoed *et al.* (1955) described that animals from feedlots frequently carry variable amounts of manure, bedding and soil on their skins when they enter the abattoir. Mud, bedding and manure adheres to the skin of the animal and may contribute to microbiological contamination of carcasses during skin removal. Microbial contamination from the skin normally includes staphylococci, micrococci, pseudomonads, yeasts and moulds. Skins may also carry as many as log 9 bacteria of soil or faecal origin per cm^2 of skin. Mud and faeces may contain food-borne pathogens like *E. coli*, *Clostridium perfringens* and *Salmonella* spp.

Chapter 3

Materials and Methods

The present study was performed HSTU ostrich farm at Sadar Upazilla in Dinajpur District of Bangladesh. Samples were taken in the bacteriology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh.

3.1 Materials

3.1.1 Study Site and period

The samples were collected from ostrich farms at Sadar Upazilla in Dinajpur District and brought to the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh during the period from January 2017 to June 2017 for laboratory analysis.

3.1.2 Sample collection

A total 75 samples comprising were orpharyngeal swab (25) , cloacal swab (25) and feaces (25) collected from different age ostriches with pre-sterilized cotton swab and immediately transferred into test tube with fuel paper containing PBS (phosphate buffer solution). Thermo flask containing ice was used to transport the samples from the collection site to Microbiology laboratory for analysis.

Fig. 1: Sample collection of cloacal swab (left) orpharyngeal swab (Right)

3.1.3 Laboratory Equipments:

The different types of sterilized equipment used for this work.

1) Distilled water
2) Sterile bent glass or plastic spreader rods.
3) Micropipette
4) Spirit lamp
5) Labeling tape
6) Experimental test tube
7) Stopper of test tube
8) Petri dish
9) Conical flask.
10) Durham's tube
11) Slide
12) Microscope
13) Cotton, Immersion Oil, Toothpick
14) Autoclave ,thermometer
15) Incubator
16) Jar ,Beaker, Cylinder
17) Electric Balance
18) Filter paper
19) Spirit lamp and
20) Bacteriological loop etc

3.1.4 Media

3.1.4.1 Media for Culture

1. Nutrient agar
2. Mac Conkey agar
3. Salmonella-Shigela Agar
4. Brillant Green Agar
5. Manitol Salt Agar
6. Soft Agar
7. Staphylococcus media No. 110

8. Mueller Hinton agar

3.1.4.2 Media for Biochemical test

1) Sugar Fermentation Broth
2) Indole Broth
3) Methyl Red Broth
4) Voges-proskauer Broth
5) Simmon's citrate Agar
6) Triple sugar iron agar
7) Motility Indole Ureas (MIU)

3.1.5 Reagent

1. Crystal violet dye
2. Grams iodine
3. Alcohol
4. Safranin
5. Saline
6. Iodine solution
7. Phosphate Buffer Solution (PBS)
8. Kovac's reagent
9. Methyl- red solution
10. 3% H_2O_2
11. P – Amino dimethylanilin oxalate
12. Phenol red

3.1.6 Media for Culture

3.1.6.1 Plate Count Agar (PCA):

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

3.1.6.2 Nutrient Agar (NA):

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

3.1.6.3 Mac Conkey Agar:

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

3.1.6.4 Salmonella Shigella Agar (SS):

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

3.1.6.5 Brilliant Green Agar (BGA):

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

3.1.6.6 Mannitol Salt Agar (MSA):

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

3.1.6.7 Soft Agar:

Soft agar is a selective medium for *Bacillus* spp.

3.2 Methods

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

3.2.1 Experimental Layout

The process of isolation and identification is presented on

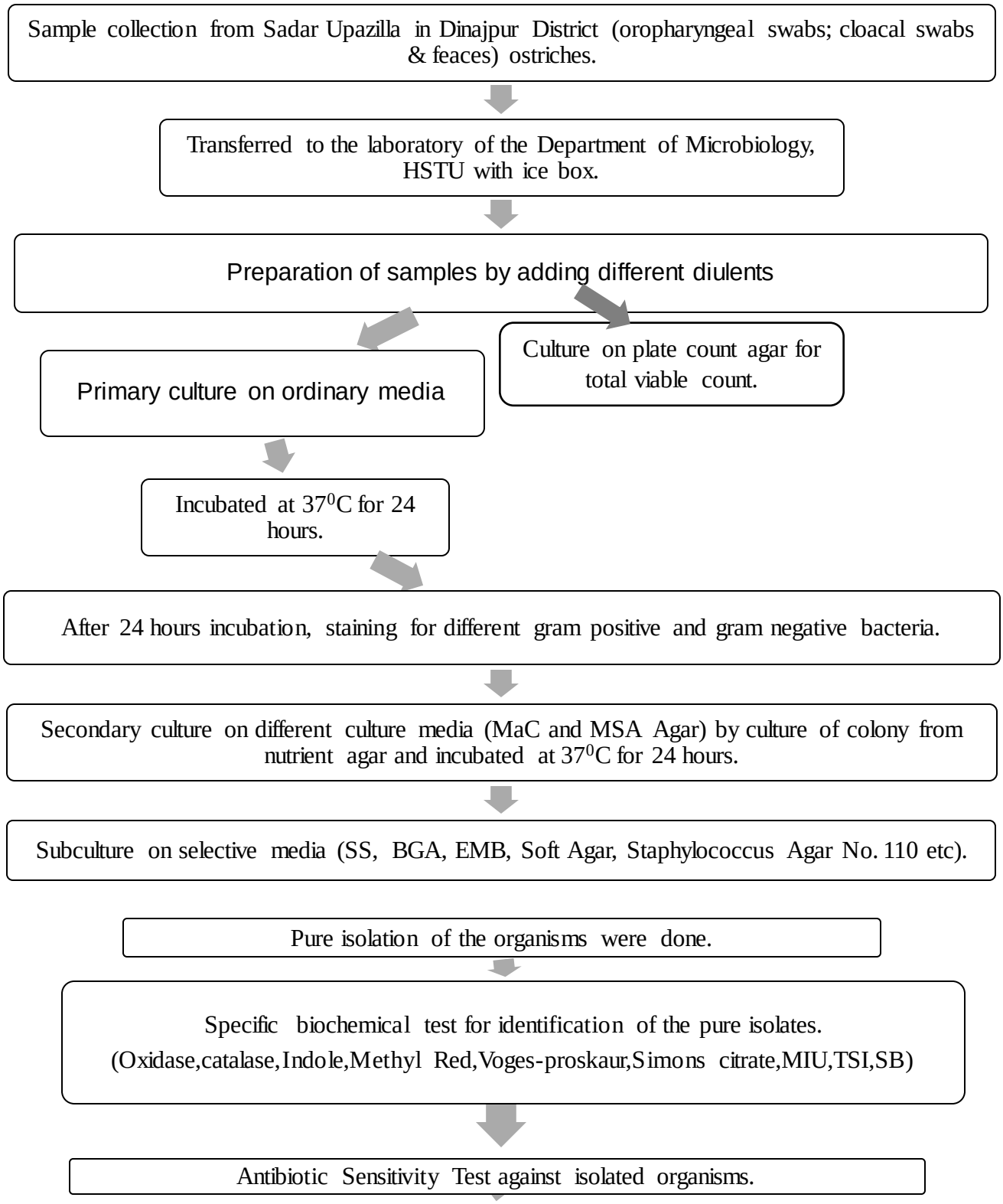


Fig. 02: Schematic illustration of the experimental layout.

3.2.3 Preparation of Culture Media:

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

3.2.3.1 Nutrient broth media:

Thirteen grams of dehydrated nutrient broth was suspended into 1000 ml of distilled water and boil to dissolve it completely. The solution was then distributed in tubes, stopper with cotton plugs and sterilized in autoclaving at 121° C and 1.2 kg/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 (Cater 1979).

3.2.3.2 Plate Count Agar (PCA):

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

3.2.3.3 Nutrient agar(NA) media

28 grams of nutrient agar powder was dissolved in 1000 ml of cold distilled water in a flask. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petri dish and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use (Cater 1979).

3.2.3.4 Eosin Methylene Blue (EMB) agar

Thirty six grams of EMB agar base was added to 1000 ml of water in a flask and boil to dissolve the medium completely. The medium was sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121°C for 15 minutes and I to 50° C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile Petri dish sized and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37° C for overnight to check their sterility and petri

dishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.5 Mac Conkey agar

51.5 grams Mac Conkey agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 45^0- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.6 Salmonella Shigela (SS) Agar:

Suspend 50g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by boiling for 5 minutes. After boiling the medium was put into water bath at 45^0- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.7 Brilliant Green Agar(BGA):

Suspend 50g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. After autoclaving the medium was put into water bath at 45^0- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large

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

3.2.3.8 Mannitol Salt Agar (MSA):

111 grams Mannitol Salt Agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 45^0- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.9 Staphylococcus Agar No. 110:

149.5 grams Staphylococcus Agar No. 110 base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 45^0- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.9 Soft Agar:

Tryptone 10 grams; Yeast extract 5 grams; NaCl 10 grams and Agar 7.5 grams Soft agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 45^0- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.4 Preparation of reagents

3.2.4.1 Methyl- Red solution

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

3.2.4.2 Methyl Red

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

3.2.4.3 Alpha- naphthol solution

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

3.2.4.4 Potassium hydroxide solution

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

3.2.4.5 Phosphate Buffered Saline solution

Eight grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate 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.

3.2.4.6 Indole reagent (Kovac's reagent)

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

3.3 Microbial assessment of the collected samples:

Samples were collected and each of the samples were diluted with distilled water as

10^{-1}, 10^{-2},10^{-3},10^{-4},10^{-5}, 10^{-6},10^{-7},10^{-8} and 10^{-9}. Then 1ml samples were taken and spread in Plate count agar(PCA) plate following the spread-plate method and incubateat 37ºC for 124 h. The number of organisms per ml of original culture is calculated by multiplying the number of colonies counted by the dilution factor:

Number of cells per ml=number of colonies × Dilution factor

Examples:

a. Colonies per plate=50
   Dilution factor=1:1×10^6(1:1000,000)
   Volume of dilution added to plate=1ml
   50×1000000=50,000,000 or (5×107) CFUs/ml
   (colony-forming units).

Record the observations and calculated bacterial counts per ml of samples. (James G. Cappuccino. 7Edition).

3.3.1 Cultivation and isolation of organisms:

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

3.3.2 Morphological characterization by Gram's staining method:

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

3.3.3 Preparation of Gram's staining solution:

The four solutions needed for the Gram staining procedure.

