ISOLATION, IDENTIFICATION & ANTIBIOGRAM OF BACTERIA ASSOCIATED WITH CAT ORAL CAVITY: A NEGLECTED ISSUE

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JUNE, 2020

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REGISTRATION NUMBER: 13-05410

A Thesis Submitted to the Faculty of Animal Science and Veterinary Medicine
Sher-e-Bangla Agricultural University, Dhaka-1207,
In Partial Fulfillment of the Requirements for the degree of

MASTER OF SCIENCE (MS) IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

SEMESTER: JANUARY-JUNE/2020

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CERTIFICATE

*This is to certify that the thesis entitled "**Isolation, Identification & Antibiogram of Bacteria Associated with Cat Oral Cavity: A Neglected Issue**" submitted to the department of Microbiology and Parasitology, faculty of Animal Science & Veterinary Medicine, Sher - e - Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka - 1207, in partial fulfillment of the requirements for the degree of **Master of Science (MS) in Microbiology**, embodies the result of a piece of bona fide research work carried out by **Shamima Ahmed**, registration no. : **13-05410**, under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

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Dedicated to

My Beloved Parents

ACKNOWLEDGEMENT

At the beginning, the author bows the grace and mercy of the "Almighty Allah", who enabled her to complete this research. The author must express her grateful indebtedness to her beloved father, ***Rashid Ahmed*** *and mother,* ***Ful Nahar Begum*** *for their ending prayer, encouragement, sacrifice and dedicated supports to educate her this level.*

The author with a sense of respect, expresses heart gratitude to her Supervisor ***Dr. Uday Kumar Mohanta****, Chairman & Associate Professor, Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh, for his supervision, untiring guidance, invaluable suggestions, timely instructions, inspirations and constructive criticism throughout the tenure of research work.*

The author with a sense of respect expresses her gratitude to her Co-Supervisor ***Dr. Jahangir Alam****, Chief Scientific Officer (CSO), Animal Biotechnology Division, National Institute of Biotechnology (NIB), Ganakbari, Ashulia, Savar, Dhaka-1349, Bangladesh, for his untiring guidance, relentless co-operation, invaluable suggestions, and constructive criticism throughout the study. The study was supported by a KrishiGobeshona Foundation (KGF) financed project entitled "Study on zoonotic diseases of pets and assessment of risk factors for human for commonly occurred zoonoses from pet: Implication for better management" awarded to Dr. Jahangir Alam.*

The author is also grateful to ***Mirza Synthia Sabrin****, Assistant Professor, Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka- 1207, for her kind advice and co-operation in the completion of the study.*

The author is grateful to ***Dr. Forhad Hossain,*** *former Chief Veterinary Officer, CVH, Dhaka-1200, for his kind support and co-operation during sample collection.*

The author is ever grateful to Ministry of Science and Technology, Government of the People's Republic of Bangladesh for providing the NST fellowship.

The author is also boundless grateful to the staffs of the Animal Biotechnology Division and Environmental Biotechnology Division, NIB; the staffs of the Central Veterinary Hospital, Dhaka and the staffs of the Department of Microbiology and Parasitology, SAU for their cooperation. The author expresses her boundless gratefulness to her brothers, relatives, friends and senior fellows for their moral support and encouragement a lot in the process of building the academic career which can never repaid.

The Author

ISOLATION, IDENTIFICATION & ANTIBIOGRAM OF BACTERIA ASSOCIATED WITH CAT ORAL CAVITY: A NEGLECTED ISSUE

ABSTRACT

This research work was conducted to isolate, identify and antibiotic sensitivity profiling of bacteria found in oral cavity of pet cat in Dhaka city. A total number of 40 samples were collected aseptically from Central Veterinary Hospital (CVH), Dhaka and transported to National Institute of Biotechnology laboratory, Savar. Total viable count (TVC) of bacteria from all samples (n = 40) was determined. Hundred percent prevalence of organisms was noted with the highest TVC, 1.199×10^{13} and the lowest TVC, 3.60×10^{2}. The isolation and identification of bacterial genera/species were performed by cultural characteristics, Gram's staining, biochemical tests and molecular identification to some extent. The prevalence of *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Staphylococcus epidermidis* were 100%, 5 %, 57.5% and 7.5 % respectively. Both pathogenic and non-pathogenic *E. coli* were confirmed by PCR and DNA sequencing. Antibiotic sensitivity test by disc diffusion method was performed against seven different antibiotics. *E. coli* isolates showed the highest sensitive to gentamycin (80%) followed by azithromycin (70%) and the highest resistant to ampicillin. Isolates of *Salmonella* isolates were found to be highest sensitive against gentamycin (100%) followed by azithromycin (50%). Highest resistant pattern of *Salmonella* spp. was showed against ampicillin (100%). *Salmonella* spp. showed 50% resistant to erythromycin, streptomycin and tetracycline. *S. aureus* showed the highest sensitivity to gentamycin (80%), followed by co-trimoxazole (60%) and the highest resistance pattern was shown against ampicillin (100%), followed by erythromycin (80%) and tetracycline (60%). *S. epidermidis* showed the highest (100%) resistant against ampicillin and the highest (100%) sensitive to gentamycin. The findings from current study recommend that pet cats in Dhaka city contain multi-drug resistant *E.coli, Salmonella* spp., *S. aureus.* Only *S. epidermidis* was not found as multidrug resistant. This multi-drug resistant phenomenon can cause a potential public health hazard through transmission to humans by direct contact or the food chain or the evolved way of life.

Keywords: TVC, prevalence, antibiogram, multi-drug resistant, public health hazard

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LIST OF ABBREVIATIONS

ABBREVIATION	FULL WORD
µg	Microgram
µl	Microliter
AMP	Ampicillin
Approx.	Approximately
AZM	Azithromycin
CFU	Colony Forming Unit
COT	Co-trimoxazole
DNA	Deoxyribonucleic acid
DX	Dextrose
E	Erythromycin
E. coli	*Escherichia coli*
EMB	Eosin Methylene Blue
ESBL	Extended-Spectrum Beta-Lactamase
et al.	and others
etc.	Etcetra
G	Glucose
GEN	Gentamicin
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen Sulphide
hrs.	Hours
IN	Intermediate
L	Lactose
Lbs	Pound
Ltd.	Limited
MC	MacConkey
MH	Muller Hinton
Min.	Minute
ML	Maltose
Ml	Milliliter
MN	Mannitol
MS	Mannitol Salt

ABBREVIATION	FULL WORD
NIB	National Institute of Biotechnology
NO.	Number
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
R	Resistant
S	Sensitive/ Streptomycin
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SAU	Sher-e-Bangla Agricultural University
SC	Simmons Citrate
Spp.	Species
SS	Salmonella-Shigella
TE	Tetracycline
TVC	Total Viable count
V-P	Voges-Proskauer

CHAPTER 1

INTRODUCTION

Rearing pet animal has the significance in the history of human civilization. In Bangladesh, people have been keeping cats and dogs as pet animals since early days. But most of them were not fully indoor animals rather they were more outdoors/stray. Currently, the tendency of keeping pet as indoor animal is increasing significantly day by day. But local breeds or stray pets are also very common both in urban and rural areas. At present, almost all classes of people have pet. Among all pet animals, cat is the most popular pet in Bangladesh. Now-a-days, people live in nuclear family. Therefore, the senior citizens and young people mostly pass their time alone. They certainly need continuous and safe company. Hence, in upcoming days the number of cat in home will be increased gradually, and the scenario matches with other countries as well. Owners treat their cats like other family members. Cats supports the owners emotionally, and relives their stress. Owners play with their cats and look after them happily which is indirectly a pleasant source of physical and mental exercise. The owners often kiss their cats, share food and bed with cats in day to day life. People are often aware of fecal and urine contamination from cat, but most of the time the owners are not much conscious of hygiene in case of cat saliva infection. Previous study showed that, cat oral cavity contained a huge number of bacteria, more than 20 types of bacteria. The bacteria did not affect human severely, that means zoonotic importance was comparatively less. On the other hand, only from cat scratch disease approximately 25000 people affected annually in the United States and almost 12000 people die (Jackson *et al.*, 1993). Hence, Cat's oral swab might be a potential source of infections.

Again, sometimes cat bite may cause mild or serious wound developed on surface of skin. However, delayed presentation may lead to significant morbidity or even mortality. It is recommended that a deep cleaning of the wound and the application of a broad spectrum antibiotic therapy is essential after a cat bite, as animal bites potentially deliver a polymicrobial infection (Talan *et al*., 1999; Weber *et al*., 1984; Wilson and Ho, 2013). The importance of a prompt antibiotic administration must be considered seriously with bacterial contamination. So, identification of the antibiotic sensitivity is essential for choosing the correct antibiotic for effective treatment.

Antimicrobial resistance is one of the main causes of failure in antimicrobial therapy. This mechanism of survival presented by the microorganisms occur naturally or can be acquired. However, acquired resistance is more important due to the fact that it limits viable options of drugs. In recent years, interest in antimicrobial resistance in companion animals has also increased. This is due, in part, to an increasing number of reports of companion animals infected or colonized with clinically and epidemiologically important multiple-drug resistant organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Vengust *et al*., 2006; Moodley *et al*., 2006). A comprehensive epidemiological research of antimicrobial resistance in animals should involve the investigation of 3 areas: veterinary pathogens, zoonotic pathogens, and indicator bacteria (Caprioli, 2000). The study of zoonotic pathogens, such as *Salmonella* spp. and MRSA, in companion animals provides information of special relevance to public health. Companion animals have been a reservoir of *Salmonella* spp. for humans through direct contact with pets, contact with feces from pets, preparation of raw meat and bones for pet consumption, and the handling of commercial pet treats (Cherry et al., 2004). Similarly, pets have been colonized and infected with MRSA and have acted as reservoirs of infection for their human contacts (Manian, 2003). The number of multi-drug resistant *E. coli* are continuously increasing although various antimicrobial agents are being used (Hussain *et al*., 1982). Uncontrolled use of antibiotics in medicine and animal husbandry for both treatment and prevention of bacterial diseases over the course of decades has fostered the selection of resistant bacteria (Tomasz, 1994 and Singer *et al*., 2003).

The popularity of cat as pet has already been increased all over the Bangladesh and it will be more popular in upcoming days. Therefore, the chances of spreading infectious diseases from cat will be increased as well. Again, the number of outdoor pet cat is more common than indoor cat in Bangladesh. If the cat is completely indoor since birth, the chances of infection might be less but it is not certain. Therefore, like feces, urine, fur, cat saliva etc. may be a direct or indirect source of human infections. A wider awareness of this problem may be useful in order to prevent life-threatening conditions after cat bite. A study found that, approximately 28% to 80% of cat bites are source of infections and most clinically infected cat bite wounds are mixed infections of aerobic and anaerobic bacteria (John, 2006).

Unfortunately, there has been only three studies (two survey and one parasitological) but no microbiological research on cat yet in Bangladesh. Therefore, it is essential to study the bacteria found in cat in Bangladesh. Hence, bacteria identification from oral cavity of pet cat has been chosen for the current study.

Considering the above facts, this study was carried out with the following objectives:

I. To isolate and identify the prevalence of bacterial flora in cat's saliva.
II. To investigate the antibiotic sensitivity patterns of the isolated bacteria.

CHAPTER 2

REVIEW OF LITERATURE

Isolation, characterization and antibiotic sensitivity determination of the bacteria observed in oral swabs of companion cat was performed using the knowledge gathered from the subsequent related review of literature.

2.1 Cat and Public health relation

Domestic cats (*Felis catus*) are a common household pet and also a notorious invasive species around the world. Because cat numbers have been increasing in many locations it is critical to work on management solutions that help to reduce threats posed by cats. With regard to cat behavior, one of the threats both to cats themselves and the species that they interact with is disease transmission. As part of a broader overview on applying cat behavior to management the focus of this review is to consider different types of cat behaviors and highlight how they relate to disease as a means to help inform management. Specifically, a research focused on cat movement, foraging, and cat–human interactions as broad classes of cat behavior that can lead to acquisition and transmission of diseases. In addition, they review the diseases that are commonly harbored by cats, are of growing human health concern, and for which we have reasonable information. Finally, they review the main forms of cat management in order to provide a set of recommendations for use in addressing cat diseases, such as, many diseases found in cats are zoonotic and of concern to human health; cats engage in behaviors that can lead to disease acquisition and transmission (Lepczyk *et al*., 2015).

Molecular evidence is restricted for the hypothesis that humans, dogs, and cats can become colonized and infected with similar virulent *E. coli* strains. To further assess this possibility, archived *E. coli* O6 isolates (n = 130) from humans (n = 55), dogs (n = 59), and cats (n = 16), representing the three main H (flagellar) types within serogroup O6 (H1, H7, and H31), were analyzed, alongside selected reference strains. Isolates underwent PCR-based phylotyping, multilocus sequence typing, PCR-based detection of 55 virulence-associated genes, and XbaI pulsed-field gel electrophoresis (PFGE) profiling. Three major sequence types (STs), which corresponded closely with H types, accounted for 99% of the 130 O6 isolates. Each ST included human, dog, and cat isolates; two included reference

pyelonephritis isolates CFT073 (O6:K2:H1) and 536 (O6:K15:H31). Virulence genotypes overlapped considerably among host species, despite statistically significant differences between human and pet isolates. Several human and dog isolates from ST127 (O6:H31) exhibited identical virulence genotypes and highly similar PFGE profiles, according to cross-species exchange of specific *E. coli* clones. The close similarity within the genomic backbone and virulence genotype between certain human- and animal-source *E. coli* isolates within serogroup O6 supports the hypothesis of zoonotic potential (Johnson *et al*., 2007).

There is no dispute over the role of *P. multocida* in cat-bite infections of man **(**Tindall and Harrison, 1972) from which the organism can be isolated in pure culture. The many studies in which modern anaerobic-culture techniques were used have revealed the diversity of human infections that are caused by anaerobes or mixtures of anaerobes (Bartlett and Finegold, 1974; Wren et al., 1977). In such human infections, gram-negative anaerobic bacilli were the most common isolates and *B. fragilis* was the species most commonly encountered. In cat abscesses it was found that the species isolated most frequently was Pepto- streptococcus anaerobius. This may reflect the predominance of anaerobic gram-positive cocci in the normal flora of the oral cavity. (Love *at el*, 1990)

Studies on the normal flora of the human oral cavity (Sutter, 1974) have revealed the following anaerobes, listed in order of prevalence: cocci, gram-negative bacilli (*Bacteroides* spp. and *Fusobacterium* spp.), gram-positive non-spore forming bacilli and clostridia. The normal flora of the feline mouth has not been described clearly due to insufficient knowledge, but it is assumed that it is likely to bear some resemblance to that of the human mouth.

Bartonella spp. are vector-borne blood-borne pathogens that have mainly been recognized within the last 30 years as a source of human zoonoses, especially for *Bartonella henselae*, the agent of cat scratch disease (Chomel and Kastel, 2010).

2.2 Cat diseases prevalence in Bangladesh

In Bangladesh there was a research conducted on pet disease where cat was also included. Researchers observed that among 200 pet cats 145 cats were diseased and a total of 5 categories of diseases were recorded. The prevalence of the diseases in one

year study period from high to low rates included- Skin Diseases, Salmonellosis, Conjunctivitis, Feline panleukopenia (FPL) and Toxoplasmosis (Runa *et al.*, 2016)

Another study was done on 361 cats in Chattogram, Bangladesh. This study reported that the endoparasitic infestation was highly prevalent in cats (91.53%) significantly ($p = 0.003$), which were ≤1 year of age. Prevalence of wound in cats were substantially higher (p=0.05) in the winter (Hasib *et al*., 2020).

A period of two months cross sectional prospective study was conducted at Central Veterinary Hospital, Dhaka to estimate the prevalence of clinical conditions in dogs and cats from June to July 2014 where 150 (25%) cats were observed with different clinical conditions. In that study prevalence of clinical conditions was analyzed on the basis of age, sex and breed. It was revealed that 103 (68.67%) cats occupied in medicinal cases followed by surgical cases 24 (16%) in cats and vaccination and health checkup 23 (15.33%) in cats. Among of the medicinal cases special sense organ diseases occupied highest prevalence 25 (16.67%) in cats. Another prevalence of non-infectious diseases in exotic breed and male cats was higher ($P \leq 0.05$). These findings address the vaccination practice in cats, variation of management within different topography in Dhaka and socio economic condition of owners (Sarker *et al.*, 2015)

2.3 Bacterial prevalence in cat

A study was carried out to identify the various bacterial species in the oral cavity of cats in two human hospitals in Sokoto, Nigeria. The buccal cavities of 26 cats (14 from Hospital A and 12 from Hospital B were liberally swabbed for bacterial evaluation. The samples were enriched in peptone water, inoculated on McConkey and Blood agar, and incubated aerobically at 37°C for 24hrs. The isolates were Gram stained and subjected to biochemical characterization for identification. A total of 51 bacterial isolates were made. There were *Staphylococcus aureus* 18 (35.3%), *Micrococcus* spp. 9 (17.7%), *Pasteurella* spp. 5 (9.8%), *Streptococcus* spp. *5 (9.8%)*, Yersinia spp. 4 (7.8%), *Bacillus* spp. 4 (7.8%), *Listeria* spp. 3 (5.4%), and *Corynebacterium* spp. 3 (5.9%). *Staphylococcus aureus* has the highest frequency of isolation 18 (35.3%) (Magaji *et al*., 2008).

