Study on antibiotic sensitivity pattern of Gram-positive bacteria isolated from cats in Chattogram Metropolitan Area

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Medicine

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DR. Ajoy Dev Nath

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Abbreviations	Elaborations
%	Percentage
≥	Greater than or equal to
≤	Less than or equal to
95% CI	95% confidence interval
AMR	Antimicrobial resistance
BA	Blood agar
CVASU	Chattogram Veterinary and Animal Sciences University
MRSA	Methicillin resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
Mbp	Mega base pairs
PCR	Polymerase chain reaction
SAQTVH	Shahedul Alam Quadary Teaching Veterinary Hospital

ABSTRACT

Globally, antibiotic resistant pathogens have become an emerging problem in animals with the great significant of public health issue. In developing countries like Bangladesh, indiscriminate use of antibiotics is very common and leading to widespread development of antimicrobial resistance. A 12 months long observational study was conducted to identify the gram positive bacterial infection in upper respiratory tract of cat and to determine their antimicrobial susceptibility pattern during March 2019 to March 2020. A total of 100 nasal swab samples were collected from cats admitted to Shahedul Alam Quadary Teaching Veterinary Hospital (SAQTVH), Chattogram Veterinary and Animal Sciences University following standard procedure. Animal related data were recorded through face to face interview using a structured questionnaire. Then the bacteria were isolated through the conventional bacteriological procedure and finally identified by polymerase chain reaction (PCR). The identified bacteria were investigated for antimicrobial susceptibility testing using disc diffusion technique. Out of the 100 cats, 40 (40%, 95% C.I.) and 18(18%, 95% C.I.) were positive for *Streptococcus* species and *Staphylococcus* species respectively. A total of 9 isolates were confirmed as *staphylococcus aureua* (9%). The result of antimicrobial antibiotic susceptibility showed that *Streptococcus* spp. and *Staphylococcus* spp. displayed resistance to Penicillin (62.5%, 38.9%) and Ampicillin (35%, 44.4%) ($p< 0.05$). All isolates were sensitive to Ciprofloxacin. *Streptococcus* infection show less than 20% Resistant to the oxytetracycline and doxycycline and Staphylococcus infection 100% susceptible to the oxytetracycline and doxycycline. The analysis of Univariable logistic regression found that young cat less than 2 years of age were more susceptible to the *Streptococcus* and *Staphylococcus* infection rather than aged cats. Similarly, local breed almost 3 times more prone to the Streptococci and Staphylococci infection. No remarkable and significant association was found in regarding sex. Presence of this zoonotic pathogen like *Streptococcus* spp. and *S. aureus* could be interrelated with the possibility for the exchange of antimicrobial resistance gene between human and cat population. Public awareness about the resistance of bacteria to the antibiotic should

be increased and proper legislative action should be implemented about ensuring prudent use of antibiotic.

Keywords: antimicrobial resistance, Streptococcus, Staphylococcus, *Streptococcus canis, Staphylococcus aureus*

Chapter-1: Introduction

Surveillance of antimicrobial resistance (AMR) is a major concern in both human and veterinary medicine and consistently assist clinicians and veterinarians to make justified antibiotic choices. The empirical use of antibiotics leads to the emergence of antibiotic resistant bacterial strains to constitute a great challenge for clinicians and limits the medication choices. The similar situation also present in use of antibiotics in pet animals making it difficult to treat infections. However, development of resistance is problematic in many pathogens and commensals, including Streptococci and Staphylococci (Hancock, 2005). Now, this creates pressure to use antimicrobials that are important in human medicine. It is difficult to understand the extent and importance of AMR in companion animals because of limited surveillance.