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

3.3.4 Gram staining procedure:

1. Obtain clean glass slides.
2. Using sterile technique, prepared a smears of each of the organisms. Did this by placing a drop of water on the slide, and then transferring each organisms separately to the drop water with a sterile, cooled loop .Mixed and speeded organism by means of a circular motion of the inoculating loop.
3. Allowed smears to air –dry and then heat fixed in the usual manner.
4. Gently flooded smears with crystal violet and let stood for 1 min gently washed with tap water.
5. Gently flooded smears with Grams iodine mordant and let stood for 1 min .Gently washed with tap water.
6. Decolorized with 95% ethyl alcohol .Gently washed with tap water.
7. Counter stain with safranin for 30 sec.
8. Gently washed with tap water.
9. Examined under oil immersion. (James G. Cuppuccion , Natalie Sherman ,1996*)*

3.3.5 Biochemical examination:

Isolated organism with supporting growth characteristics of suspected identified by biochemical test are performed Sugar fermentation test, Oxidase test, Catalase test, Indole test , MR Test, Voges-proskauer test, Simmon's citrate, Triple sugar iron agar(TSI), Mortility Indole Urease **(**MIU) test, Selenite broth.

3.3.5.1 Sugar fermentation test

The sugar fermentation test was performed by inoculating a loop full of NB culture of the organisms into each tube containing five basic sugars (e.g. dextrose, sucrose, lactose, maltose and Mannitol) separately and incubated for 24 hours at 37°c Acid production was indicated by the color change from reddish to yellow in the medium and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tube (Cheesbrough, 1985).

3.3.5.2 Oxidase test

The oxidase test uses Kovac's reagent (a 1% [wt/vol] solution of N, N, N', N' –tetramethyl-ρ-phenylenediamine dihydrochloride) to detect the presence of cytochrome c in a bacterial

organism's respiratory chain; if the oxidase reagent is catalyzed, it turns purple. The oxidase test can be performed on filter paper or on a swab (Cheesbrough, 1985).

3.3.5.3 Catalase test

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

3.3.5.4 Indole test

Two milliliter of peptone water was inoculated with the 5 ml of bacterial culture and incubated at 37°c for 48 hours. Kovac's reagents (0.5ml) were added, shake well and examined after 1 minute. A red color in the reagent layer indicated Indole test positive. In negative case there is no development of red color *(*Cheesbrough, 1985).

3.3.5.5 Methyl Red test (MR)

Sterile MR-VP broth was inoculated with the test organism and following incubation at 37°c for 24 hours. if the organism would ferment glucose via the mixed acid fermentation pathway like lactic, acetic, which decreases the PH ,hence upon the addition of the indicator methyl red the broth becomes red in color and yellow color indicated a negative result (Cheesbrough, 1985).

3.3.5.6 Voges-Proskauer test (VP)

Voges Proskauer Test – If the organism would ferment glucose via the butylenes glycol pathway, an intermediate product, acetyl methyl carbinol or acetone which is neutral is converted to diacetyl upon the addition of the VP – Reagent –B (40% KOH with 0.3% creatine) in the presence of VP – Reagent – A (5% alpha- naphthol in abs. methyl alcohol). Diacetyl is red in color. Negative is yellow color (Cheesbrough, 1985).

3.3.5.7 Simmon's Citrate Agar (SCA)

This tube medium is used to identify Gram negative enteric bacilli based on the ability of the organisms to utilize citrate s the sole source of carbon. (Citrate utilization test).The organism

which utilizes citrate as its source of carbon degrades it to ammonia and subsequently converts it to ammonium hydroxide. The pH of the medium is then increased and this is indicated by a change in color from green to blue (Cheesbrough, 1985).

3.3.5.8 Triple Sugar Iron Agar (TSI)

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

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

3.3.5.9 Mortility Indole Urease (MIU) test

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

3.4 Antibiotic sensitivity test against isolated microbes:

To determine the drug Sensitivity and resistance patterns of isolated organisms used different types of commercially available antimicrobial discs, (Mast diagnostics Mersey side, UK.) Which were showed in (Table 1). The antibiotic resistance was determined by Kirby-Bauer disc diffusion technique using Mueller-Hinton agar (Difco), according to the recommendations of National Committee for Clinical Laboratory Standards (CLSI 2011). After overnight incubation at 37 °C, the diameter in millimeters of the zones of inhibition around each of the antimicrobial discs was recorded and categorized as resistant or sensitive in accordance with company recommendations.(Cappuccino 2005*). E.coli, Salmonella spp, Staphylococcus spp,* and *Bacillus spp* isolates were tested for sensitivity to (12 of routine and

practical antibiotics) Levofloxacin (5µg),Penicillin (10units), amoxicillin(30 µg), Cefxime (5 µg), Feridoxin(10 µg) Ceftriaxone (30 µg), Chloramphenicol (30 µg),Gentamicin (10 µg), Nalidixic acid (30 µg) Vancomycine (30 µg)and Azithromycine (15 µg), Eryhromycine (15 µg)and tetracycline(30 µg) .The disks were purchased from national company. The results were interpreted by special manufacturer's tables

Table 1: Antimicrobial agents with their disc concentration

Antimicrobial agents	Symbol	Disc concentration (µg/disc)
Levofloxacin	LE	5µg
Penicillin	P	10units
Amoxicillin	AMX	30 µg
Feridoxin	FD	10 µg
Chloramphenicol	C	30 µg
Gentamicin	GEN	10 µg
Nalidixic acid	NA	30 µg
Azithromycine	AZM	15 µg
Erythromycine	E	15 µg
Tetracycline	TE	30 µg
Ceftriaxone	CTR	30 µg

Notes: µg =microgram.

3.4.1 Recording and interpreting results of antibiogram study:

The zones of growth inhibition was compared with the zone-size interpritative table no 2,3,4,5 standard for *E.coli ,Salmonella spp Staphylococcus SppBacillus spp*

Table 2: Zone diameter imperative standards for *Staphylococcus spp*

Antimicrobial agents	Zone diameter		
	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Levofloxacin	**≤22**	**23-29**	**≥30**
Penicillin	**≤28**	**-**	**≥29**
Amoxicillin	**≤19**	**-**	**≥20**
Feridoxin	**≤24**	**25-30**	**≥31**
Cefxime	**≤21**	**-**	**≥22**

Chloramphenicol	**≤12**	**13-17**	**≥18**
Gentamicin	**≤20**	**21-28**	**≥29**
Nalidixic acid	**≤13**	**14-18**	**≥19**
Azithromycine	**≤13**	**14-17**	**≥18**
Erythromycine	**≤13**	**14-22**	**≥23**
Tetracycline	**≤14**	**15-18**	**≥19**
Ceftriaxone	**-**	**-**	**≥24**

[Note: LE=Levofloxacin; P=Penicillin; AMX=Amoxicillin; FD=Feridoxin; CFM=Cefxime C=Chloramphenicol; GEN=Gentamicin; NA=Nalidixic acid; AZM=Azithromycine E=Erythromycine; TE= Tetracycline; CTR=Ceftriaxone; VE=Vancomycine]

Table 3: Zone diameter imperative standerds for *Salmoneiia spp*

Antimicrobial agents	**Zone diameter**		
	Resistant (mm)	**Intermediate (mm)**	**Sensitive (mm)**
Levofloxacin	**≤23**	**24-30**	**≥31**
Amoxicillin	**≤19**	**-**	**≥20**
Feridoxin	**≤24**	**25-30**	**≥31**
Cefxime	**≤21**	**-**	**≥22**
Chloramphenicol	**≤12**	**13-17**	**≥18**
Gentamicin	**≤20**	**21-28**	**≥29**
Nalidixic acid	**≤13**	**14-18**	**≥19**
Azithromycine	**≤13**	**14-17**	**≥18**
Erythromycine	**≤13**	**14-22**	**≥23**
Tetracycline	**≤14**	**15-18**	**≥19**
Ceftriaxone	**-**	**-**	**≥24**

[Note: LE=Levofloxacin; AMX=Amoxicillin; FD=Feridoxin; CFM=Cefxime C=Chloramphenicol; GEN=Gentamicin; NA=Nalidixic acid; AZM=Azithromycine E=Erythromycine; TE= Tetracycline; CTR=Ceftriaxone; VE=Vancomycine]

Table 4: Zone diameter imperative standerds for *Escherichia coli*

Antimicrobial agents	Zone diameter		
	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Levofloxacin	≤28	29-37	≥38
Amoxicillin	≤19	-	≥20
Feridoxin	≤29	30-40	≥41
Vancomycine	≤21	-	≥22
Chloramphenicol	≤12	13-17	≥18
Gentamicin	≤20	21-28	≥29
Nalidixic acid	≤13	14-18	≥19
Azithromycine	≤13	-	≥20
Erythromycine	≤13	14-22	≥23
Tetracycline	≤14	15-18	≥19
Ceftriaxone	-	-	≥24

[Note: LE=Livofloxacin; P=Penicillin; AMX=Amoxicillin; FD=Feridoxin; CFM=Cefxime C=Chloramphenicol; GEN=Gentamicin; NA=Nalidixic acid; AZM=Azithromycine, E=Erythromycine; TE= Tetracycline; CTR=Ceftriaxone; VE=Vancomycine]

Table 5: Zone diameter imperative standerds for *Bacillus spp*

Antimicrobial agents	Zone diameter		
	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Levofloxacin	≤28	29-37	≥30
Penicillin	≤28	-	≥29
Amoxicillin	≤19	-	≥20
Feridoxin	≤29	30-40	≥41
Cefxime	≤21	-	≥22
Chloramphenicol	≤12	13-17	≥18
Gentamicin	≤20	21-28	≥29

Nalidixic acid	**≤13**	**14-18**	**≥19**
Azithromycine	**-**	**-**	**≥12**
Erythromycine	**≤13**	**14-22**	**≥23**
Tetracycline	**≤14**	**15-18**	**≥19**
Ceftriaxone	**-**	**-**	**≥24**

Source: CLSIFDA- 2013 table –update pdf.

[Note: LE=Levofloxacin; P=Penicillin; AMX=Amoxicillin; FD=Feridoxin; CFM=Cefxime C=Chloramphenicol; GEN=Gentamicin; NA=Nalidixic acid; AZM=Azithromycine, E=Erythromycine; TE= Tetracycline; CTR=Ceftriaxone; VE=Vancomycine].

3.5 Statistical analysis:

Data were analyzed using SPSS version 21. The chi-square (χ^2) test was used to assess statistical differences between the groups. A *p*-value less than 0.05 were statistically considered significant.

Chapter 4

Results

The present study was microbial assessment of ostrich at HSTU ostrich farm in Dinajpur with their antibiogram study. The samples (orapharyngeal swab, cloacal swab and feces) collected were subjected to various bacteriological, biochemical examination in the laboratory of the department of microbiology, HSTU, Dinajpur. For this a total 75 (25 orapharyngeal swab, 25 cloacal swab and 25 feces) samples were collected from different ages of ostrich.