In another study 10 swabs were taken from the buccal cavities of some domestic cats and the following bacteria were isolated from cats: *Staphylococcus aureus*, *Bacillus* spp., *Clostridium* spp., *Pseudomonas aeruginosa, Pasteurella multocida* and *Citrobacter* spp. (Umaru *et al*., 2002).

Thirty-six closed abscesses in the subcutis of cats were examined in a study. 168 bacterial strains isolated, 121 (72 %) were anaerobes and 47 (28 %) were facultative anaerobes. Twenty-six abscesses contained mixtures of facultative anaerobes and anaerobes, six contained anaerobes only and four contained facultative anaerobes only. Bacteroides was the genus most commonly isolated (28.6 % of all isolates) followed by Fusobacterium (19.0 %) and *P. multocida* (13.1 %). *Peptostreptococcus anaerobius* was the most commonly isolated anaerobic species (13.2 % of anaerobic isolates and 9.5 % of all isolates) and *P. multocida* was the most commonly isolated facultative anaerobe (46.8 %; 13.1 % of all isolates) (Love *et al*., 2006).

Microbiota of periodontally healthy cat were distinguishable from diseased cats. Most of the genera known to be related to periodontitis were also identified in healthy cats, they were present at significantly lower relative abundance. Remarkably, alpha diversity was found to be higher within the disease groups compared to healthy animals. The complexity of the subgingival microbiota of the house cat and reveal both differences and similarities among periodontally healthy and diseased cats (Rodrigues *et al*., 2019).

Cat Samples from the gingival margins of 14 cats considered normal on clinical examination were cultured for facultative and obligate anaerobic bacteria. All mouths were free from any gingival marginal inflammation and tartar build-up; all cats were between 6 and 12 months aged. A mixed growth was obtained from all samples. The mean number of bacterial species per sample was 10.7 with a variety of 7–16 isolates. Of the 150 isolates processed, 109 (72.66%) were obligate anaerobes. Of the facultatively anaerobic species, *Actinomyces* (including A. viscosus, A. hordeovulneris and A. denticolens) comprised 12%, *Pasteurella multocida* 9.33% of isolates and *Propionibacterium* species 6% of all isolates. Gram-negative bacilli belonging to the genera *Bacteroides* and *Fusobacterium* were isolated from 12 of the 14 samples, and comprised 77% of the obligate

anaerobes isolated. *Clostridium villosum* comprised 10.1% of obligately anaerobic isolates, *Wolinella* species made up 6.42%, while 4.58% were *Peptostreptococcus anaerobius*. The foremost commonly isolated obligate anaerobic species was *C. villosum* and therefore the most ordinarily isolated facultative anaerobic species was *P. multocida*. These findings show a bacterial flora of the traditional feline mouth which is extremely similar in composition thereto of cat fight abscesses and feline pyothorax (Love *et al*., 1990).

Any kind of puncture wounds, especially from cats, frequently become infected with various bacterial species. These include *Staph. aureus*, *Staph. intermedius*, *Strep. pyogenes*, *Strep. canis*, *Strep. oralis*, *Corynebacterium spp*., *Listeria* spp. and *Pasteurella multocida* (Barrow and Feltham, 1993).

Usually in research cats are routinely ignored as a possible source of *salmonella* infection. But over a period of 18 months, 142 cats received from commercial vendors to be used in research were screened for enteric *Salmonella*. *Salmonella* was isolated from 15 animals, an incidence of 10.6%. Five (29%) of the 17 shipments contained animals that were positive for Salmonella. The serotypes isolated were *Salmonella derby, Salmonella typhimurium, Salmonella anatum, Salmonella enteritidis and Salmonella bredeney* (Fox and Beaucage, 1979).

The presence and variety of *staphylococcus* spp. in healthy domestic cats was wider than in feral or sick cats. In that research it was also observed that cats were carriers of both coagulase-positive (CoPS) and coagulase-negative *Staphylococcus* species (CoNS) (Gandolfi *et al*., 2013).

Bartonella species are being recognized as increasingly important bacterial pathogens in veterinary and human medicine. These organisms are often transmitted by an arthropod vector or alternatively by animal scratches or bites. During the period of this study *B. henselae* was detected in 10.9% of saliva samples (12/110) from pet cats. *B. henselae* wasn't detected in nail samples of pet cats (n=110), and in any feral cats' saliva and nail samples (n=30) (Ohad *et al*., 2010).

P. multocida was cultured from the nasopharynx of 94% of normal cats by (Smith, 1964), who considered that it was invariably present in abscesses that occurred as a result of fighting; noted that beta-haemolytic streptococci and anaerobic fusiform bacilli were often present with *P. multocida*.

2.4 Features of *Escherichia coli*

Escherichia coli may be a facultative anaerobe that could grow from 7ºC to 50ºC but the optimum temperature is 37ºC, although there are reports of some Enterotoxigenic *E. coli (*ETEC) strains growing at temperatures as low as 4ºC. *E. coli* are often differentiated from other members of the Enterobacteriaceae on the idea of variety of sugar-fermentation and other biochemical tests. Classically a crucial group of tests used for this purpose are known by the acronym IMViC. These tested for the power to produce: indole from tryptophan (I); sufficient acid to scale back the medium pH below 4.4, the break point of the indicator methyl red (M); acetoin (acetylmethyl carbinol) (V); and therefore the ability to utilise citrate (C) (Adams and Moss, 2008).

Despite *E. coli* are often identified with a spread of biochemical reactions, the indole test remains the foremost useful method to differentiate lack of production of β-glucuronidase. Sorbitol non fermenting strains of E. coli O157:H7 are related to colitis and hemolytic uremic syndrome (HUS) (Besser *et al.*, 1999).

E. coli is transmitted by ingestion of contaminated food and water, direct contact with animals, feces, contaminated soil and cross contamination directly from one person to a different person. (CDC, 2008 and Denny *et al.,* 2008)

2.5 Features of *Salmonella* spp.

Salmonella spp. constitutes a major public health burden and represents a significant cost in many countries. Millions of human cases are reported worldwide every year and the disease results in thousands of deaths. *Salmonella* is a Gram-negative facultative anaerobic rod-shaped bacterium in the family of Enterobacteriaceae, also known as enteric bacteria. *Salmonella* is a motile bacterium with the exception of *S. gallinarum* and *S. pullorum* and they are all non-spore forming. There is a widespread occurrence of Salmonellosis in animals, especially poultry (FDA, 1998).

The most commonly used media selective for *Salmonella* spp. are Salmonella-Shigella (SS) agar, bismuth sulfite agar, Hektoen enteric (HE) medium, brilliant green agar, xyloselysine-deoxycholate (XLD), and MacConkey agar. All these media contain both selective and differential ingredients (Edwards and Ewing, 1972).

2.6 Features of *Staphylococcus* spp.

The genus Staphylococcus comprises of different species which have been classified and differentiated on the basis of a variety of phenotypic characteristics such as morphology, and biochemical reactions. Pigment was the initial criterion used to classify staphylococcal species, and in 1885, Rosenbach recognized members of the genus Staphylococcus based on the color of colonies. Staphylococci forming orange-yellow colonies were named *S. aureus* by Rosenbach, while staphylococci forming white colonies were named *S. albus* (Kloos, 1980). Another characteristic feature which was described for differentiation between staphylococci was the coagulase test which involves the investigation of the ability of *S. aureus* to clot blood plasma (Kloos, 1980) which paved way for the separation of Staphylococci into two main groups Coagulase positive *S. aureus* (CoPS) and Coagulase negative *S. aureus* (CoNS). Based on different studies carried by different researchers, at present the genus Staphylococcus comprises of 37 species and 17 subspecies (Kloos and Schleifer, 1975).

S. aureus is a gram-positive, catalase-positive, usually oxidase-negative, facultative anaerobic coccus, which belongs to the family of Micrococcaceae and the group of Staphylococci. Different phenotypic methods are been proposed to identify *S. aureus* isolates from humans and animals from other species of Staphylococcus. These methods include anaerobic fermentation of mannitol, production of coagulase, production of heat stable thermonuclease and production of acetoin from glucose (Devriese, 1981; Roberson *et al*., 1992).

2.7 Mechanism and origin of antibiotic resistance

Though the antibiotics were more successful as therapeutics against many bacterial infection in the history of medicine, their irrational and indiscriminate use has created enormous pressure resulting in the development of antibiotic resistance in

bacteria (Witte, 1998). Antibiotic resistance can be an intrinsic property of bacteria themselves or it can be acquired later. In natural or intrinsic resistance to a drug occurs without any additional changes in their genetic elements, whereas acquired resistance results through random mutations or acquisition of foreign genetic material carrying resistance determinants (Hollenbeck and Rice, 2012), The antimicrobial agent becomes effective against a target bacterial species only when a susceptible antibiotic target site exists in the cell, the antibiotic reaches the target in sufficient quantity and the antibiotic is not inactivated or modified by the bacterial cell wall (Sutcliffe *et al*., 1999). So, the unavailability or any change in these conditions trigger the cells to acquire resistance. The mode of acquiring resistance to an antimicrobial drug in bacterial species is categorized broadly into two groups based on biochemical and genetic aspect (Senka *et al*., 2008).

2.8 Antimicrobial sensitivity pattern of *E. coli*, *Salmonella* spp. and *Staphylococcus* spp.

The prevalence and patterns of antimicrobial susceptibility of fecal *E coli*, *Salmonella* spp., extended β-lactamase producing *E. coli* (ESBL-E. coli), methicillin-resistant *Staphylococcus aureus* (MRSA), and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) were determined for healthy cats (n = 39) from veterinary hospitals in southern Ontario that had not had recent exposure to antimicrobials. The prevalence of antimicrobial resistance in *E. coli* was as follows: streptomycin (cats — 2%), ampicillin (cats — 4%), cephalothin (cats — < 1%), and tetracycline (cats — 2%). 15% of cats had isolates that were immune to a minimum of 2 antimicrobials. The observed prevalence of resistance in commensal E. coli from this population was less than that previously reported in companion animals (Murphy *et al*., 2009).

(Leelaporn et al., 2003) performed antimicrobial susceptibility tests of *E. coli* isolates in Bangkok, by disc diffusion method. All the isolates were reported susceptible to cefaclor, ceftriaxone, imipenem, netilmicin, norfloxacin, ciptofloxacin, nalidixic acid, and forfomycin. More than 90% of the isolates were susceptible to cefdinir, gentamycin, neomycin and chlorophenicol. Resistance rates to ampicillin, co-trimoxazole and tetracycline were 17, 39, and 65 percent respectively.

Shiga-toxin producing *E. coli* (STEC) isolates (12 of animals, 1 of human and 4 of food samples) from a total of 876 samples (330 of animals, 184 of humans and 362 food samples) were reported uniformly sensitive to common antibiotics, except tetracycline, dicloxicillin, erythromycin, cephalaxin and linomycin (Chattopadhya *et al*., 2001).

A study determined that the antimicrobial resistance patterns of 138 *E. coli* isolated from humans in Japan. About 31 isolates showed the resistance to one or more antimicrobial agents. 24 of the isolates were resistant to tetracycline, 23 to streptomycin, 12 to ampicillin, 7 to chloramphenicol and kanamycin, 3 to nalidixic acid, 1 to gentamycin and 1 to cefuroxime (Hiroi *et al*., 2012).

12 isolates of Entero haemorrhagic *E. coli* (EHEC) which were isolated from lambs (4), calves (4) and fish (4) in Egypt to determine the frequency of resistance to commonly used antimicrobial agents in veterinary field. Results showed that among the antimicrobial discs tested, ampicillin was the most common antibiotic that the isolates were resistant to (91.6%), followed by tetracycline (83.3%) (Mahmoud *et al*., 2013).

E. coli from 50 (83.33%) samples and *Salmonella* spp. from 18 (31.66%) samples by using standard bacteriological techniques. Furthermore, the isolates were subjected to antibiogram studies by disk diffusion method using eight commonly used antibiotics. Antibiogram studies revealed that gentamycin, ciprofloxacin, and norfloxacin were highly sensitive against all the isolated bacteria, whereas most of the isolates were resistant to amoxicillin, erythromycin, and tetracycline. Out of all the isolates, 5 isolates of *E. coli* and 3 isolates of Salmonella were found multidrug resistant (Al-Salauddin *et al*., 2015).

30 samples out of 65, 17 from chicken and 13 from goat were positive for Staphylococci with the prevalence rate of 48.57% from chicken and 43.33% from goat. Staphylococcal isolates were found variably resistant to the antibiotics tested. 80% of the isolates were positive for at least one of the antibiotics used in this study. The isolates showed maximum resistance for penicillin (73.33%) which is followed by erythromycin (36.66%), tetracycline (26.66%), oxacillin (23.33%), ciprofloxacin (16.66%), chloramphenicol (10%), vancomycin (3.33%) (Das et al., 2016).

To determine whether cats were a risk for transmission of *Salmonella* to humans, the researchers evaluated the excretion of *Salmonella* by pet cats. Rectal-swab specimens were taken from 278 healthy house cats, from 58 cats that died of disease, and from 35 group-housed cats. Group-housed cats were kept in one room with three cat trays and a common water and feed tray. Eighteen (51.4%) of 35 group-housed cats, 5 (8.6%) of 58 diseased cats, and 1 (0.36%) of 278 healthy house cats excreted *Salmonella*. *Salmonella* isolates were of serotypes Typhimurium, Enteritidis, Bovismorbificans. Acquired antimicrobial resistance was found in serotype Typhimurium (resistance to ampicillin, chloramphenicol, and tetracycline; to ampicillin; and to chloramphenicol). Cats that excrete *Salmonella* can pose a public health hazard to people who are highly susceptible to *Salmonella*, such as children, the elderly, and immunocompromised persons (Van *et al*., 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

To conduct the study, a number of materials were used. The list of materials is as follows:

3.1.1 Samples

Total 40 oral swab samples were collected from pet cat handled at Central Veterinary Hospital, Dhaka, Bangladesh.

Table 1. Oral swab from pet cat

Sample No. & ID	Collection zone	Gender	Age	Breed	Feed habit (boiled, raw)	Sample type
1. S1	CVH, Dhaka	Female	2.5 Years	Local	Boiled	Oral swab
2. S2	CVH, Dhaka	Female	2.5 Years	Local	Boiled	Oral swab
3. S3	CVH, Dhaka	Male	1.5 years	Exotic	Both	Oral swab
4. S4	CVH, Dhaka	Female	6 months	Exotic	Boiled	Oral swab
5. S5	CVH, Dhaka	Female	2 months	Local	Boiled	Oral swab
6. S6	CVH, Dhaka	Female	6 months	Exotic	Boiled	Oral swab
7. S7	CVH, Dhaka	Male	1.9 years	Local	Raw	Oral swab
8. S8	CVH, Dhaka	Male	3 month	Local	Boiled	Oral swab
9. S9	CVH, Dhaka	Male	1.1 years	Local	Both	Oral swab
10. S10	CVH, Dhaka	Male	3 years	Mixed	Boiled	Oral swab
11. S11	CVH, Dhaka	Male	2.9 years	Local	Raw	Oral swab
12. S12	CVH, Dhaka	Male	8 months	Local	Boiled	Oral swab
13. S13	CVH, Dhaka	Male	2.8 years	Exotic	Raw	Oral swab
14. S14	CVH, Dhaka	Female	1.5 years	Local	Boiled	Oral swab
15. S15	CVH, Dhaka	Male	1.3 year	Mixed	Boiled	Oral swab
16. S16	CVH, Dhaka	Male	1 year	Local	Raw	Oral swab
17. S17	CVH, Dhaka	Female	9 months	Local	Boiled	Oral swab
18. S18	CVH, Dhaka	Male	1.1 years	Exotic	Boiled	Oral swab
19. S19	CVH, Dhaka	Female	3.5 years	Local	Boiled	Oral swab

20. S20	CVH, Dhaka	Female	2 years	Local	Boiled	Oral swab
21. S21	CVH, Dhaka	Female	3 months	Local	Both	Oral swab
22. S22	CVH, Dhaka	Male	8 months	Exotic	Boiled	Oral swab
23. S23	CVH, Dhaka	Male	2 years	Local	Boiled	Oral swab
24. S24	CVH, Dhaka	Male	10 months	Local	Raw	Oral swab
25. S25	CVH, Dhaka	Female	1.5 years	Local	Both	Oral swab
26. S26	CVH, Dhaka	Female	1.7 years	Local	Both	Oral swab
27. S27	CVH, Dhaka	Male	1.5 years	Exotic	Boiled	Oral swab
28. S28	CVH, Dhaka	Female	1.9 years	Mixed	Both	Oral swab
29. S29	CVH, Dhaka	Male	9 months	Exotic	Boiled	Oral swab
30. S30	CVH, Dhaka	Male	6 months	Exotic	Boiled	Oral swab
31. S31	CVH, Dhaka	Female	1.1 year	Exotic	Both	Oral swab
32. S32	CVH, Dhaka	Female	2 years	Exotic	Boiled	Oral swab
33. S33	CVH, Dhaka	Female	2 month	Local	Both	Oral swab
34. S34	CVH, Dhaka	Male	1.7 years	Mixed	Both	Oral swab
35. S35	CVH, Dhaka	Female	2 years	Local	Raw	Oral swab
36. S36	CVH, Dhaka	Male	3 years	Local	Boiled	Oral swab
37. S37	CVH, Dhaka	Female	1.7 years	Local	Raw	Oral swab
38. S38	CVH, Dhaka	Female	1.1 year	Local	Boiled	Oral swab
39. S39	CVH, Dhaka	Female	9 months	Local	Raw	Oral swab
40. S40	CVH, Dhaka	Male	9 months	Exotic	Boiled	Oral swab

3.1.2 Bacteriological media

3.1.2.1 Agar media

Different agar media were used for bacteriological study, such as, Nutrient agar, MacConkey (MC) agar, Eosin Methylene Blue (EMB) agar, Mannitol Salt (MS) agar, Salmonella-Shigella (SS) agar, SIM test agar media and Simmons Citrate (SC) Agar and Muller Hinton (MH) agar.