Evaluation of the scope of AMR in companion animals is difficult, as there are few formal surveillance programs, particularly compared to the data available for food animal species. Furthermore, the occurrence of AMR data in bacteria from animals, foods and humans are published every year by the European Food Safety Authority. EU member states reported the data in each year. This reporting system publishes the data for many different species but the occurrence of AMR in bacteria from companion animals are absent or scare. Antimicrobial resistance in bacteria has become a significant issue for human health nowadays. Pet animals act as a reservoir of various antimicrobial resistant bacteria (Guardabassi et al., 2004; Lloyd, 2007; Weese, 2008) sharing environment of humans and companion animals, transfer of resistant bacteria or motile resistance determinants between companion animals and humans is likely to occur and has been indicated in multiple previous studies (Simjee et al., 2002; Guardabassi et al., 2004; Marshall and Levy, 2011; Lozano et al., 2017) but the extent to which this occurs is still largely unknown (Weese et al., 2006; Zhang et al., 2011).

Various longitudinal and retrospective studies in Europe and the United States have reported an increase in the prevalence of AMR in different bacterial species isolated

from pet animals (Pedersen and Wegener, 1995; Lloyd et al., 1996). So, considering this emerging situation and limiting studies in companion animals this study mainly focused on comprehensively describing *Streptococcus* and *Staphylococcus* spp.

In Bangladesh, numerous studies of antimicrobial resistance for different pathogens of food animals have already been published (Sarker and Samad, 2011; Islam et al., 2016) but no report of companion animals such as cat yet published to the best of the authors knowledge. All groups of antimicrobials are widely used in treatment of animal diseases but some of them are regarded as reserve group for use in humans. Fluoroquinolones and Cephalosporins are the major antimicrobial drugs ranked by the U.S. Food and Drug Administration as critically important in human medicine, and for which emergence of resistant bacteria is especially undesirable (Food, 2003).

In Denmark, A comparatively small number of companion animals like 550,000 dogs and 650,000 cats consume approximately the same amount of Fluoroquinolones and Cephalosporins as consumed annually in the much larger population of food animals (Heuer et al., 2005). The total sales of veterinary antimicrobial formulations approved for use in pet animals in Sweden and Norway are also increased day by day. It is 3% of all veterinary antimicrobial formulations in 1990 to 8% and 7% in 1998 (Odensvik et al., 2001). In the UK, therapeutic antimicrobials indicated for use in companion animals represent approximately 6% of the total amount used in animals, particularly in dog and cat (Directorate, 2002).

Many countries are consistently attempting to develop guidelines for the judicious use of antimicrobials in companion animals. Considerable attention is being given to antimicrobials resistance regarding public and animal health in the European commission. The Heads of Medicines Agencies, the Federation of Veterinarians of Europe and a number of Member States and veterinary organizations all issuing strategies and action plans for the treatment of pet animal (De Briyne et al., 2014a; De Briyne et al., 2014b) so it is an essential to develop a guideline for the use of antibiotic in other countries. The present study mainly reported on the prevalence of *Streptococcus* and *Staphylococcus* spp. in cat with their antibiotic sensitivity pattern.

In recent years rearing of cat has become a trend in Bangladesh. Pet populations are increasing day by day. This number mainly increases in the metropolitan area among the literate people of Bangladesh. A large number of pet clinics are opened in main cities of Bangladesh. All type of antibiotics is used in treatment of pet animal in Bangladesh indiscriminately. Infection with the multi-drug resistant bacteria from cat in human has become a growing concern(Guardabassi et al., 2004). Therefore, there is a need to explore the antibiotic resistance in cat. On this background, this study was aimed to achieve following objectives.

1.1.Objectives

So the present study was conducted to meet following objectives:

a) To isolate and identify the common Gram-positive bacteria from cat in Chattogram Metropolitan Area
b) To study the risk factors associated with bacterial pathogens isolated from cats
c) To study the antimicrobial sensitivity pattern of the bacteria isolated from cat.