4.1 Microbial assessment of collected samples by total viable counts

During the study period a total 75 (25 orapharyngeal swab, 25 cloacal swab and 25 feces) samples were collected from different ages of ostrich for Total viable counts expressed as CFU which were shown in Table 06. In every case the average colony(CFU)were counted at 30 days interval. In case of orapharyngeal swab the average colony count on 90 days(3months)were $1.87\times10^{9}\pm0.037$ followed by days 120 were $1.52\times10^{9}\pm0.054$days150 were $1.38\times10^{9}\pm0.029$ days 180 were $1.38\times10^{9}\pm0.029$ and days 210 were $0.9\times10^{9}\pm0.04$ respectively. In case of cloacal swab the average colony count on 90 days (3months) were $1.54\times10^{9}\pm0.022$ followed by days 120 were $1.52\times10^{9}\pm0.054$ days 150 were $1.39\times10^{9}\pm0.01$ days 180 were $1.30\times10^{9}\pm0.04$ and days 210 were $0.69\times10^{9}\pm0.019$ respectively. In case of feces swab the average colony count on 90 days(3months)were $1.13\times10^{9}\pm0.027$ followed by days 120 were $1.29\times10^{9}\pm0.038$ days 150 were $1.52\times10^{9}\pm0.027$ days 180 were $1.62\times10^{9}\pm0.03$ and days 210 were $1.83\times10^{9}\pm0.04$ respectively.

Table 06: Total viable counts expressed as CFU for 75 samples (orapharyngeal swab, cloacal swab and feces) of ostrichesin different age group.

Sample and sample size	Age of ostrich					Mean± SEM N=25	t test	P value
	3 months n=5 (Mean± S SEM)	4 months n=5 (Mean± SEM)	5 months n=5 (Mean± SEM)	6 months n=5 (Mean± S SEM)	7 months n=5 (Mean± SEM)			
oropharyngeal swab (n=25)	1.87×10^{9} ±0.037	1.52×10^{9} ±0.054	1.38×10^{9} ±0.029	1.11×10^{9}±0.024	0.9×10^{9}± 0.04	1.36×10^{9}±0.38	8.10	0.001
Cloacal swab (n=25)	1.54×10^{9} ±0.022	1.47×10^{9} ±0.057	1.39×10^{9} ±0.01	1.30×10^{9}±0.04	0.69×10^{9} ±0.019	1.28×10^{9}±0.34	8.39	0.001
Faeces (n=25)	1.13×10^{9} ±0.027	1.29×10^{9} ±0.038	1.52×10^{9} ±0.027	1.62×10^{9}±0.03	1.83×10^{9} ±0.04	1.48×10^{9}±0.27	12.02	0.00

[SEM means Standard Error Mean]

P value <0.01 means significant at 1% level of significance

4.2 Result of Isolation of organism

The result of isolation of organism from ostrich orapharyngeal swab, cloacal swab and feces were shown in Table no2, 3,4 and 5. During the study period a total 75 (25 orapharyngeal swab, 25 cloacal swab and 25 feces) samples were collected from different ages of ostrich for isolation of organisms. In case of orapharyngeal swabs 15(60%) positive for *Staphylococcus spp*. 3(12%) positive for *Salmonella spp* .6(24%) positive for *Escherichia coli* .1(04 %)were positive for *Bacillus spp* respectively. In case of cloacal swabs 3 (12%) positive for *Staphylococcus spp*. 32 % (8) positive for *Salmonella spp* . 44% (11) positive for *Escherichia coli* . 12% (3)were positive for *Bacillus spp* respectively. In case of feces 08 % (2) positive for *Staphylococcus spp*. 40% (10) positive for *Salmonella spp* . 48 % (12) positive for *Escherichia coli* . 04% (1) were positive for *Bacillus spp* respectively. Among seventy five sampls, 29 (38.66%) *Escherichia coli*, 21 (28%) *Salmonella spp, 20 (26.67%) Staphylococcus spp.* 5 (6.6%) *Bacillus spp.* were identified. In twenty five oropharyngeal

swab sampls, *Staphylococcus spp* was found higher 15 (60%) than other organisms but *Escherichia coli* were found higher both in cloacal 11(44%) and faecal 12(48%) samples.

Table 07: Distribution bacterial isolates identified on ostrich oropharyngial swab, clocal swab & feaces of ostrich

Bacterial isolets	Oropharyngeal swab	Cloacal swab	Feaces swab	Percentage (%)
Staphylococcus spp.	60 % (15)	12% (3)	08 % (2)	26.67%
Salmonella spp.	12 % (3)	32 % (8)	40% (10)	28 %
Escherichia coli	24 % (6)	44% (11)	48 % (12)	38.66 %
Bacillus spp.	04 % (1)	12% (3)	04% (1)	06.67 %
Total number of isolates identified	25	25	25	100

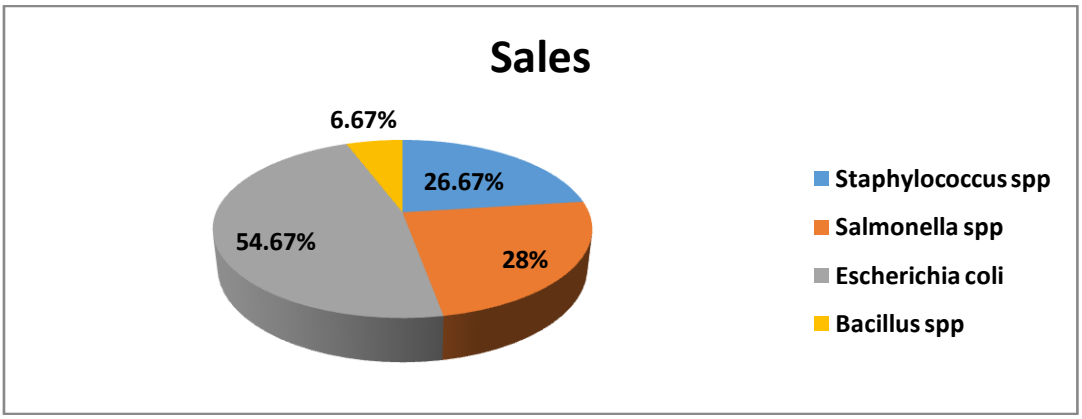


Figure 03: Frequency of bacterial isolates identified on ostrich oropharyngial swab, clocal swab & feaces of ostrich

Table 8: Bacterial species isolated from oropharyngeal swab samples of ostrich

Sample size	Isolated bacteria	Positive isolate	Prevalence (%)	χ^2 value	P value
25	*Staphylococcus spp.*	**15**	**60 %**	**24.48**	**0.00**
	Salmonella spp.	**3**	**12 %**		
	Escherichia coli	**6**	**24 %**		
	Bacillus spp.	**1**	**04 %**		

P value <0.01 means significant at 1% level of significance

Table 9: Bacterial species isolated from Cloacal swab samples of ostrich

Sample size	Isolated bacteria	Positive	Prevalence (%)	χ^2 value	P value
25	*Staphylococcus spp.*	**3**	**12 %**	**9.97**	**0.019**
	Salmonella spp.	**8**	**32 %**		
	Escherichia coli	**11**	**44 %**		
	Bacillus spp.	**3**	**12 %**		

P value >0.01 means significant at 5% level of significance

Table 10: Bacterial species isolated from faces samples of ostrich

Sample size	Isolated bacteria	Positive isolate	Prevalence (%)	χ^2 value	P value
25	*Staphylococcus spp.*	**2**	**08 %**	**19.79**	**0.00**
	Salmonella spp.	**10**	**40 %**		
	Escherichia coli	**12**	**48 %**		
	Bacillus spp.	**1**	**04 %**		

P value <0.01 means significant at 1% level of significance

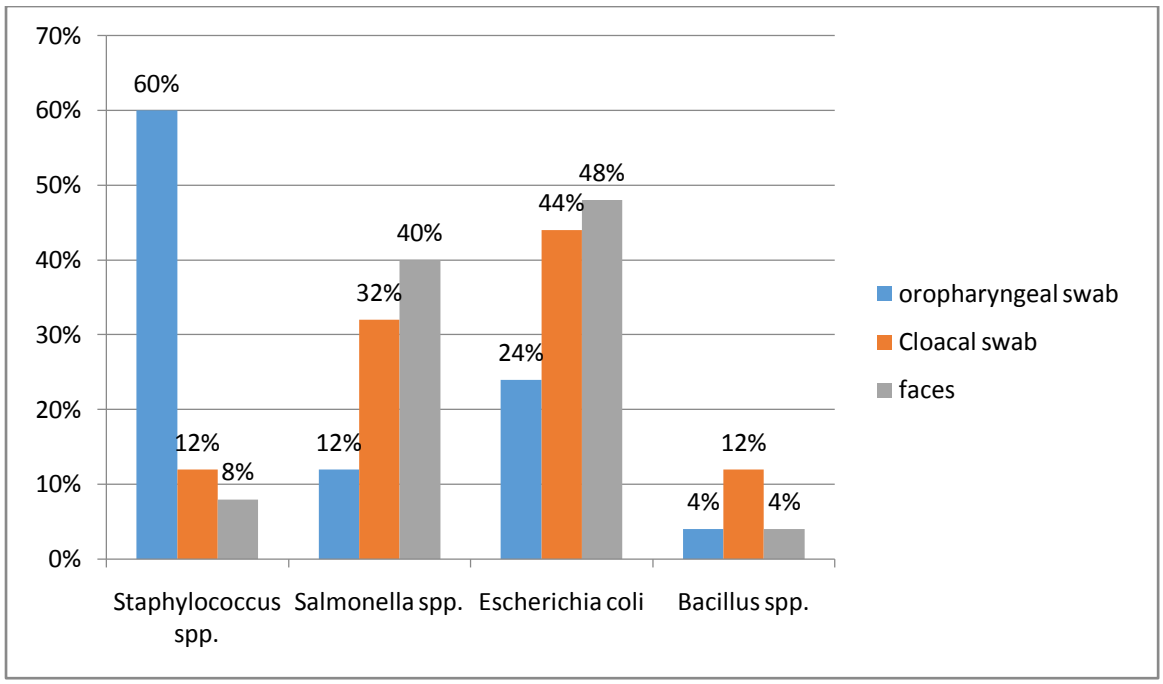


Figure: Distribution of Bacterial species Isolated from orpharungeal swab, Cloacal swab and faces sample of ostrics

4.3 Identification of organism by different bacteriological methods:

4.3.1 Results of Cultural Examination:

The cultural characteristics of *E. coli*, *Salmonella* spp. *Staphylococcus*, and *Bacillus* spp., on various selective media are presented in Table

Table 11: The result of cultural characteristics of the organisms which are isolated from orapharyngeal swab, cloacal swab and feces of ostrich

Serial No	Name of bacteria	Name of media	Colony characteristics
01	*E. coli*	Nutrient Agar	Large, mucoid, white colony
		Mac-Conkey's Agar	Produce large mucoid rose pink colony
		EMB agar	Metallic sheen(greenish black) colony
02	*Staphylococcus spp.*	Nutrient Agar	Black colour/ non-colour smooth, glistening colony.
		Staphylococcus	Yellow colony

		Agar No.110	
		Blood Agar	β-hemolytic colony
03	*Salmonella* spp.	Mac-Conkey agar	Small, white, translucent dew drop like colony.
		S.S agar	Opaque, smooth, round with black centered colony
04	*Bacillus* spp.	Nutrient Agar	Grayish white color with Medusa head colony
		Soft Agar	Thick, grayish white or cream colored colony

4.3.1.1 Nutrient Agar:

Nutrient agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and were indicated by the growth of circular, small smooth, convex and gray white or yellowish colonies.