3.1.2.2 Liquid media

The liquid media used in this study were Nutrient broth, Peptone broth, Methyl-Red and Voges-Proskauer broth (MR-VP broth) and Sugar media (glucose, dextrose, maltose, lactose and mannitol).

3.1.3 Chemicals and reagents

The chemicals and reagents used for this study were 0.1% Peptone water, Phosphate buffered saline (PBS), reagents for Gram's staining (Crystal Violate, Gram's iodine, Safranin, Acetone alcohol), 3% Hydrogen peroxide, Phenol red, Methyl red, 10% Potassium hydroxide, Kovac's indole reagent (4-dimethylamino-benzaldehyde, concentrated HCL), Mineral oil, Normal saline and other common laboratory chemicals and reagents.

3.1.4 Glass wares and other appliances

The following glass wares and appliances were used during the course of the experiment. Test tubes (with or without Durham's fermentation tube and stopper), petri dishes, conical flasks, pipettes (1 ml, 2 ml, 5 ml, 10 ml) & micro-pipettes (10 µl, 100 µl, 200 µl, 1000 µl) slides and cover slips, hanging drop slides, immersion oil, compound microscope, bacteriological loop, sterilized cotton, cotton plug, test tube stand, water bath, bacteriological incubator, refrigerator, sterilizing instruments, thermometer, ice carrier, hand gloves, spirit lamp, match lighter, laminar air flow, hot air oven, centrifuge tubes and machine, PCR machine, thermos scientific nano drop spectrophotometer, UV trans illuminator, Gel documentation machine, electronic balance, syringe and needle, tray, forceps, scalpel, scissors etc.

3.1.5 Antimicrobial discs

Commercially available antimicrobial discs (OXOID Limited, Canada) were used to test the drug sensitivity and resistance pattern. This method is allowed for the rapid detection of drugs efficacy against the test organisms by measuring the diameter of the zone of inhibition.

The following antimicrobial agents with their disc concentration were used to test the sensitivity and resistance pattern of the selected *E. coli, Salmonella* spp., *S. aureus and S. epidermidis* isolates from oral swab of cat.

Table 2. Antibiotics with disc concentrations

Sl. No.	Antimicrobial agents	Disc concentration (µg)
1.	Gentamycin (GEN)	10
2.	Erythromycin (E)	15
3.	Tetracycline (TE)	30
4.	Co-Trimoxazole (COT)	25
5.	Ampicillin (AMP)	25
6.	Streptomycin (S)	10
7.	Azithromycin (AZM)	15

3.2 Methods

3.2.1 Brief description of the experimental design

The entire experiment was categorized into two principal steps:

I. The first step included selection of sources, collection of samples, and isolation, identification and characterization of microorganisms on the basis of their colony morphology, motility, biochemical characteristics and molecular identification (only for *E. coli*)

II. In the second step, the current status of drug sensitivity and resistance pattern of the isolated bacteria were determined.

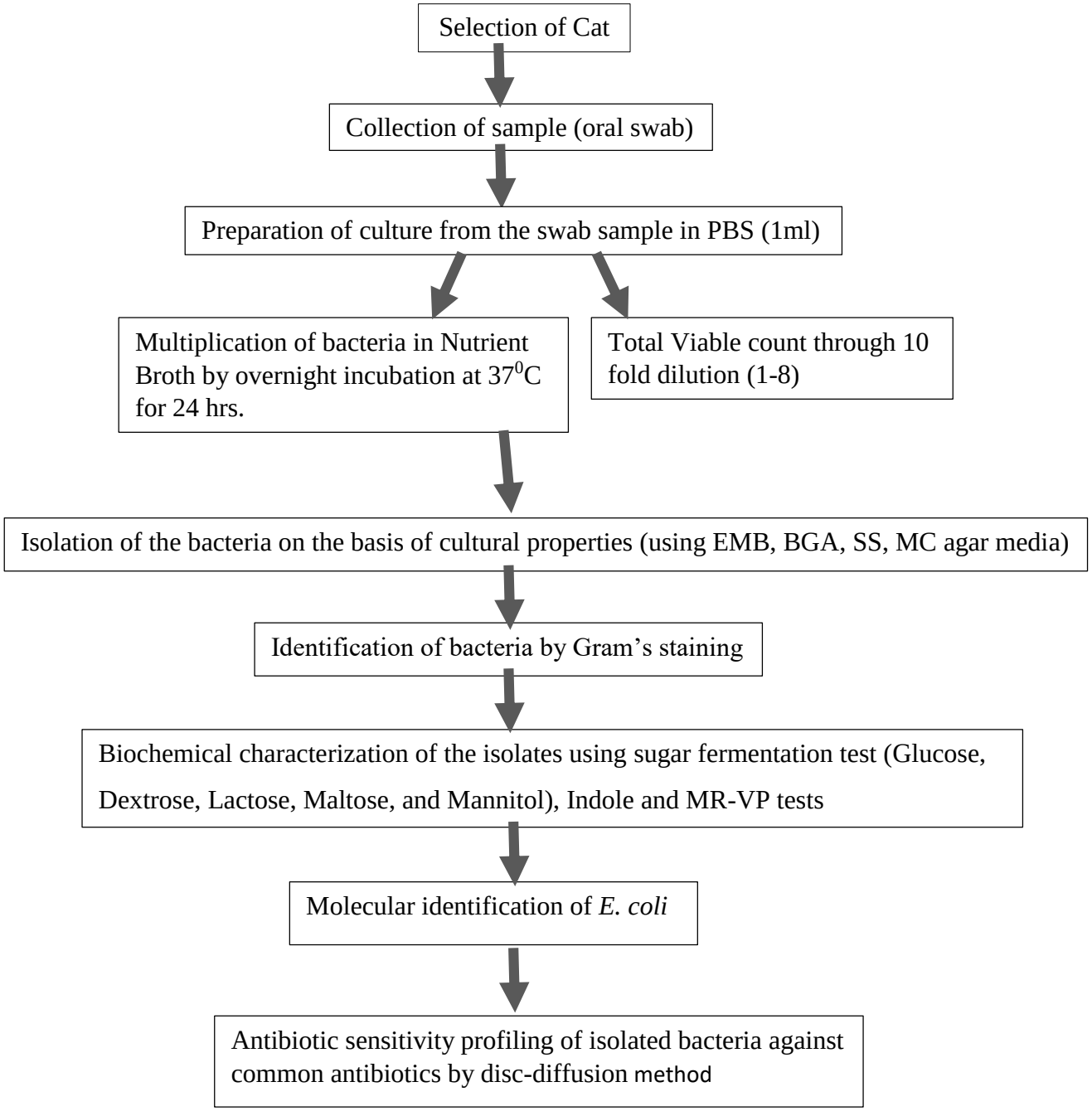


Figure 1: Layout of the experiment

3.2.2 Collection and transportation of samples

40 oral swab samples of cats were collected using sterile swab stick in 2 ml Eppendorf tube filled with 1 ml PBS from the cat from Central Veterinary Hospital (CVH), Dhaka. The collected samples were immediately carried to the laboratory maintaining proper cool chain.

Figure 2: Sample collection from Central Veterinary Hospital (CVH)

3.2.3 Preparation of bacteriological culture media

3.2.3.1 Nutrient broth

Nutrient Broth was prepared by Suspending 25.0 grams in 1000 ml purified/distilled water. Heat was applied to dissolve the medium completely. Sterilizing was done by autoclaving at 15 lbs pressure (121°C) for 30 minutes. The broth was filled in test tubes and incubated at 37ºC for overnight to check their sterility, and stored at 4ºC in the refrigerator until used.

3.2.3.2 Nutrient agar

Nutrient agar was prepared by dissolving 28.0 grams of dehydrated nutrient agar (HiMedia, India) in to 1000 ml distilled water, and was sterilized by autoclaving at 121ºC under 15 lb pressure per square inch for 15 minutes. Then the agar was dispensed into petri dishes, and was incubated overnight at 37ºC to check their sterility, and stored at 4ºC in the refrigerator until used.

3.2.3.3 MacConkey agar

49.53 grams of Bacto MacConkey agar (HiMedia, India) was suspended in to 1000 ml distilled water and was heated to dissolve the medium completely. It was then poured in to sterile petri dishes, and was allowed to solidify. After solidification of the media, the plates were then incubated overnight at 37ºC to check their sterility, and stored at 4ºC in the refrigerator until used.

3.2.3.4 Eosine Methylene Blue (EMB) agar

36.0 grams powder of EMB agar base (HiMedia, India) was suspended in 1000 ml of distilled water. The suspension was heated to dissolve the powder completely. The medium was autoclaved for 30 minutes to make it sterile. After autoclaving the medium was put in to water bath at 45ºC. From water bath 10-20 ml of medium was poured in to medium sized sterile petri dishes to make EMB agar plates. After solidification of the medium in the plates, the plates were incubated at 37ºC for overnight to check their sterility, and stored at 4ºC in the refrigerator until used.

3.2.3.5 Salmonella-Shigella agar

60 grams SS agar (HiMedia, India) powder was suspended in 1000 ml distilled water, and heated to dissolve the medium completely. The medium was sterilized by autoclaving. Then the medium was put in to water bath of 45ºC to decrease. After solidification of the medium in the petri dishes, the petri dishes were allowed for incubation at 37ºC for overnight to check their sterility, and then stored at 4ºC in a refrigerator for further use.

3.2.3.6 Mannitol salt (MS) agar

11.1 grams MS agar base (Hi-media, India) powder was suspended in 100 ml of distilled water and heated to dissolve the powder completely. The medium was autoclaved for 30 minutes under 15 lbs pressure. Then the medium was put into water bath maintaining 45°C and poured in to petri dishes to make MS agar plates. After solidifying the medium, the plates were kept in the incubator at 37°C for overnight to check their sterility, and then stored at 4ºC in a refrigerator for further use.

3.2.3.7 Mueller Hinton agar

38.0 grams MH agar powder was suspended in 1000 ml distilled water and heated to dissolve the medium completely. After the sterilization by autoclaving at 15 lbs pressure at 121°C for 15 minutes. Cooling was done to 45-50°C.Then it was mixed well and poured into sterile Petri dishes. After solidification of the medium in the petri dishes, the petri dishes were allowed for incubation at 37ºC for overnight to check their sterility and then stored at 4ºC in a refrigerator for future use.

3.2.3.8 Phosphate Buffered Saline (PBS)

To prepare phosphate buffered saline, 8.0 gm of sodium chloride (NaCl), 2.89 gm of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), 0.2 gm of potassium chloride (KCl) and 0.2 gm of potassium hydrogen phosphate (KH_2PO_4) were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely and pH was adjusted with the help of pH meter. The solution was then sterilized by autoclaving and stored at 4ºC for future use.

3.2.3.9 Simmons Citrate (SC) agar

5.0 gm sodium chloride (NaCl), 2.0 gm sodium citrate (dehydrate), 1.0 gm ammonium dihydrogen phosphate, 1.0 gm dipotassium phosphate, 0.2 gm magnesium sulfate (heptahydrate) were dissolved in 1000 ml distilled water. The pH was adjusted to 6.9. Then agar and bromothymol blue were added. Gently heat with shaking until agar is dissolved. The media were dispensed 5.0 ml into each test tubes. Autoclave at 121°C under 15 lbs pressure for 15 minutes. Cooling in slanted position (slant and butt). The uninoculated medium will be a deep forest green due to the pH of the sample and the bromothymol blue. During inoculation, the surface of the medium is lightly inoculated by streaking and, where slopes are used, the butt of medium is inoculated by stabbing.

3.2.3.10 SIM (Sulfide, Indole, Motility) media

SIM media was prepared by suspending 36.23 grams in 1000 ml distilled water. Heat to dissolve the media completely. Dispensed in tubes. Sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes and stored at 4ºC for future use.

3.2.3.11 Methyl Red and Voges–Proskauer (MR-VP) broth

MR-VP broth was prepared by suspending 3.4 grams of MR-VP media (HiMedia, India) in 250 ml of distilled water, distributed in 5.0 ml in each test tube and then autoclaved. After autoclaving, incubated overnight at 37ºC to check their sterility, and then stored at 4ºC for future use.

3.2.3.12 Sugar solutions

To prepare the media, fermentable sugars were added with 1% peptone water. Peptone water was prepared by adding 1.0 gram of Bacto peptone (Difco, USA) and 0.5 grams of sodium chloride in 100 ml distilled water, boiled for 5 minutes, adjusted to pH 7.6 by phenol red (0.02%) indicator, cooled and then filtered through filter paper. The solutions were then dispensed in 5.0 ml amount into cotton plugged test tubes containing invertedly placed Durham's fermentation tubes. Then the sugars, glucose, dextrose, maltose, lactose, and mannitol for the fermentation were prepared separately as 10 percent solutions in distilled water. A little heat was necessary to dissolve the sugar. These were then sterilized by autoclaving for 15 minutes. The sugar solutions were sterilized in Arnold's steam sterilizer at 100ºC for 30 minutes for three consecutive days. An amount of 5.0 ml of sterile sugar solution was added aseptically in each culture tubes containing sterile peptone water. The sugar solutions were incubated at 37ºC for 24 hours to check sterility.

3.2.4 Isolation of bacteria

3.2.4.1 Preparation of sample

The raw samples were kept in 1.0 ml PBS, further, any chemical was not used/added for sample preparation.

3.2.4.2 Serial dilution for bacterial culture (10 fold dilution method)

Serial dilution of the stock sample was done for reduction the bacterial concentration for easy total viable count (TVC). This process was conveyed by taking 8 (1-8) Eppendorf tube filled with 450µl of PBS. 50µl of stock sample was transferred from the stock tube (1ml) to the Eppendorf tube next to the stock tube. Then 50µl of diluted sample is transferred from the first Eppendorf tube to the next. Successive dilution

should be made in the same way to the last tube and from the last tube 50µl of diluted sample was discarded. From all the tube, 25µl of liquid sample was poured to the nutrient agar media by spreading technique for the total viable count.

3.2.4.3 Primary culture of microorganism

Primary growth of all kinds of bacteria present in the collected samples was performed in nutrient broth. The samples were inoculated in to nutrient broth, and incubated overnight at 37ºC for the growth of the organisms.

3.2.4.4 Isolation in culture media

After primary culture of the organism, a small amount of inoculums from Nutrient broth was streaked on the MacConkey, Salmonella-Shigella and Mannitol Salt agar to observe the colony morphology of the isolates. Characteristic colony morphology of the organisms selected for subculture on selective media, such as, Eosine Methylene Blue agar and MacConkey agar for *E. coli*, Salmonella-Shigella agar and MacConkey agar for *Salmonella* spp., and Mannitol Salt agar for *S. aureus and S. epidermidis*. Morphological characteristics (shape, size, surface texture, edge and elevation, color, opacity etc.) of the suspected colonies on different agar media were carefully recorded.

3.2.5 Microscopic identification of the suspected colonies by Gram's staining method

Gram's staining of the pure culture was performed according to method described by (Cheesbrough, 2006). Briefly, a single colony was picked up with a bacteriological loop, smeared on a glass slide and fixed by gentle heating. Crystal violate was then applied onto smear to stain for two minutes and then washed with running tap water. Few drops of Gram's iodine were then added for few seconds. After washing with water, Safranin was added as counter stain and allowed to stain for 2 minutes. The slides were then washed with water, blotted and dried in air and then examined under light microscope (100X) using immersion oil

3.2.6 Identification of isolates by different biochemical tests

Several biochemical tests were performed for confirmation of *E. coli, Salmonella* spp., *S. aureus and S. epidermidis.*

3.2.6.1 Carbohydrate fermentation test

The carbohydrate fermentation test was performed by inoculating 0.2 ml of nutrient broth culture of the isolated organisms into the tubes containing different sugar media (five basic sugars such as glucose, dextrose, maltose, lactose, and mannitol) and incubated for 24 hours at 37ºC. Acid production was indicated by the color change from red to yellow, and gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube (Cheesbrough, 2006).

3.2.6.2 Methyl Red test

The test was conducted by inoculating single colony from the pure culture of the test organism in 5 ml sterile MR-VP broth. After 5 days incubation at 37ºC, 5 drops of methyl red solution was added and observed for color formation. Development of red color was positive and indicated an acid pH of 4.5-6 resulting from the fermentation of glucose. Development of yellow color indicated negative result (Cheesbrough, 2006).

3.2.6.3 Voges-Proskauer (V-P) test

The test E. coli organisms were grown in 3 ml of sterile MR-VP broth at 37ºC for 48 hours. Then 0.6 ml of 5% alpha-napthol and 0.2 ml of 40% potassium hydroxide containing 0.3% creatine was added per ml of broth culture of the test organism. Then shaking well and allowed to stand for 5-10 minutes to observe the color formation. Positive case was indicated by the development of a bright orange red color. In negative cases, there was no development of pink color (Cheesbrough, 2006).