Chapter-2: Review of literature

Among the carnivorous animal, cat is the most widely domesticated species under the family felidae. It often referred to as the domestic cat to distinguish it from wild members of the family. Cats are common pets in all continents of the world except Antarctica. Although, global cat population is difficult to ascertain, it is roughly estimated that ranging from anywhere between 200 million to 600 million (Gehrt et al., 2010). In Europe more than 102 million and 86 million households have their own cats and dogs, respectively. Multifarious bacterial infection in cat is very common and hence they get exposure with almost all types of antibiotics. So, there is strong possibilities of developing and exchange of antibiotic resistant bacteria. The main purpose of this chapter is to provide latest information concerning the research work which is addressed here. Important information related to the isolated bacterial species has been reviewed under the following headings and sub-headings.

2.1. Risk factor

Disease associated with bacterial infection in respiratory tract (RTI) can occur infection with certain bacterial pathogens which have devastating effect on respiratory tract or proliferation of the normal bacterial inhabitants of the respiratory tract if the natural defense mechanisms are impaired (Schulz et al., 2006). Predisposing factors include concurrent virus infection, toxoplasmosis, lungworm infection, trauma, aspiration pneumonia, neoplasia, tooth root infection, nasopharyngeal polyps, congenital anomalies, otitis media or interna, metabolic dysfunctions and immunosuppressive therapy (Van Pelt and Lappin, 1994). Previous reports identified *Pasteurella, Streptococcus, Staphylococcus* species and *Escherichia coli* (Dossin et al., 1998) as most frequent pathogens inhabited in nasal samples. On the other hand, *Pasteurella* species, *E coli, Streptococcus, Enterobacter* and *Staphylococcus* species are commonly isolated bacteria from the lower airway samples (Macdonald et al., 2003).Young cat are more susceptible than the older cat due to limited exposure to the infection and possible exchange of maternal antibody (Pesavento and Murphy, 2014).

2.2. Streptococcaceae

Among the gram positive bacteria streptococcus is a spherical shaped bacteria belonging to the phylum Firmicutes and the order Lactobacillales (lactic acid bacteria). Species of β hemolytic Streptococcus are typically pathogenic for all animal species (Markey et al., 2013). Due to single axis cell division of these bacteria, they grow in chains or pairs. Different streptococci have been rarely isolated from cats, including *Streptococcus agalactiae, S pneumoniae* and *S suis*. The most prevalent species is *S equi* sub species *zooepidemicus* has been recognized as an emerging pathogen in dogs, and also recently in cats (Chalker et al., 2003). However, some pathogenic species, such as *S. agalactiae, S. equisimilis*, have variable hemolytic patterns. Because of cell wall polysaccharides (C-substance), some species can be categorized into alphabetically designated Lancefield groups. Lancefield groups B, C, D, or G are the main pathogenic species of Streptococcal infection in cat (Frymus et al., 2015).

2.2.1. Morphology of Streptococci

Most of the streptococcus is oxidase and catalase negative, and many are facultative anaerobes. In routine Gram stains, cytologic preparations, and histological sections *Streptococcus* spp. *a*re Gram positive bacterial cocci that often appear in pairs or chains. *Streptococcus* spp. are easily cultivated on blood agar that are categorized on the basis of their hemolytic pattern on as α hemolytic, β hemolytic or g hemolytic (non hemolytic) (Lamm et al., 2010). The Lancefield groups A, C, E, and G identified in domesticated cat among the four sero groups of hemolytic streptococci (Biberstein et al., 1980). Of these four, *Streptococcus* spp. is described as belonging to group G due to it being a beta-hemolytic and aesculin negative *Streptococcus* that is able to ferment lactose. These bacteria are essentially known to be part of the existent natural flora of the respiratory tract of cats.