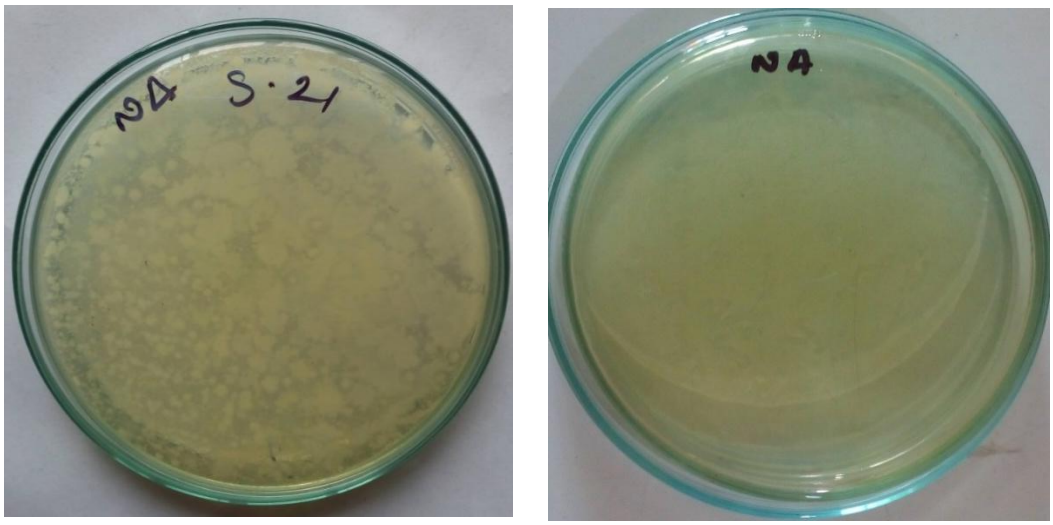


Plate 4.1: Culture of organism on Nutrient's Agar (left); Control of Nutrient's Agar (right).

4.3.1.2 Mac Conkey Agar:

Mac Conkey Agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and were indicated the colorless colonies after prolonged incubation pink color colonies.

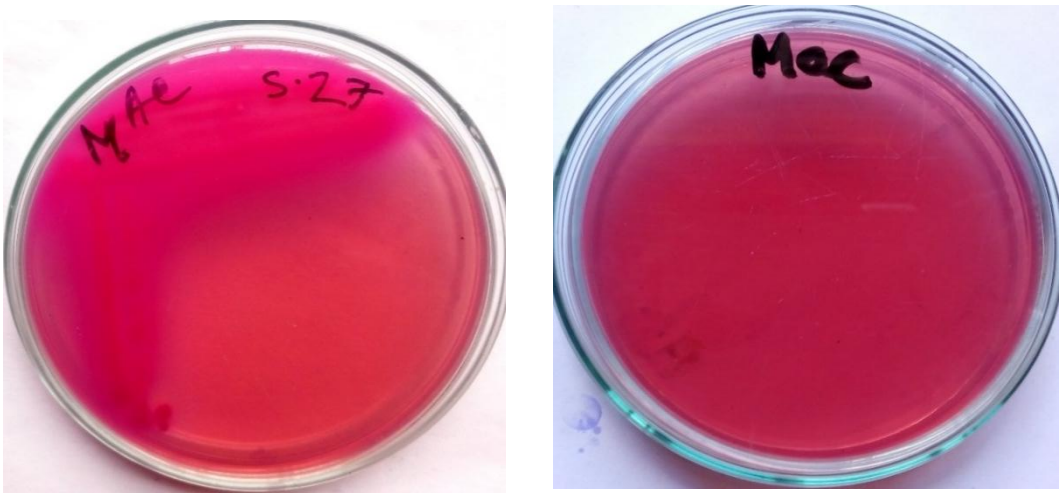


Plate 4.2: *E.coli* on Mac Conkey Agar (left); Control of Mac Conkey Agar(right)

4.3.1.3 Salmonella-Shigella Agar:

Salmonella-Shigella Agarplates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and were indicated by the Clear, black center colony, transparent.

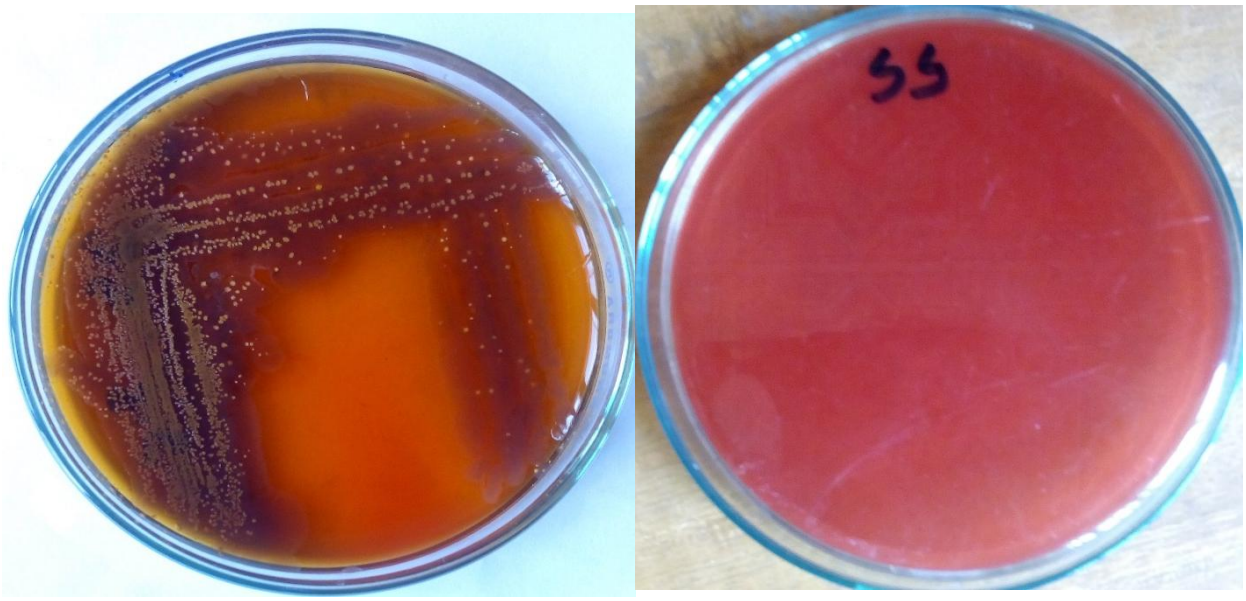


Plate 4.3: *Salmonella* spp. on Salmonella-Shigella Agar (left); Control of Salmonella-Shigella Agar (right)

4.3.1.4 Brilliant Green Agar:

Brilliant Green Agarstreaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and observed red, pink white colonies.

Plate 4.4: *Salmonella spp*. on Brilliant Green Agar (left); Control of Brilliant Green Agar (right)

4.3.1.5 Manitol Salt Agar:

Manitol salt agarplates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^0C a8erobically and were indicated by the pink color.

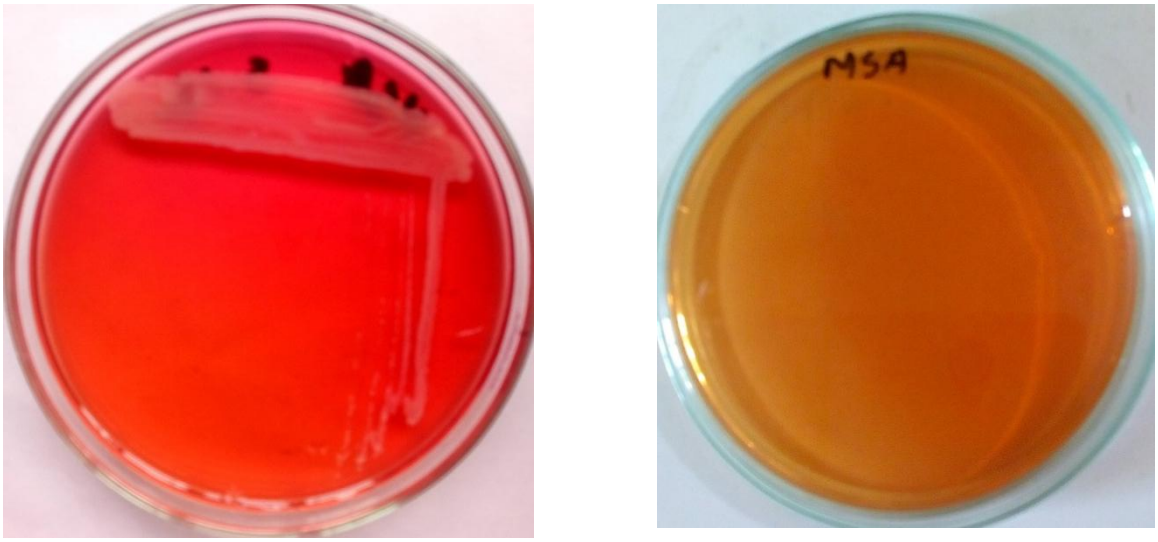


Plate 4.5: *Staphylococcus* spp. on Manitol Salt Agar(left); Control of Manitol Salt Agar (right)

4.3.1.6 Soft Agar:

Soft agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and were indicated by the

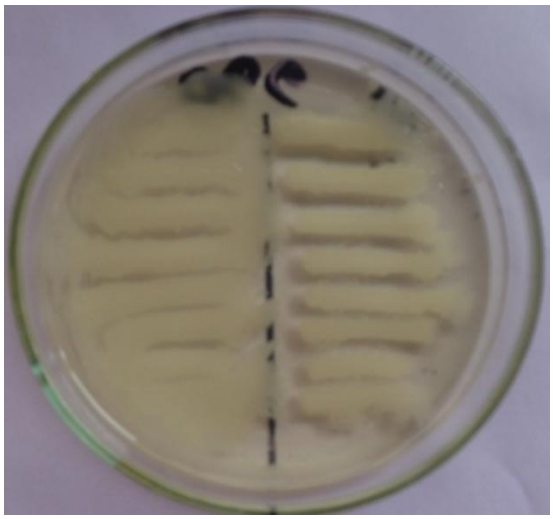


Plate 4.6: *Bacillus* spp. on Soft Agar

4.3.1.7 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.