3.2.6.4 Oxidase test

Moisten the paper with a sterile distilled water. Pick the colony to be tested with wooden or platinum loop and smear in the filter paper. Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds.

3.2.6.5 Catalase test

For this study 3.0 ml of catalase reagent (3% H_2O_2) was taken in a test tube. Single colony from the pure culture of the organisms was taken with a glass rod and merged in the reagent. The glass slide was observed for bubble formation. Formation of bubble

within few seconds was the indication of the positive test, while the absence of bubble formation indicated negative result (Cheesbrough, 2006).

3.2.6.6 SIM (Sulfide, Indole, Motility) test

The formulation of SIM Medium is designed to allow the detection of sulfide production, indole formation and motility. The medium contains ferrous ammonium sulfate and sodium thiosulfate, which together serve as indicators for the production of hydrogen sulfide. H2S Production: Positive (+): Blackening along stab line, Negative (-): No blackening Indole Production. Indole positive (+): Red color change after Kovac's, reagent Negative (-): No color change after Kovac's reagent (reagent remains yellow), motility: Positive (+): Diffuse growth outward, Negative (-): Growth only along the stab line

3.2.6.7 Simmons Citrate agar test

Positive growth (i.e. citrate utilization) produces an alkaline reaction and changes the color of the medium from green to bright blue. In case of negative test, (i.e. no citrate utilization) the color of the medium remains unchanged.

3.2.7 Molecular identification of the isolates (only *E.coli*)

3.2.7.1 Bacterial DNA isolation

a. Materials:

TE buffer 10% (w/v)
Sodium dodecyl sulfate (DSS)
20 mg/ml proteinase K (stored in small single-use aliquots -200C)
3 M sodium acetate, pH 5.2
25:24:1 phenol/chloroform/isoamyl alcohol
Isopropanol
70% ethanol
90% ethanol
1.5 ml micro centrifuge tube

b. Procedure:

25 ml of liquid culture with the bacterial strain of interest was inoculated. Grown in conditions appropriate for that strain until the culture is saturated.

↓

1.0 ml of overnight culture was spin in a micro centrifuge tube for 5 minutes at 10000 rpm. The supernatant was discarded.

↓

The step was repeated

↓

The supernatant was resuspended in 467µl TE Buffer by pipetting. 30 µl of 10% SDS and 3µl of proteinase K was added to give a final concentration of 100µg/ml proteinase K in 0.5% SDS. It was thoroughly mixed and incubated for 30min. to 1hr. at 37^0C.

↓

Approximately equal volume of (500µl) of phenol/chloroform/isoamyl alcohol was added. It was mixed thoroughly but carefully to avoid the mixing of DNA, by inverting the tube until the phases are completely mixed.

↓

Then the centrifuge of the tubes was done at 12000 rpm for 10 minutes.

↓

Aqueous, viscous supernatant (~450 µl) was collected to a fresh micro centrifuge tube, leaving the interface behind. An equal volume of phenol/chloroform/isoamyl alcohol was added, was extract thoroughly and was spin in a micro centrifuge at 10000 rpm for 5 min.

↓

The supernatant was transferred to a fresh tube (~400 µl).

↓

1/10th volume of 3m sodium acetate was added and mixed

↓

0.6 volumes of isopropanol was added to precipitate the nucleic acids, keep on ice for 10 minutes.

Centrifuge was done at 13500 rpm for 15 minutes.

The supernatant was decanted

The obtained pellet was washed with 1ml of 95% ethanol for 5 minutes. Then centrifuge was done at 12000 rpm for 10 minutes.

The supernatant was decanted

The pellets had been dried until there were no alcohol.

The pellet was resuspended in 50µl of TE and stored DNA at 40C for short time and -200C for long term.

Figure 3: DNA isolation in the laboratory

3.2.7.2 Polymerase Chain Reaction (PCR)

a) Primer used for PCR

A genus specific PCR was performed to amplify 16S rRNA of *E.coli* using previously designed primers (Tsen et al., 1998) in Table 3. Primer E1 and E2 can amplify 584bp fragments from the pathogenic *E. coli*. Primer E1 and E3 can amplify same from non-pathogenic organism.

Table 3. Primer sequence with PCR product size and reference

Primer	Sequence(5´-3´)	Size (bp)	Reference
E. coli 16E1(F)	GGGAGTAAAGTTAATCCTTTGCTC		
E. coli 16E2(R)	TTCCCGAAGGCACATTCT	584	Tsen et al., 1998
E. coli 16E3(R)	TTCCCGAAGGCACCATC		

b) Preparation for a PCR mixture

PCR mixture (25 µl) was prepared as follows:

Nuclease free water:	8.5µl
2x master PCR mix:	12.5µl
Forward primer:	1.0µl (20pmole/µl)
Reverse primer:	1.0µl (20pmole/µl)
DNA template:	2.0µl

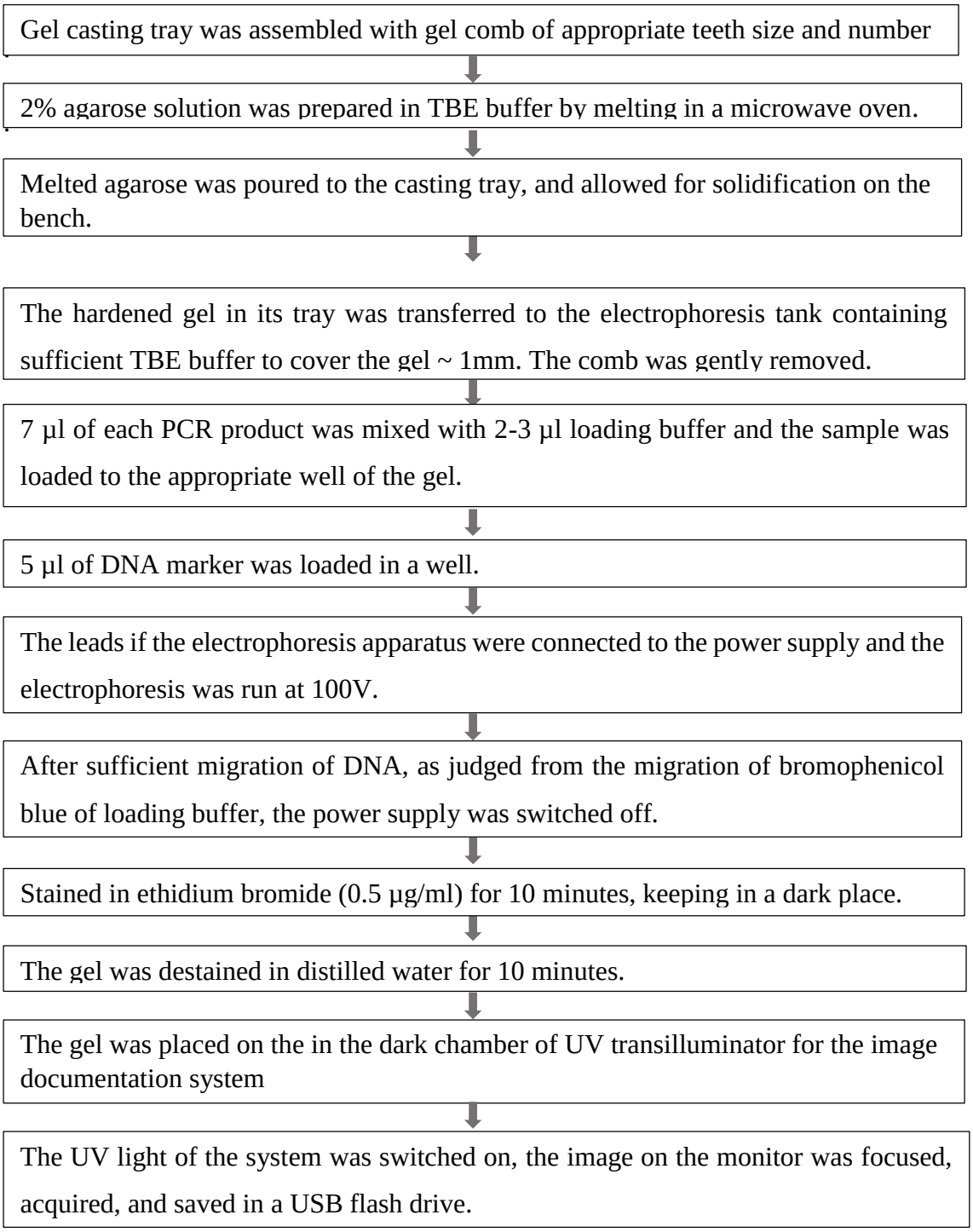
c) Thermal cycles

PCR reaction profile was prepared as follows:

Initial denaturation	95^{o}C for 5 minute	
Denaturation	94^{o}C for 30 sec	35 cycles
Annealing	60^{o}C for 30 sec	
Extension	72^{o}C for 30 sec	
Final extension	72^{o}C for 5 min	

d) Electrophoresis

2% agarose (Sigma) gel was used for gel electrophoresis of the PCR products. The procedure of gel electrophoresis is as follows:



3.2.7.3 Sequencing and phylogenetic analysis

DNA from a total of six samples was sequenced by Genetic Analyzer 3130 (Applied Biosystems) using dideoxy chain termination method (Sanger and Coulson, 1975). Sequencing was done using both directions. Obtained sequences were edited and analyzed by Molecular Evolutionary Genetics Analysis (MEGA-X) software (Kumar, et al., 2018). Phylogenetic tree was made using neighbor joining method with 1000 bootstrap replication (Felsenstein, 1985; Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura et al., 2004).

3.2.7.4 Gene Bank accession number

The nucleotide sequences (n=6) were submitted to Gene Bank under the accession number MW116768 (CVH/Cat/Oralswab-7/2019 (E1+E3)), MW116769 (CVH/Cat/Oralswab-13/2019 (E1+E2)), MW116770 (CVH/Cat/Oralswab-26/2019 (E1+E2)), MW116771 (CVH/Cat/Oralswab-33/2019 (E1+E2)), MW116772 (CVH/Cat/Oralswab-33/2019 (E1+E3)) and MW116773 (CVH/Cat/Oralswab-39/2019 (E1+E2))

3.2.8 Maintenance of stock culture

Stock culture of raw samples and specific isolates were prepared by adding 0.5 ml of NB and 0.5 ml of 99% sterilized glycerol respectively in pellet of culture and it was stored in -20º C.

3.2.9 Antimicrobial sensitivity pattern of *E. coli, Salmonella* spp.*, S. aureus* and *S. epidermidis* isolated from oral swab of cat

The antimicrobial sensitivity testing of each isolate was carried out by the Kirby-Bauer disc diffusion method (Bauer et al., 1966) according to National Committee for Clinical Laboratory Standards (NCCLS) procedures. Antibiotic sensitivity discs used were gentamicin (GEN), azithromycin (AZM), tetracycline (TE), ampicillin (AMP), streptomycin (S), erythromycin (E) and co-trimoxazole (COT). This method allowed for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition.

3.2.9.1 Disc diffusion method

The suspension of the test organism was prepared in a test tube containing 5 ml nutrient broth by overnight incubation in shaking incubator. By micropipette 100µl of broth culture of the test organism was poured on Muller-Hinton agar plate. Sterile glass spreader was used to spread the culture homogenously on the medium. Inoculated plates were closed and allowed to dry for approximately 3-5 minutes. Then the antibiotic discs were applied aseptically to the surface of the inoculated agar plates at a special arrangement with the help of a sterile forceps. The plates were then inverted and incubated at 37ºC for 24 hours.

After incubation, the plates were examined and the diameter of the zone of complete inhibition was measured by mm scale. The zone diameters for individual antimicrobial agents were translated in to sensitive, intermediate and resistant categories by referring to an interpretation table.

Antimicrobial testing results were recorded as susceptible, intermediate and resistant according to zone diameter interpretive standards provided by CLSI (2016).

Table 4. The zone-size (mm) for Enterobacteriaceae family interpretative table

Antimicrobial agents	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Azithromycin	≤ 12	-	≥ 13
Erythromycin	≤ 13	14-17	≥ 18
Streptomycin	≤15	16-21	≥22
Ampicillin	≤12	13-19	≥20
Co-trimoxazole	≤ 10	11-15	≥16
Tetracycline	≤ 11	12-14	≥ 15
Gentamycin	≤ 12	13-14	≥ 15

Table 5. The zone-size (mm) for *Staphylococcus* spp. interpretative table

Antimicrobial agents	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Azithromycin	≤ 13	14-17	≥ 18
Erythromycin	≤ 13	14-17	≥ 18
Streptomycin	≤16	17-21	≥22
Ampicillin	≤12	13-19	≥20
Co-trimoxazole	≤10	11-15	≥16
Gentamycin	≤ 12	13-14	≥ 15
Tetracycline	≤ 14	15-18	≥ 19

CHAPTER 4

RESULTS & DISCUSSION

4.1 Results

The results presented below demonstrated the isolation and identification of bacteria from oral swab samples of cat collected from Central Veterinary Hospital, Dhaka. The results also focused on the sensitivity and resistance pattern of the isolates in different drugs.

4.1.1 Total viable count

Total viable count of all bacterial samples were done in the laboratory where S39 contained the highest TVC (1.199×10^{13}) and the lowest TVC was found in S6 (3.60×10^{2}). The result of TVC of all 40 samples is shown in Table 6.

Table 6. Total viable count of bacteria

Serial No.	Sample ID	Total Viable Count (TVC)(CFU/ml)
1.	S1	9.20×10^{2}
2.	S2	5.08×10^{3}
3.	S3	1.80×10^{3}
4.	S4	9.76×10^{3}
5.	S5	7.84×10^{3}
6.	S6	3.60×10^{2}
7.	S7	4.00×10^{2}
8.	S8	6.00×10^{2}
9.	S9	1.696×10^{5}
10.	S10	3.95×10^{6}
11.	S11	8.58×10^{5}
12.	S12	1.48×10^{3}
13.	S13	6.04×10^{7}
14.	S14	2.88×10^{4}
15.	S15	7.5×10^{6}
16.	S16	5.01×10^{5}

17.	S17	6.48×10^{3}
18.	S18	1.36×10^{3}
19.	S19	1.104×10^{4}
20.	S20	6.44×10^{6}
21.	S21	2.16×10^{6}
22.	S22	2.44×10^{9}
23.	S23	3.54×10^{5}
24.	S24	1.49×10^{6}
25.	S25	1.465×10^{6}
26.	S26	4.76×10^{6}
27.	S27	3.56×10^{5}
28.	S28	6.6×106
29.	S29	5.6×10^{5}
30.	S30	2.04×10^{7}
31.	S31	5.72×10^{4}
32.	S32	6.40×10^{2}
33.	S33	3.56×10^{5}
34.	S34	3.72×10^{6}
35.	S35	5.52×10^{3}
36.	S36	2.16×10^{7}
37.	S37	1.96×10^{3}
38.	S38	1.188×10^{6}
39.	S39	1.199×10^{13}
40.	S40	4.96×10^{6}

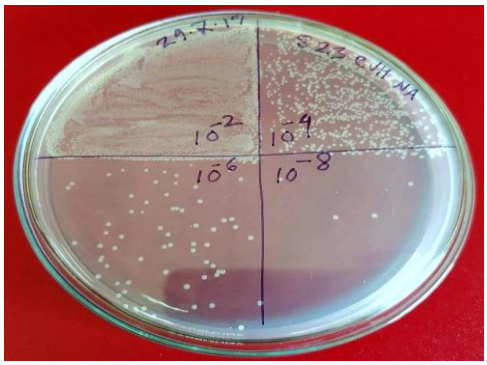


Figure 4: Total Viable count by 10 fold dilution method

4.1.2 Prevalence of microorganisms in cat saliva

The prevalence of organisms was 100%.

4.1.3 Results of isolation and identification of *E.coli, Salmonella* spp., *S. aureus and S. epidermidis*

4.1.3.1 Results of cultural examination

4.1.3.1.1 Culture in nutrient broth

All the isolates produced turbidity in nutrient broth.

4.1.3.1.2 Culture in different selective media

From nutrient broth culture was done in the selective media, and incubated for 24 hrs. at 37°C. Colony morphology was observed for cultural characteristics for particular suspected bacteria.