2.3. Cat diseases caused by *Streptococcus* spp.

Multifarious infections in cat are caused by *streptococcus* spp. It is generally considered that the *S. canis, S. zooepidemicus* is not part of the normal micro flora of cats (Devriese et al., 1992). Nevertheless, both canine and feline sub - clinical infections have been observed (Feng and Hu, 1977; Abbott et al., 2010). The

bacterium may also act as a primary cause of feline pneumonia in per acute course, although experimental infections have not been performed (Gower and Payne, 2012). It has been postulated that close confinement of animals, such as in shelters, research laboratories and other facilities may be the major risk factor for the development of *S zooepidemicus* associated disease in dogs and cats (Britton and Davies, 2010). In older cats, the infection is usually opportunistic, as a result of wounds, surgery and immunosuppressant stage (Pesavento and Murphy, 2014), Conditions associated with this pathogen include abscesses, pneumonia, polyarthritis, urogenital infections, necrotizing fasciitis (toxic shock syndrome), sinusitis and meningitis. Outbreaks of fatal disease in cats have been reported in shelters and breeding colonies, or viral infection. In those cats which suffering from chronic upper respiratory tract disease, *Streptococcus canis* can be isolated from the nasal cavity of those cats (Pesavento and Murphy, 2014), especially of the lung and heart (Frymus et al., 2015). *S. canis* was isolated mainly from dogs and less in cats, and in rare cases it was also isolated from the udders of lactating cows and other animal species (Hassan et al., 2005).

2.4. Pathogenesis of streptococcus

In many small animal, the rapid onset of disease and clinical signs are similar to human toxic shock syndrome caused by *Streptococcus pyogenes* (Priestnall et al., 2010). Toxic shock syndrome is characterized by a hyper reactive inflammatory response. As a result of increase in vascular permeability follows vasodilatation and increased coagulation and finally lead to migration of inflammatory cells to the site of infection (Lappin and Ferguson, 2009). Additionally, pyrogenic exotoxins produced by some streptococci act as super antigens by binding simultaneously to major histocompatibility complex class II receptors on macrophages and T-cell receptors, by passing conventional antigen presentation, and help to the activation of a large proportion of T lymphocytes (Fraser and Proft, 2008). So far, no clinical signs like toxic shock syndrome have been described in cats and Group C and G streptococci (GCS and GGS) are commonly regarded as commensals because they are often found in association with the normal flora of human skin, pharynx and the intestine (Johnson, 2000). Mortality from *Streptococcus* spp. in humans is very low with only a few reported cases. This species, in general, is highly susceptible to antibiotics, and

there are plans to develop a vaccine to prevent human infections are currently being considered (Yang et al., 2010). In general, *Streptococcus* spp. is known to infect a variety of mammal including dogs, cats, mink, mice, rabbits, foxes, cattle and even humans (Devriese et al., 1986).

2.5. Staphylococcaceae

The Staphylococcaceae family is a Gram-positive bacterium that includes the genus *Staphylococcus*, noted for encompassing several medically significant pathogens. Staphylococci are Gram-positive cocci, which are non-motile, non-spore forming and facultative anaerobes that are commonly found on the skin of mammals. Thirty-seven species have been identified (Euzéby, 1997) and all species are part of the normal micro flora of the skin and mucosal surfaces of the upper respiratory tract of man and animals. In dog and cat, *Staphylococcus aureus* and *Staphylococcus schleiferi* are common emerging pathogenic species in small animals (Bes et al., 2002; Frank et al., 2003; Yamashita et al., 2005). Generally Staphylococci do not cause any significant diseases in cats (Igimi et al., 1994) but cases of superficial dermatitis, bacterial folliculitis, respiratory infection and superficial pyoderma caused by *Staphylococcus intermedius* have been reported (Scott, 1980).