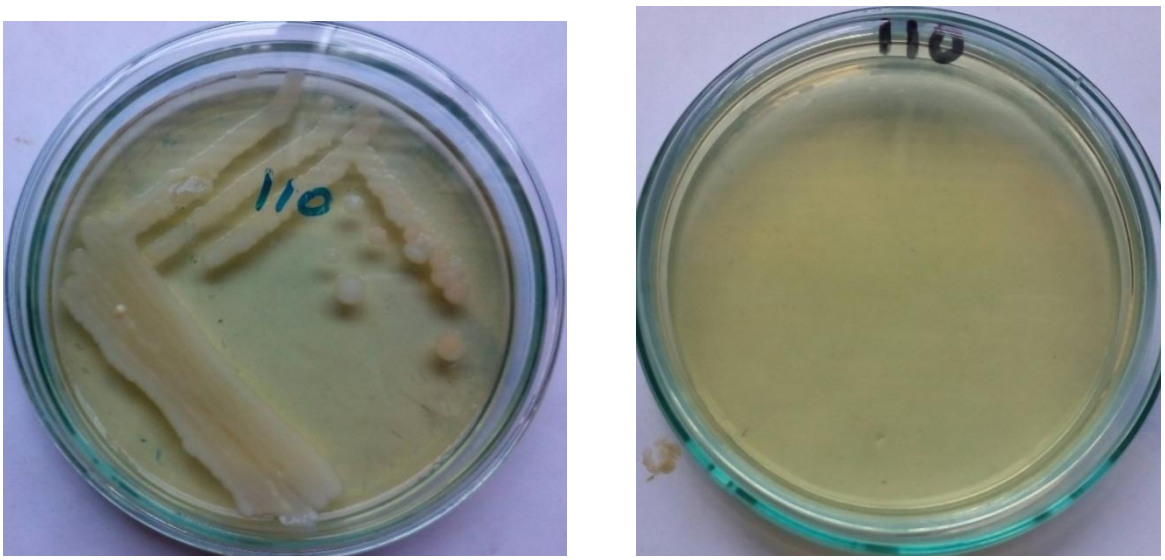


Plate 4.7: *Staphylococcus spp*. on Staphylococcus Agar No. 110. (left); Control of Staphylococcus Agar No. 110 (right)

4.3.2 Microscopic examination:

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

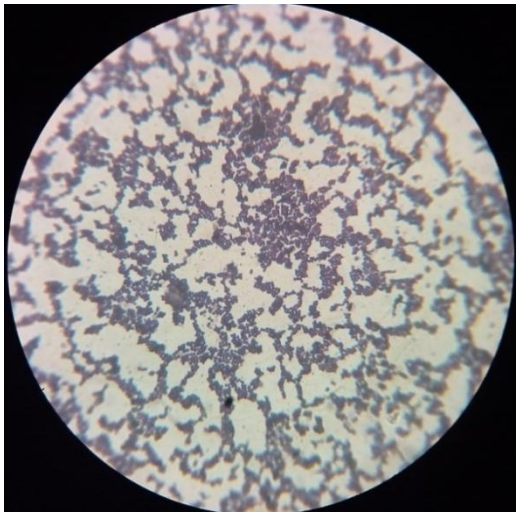


Plate 4.8: Gram positive Grape like *Staphylococcus spp.*

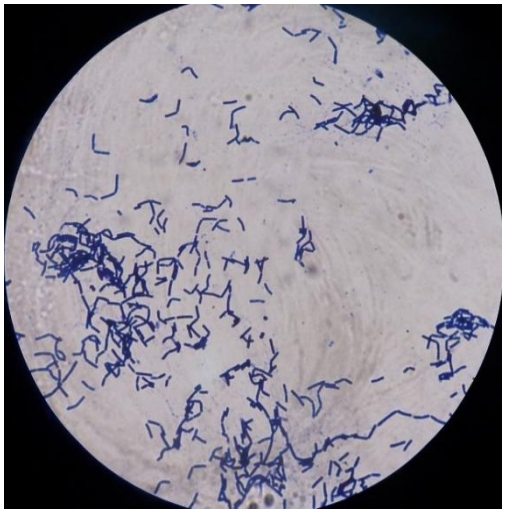


Plate 4.9: Gram positive large rod long chain *Bacillus spp.*

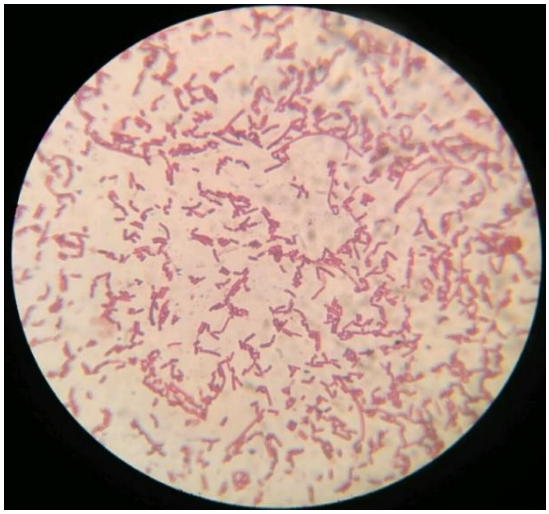


Plate 4.10: Gram negative large rod pink colour *E. coli*

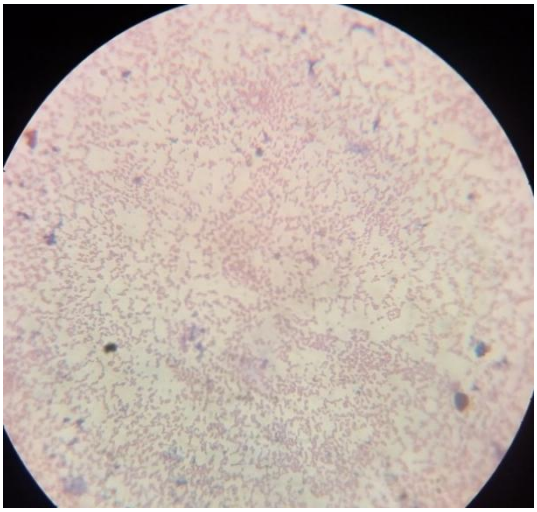


Plate 4.11: Gram negative Small rod pink colour *Salmonella spp*

4.3.3 Biochemical Results:

Table 12:Result of biochemical test Enrichment results of the representative test isolates

Serial No	OXI	CT	Ind	MR	VP	SC	TSI	MIU	SB	*Result*
1	-	+	+	+	-	-	YY	+	+	*E.coli*
2	-	+	-	+	+	+	YR	+	+	*Salmonella*
3	-	+	-	+	-	-	YR	+	_	*Staphylococcus spp*
4	-	+	-	-	-	-	YR	+	_	*Bacillus spp*

[+ = positive, - = negative, OXI= Oxidase, CT= Catalase, Ind= Indole, MR= Methyl Red, VP= Voges-Proskaur, SC= Simmons Citrate, TSI= Triple Sugar Iron, MIU= Motility Indole Urease, SB=selenite broth]

4.3.3.1 Oxidase test

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

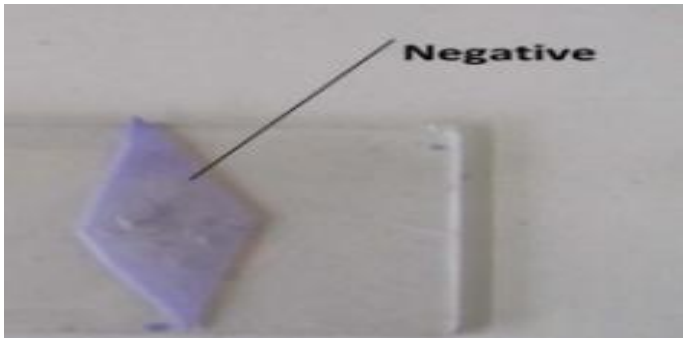


Plate 4.12 : Oxidase Test

4.3.3.2 Catalase test

All isolates were positive for catalase testwith gas bubble formation.

Plate 4.13 : Catalase Test

4.3.3.3 Methyl Red

The *E.coli, Salmonella, Staphylococcus* were positive and *Bacillus spp was negative for* methyl red test.

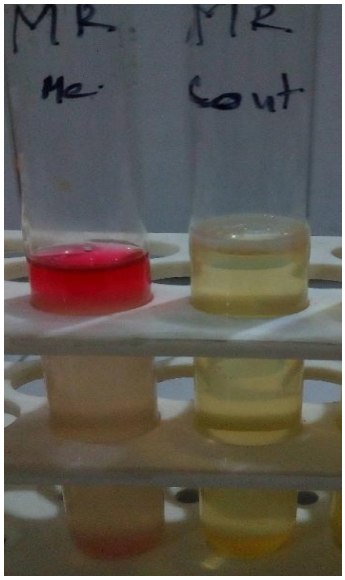


Plate 4.14 : **Methyl-Red test** for ***E. coli.*** indicated positive by the changed of medium to bright red colour inoculated (left) and control (right).

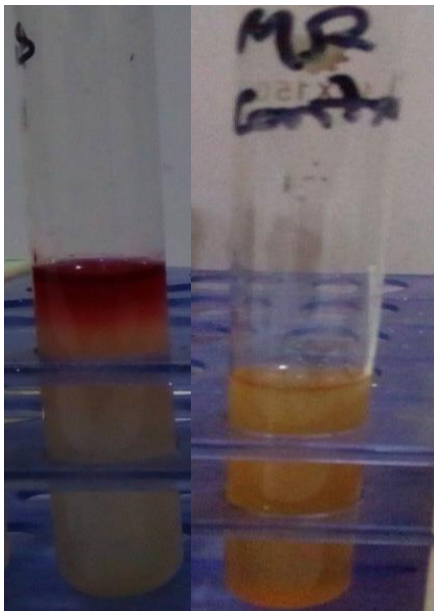


Plate 4.15 : **Methyl-Red test** for ***Staphylococcus spp*** indicated positive by the changed of medium to bright red colour inoculated (left) and control (right).

Plate 4.16: **Methyl-Red test** for ***Salmonella spp*** indicated by the changed of medium to bright red colour inoculated (left) and control (right).

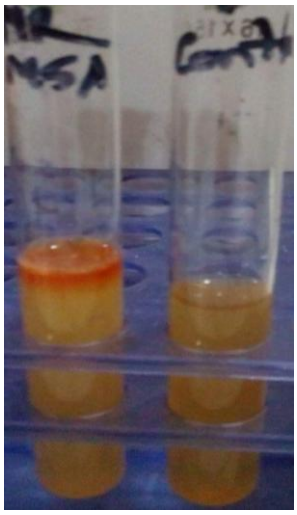


Plate 4.17: **Methyl-Red test** for ***Bacillus spp*** indicated negative by no changed of medium to bright red colour inoculated (left) and control (right).

4.3.3.4 Voges-Proskauer Test

The *E.coli, Staphylococcus spp, Bacillus spp* were negative and *Salmonella spp was positive for* voges-proskauer test

Plate 4.18: **Voges-Proskauer test** for ***E. coli*** showing negative result by no changed of medium to rose red colour inoculated (right) and control (left).

Plate 4.19: **Voges-Proskauer test** for ***Staphylococcus spp*** showing negative result by no changed of medium to rose red colour inoculated (left) and control (right).

Plate 4.20: **Voges-Proskauer test** for ***Bacillus spp*** showing negative result by no changed of medium to rose red colour inoculated (left) and control (right).

Plate 4. 21: **Voges-Proskauer test** for ***Salmonella spp*** showing positive result by the changed of medium to rose red colour inoculated (left) and control (right).