Table 7. Cultural characteristics of *E. coli* in selective agar

Sources of *E. coli*	Colony characteristics in different agar media	
	MC agar	**EMB agar**
S1-S40	Smooth, circular and rose pink colored colonies	Greenish colonies with metallic sheen

Figure 5: *E.coli* in MC agar media

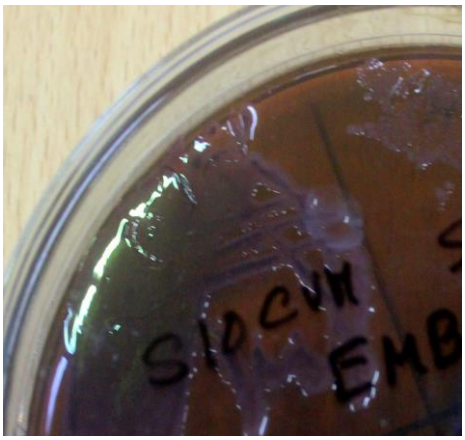


Figure 6: *E.coli* in EMB agar media

Table 8. Cultural characteristics of *Salmonella* spp. in selective agar

Sources of *Salmonella* spp.	Colony characteristics in different agar media	
	MC agar	**SS agar**
S11, S13	Red colonies surrounded by brilliant red zones	smooth, circular, black color colonies

Figure 7: *Salmonella* spp. in MC agar

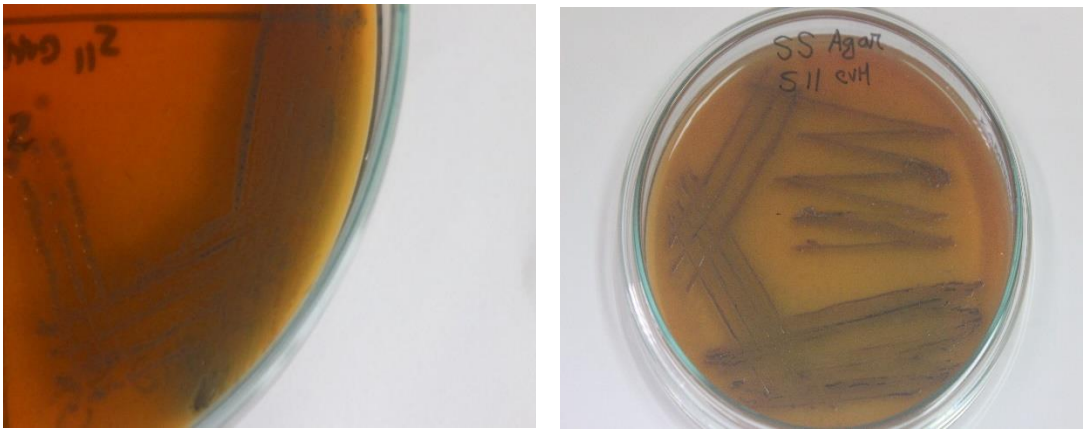


Figure 8: *Salmonella* spp. in SS agar

Table 9. Cultural characteristics of *S. aureus* in selective agar media

Sources of *S. aureus*	**Colony characteristics in MS agar media**
S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S17, S19, S21, S25, S26, S28, S30, S31, S32, S33, S35, S36, S39	*S. aureus* on MS agar was indicated by smooth, circular, small whitish or yellowish colony

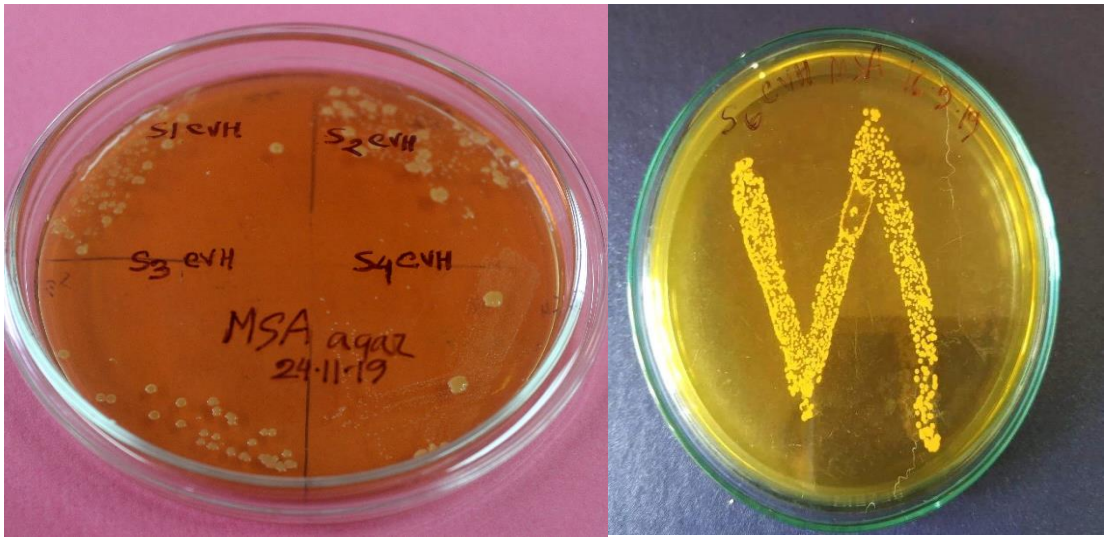


Figure 9: *S. aureus* in MS Agar

Table 10. Cultural characteristics of *S. epidermidis* in selective agar media

Sources of *S. epidermidis*	**Colony characteristics in MS agar media**
S27, S31, S35	*S. epidermidis* on MS agar was indicated by smooth, circular, small pink colony

Figure 10: *S. epidermidis* in MS Agar

(Left: *S. epidermidis* mixed with *S. aureus*, Right: pure *S. epidermidis*)

4.1.3.2 Results of Gram's staining technique

Results of light microscopic examination (100X) after Gram's staining of suspected isolates are shown in Table 11 and in Figure 11, 12, 13, 14.

Table 11. Morphology and staining characteristics of different isolates

Isolates	Morphology and staining characteristics
E. coli	Gram negative rod shaped organism and arranged as single or in pair
Salmonella spp.	Gram negative short plump rod shaped organism and arranged as single or in pair
S. aureus	Gram positive cocci shaped bacteria and arranged in grapes like cluster
S. epidermidis	Gram positive cocci shaped bacteria and arranged in grapes like cluster

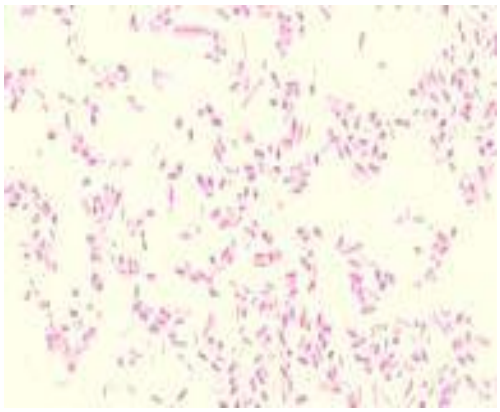


Figure 11: Gram negative, rod shape organism (*E. coli*) at 100X magnification

Figure 12: Gram negative, rod shape organism (*Salmonella* spp.) at 100X magnification

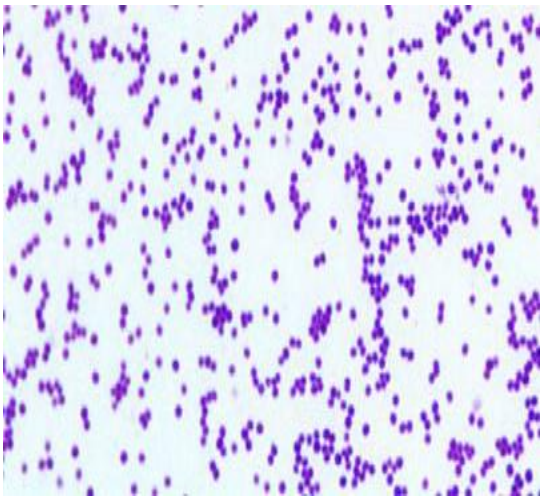


Figure 13: Gram positive, circular, and cluster organism (*S. aureus*) at 100X magnification

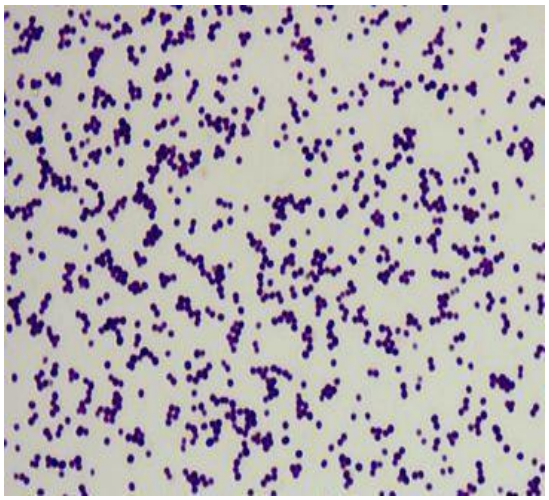


Figure 14: Gram positive, circular, and clustered organism (*S. epidermidis*) at 100X magnification

4.1.3.3 Results of Biochemical test

4.1.3.3.1 Sugar fermentation test

All the isolates of *E. coli* produced both acid and gas in glucose, lactose, dextrose, maltose and mannitol. All the isolates of *Salmonella* spp. fermented glucose, dextrose and mannitol by acid production. Production of less acid was observed in maltose

fermentation. On the other hand, no acid and gas were produced by *Salmonella* spp. in lactose. All the isolates of *S. aureus* fermented glucose, lactose, dextrose, maltose and mannitol by acid production. All the isolates of *S. epidermidis* fermented glucose, lactose, dextrose, maltose by acid production except mannitol.

Acid production was indicated by the color change of the sugar media from reddish to golden yellow and the gas production was indicated by the accumulation of gas bubbles in the Durham's tubes which are shown in Table 12 and Figure 15, 16, 17, 18, 19.

Table 12. Results of carbohydrate fermentation test

Organisms	**Fermentation properties with five basic sugars**				
	G	**L**	**DX**	**ML**	**MN**
E. coli	AG	AG	AG	AG	AG
***Salmonella* spp.**	A	-	A	A↓	A
S. aureus	A	A	A	A	A
S. epidermidis	A	A	A	A	-

Legends: G = Glucose; L = Lactose; DX = Dextrose; ML = Maltose; MN = Mannitol; A = Acid production; G = Gas production; A↓ = Less acid production; G↓ = Less Gas production; + = Positive reaction; - = Negative reaction

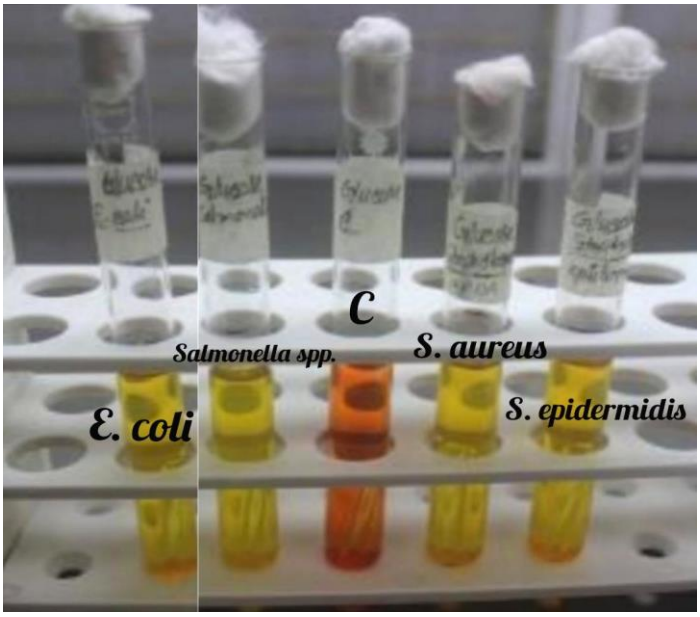


Figure 15: Results of Sugar (Glucose) fermentation test

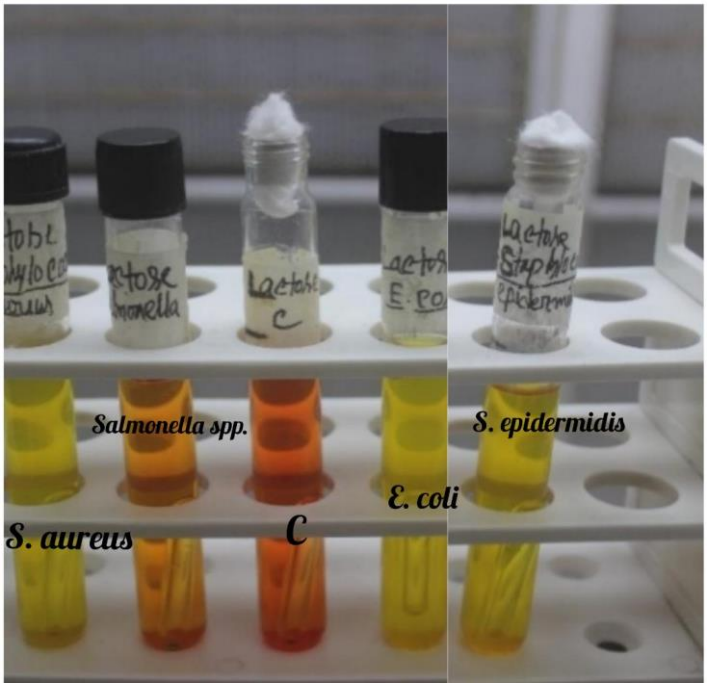


Figure 16: Results of Sugar (Lactose) fermentation test

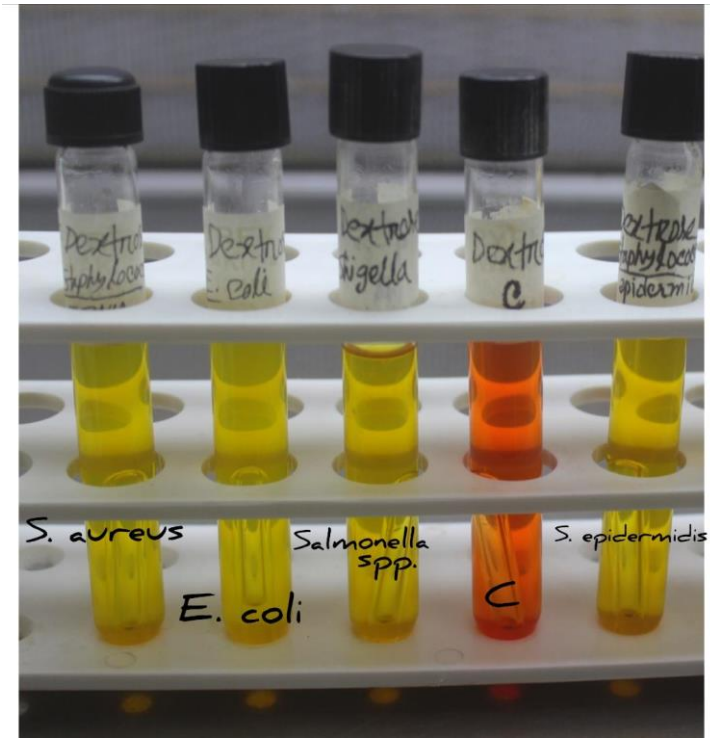


Figure 17: Results of Sugar (Dextrose) fermentation test

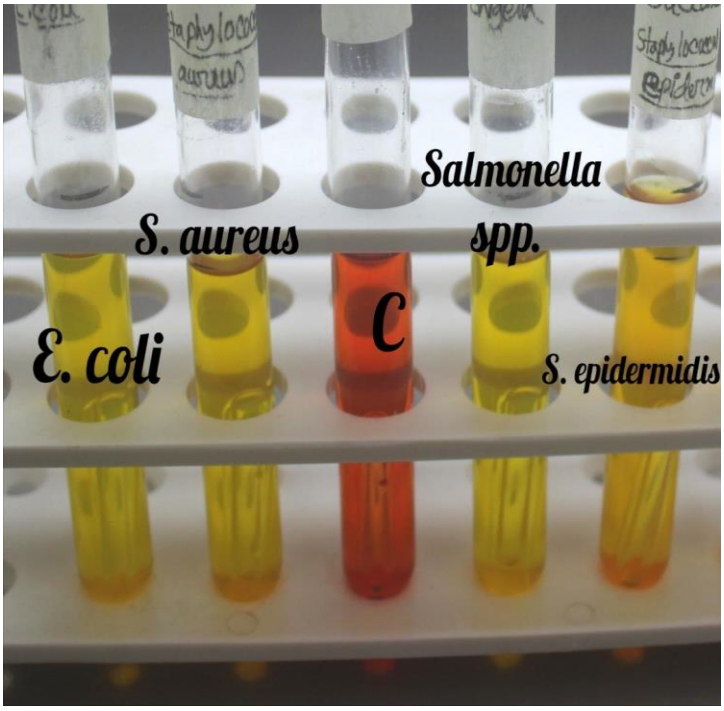


Figure 18: Results of Sugar (Maltose) fermentation test

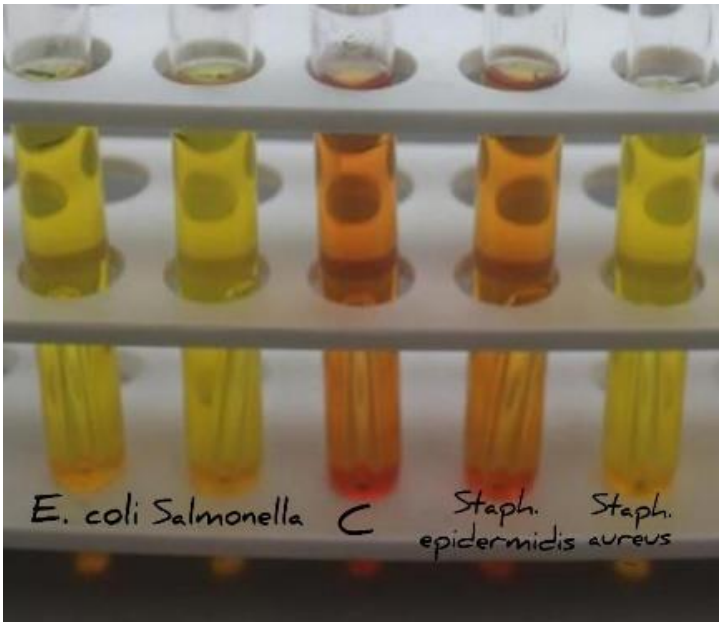


Figure 18: Results of Sugar (Mannitol) fermentation test

4.1.3.3.2 MR test, V-P test, Oxidase test, Catalase test, SIM test and Simmons Citrate agar test

All isolates showed positive results for MR test by formation of red color ring. The result of MR test was demonstrated in Table 13 and Figure 20.