4.3.3.5 Indole Test

The *E.coli was* positive and *Salmonella, Staphylococcus Bacillus spp were negative for* indole test.

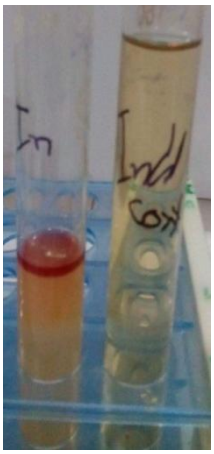


Plate 4.22: **Indole test** for ***E. coli spp*** showing positive result by red coloration of the medium colour inoculated (left) and control (right).

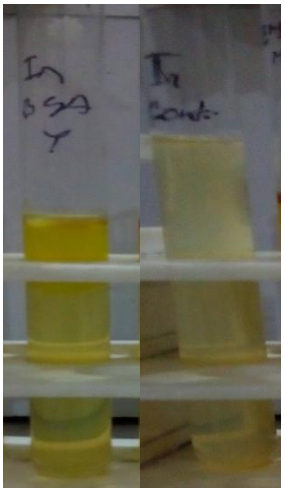


Plate 4.23: **Indole test** for ***Salmonella spp*** showing negative result by no red coloration of the medium colour inoculated (left) and control (right).

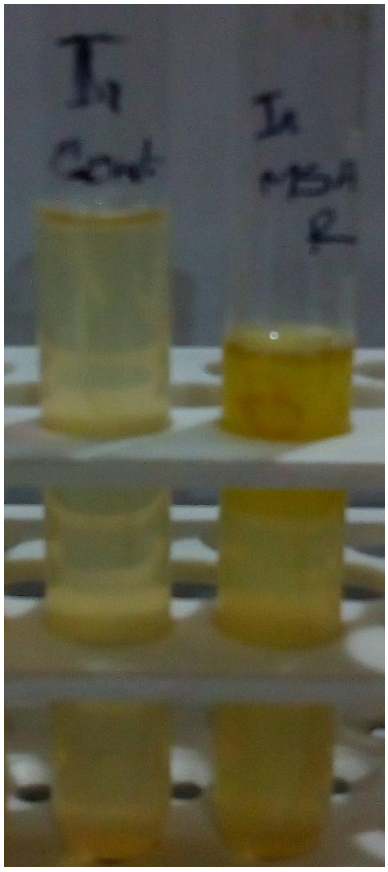


Plate 4.24: **Indole test** for ***Staphylococcus spp*** showing negative result by no red coloration of the medium colour inoculated (left) and control (right).

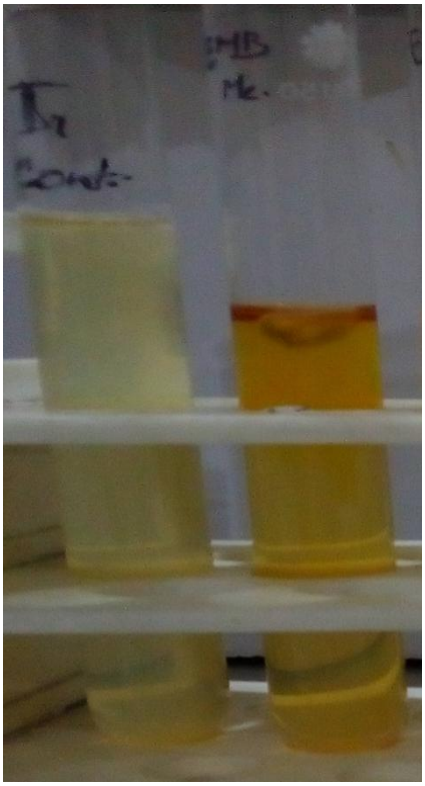


Plate 4.25: **Indole test** for ***Bacillus spp*** showing negative result by no red coloration of the medium colour inoculated (left) and control (right).

4.3.3.6 Simmons Citrate

The *E.coli; Staphylococcus spp & Bacillus spp* were negative and Salmonella spp was positive for simmons citrate test.

Plate 4.26: **Simmons Citrate test** for ***E.coli*** showing negative result by no changed of medium to blue colour inoculated (left) and control (right).

Plate 4.27: **Simmons Citrate test** for ***Staphylococcus spp***showing negative result by no changed of medium to blue colour inoculated (left) and control (right).

Plate 4. 28: **Simmons Citrate test** for ***Bacillus spp*** showing negative result by no changed of medium to blue colour inoculated (left) and control (right).

Plate 4. 28: **Simmons Citrate test** for ***Bacillus spp*** showing negative result by no changed of medium to blue colour inoculated (left) and control (right).

4.3.3.7 Triple Sugar Iron (TSI) Test

The *Salmonella spp* was positive and for TSI test.

Plate 4.30: **Triple Sugar Iron test** for ***E. coli*** showing yellow colour butt & yellow colour slant inoculated (left) and control (right).

Plate 4.31: **Triple Sugar Irontest** for ***Staphylococcus spp*** showing yellow colour butt & red colour slant inoculated (left) and control

Plate 4.32: **Triple Sugar Iron test** for ***Salmonella spp*** showing yellow colour butt & red colour slant with gas and H_2S production inoculated (left) and control (right).

Plate 4.33: **Triple Sugar Iron test** for ***Bacillus spp*** showing yellow colour butt & red colour slant with gas and H_2S production inoculated (right) and control (left).

4.3.3.8 Selenite Broth

The *E.coli, Salmonella spp* were positive and *Staphylococcus spp, Bacillus spp were negative for* selenite broth test

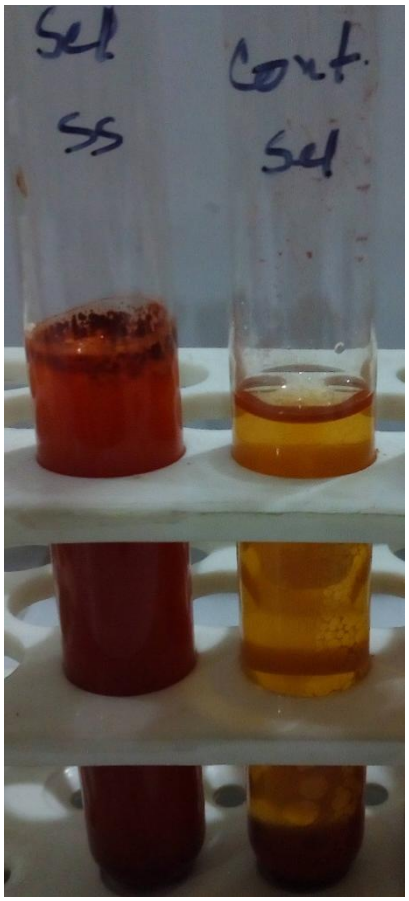


Plate 4.34: **Selenite Broth test** for ***Salmonella spp*** showing positive result by the changed of medium to blue colour inoculated (left) and control (right).

Plate 4.35: **Selenite Broth test** for ***Staphylococcus spp*** showing positive result by the changed of medium to blue colour inoculated (left) and control (right).

Plate 4.36: **Selenite Broth test** for *E.coli* showing positive result by the changed of medium to blue colour inoculated (right) and control (left).

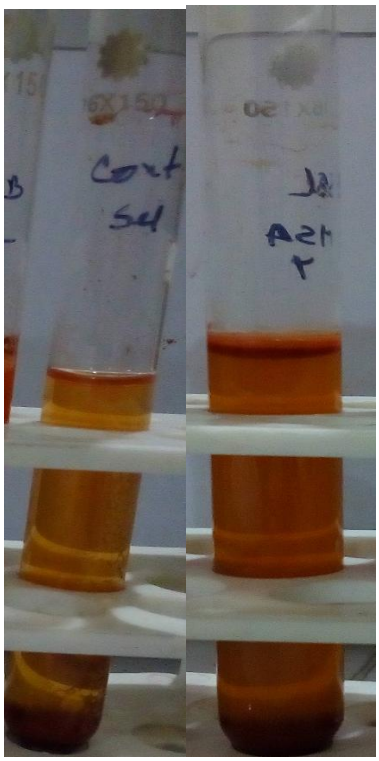


Plate 4.37: **Selenite Broth test** for ***Bacillus spp*** showing positive result by the changed of medium to blue colour inoculated (right) and control (left).

4.4 Result of Antibiotic Sensitivity Test

A total four isolates such as*E.coli* ,*Salmonella spp Staphylococcus SppBacillus spp* obtained from oropheryngial swab, clocal swab and feces samples of ostrich were subjected to Antibiotic Sensitivity assay shown in Table no 7,8,9,10. And figure..

Table 13: Antimicrobial profile of of *E.coli*

Antimicrobial agents	**Diameter of zone of inhibition(mm)**	**Interpretation**
Levofloxacin	17	S
Penicillin	-	R
Amoxicillin	-	R
Feridoxin	-	R
Cefxime	-	R
Chloramphenicol	-	R
Gentamicin	24	I
Nalidixic acid	-	R
Azithromycine	-	R
Eryhromycine	22	S
Tetracycline	-	R
Ceftriaxone	-	R
Vancomycine	15	S

Source: CLSIFDA- 2013 table –update pdf [Note: S=Sensitive, R=Resistant, I=Intermidate.]

Table 14: Antimicrobial profile of *Salmonella spp*

Antimicrobial agents	**Diameter of zone of inhibition(mm)**	**Interpretation**
Levofloxacin	-	R
Penicillin	-	R
Amoxicillin	-	R
Feridoxin	-	R
Cefxime	-	R
Chloramphenicol	-	R
Gentamicin	-	R
Nalidixic acid	-	R
Azithromycine	18	S
Eryhromycine	-	R
Tetracycline	-	R
Ceftriaxone	-	R
Vancomycine	-	R

Source: CLSIFDA- 2013 table –update pdf [Note: S=Sensitive, R=Resistant, I=Intermidate.]

Table 15:Antimicrobial profile *Staphylococcus Spp*

Antimicrobial agents	**Diameter of zone of inhibition(mm)**	**Interpretation**
Levofloxacin	18	S
Penicillin	-	R
Amoxicillin	-	R
Feridoxin	-	R
Cefxime	19	S
Chloramphenicol	-	R
Gentamicin	14	S
Nalidixic acid	-	R
Azithromycine	-	R
Eryhromycine	22	S
Tetracycline	-	R
Ceftriaxone	-	R
Vancomycine	17	S

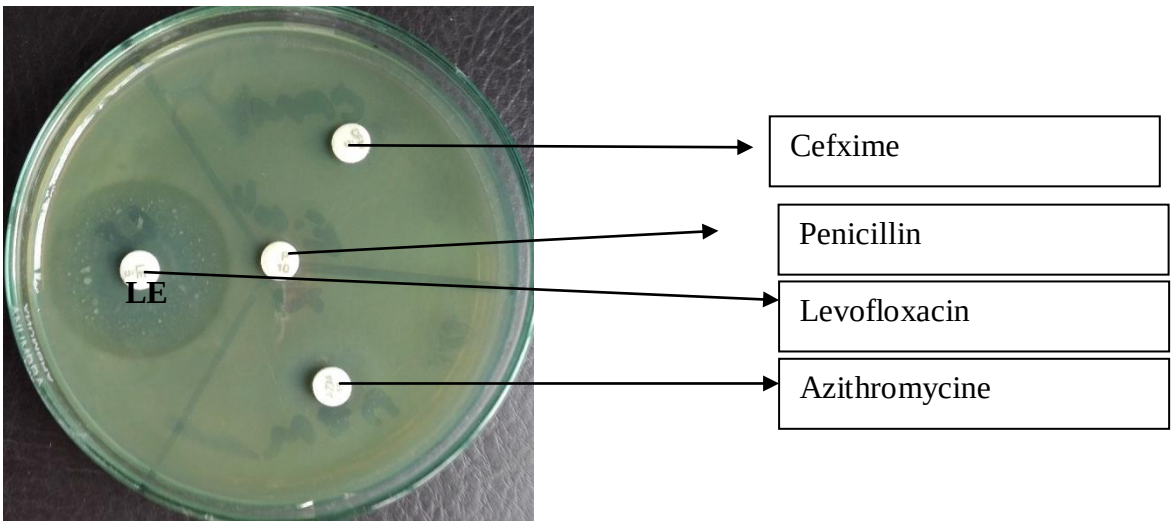
Source: CLSIFDA- 2013 table –update pdf [Note: S=Sensitive, R=Resistant, I=Intermidate.]

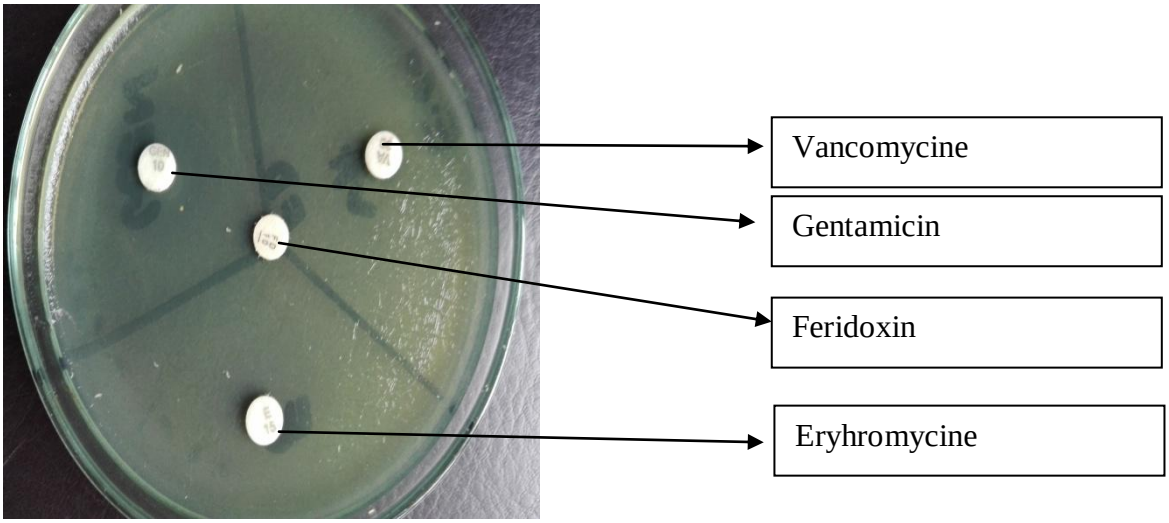
Table 16: Antimicrobial profile of *Bacillus spp*

Antimicrobial agents	**Diameter of zone of inhibition(mm)**	**Interpretation**
Livofloxacin	18	S
Penicillin	-	R
Amoxicillin	-	R
Feridoxin	-	R
Cefxime	19	S
Chloramphenicol	-	R
Gentamicin	14	S
Nalidixic acid	-	R
Azithromycine	-	R
Eryhromycine(E)	22	S
Tetracycline	-	R
Ceftriaxone	-	R
Vancomycine(VE)	17	S

Source: CLSIFDA- 2013 table –update pdf [Note: S=Sensitive, R=Resistant, I=Intermidate.]

E. coli





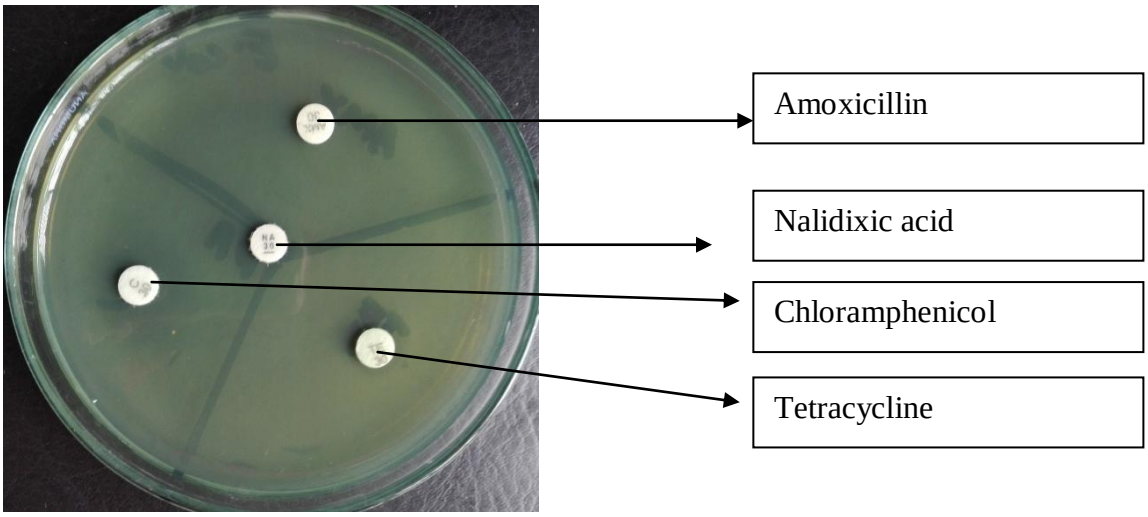


Plate 4.38: Antibiogram test of *E.coli*

Salmonella spp.