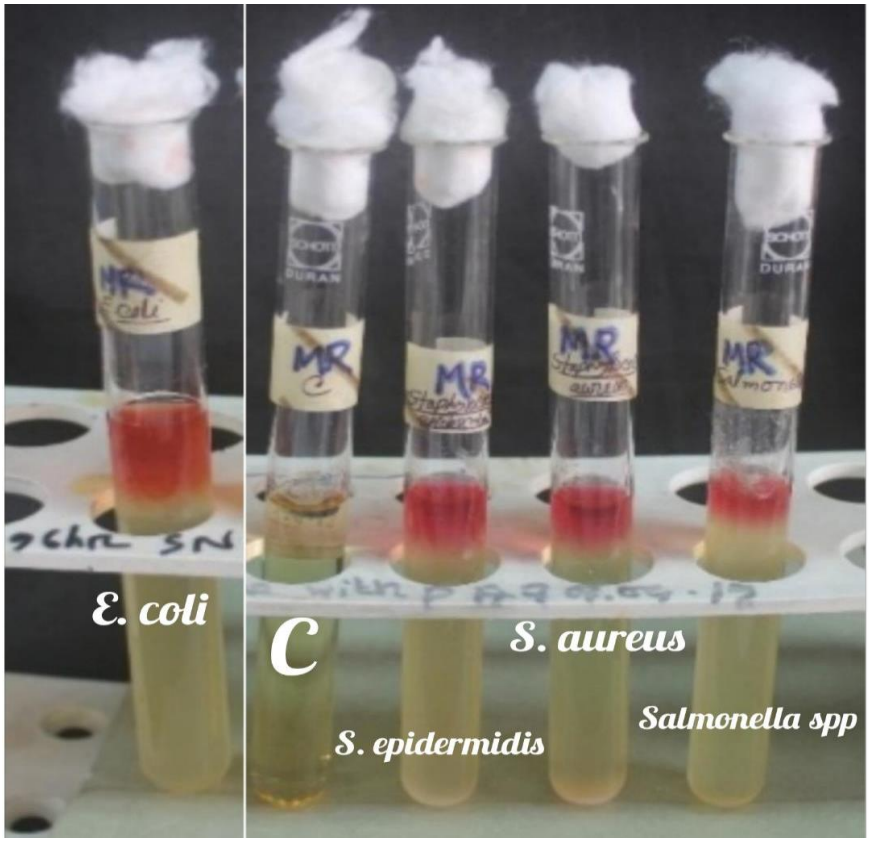


Figure 20: Result of MR test

Suspected *E. coli* and *Salmonella* spp. did nor form any ring in V-P test which meant V-P test for *E. coli*, *Salmonella* and *S. epidermidis* spp. were negative. On the contrary, suspected *S. aureus* was V-P test positive, they produce yellow color ring. The result of V-P test was demonstrated in Table 13 and Figure 21.

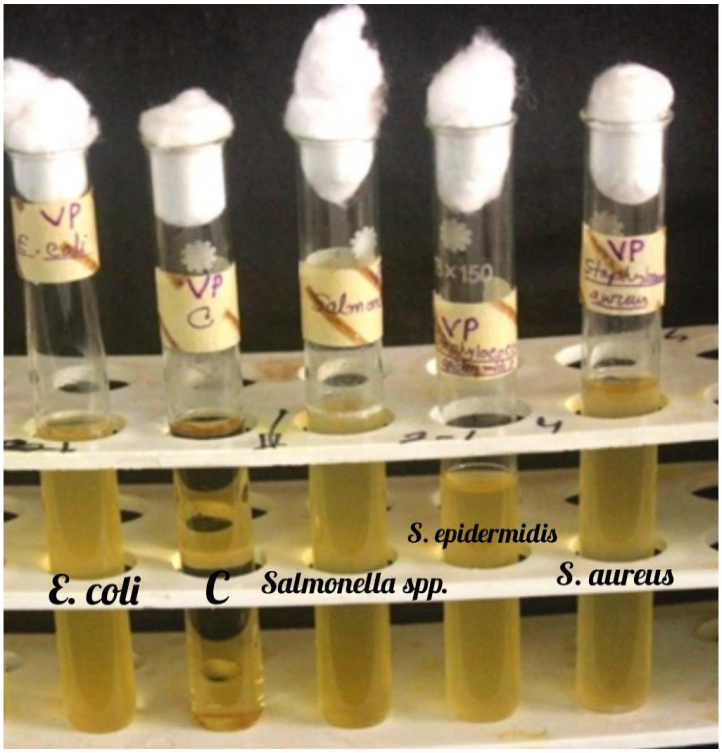


Figure 21: Result of V-P test

Oxidase test was negative (no color change) for all suspected organisms. The result of oxidase test was demonstrated in Table 13 and in Figure 22.

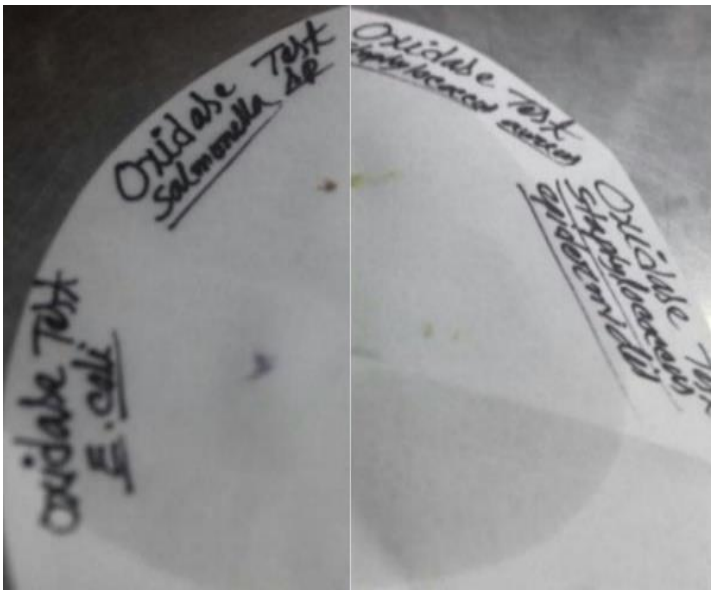


Figure 22: Result of Oxidase test

Catalase test was positive for all the isolates which was indicated by bubble formation in glass slides. The result of catalase test was demonstrated in Table 13 and in Figure 23.

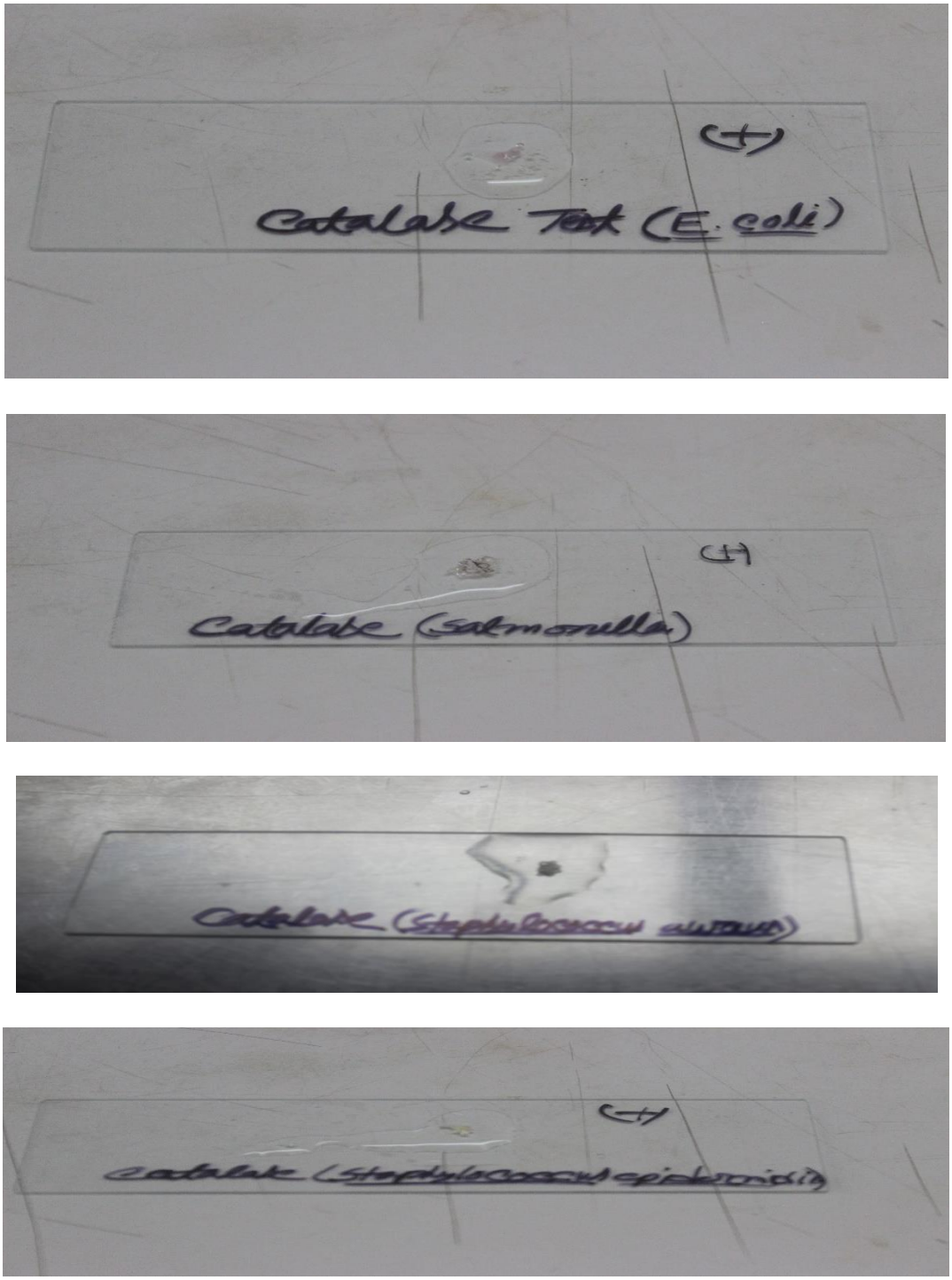


Figure 23: Result of Catalase test

The reaction of all isolates on SIM media were different for specific isolates. Red ring was only formed by *E. coli* which indicated *E. coli* was indole positive. Turbidity was found in suspected *E. coli* and *Salmonella* spp. which denoted that these two were motile. And blackening of media were found in *Salmonella* spp. and *S. epidermidis* containing test tubes which was the indication of H_2S production. The result of SIM test was displayed in Table 13 and Figure 24.

Simmons Citrate agar media green color turned in to blue in *Salmonella* spp. and *S. aureus* containing test tubes that indicated only these two were citrate positive. The result is displayed in Table 13 and Figure 25.

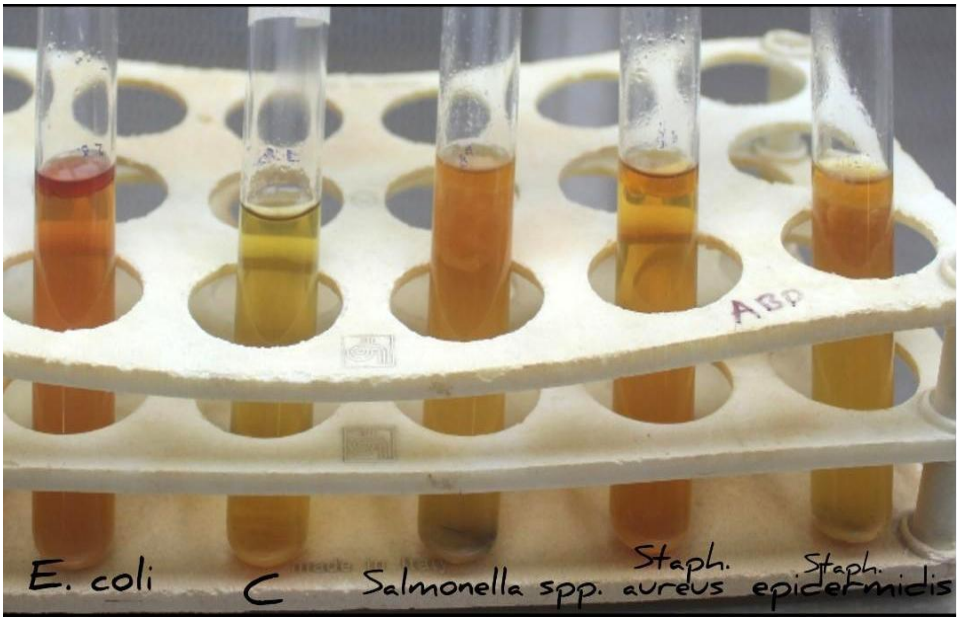


Figure 24: Result of SIM test

Figure 25: Result of SC agar test

Table 13. Results of MR test, V-P test, Oxidase test, Catalase test, SIM test and Simmons Citrate agar test

Isolates	MR test	V- P Test	Oxidase test	Catalase test	SIM test			Simmons citrate agar test
					S	I	M	
E. coli	+	−	−	+	−	+	+	−
***Salmonella* spp.**	+	−	−	+	+	−	+	+
S. aureus	+	+	−	+	−	−	−	+
S. epidermidis	+	−	−	+	+	−	−	−

Legends: + = Positive reaction; - = Negative reaction

4.1.3.4 Molecular identification of *E. coli*

4.1.3.4.1 Result of PCR

For molecular identification, DNA was extracted from isolated *E. coli*. Then PCR was performed and electrophoresis was done in 2% agarose gel. About 584bp DNA was amplified from 16S rRNA gene using the primer 16 E1 (GGGAGTAAAGTTAATCCTTTGCTC) as forward for both pathogenic and non-pathogenic *E. coli*. 16 E2 (TTCCCGAAGGCACATTCT) and 16 E3 (TTCCCGAAGGCACCATC) were used as reverse primer for the amplification of pathogenic and non-pathogenic *E. coli*, respectively.

20 isolates were selected randomly for PCR to identify both pathogenic and non-pathogenic *E. coli*. All of them were found positive in PCR. A representative figure is shown below. Positive result found for the pathogenic organism and negative result found for the other reverse primer.

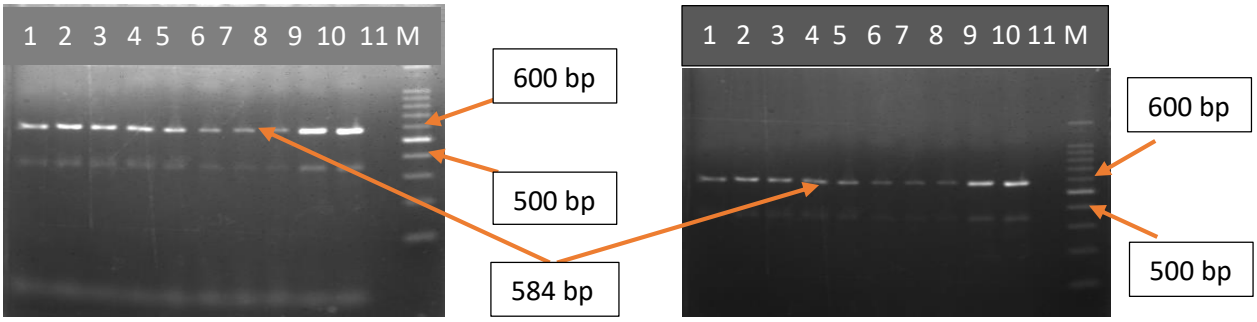


Figure 26: Amplification of 584bp DNA from 16S rRNA gene of *E. coli*

(Left: pathogenic, Right: non-pathogenic; Lane 1-10: Test sample, Lane 11: Negative control, Lane M: 100bp DNA Marker,)

4.1.3.4.2 Phylogenetic analysis

Obtained sequences were found to have high homology which ranges from 99.15 to 100% (Table 16). However, our sequences were clustered into two clusters/clades (Figure: 28).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).The optimal tree with the sum of branch length = 0.02015216 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. This analysis involved 20 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 586 positions in the final dataset. Our sequences are marked by black circle in the tree.

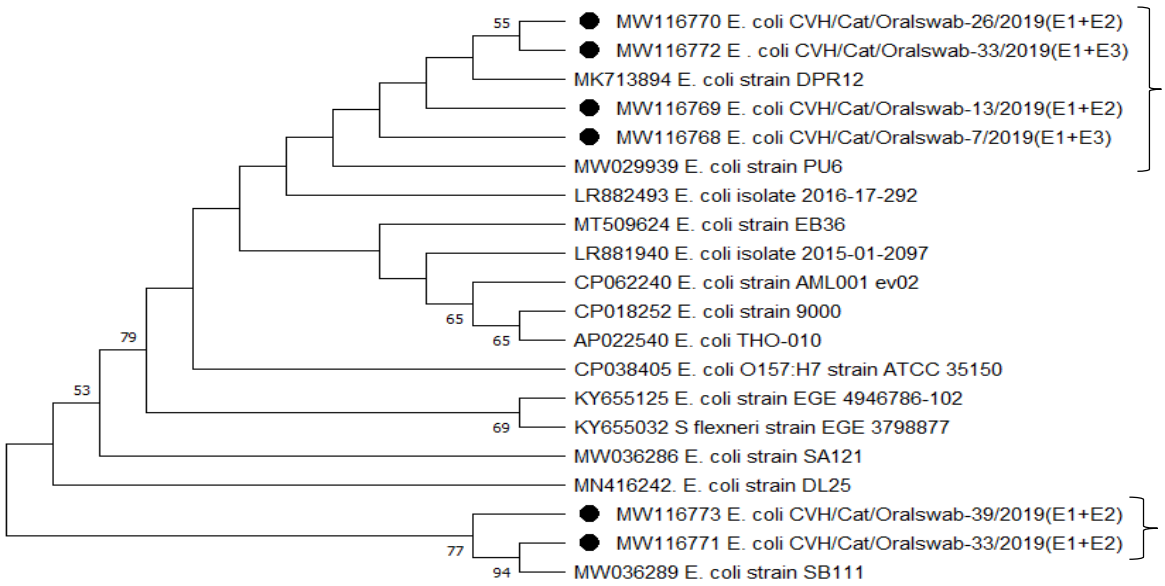


Figure 27: Phylogenetic analysis of *E. coli*

Table 14. The estimates of evolutionary divergence between sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. MW116768 E. coli CVH/Cat/Oralswab-7/2019(E1+E3)																			
2. MW116769 E. coli CVH/Cat/Oralswab-13/2019(E1+E2)	0.000																		
3. MW116770 E. coli CVH/Cat/Oralswab-26/2019(E1+E2)	0.000	0.000																	
4. MW116771 E. coli CVH/Cat/Oralswab-33/2019(E1+E2)	0.015	0.015	0.014																
5. MW116772 E . coli CVH/Cat/Oralswab-33/2019(E1+E3)	0.002	0.002	0.002	0.016															
6. MW116773 E. coli CVH/Cat/Oralswab-39/2019(E1+E2)	0.002	0.002	0.002	0.002	0.002														
7. KY655125 E. coli strain EGE 4946786-102	0.002	0.002	0.005	0.019	0.003	0.006													
8. KY655032 S flexneri strain EGE 3798877	0.002	0.002	0.005	0.019	0.003	0.006	0.000												
9. MN416242. E. coli strain DL25	0.002	0.002	0.006	0.006	0.006	0.004	0.002	0.002											
10. MW036286 E. coli strain SA121	0.000	0.000	0.004	0.004	0.004	0.004	0.000	0.000	0.000										
11. CP062240 E. coli strain AML001 ev02	0.000	0.000	0.003	0.017	0.005	0.006	0.002	0.002	0.002	0.000									
12. MW036289 E. coli strain SB111	0.015	0.015	0.017	0.003	0.019	0.006	0.016	0.016	0.002	0.000	0.014								
13. CP018252 E. coli strain 9000	0.000	0.000	0.003	0.017	0.005	0.006	0.002	0.002	0.002	0.000	0.000	0.014							
14. AP022540 E. coli THO-010	0.000	0.000	0.003	0.017	0.005	0.006	0.002	0.002	0.002	0.000	0.000	0.014	0.000						
15. MT509624 E. coli strain EB36	0.000	0.000	0.000	0.015	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.015	0.000	0.000					
16. CP038405 E. coli O157:H7 strain ATCC 35150	0.000	0.000	0.000	0.015	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.015	0.000	0.000	0.000				
17. MK713894 E. coli strain DPR12	0.000	0.000	0.000	0.015	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.015	0.000	0.000	0.000	0.000			
18. LR882493 E. coli isolate 2016-17-292	0.000	0.000	0.000	0.015	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.000		
19. LR881940 E. coli isolate 2015-01-2097	0.000	0.000	0.000	0.015	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.000	0.000	
20. MW029939 E. coli strain PU6	0.000	0.000	0.000	0.015	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.000	0.000	0.000

4.1.4 Prevalence of specific bacteria

Table 15. Prevalence of *E.coli, Salmonella* spp., *S. aureus* and *S. epidermidis*

Organism from sample	Isolated organism	Number of isolates	Prevalence
All (S1-S40)	*E.coli*	40	100
S11, S13	*Salmonella* spp.	2	5
S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S17, S19, S21, S25, S26, S28, S30, S31, S32, S33, S35, S36, S39	*S. aureus*	23	57.5
S27, S31, S35	*S. epidermidis*	3	7.5

4.1.5 Antibiotic sensitivity and resistance pattern of *E. coli, Salmonella* spp., *S. aureus* and *S. epidermidis* isolated from cat oral cavity

Drug sensitivity test was observed for 10 *E. coli* isolates (S5, S7, S11, S13, S 19, S26, S31, S33, S36 and S39) which were selected randomly from 40 isolates, all (S11, S13) *Salmonella* spp. isolates, 5 *S. aureus* isolates were selected randomly, and all (S27, S31, S35) *S. epidermidis* of cat saliva.

A large number of *E. coli* isolates from 10 samples were found sensitive to gentamycin (80%) and it was found the highest sensitive antibiotic for *E.coli.* In this study, 70% *E.coli* showed sensitivity to Azithromycin. A little number was sensitive to erythromycin (20%), streptomycin (20%), co-trimoxazole (10%), tetracycline (10%). None of the isolate showed sensitivity against ampicillin, rather they showed the highest resistance (90 %) against ampicillin. Next to ampicillin the resistance pattern were higher in co-trimoxazole (70%), tetracycline (60%), erythromycin (60%), streptomycin (50%), azithromycin (30%). A number of isolates, about 10-30 % showed intermediate resistance against all drugs except azithromycin.

Table 16. Sensitivity and resistance pattern of different *E. coli* isolates

Different drugs	Name of the samples										Resistance (%) to different drugs		
	S5	S7	S11	S13	S19	S26	S31	S33	S36	S39	R	IN	S
Azithromycin	S	R	S	S	S	R	S	S	R	S	30	0	70
Erythromycin	R	R	R	IN	IN	R	R	S	R	S	60	20	20
Streptomycin	IN	R	R	R	S	S	IN	R	R	IN	50	30	20
Ampicillin	R	R	R	R	R	R	R	IN	R	R	90	10	0
Co-trimoxazole	R	R	R	R	IN	R	R	IN	R	S	70	20	10
Gentamycin	S	S	S	S	S	IN	S	IN	S	S	0	20	80
Tetracycline	IN	IN	R	IN	R	R	R	S	IN	R	60	30	10

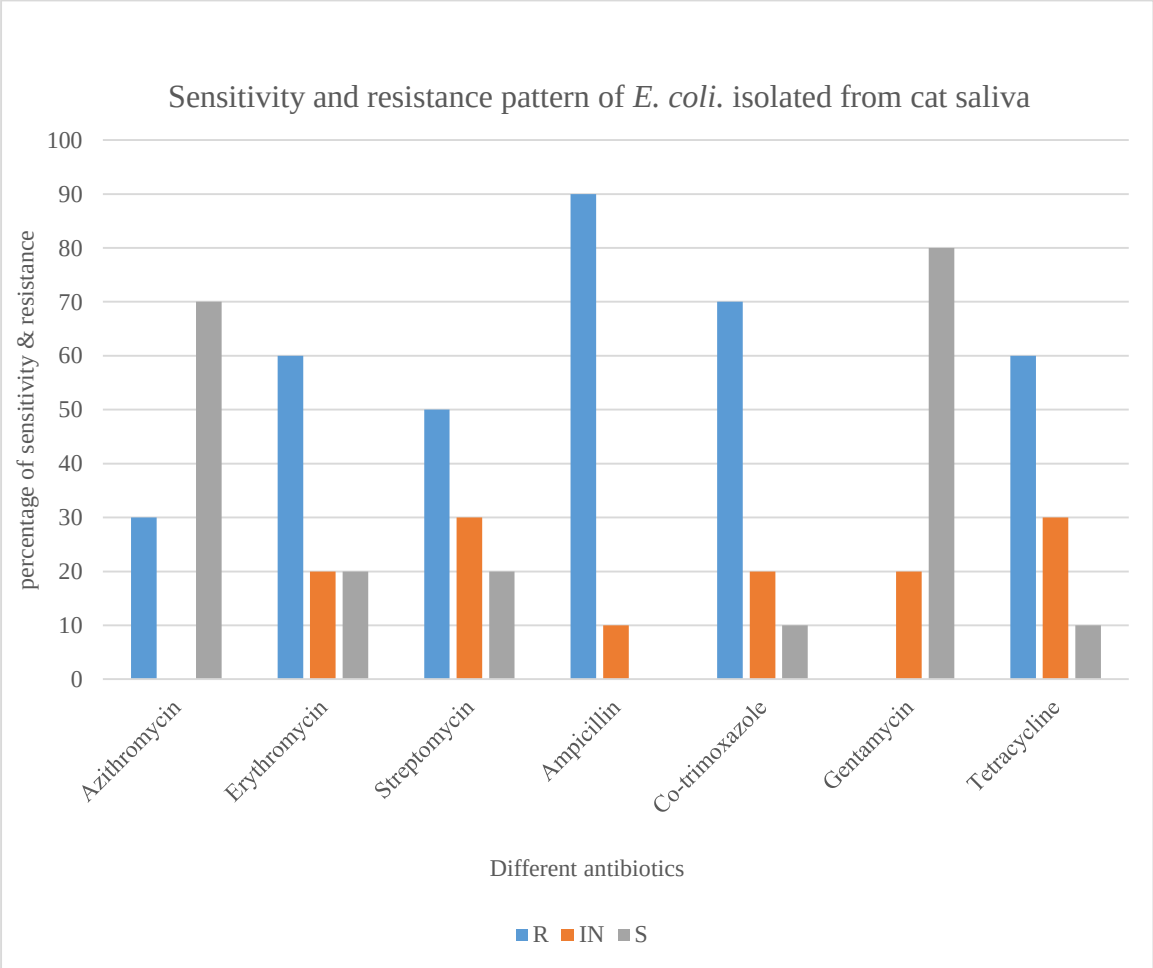


Figure 28: Diagram showing the antibiotic sensitivity and resistance pattern of *E.coli*

Salmonella isolates showed the highest (100%) sensitivity to gentamycin, followed by azithromycin (50%). Highest resistant pattern was showed by ampicillin (100%). Co-trimoxazole showed 100% intermediate resistant pattern. Furthermore, resistance and intermediate resistance pattern were showed equally in azithromycin, erythromycin, streptomycin and tetracycline.

Table 17. Sensitivity and resistance pattern of different *Salmonella* spp. isolates

Different drugs	**Name of the samples**		**Resistance (%) to different drugs**		
	S11	S13	R	IN	S
Azithromycin	IN	S	0	50	50
Erythromycin	R	IN	50	50	0
Streptomycin	IN	R	50	50	0
Ampicillin	R	R	100	0	0
Co-trimoxazole	IN	IN	0	100	0
Gentamycin	S	S	0	0	100
Tetracycline	R	IN	50	50	0

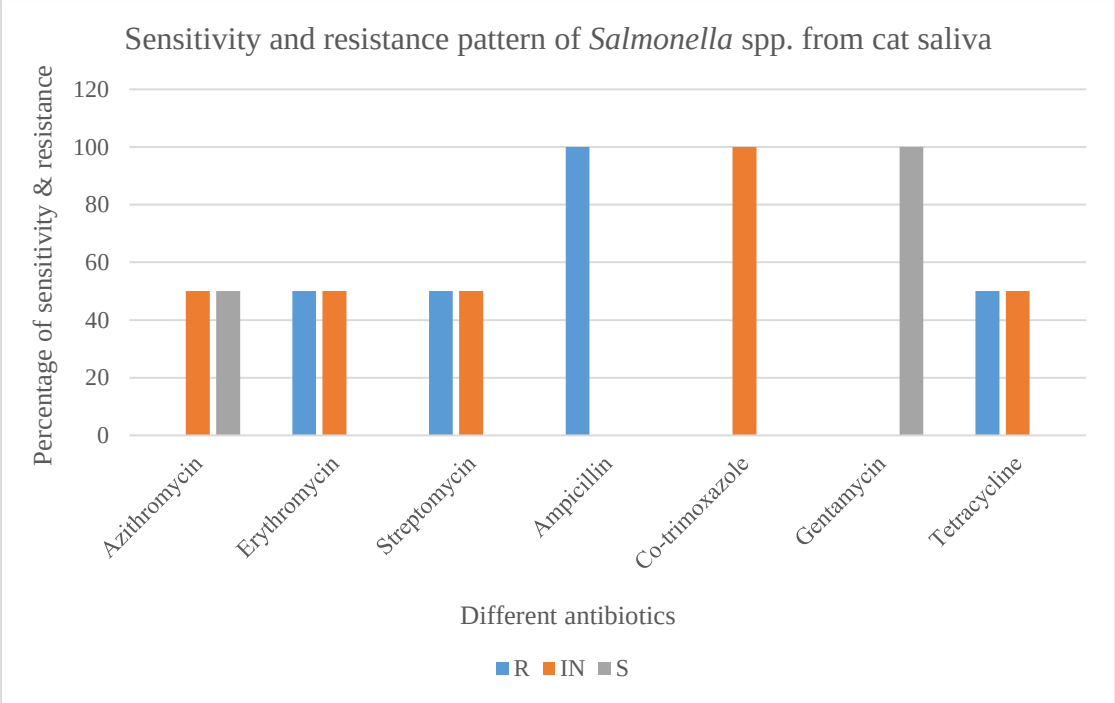


Figure 29: Diagram showing the antibiotic sensitivity and resistance pattern of *Salmonella* spp.

S. aureus isolates showed the highest sensitivity to gentamycin (80%), followed by the co-trimoxazole (60). Azithromycin (40%), streptomycin (20%) and tetracycline are found sensitive in this study. Highest resistant pattern showed by ampicillin (100%), followed by erythromycin (80%), followed by tetracycline (60%). Azythromycin and streptomycin showed equal (40%) intermediate resistance pattern.

Table 18. Sensitivity and resistance pattern of different *S. aureus* isolates

Different drugs	**Name of the samples**					**Resistance (%) to different drugs**		
	S5	S19	S25	S31	S35	R	IN	S
Azithromycin	S	IN	IN	S	R	20	40	40
Erythromycin	R	R	IN	R	R	80	20	0
Streptomycin	R	IN	S	IN	R	40	40	20
Ampicillin	R	R	R	R	R	100	0	0
Co-trimoxazole	S	S	IN	S	R	20	20	60
Gentamycin	S	S	IN	S	S	0	20	80
Tetracycline	R	IN	R	S	R	60	20	20

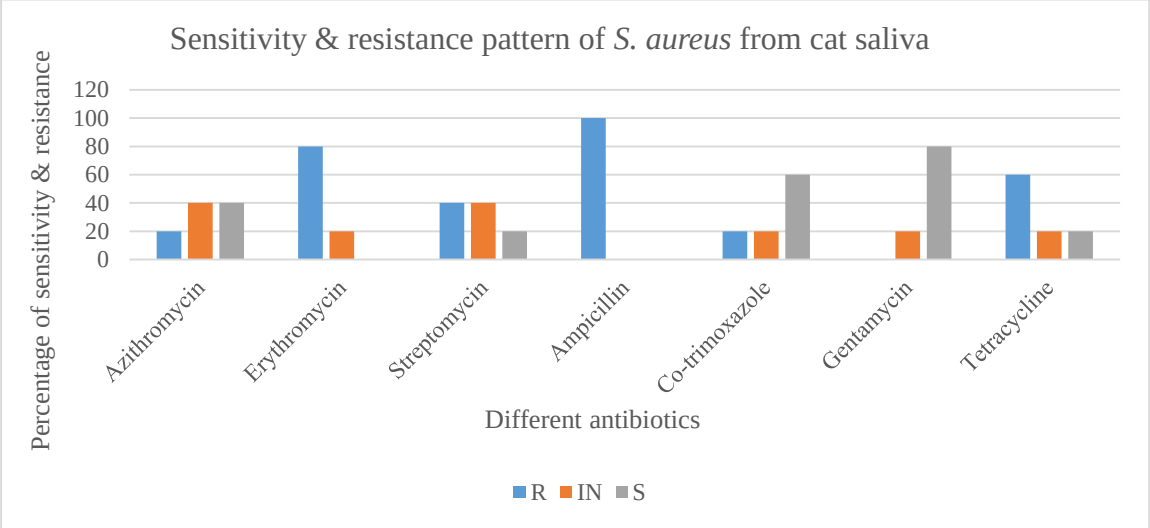


Figure 30: Diagram showing the antibiotic sensitivity and resistance pattern of *S. aureus*

S. epidermidis isolates showed the highest sensitivity to gentamycin (100%). They did not show any sensitivity to ampicillin and tetracycline. Except these two, *S. epidermidis* showed sensitivity and intermediate resistance to azithromycin, erythromycin, streptomycin and co-trimoxazole by 66.66% and 33.33% respectively. The highest resistance pattern were shown in ampicillin (100%), followed by tetracycline (66.66%)

Table 19. Sensitivity and resistance pattern of different *S. epidermidis* isolates