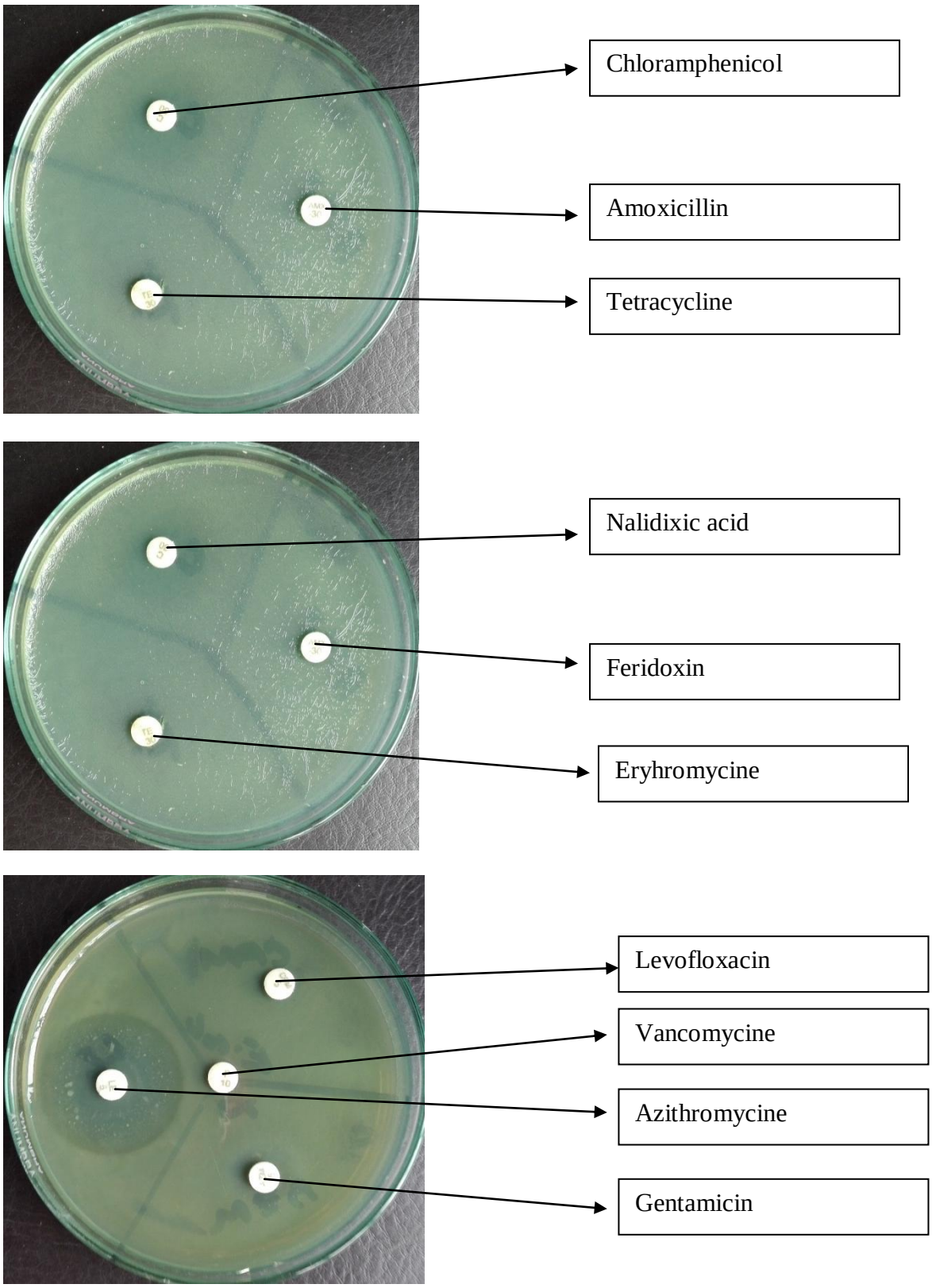


Plate 4.39: Antibiogram test of *Salmonella spp.*

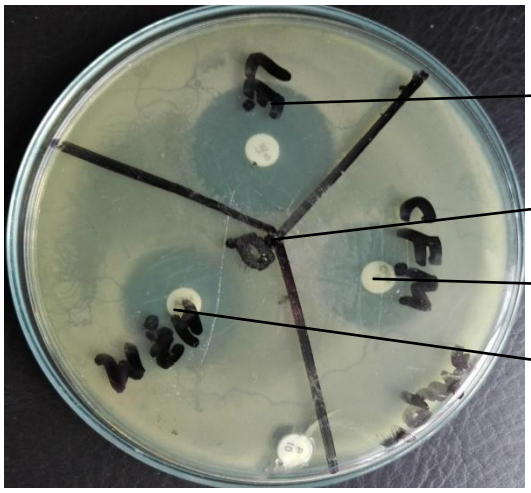
Staphylococcus spp

Vancomycine

Feridoxin

Gentamicin

Eryhromycine



Levofloxacin

Penicillin

Cefxime

Azithromycine

Tetracycline

Nalidixic acid

Amoxicillin

Chloramphenicol

Plate 4.40: Antibiogram test of *Staphylococcus spp.*

Bacillus spp

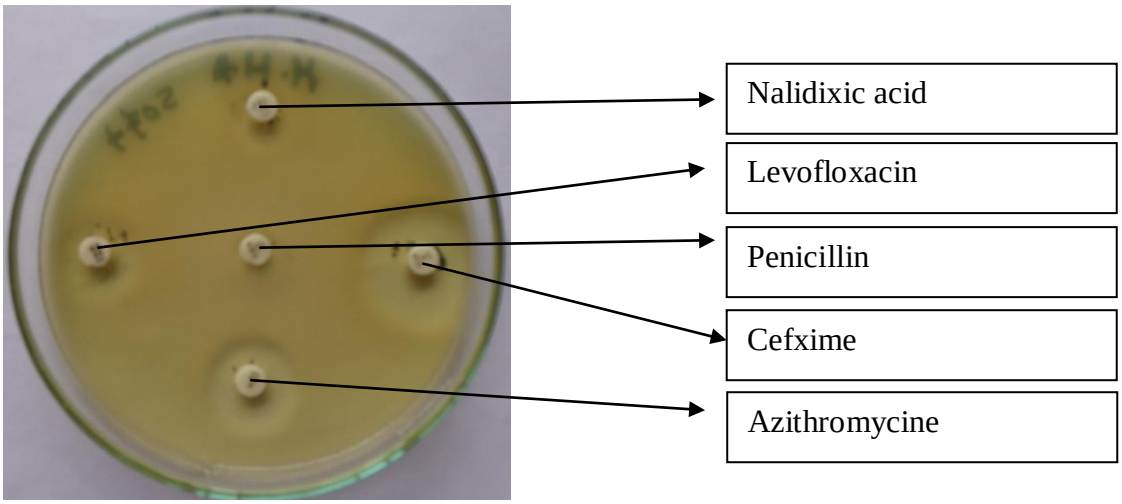


Plate 4.41: Antibiogram test of *Bacilus spp.*

Chapter 5

DISCUSSION

Ostrich farming system is not common in Bangladesh . At HSTU ostrich farms they fed many type of food items like vegetables, leaves, drink, tubewell water which are may be contaminated with different type of microorganisms leading to frequent infections. Of my known knowledge, this was the first study about ostrich in Dinajpur, Bangladesh to monitor the microbial assessment in oropharyngeal swab , cloacal swab and fecal samples of ostrich and also update knowledge on antimicrobial sensitivity of isolated organisms .A total of 75 samples comprising orapharyngeal swab(25), cloacal swab (25) and feaces (25) collected from different age of ostriches and were submitted to microbiology laboratory for analysis.

In my study the result of total viable counts were expressed by CFU (colony forming unit).In every case the average CFU was counted at 30 days interval. The mean colony counts in orapharyngeal swab, cloacal swab and feces samples were $1.36\times10^{9}\pm0.38$, $1.28\times10^{9}\pm$ 0.34 and $1.48\times10^{9}\pm0.27$ respectively. The present findings revealed that the total viable count recorded in oropharyngeal swab and cloacal swab shown higher in three (3) months age group and gradually lower in 7 months age. On the other hand the results of total viable count was increased in feces samples with the increased age of ostrich.

In a previous study there were very little information about total viable colony count in ostrich and the result a gred with (Cook *et al*. 1997). in Newzeland detected a maximum areas viable count was log 2.11 for ostrich carcass. But (Sofos *et al*. 1999b) in USA shown that counts was about log 6.0 for ostrich carcass. Gill *et al*. (2000) estimated log mean APC numbers of ostriches and emus were greater than the corresponding values estimated for beef carcasses. In this the results shown that ostriches were potential reservoirs for *salmonella* spp. *E. coli* spp. *Staphylococcus* spp. and *Bacillus spp*. that, these bacteria are important pathogens for human and other animals .

Among seventy five (75) samples, 29 (38.66%) was *Escherichia coli*, 21 (28%) *Salmonella spp, 20 (26.67%) Staphylococcus spp.* 5 (6.6%) *Bacillus spp*. Were identified. Among 25

oropharyngeal swab samples, *Staphylococcus spp* was found higher 15 (60%) than other organisms but *Escherichia coli* were found higher both in cloacal 11(44%) and faecal 12(48%) samples. In my study overall cultural prevalence of *E. coli* isolates 29 (38.66%) which was lower than the observation of Gill*et al*. 1996b, Sofos *et al*. 1999b, Ingham & Schmidt (2000). 43% (13/30) and 53% (9/17) *E. coli* were positive for ostrich carcasses during processing and post-evisceration. . *E. coli* was the most frequently isolated bacterium in the study. This finding correlates with the observation that *E. coli* is the predominant enteric bacteria isolated from ostrich chicks suffering from enteritis (Verwoerd *et al*., 1998).*E. coli* is known to form part of the normal intestinal flora (Levine, 1987) .

In my study overall cultural prevalence of *Salmonella spp, 20 (26.67%).*More(1996); Welsh et al., 1997b; Huchzermeyer, 1998; Verwoerd et al., 1998) studied in 3 different serotypes of *Salmonella* identified isolated from ostriches. (More, 1996).Cooper RG(2005) shown that meat-producing farm animals, including poultry, pigs and ostriches, can be carry different types of microbes specially *Salmonella, E.coli,* and can shed them fecally without any signs of disease. From above findings it is closely related to our study. The lower GIT of most animal species including poultry and ostriches is normally populated by large numbers of microorganisms.(Freitas Neto DE. *et al* 2009).

Concerning the occurrence of *S. aureus* which is not usually detected in the intestinal tract of ostrich. Notermans *et al*. (1982) but in my study Staphylococcus *spp*. 3(12%) observed in cloacal swab. Grau (1979), in which the evisceration process did not contribute significantly to a high aerobic count. In the present study overall cultural prevalence of 5 (6.6%) *Bacillus spp* are detected which was little lower than 11(5.5%) *Bacillus spp.* by Hassan G. C*et al.* (2016).Regarding to the above mentioned points, intestinal microbiota are referred to as commensal as they coexist without initiating inflammatory or infectious responses. It is becoming clear that these bacteria provide at least three key functions in the poultry intestine including epithelial cell health, nutrient metabolism and breakdown, and indirect mucosal defense against pathogenic bacterial strains .(Barnes EM *et.al,*1972).

Household, workers, veterinarian and persons with specific medical conditions such as a chronic illness, immunodeficiency and pregnancy may be at higher risk of developing disease or complications from a zoonotic bacterial disease by contact with poultry and ostriches at the household and the industrial level. The obtained results indicated that ostrich excreta is one of

the most important sources of *Escherichia coli, Staphylococcus spp, Salmonella spp, Bacillus spp*. in ostrich farms.

On antibiogram study isolated *Escherichia coli* were more sensitive to Levofloxacin, while more resistant to Tetracycline, Penicillin, Chloramphenicol, Erythromycin, Feridoxin, Amoxicillin, Gentamicin, Ceftriaxone, Nalidixic acid, Azithromycine , Vancomycine. S. Sahinduran (2004) Stated in his research isolated *Escherichia coli* were the most susceptible to amoxycillin and clavulanic acid combinations.

In my study isolated *Salmonella spp* were sensitive to Azithromycine, while resistant to Tetracycline, Penicillin ,Chloramphenicol, Erythromycin, amoxicillin, Amoxicillin, Gentamicin, Ceftriaxone, Nalidixic acid, Levofloxacin and Vancomycine. But in a previous study by Yadav, Saroj Kumar *et al.*(2017) at Chittagong, Bangladesh *Salmonella* isolates from ostriches were foundresistance to amoxicillin, ampicillin, oxytetracycline, cotrimoxazole, azithromycin and erythromycin followed by colistin sulfate 83.33%, pefloxacin 38.88%, enrofloxacin 38.88%, gentamycin 11.1% and Ceftriaxone 0% which was cent present relevant to the study. Antibiogram was applied on *Salmonella* positive samples for 12 different antimicrobials. Cent percent resistance was found to amoxicillin, ampicillin, oxytetracycline, cotrimoxazole, azithromycin and erythromycin followed by colistin sulfate 83.33%, pefloxacin 38.88%, enrofloxacin 38.88%, gentamycin 11.1% and Ceftriaxone 0%.
In a previous study by Hassan et al. (2014) at Chittagong, Bangladesh. *Salmonella* isolates from layer poultry were found 100% resistant to amoxicillin and tetracycline followed by enrofloxacin (87.5%), ciprofloxacin (87.5%), pefloxacin (87.5%), doxycycline (50%), colistin (50%) and Kanamycin (50%) and isolates showed high sensitivity (100%) to gentamicin and neomycin are similar to our findings. In my study isolated *Staphylococcus spp*. and *Bacillus spp* were more sensitive to Levofloxacin, Azithromycin, Cefxime, Erythromycin, Vancomycine and Erythromycin respectively while more resistant to Tetracycline, Penicillin ,Chloramphenicol, amoxicillin and amoxicillin respectively. S. Sahinduran (2004) also state that on his antibiogram results, isolated microbial agents were the most susceptible to amoxycillin and clavulanic acid combinations.

In my study, a range of bacterial flora was isolated from the samples, indicating the presence of these organisms in the healthy ostriches that living in HSTU ostrich farm. These findings

was in accordance with some report about birds and ruminant that reported as potential pathogens for humans and animals. (Bengmark S.1998).

In order to obtain the appropriate microbial assessment in different samples of ostrich, this study should be continued by high population in different farms with defined variable into the future.

Chapter 6

CONCLUSION

The results collected during this study indicate that ostriches are susceptible to a number of microbial agents which are common to other avian species. Ostrich oropharyngeal swab is one of the most important sources of *Staphylococcus spp,* cloacal swab and feaces samples are the sources of *E.coli and Salmonella* spp, few percentage of *Bacillus spp* found every sample in ostrich farm. These isolated organisms may be transmits to human. The prevalence of these microorganism in ostrich environment depend mainly on the degree of the hygienic measures used in each farms. Poor sanitation and handling of captive ostrich could be a source of contamination. Antibiotic resistance of isolated organisms are also considered as an important problem. Proper choice of antimicrobials and strict bio-security measure should be relevant for the ostrich farms. As ostrich farming is gaining attention as an emerging industry in Bangladesh, therefore this type of study will be beneficial for the stockholders and prescribers and also ostrich owners. Finally, it may be concludes that the logical use of antibiotics must be adopted in ostrich farms reared in Bangladesh for prevention the appearance of multi drug resistance bacteria. Moreover proper measures should be taken to ward off zoonotic diseases in peoples who are related to ostrich farming.

Further study in connection with this research work might be:

- Characterizations of isolated *Salmonella spp* serotypes from different organs and swabs of ostriches.
- Molecular differentiation of isolated *Salmonella spp* by PCR.
- Development of vaccine against isolated *Salmonella spp*.

References

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