Different drugs	**Name of the samples**			**Resistance (%) to different drugs**		
	S27	S31	S35	R	IN	S
Azithromycin	S	IN	S	0	33.33	66.66
Erythromycin	S	S	IN	0	33.33	66.66
Streptomycin	S	S	IN	0	33.33	66.66
Ampicillin	R	R	R	100	0	0
Co-trimoxazole	S	S	IN	0	33.33	66.66
Gentamycin	S	S	R	0	0	100
Tetracycline	R	IN	R	66.66	33.33	0

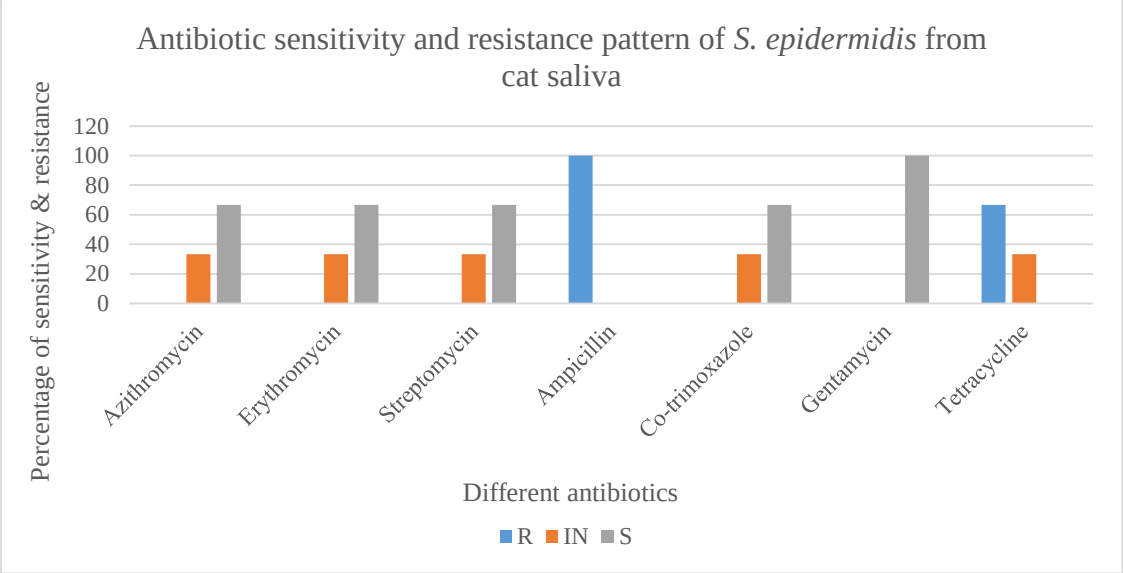


Figure 31: Diagram showing the antibiotic sensitivity and resistance pattern of *S. epidermidis*

4.2 Discussion

This research was conducted to isolate and identify a number of common bacteria species from swab samples of pet cat in Dhaka city and figure out the current status of drug sensitivity and resistance pattern of isolated bacteria so that a veterinarian/ physician can apply the appropriate drug for therapeutic purpose.

The isolation and identification of *E. coli*, *Salmonella* spp., *S. aureus* and *S. epidermidis* were conveyed by cultural properties, staining characteristics, biochemical tests. For the confirmation of *E. coli* molecular test (PCR and sequencing) was additionally done. Furthermore, in vitro antibiotics sensitivity test of isolated bacteria was performed against randomly selected antibiotics. Different kinds of culture media including enriched media and selective media were used to facilitate the growth of *E. coli*, *Salmonella spp., Staphylococcus aureus* and *Staphylococcus epidermidis.* The media used in this study were selected considering the experience of the past researcher worked in various fields relevant to the present study by (Nazir *et al*., 2005; Hassan *et al.,* 2014).

The cultural properties of *E. coli* were the production of metallic sheen on EMB agar and rose pink colored colony on the MacConkey agar, which was similar with the findings of other researches (Kabir *et al*., 2017; Parvej *et al.,* 2018). The morphology of the isolated *E. coli* was exhibited as Gram negative, short plump rod arranged in single, paired or in short chain in gram's staining which was reported in previous study (Mamun *et al*., 2016; Kabir *et al*., 2017; Parvej *et al.,* 2018). *E. coli* isolates were able to ferment the five basic sugars by producing both acid and gas which was supported by (Beutin *et al.,* 1997; Sandhu *et al*., 1996). The isolated *E. coli* were found MR and indole test positive but VP test negative. Those similar results were reported by different investigators (Mishra *et al.,* 2002; Ali *et al.,* 1998). Molecular identification was done for *E. coli*, *E. coli* identification showed positive result 16S rRNA identification band at 584bp, similar result was found in a previous study by (Rawal *et al*., 2013) from lake water. PCR products of a total of six *E. coli* isolates were sequenced from both forward and reverse direction. Sequences were edited and aligned using freely available software (MEGA-X). When compared with sequences from Gene Bank one cluster was found to have closer relation (high homology) with the sequences of *E. coli* strain DPR12 (accession number MK713894) Hyderabad, Telangana, India. This

strain was isolated from poultry. On the other hand our two isolates (MW116771 and MW116773) were clustered with *E. coli* strain SB111 (MW036289) isolated from cow in China. Other bacteria used in phylogenetic analysis were also found to carry high sequence homology. The organisms were derived from various sources of different countries including microbial type culture collection (MW029939), red fox (LR882493), from meat processing plant in Egypt (MT509624), from USA (CP062240), cattle feces (CP018252), blood (extra intestinal pathogenic *E. coli*) from Turkey (KY655125), human in Japan (AP022540).

Specific enriched media like SS as described earlier by others was used for the isolation and identification of *Salmonella* spp. (Kabir *et al.*, 2017; Habrun and Mitak., 2003; Fathpour *et al*., 2003; Núñez *et al*., 1996). Organisms grown on the selective media for *Salmonella* spp. were further subjected to detailed study on morphology, colony characteristics and biochemical properties. The colony characteristics of the organisms grown on SS agar media for *Salmonella* spp. was black color that was in accordance with the findings reported by other authors as characteristics for *Salmonella* spp. (Habrun and Mitak., 2003; Hossain, 2002). In MacConkey agar media, *Salmonella* isolates were able to produce red to pink-white colonies surrounded by brilliant red zones in MacConkey agar. The isolates were revealed as motile as well. These findings were supported by several authors such as (Buxton and Fraser, 1977; Freeman, 1985; Jones *et al.*, 1987) In addition, the isolated organism was Gram's negative, short plump rod arranged singly or in pair which also indicative of *Salmonella* (Musa *et al.,* 2017; Kamal *et al.,* 2018). Furthermore, suspected *Salmonella* spp. were able to ferment glucose, dextrose, maltose and mannitol with the production of both acid and gas but did not ferment lactose, and those characteristics of *Salmonella* spp. were satisfied the statement of (Han *et al.,* 2011 and Musa *et al.,* 2017). The isolated *Salmonella* spp. were found MR test positive but indole and VP test negative that satisfied the statement of (OIE, 2000). These all cultural, morphological and biochemical properties indicated that the isolated organism as species belonging to the genus *Salmonella.*

The colonies of *S. aureus* on mannitol salt agar showed colonies that fermented mannitol and appeared golden yellow were characteristically similar to those reported previously (Shapna *et al.,* 2018; Das *et al.,* 2019; Haque *et al.,* 2018; Nakuleshwar *et al*., 2013). The colonies of *S. epidermidis* showed pink color colonies

which means it could no ferment mannitol and this feature was supported by other research (Nakuleshwar *et al*, 2013). Microscopically gram's stained smear of both species of *Staphylococcus* spp. were gram positive cocci arranged in grape like cluster reported by (Kabir *et al.*, 2017). The isolated *S. aureus* showed glucose, dextrose, maltose, lactose, and mannitol fermentation with only acid. These findings are in close agreement with (Haider *et al.*, 2018; Shapna *et al.*, 2018). However, *S. epidermidis* could not ferment mannitol but fermented rest of the sugars and produced acid (Nakuleshwar et al., 2013). Both *S. aureus* and *S. epidermidis* were positive in Methyl Red test, catalase test and Voges-Proskauer test but negative in oxidase test. Previous researches found out that *S. aureus* was citrate positive and *S. epidermidis* was citrate negative. Additionally, *S. epidermidis* produced H_2S but Staphylococcus aureus did not (Nakuleshwar *et al*, 2013). Only by cultural and biochemical properties it is tough to differentiate between two species of Staphylococcus genus. But the cultural properties on MS agar, Simmons Citrate agar reaction and H_2S production capability/incapability clearly differentiated the presence of Staphylococcus aureus and Staphylococcus epidermidis in cat saliva sample.

In this study, the prevalence of organisms was 100%. All 40 cat saliva sample bacterial contamination in NA. The infection with E.coli was highest (100%), followed by *S. aureus* (57.5%), followed by *S. epidermidis* (7.5 %) and the infection with Salmonella spp. was the lowest (5%). *S. aureus* was found 35.3% in oral cavity of cat in a previous study which was slightly lower than current finding (Magaji *et al*., 2008). An incidence of 10.6%. *Salmonella spp.* was reported in an investigation that is higher than this research (Fox and Beaucage, 1979). But in this study, 5% *Salmonella* spp. was found. These variation might be due to variant food habit, environment, health status and more on.

The prevalence of bacteria is 100 % which indicates bacterial presence is observed in all gender, age, breed and feed habit of cat irrespectively.

E. coli is also present in all bacterial sample. The presence of E. coli indicates that the cat were in touch of environmental or fecal contamination which is similar to history. All cat have licking habit and most of them are not fully indoor. In case, of *Salmonella spp,* it was observed in those animal who were male, ate raw feed, and both of them are above 2 years though one of cats were local breed and another one is exotic. On the

other hand, *S. aureus* and *S. epidermidis* both were found more in female cat than male cat. In case of, gender, breed and feed habit *S. aureus* and *S. epidermidis* was found irrespectively.

Frequent use of broad spectrum antibiotics for any disease is commonly practiced in Bangladesh which is clear sign of the development of multi-drug resistant organisms. Moreover, due to mutation bacteria show resistance to certain antibiotic as well. And these antibiotic resistant bacteria could transmit from cat to human directly via saliva or other ways of lifestyle. Therefore, in this study antibiotic sensitivity of isolated bacteria was observed. A total seven different antibiotics available in the market were used to study antimicrobial susceptibility and resistance profiles of the *E. coli*, *Salmonella* spp. and *S. aureus* were found to grow multidrug resistant (resistant against 4-6 antibiotics) and *S. epidermidis* were found to grow resistance against 2 antibiotics. From that study, it was revealed that the isolated *E. coli* were susceptible to gentamicin, azithromycin and resistant to tetracycline which was supported fully by (Akond *et al.,* 2009). They were resistant to ampicillin, streptomycin which was supported by (Al-Ghamdi *et al.,* 2001). *Salmonella* spp. were susceptible to gentamycin and azithromycin which was supported by (Al-Ferdous *et al.,* 2013). They were found resistant against ampicillin, tetracycline, erythromycin and streptomycin. *Staphylococcus* spp. were found sensitive to gentamicin, streptomycin, azithromycin which was not completely similar the report of (Haider *et al.,* 2018 and Shapna *et al.,* 2018) who reported that *Staphylococcus* spp. was resistant to azithromycin and was sensitive to ampicillin.

Cat is gradually becoming more popular pet animal in Bangladesh. Therefore, the interaction between human and cat is increasing. But the presence of different pathogenic bacteria which are resistant to multiple antibiotics is threating for human health. To reduce this problem, proper hygienic management is essential to maintain by the cat owner.

CHAPTER 5
SUMMARY AND CONCLUSION

The current study was conducted to isolate the bacteria from the oral cavity of pet cat in Dhaka city and additionally to perform a comparative study to observe the sensitivity and resistance patterns of the isolates to different antimicrobial agents from January, 2019 to December, 2019. A total of 40 cat oral swab samples were collected. The samples were immediately transferred to the laboratory of the National Institute of Biotechnology (NIB) maintaining proper cool chain for bacterial analysis.

In the laboratory, firstly, total viable count (TVC) of raw bacterial samples was done by 10 fold dilution method where 100% prevalence of bacteria was found. The highest TVC (1.199×10^{13}) and the lowest TVC was found in S6 (3.60×10^{2}). The samples were subjected to various tests and experiments accordingly for isolation and identification of organisms in cat. The prevalence of *E. coli*, *Salmonella* spp. and *S. aureus and S. epidermidis* were 100%, 5%, 57.5% and 7.5 % respectively.

Primary isolation of these bacteria were performed by propagating the organisms in nutrient broth followed by culture on different selective agar media such as MacConkey agar, EMB agar, SS agar and MS agar for the determination of their distinctive colony characteristics. They were identified on the basis of colony morphology. Gram's staining technique; and Simmons Citrate agar reaction was observed. In addition to, biochemical properties of the isolates were studied by fermentation test with five basic sugars and also by Catalase test, MR test, V-P test and SIM test. For the confirmation, PCR and sequencing of *E. coli* were done.

The study was further extended to investigate sensitivity and resistance pattern of the identified isolates to different antibiotics. This in vitro test revealed that there were considerable variations among the isolates of different sources in respect of antibiotics sensitivity and resistance pattern.

All isolates showed a higher sensitivity to GEN and higher resistance against AMP. High percentage of *E. coli* isolates from the pet cat were sensitive to GEN and AZM while most of the *E. coli* isolates were resistant to AMP, COT, TE, E and S. In case of *Salmonella* isolated good sensitivity found only against GEN while AMP and COT were 100% resistant to the *Salmonella* spp. *S. aureus* isolates showed the highest

sensitivity to GEN, followed by COT, followed by AZM. AMP showed the highest resistance against *S. aureus* isolates as well. Among all isolates *S. epidermidis* showed more sensitivity to multiple drugs and they were GEN, AZM, E, S and COT respectively. However, *S. epidermidis* showed complete resistance against AMP. It is assumed that one or more drug resistant clones have gradually acquired resistance to other drugs by conjugation with multi-drug resistant strains.

From the present study it may be concluded that

(a) The prevalence of identified bacteria *E. coli, Salmonella* spp. and *S. aureus and S. epidermidis* were 100%, 5%, 57.5% and 7.5 % respectively.

(b) *E. coli* isolates showed the highest sensitive to gentamycin (80%) azithromycin (70%) and the highest resistant to ampicillin. Isolates of *Salmonella* were found to be highest sensitive against gentamycin (100%) followed by followed by azithromycin (50%). Highest resistant pattern of *Salmonella* spp. was showed against ampicillin (100%). *Salmonella* spp. showed 50% resistant to erythromycin, streptomycin and tetracycline. *S. aureus* showed the highest sensitivity to gentamycin (80%), followed by co-trimoxazole (60%) and the highest resistance pattern was shown against ampicillin (100%), followed by erythromycin (80%) and tetracycline (60%). *S. epidermidis* showed the highest (100%) resistant against ampicillin and the highest (100%) sensitive to gentamycin. So, the study found multi-drug resistant *E. coli, Salmonella* spp., *S. aureus.* Only *S. epidermidis* was not found as multidrug resistant.

(c) Treatment of *E. coli, Salmonella* spp., *S. aureus* and *S. epidermidis* should be carried out with gentamycin in infectious condition of cat.

(d) Indiscriminate use of antimicrobial agents should be avoided in order to eliminate health hazards in man and animals caused by *E. coli, Salmonella* spp., *S. aureus* and *S. epidermidis* through preventing the development of multi-drug resistant mutants in nature.

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APPENDIX I

Composition of different media

1. Nutrient Broth

peptic digest of animal tissue	5.0 gm
Sodium chloride	5.0 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Distilled water	1000 ml
Final pH (at 25ºC)	7.4 ± 0.2

2. Nutrient Agar

Peptone	5.00 gm
Sodium chloride	5.00 gm
HM peptone	1.50 gm
Yeast extract	1.50 gm
Agar	15.0 gm
Final pH (at 25°C)	7.4±0.2

3. MacConkey Agar

Peptones (meat and casein)	3.00 gm
Pancreatic digest of gelatin	17.00 gm
Lactose monohydrate	10.00 gm
Bile salts	1.50 gm
Sodium chloride	5.00 gm
Crystal violet	0.001 gm
Neutral red	0.030 gm
Agar	13.50 gm
Final pH (at 25°C)	7.1±0.2

4. Eosin Methylene Blue Agar

Peptic digest of animal tissue	10.000 gm
Dipotassium phosphate	2.000 gm
Lactose	5.000 gm
Sucrose	5.000 gm
Eosin - Y	0.400 gm
Methylene blue	0.065 gm
Agar	13.500 gm
Final pH (at 25°C)	7.2±0.2

5. Mueller Hinton Agar

HM infusion B from	300.000
Acicase	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.4±0.1

6. Methyl Red Indicator

Methyl red	0.200 gm
Ethyl alcohol	60.000 ml
Distilled water	40.000 ml

7. MR-VP Broth

Buffered peptone	7.000
Dextrose	5.000
Dipotassium phosphate	5.000
Final pH (at 25°C)	6.9±0.2

8. Phosphate Buffer Saline

Sodium chloride	8.0 gm
Disodium hydrogen phosphate	2.8 gm
Potassium chloride	0.2 gm
Potassium hydrogen phosphate	0.2 gm
Distilled water to make	1000 m