2.5.1. Morphology

Staphylococci are members of the family Staphylococcaceae. This is a non-motile, non-spore forming bacterium. Based on ability to produce the extracellular enzyme coagulase, species of staphylococci are separated into two large groups. Organisms that produce coagulase are known as coagulase-positive staphylococci mainly *S. aureus* (Kloos, 1997) and organisms that cannot produce coagulase are referred to as coagulase negative Staphylococcus or non-aureus Staphylococci. Surgeon Sir Alexander Ogston first discovered *Staphylococcus* spp. in Aberdeen, Scotland in 1880 in pus from surgical abscesses. Staphylococci grow best in an aerobic environment, as well as anaerobic environment. They grow readily on most routine laboratory media, specific for Muller Hinton agar usually are isolated from clinical specimens using sheep blood agar. Gram staining reveals that gram-positive cocci are 0.7 to 1.2 mm in diameter that is usually visible in irregular grapelike clusters with yellow color colony. The golden pigmentation is caused by the presence of carotenoids and has

been reported to be a virulence factor protecting the pathogen against oxidants produced by the immune system (Harris et al., 2002). All staphylococcal genomes are approximately 2.8 Mbp (mega base pairs) in size with a relatively low G and C content. Comparative analysis revealed that most regions of the staphylococcal genome are well conserved, whereas several large sequence blocks contain high variability (Baba et al., 2008). The cell wall of *Staphylococcus* spp. is a tough protective coat, which is relatively amorphous in appearance and the thickness is about 20-40 nm. Underneath the cell wall is the cytoplasm that is enclosed by the cytoplasmic membrane. Peptidoglycan is the basic component of the cell wall. It makes up 50% of the cell wall mass. Another cell wall constituent is a group of phosphate containing polymers called teichoic acids, which contribute about 40% of cell wall mass. There are two types of teichoic acids one of them is cell wall teichoic acid and another is cell membrane associated lipoteichoic acid; bound covalently to the peptidoglycan or inserted in the lipid membrane of the bacteria. Teichoic acids contribute a negative charge to the staphylococcal cell surface and play a role in the acquisition and localization of metal ions, particularly divalent cations, and the activities of autolytic enzymes. About 90% of the weight of the cell wall mainly composed of Peptidoglycan and teichoic acid together and the rest is composed of surface proteins, exoproteins and peptidoglycan hydrolases (autolysins). Some of these components are involved in attaching the bacteria to surfaces and are virulence determinants (Harris et al., 2002).

2.6. Animal infections by *Staphylococcus* spp.

A variety of coagulase-positive and coagulase-negative staphylococci have been identified in cats (Duquette and Nuttall, 2004). Staphylococcus has been isolated from all species of animal. Molecular analyses of isolates from different animals have revealed that there are some strains that appear to be host adapted to a particular animal species such as horses, cattle, pigs, sheep, chickens, or humans and other strains can colonize multiple species of animals (Cuny et al., 2010). However, it is also an opportunistic pathogen that can cause multifarious infectious diseases with diverse severity. *Staphylococcus* is one of the most frequently isolated genera of opportunistic bacteria in animals and human beings. In mammals, staphylococci

physiologically inhabit primarily the skin, mucous membrane of the nasal cavity, throat, and anus (Nagase et al., 2002; Wertheim et al., 2005). Staphylococci in mammals mostly inhabit the skin and mucous membranes. Specifically, coagulase negative staphylococcus cause upper respiratory tract infection (Schulz et al., 2006). Intimate association between potential hosts can enhance staphylococcal infection as they are easily spread by skin to skin contact, aerosols from sneezing and coughing and also through saliva.

2.7. Pathogenesis of staphylococcus infection

Staphylococcus spp. is a ubiquitous commensally bacterium on skins and anterior nares, but frequently causes severe infections. Rapid and direct identification of *Staphylococcus* spp. is crucial for proper management of patients with skin infections, abscesses, septicemia/bacteremia, gastroenteritis, endocarditis, toxic shock syndrome and certain food intoxications(Kateete et al., 2010). Staphylococcal food poisoning includes symptoms such as sudden onset of nausea, vomiting, abdominal cramps and diarrhea (Balaban and Rasooly, 2000). Staphylococcal enterotoxins are highly heat resistant and are thought to be more heat resistant in foodstuffs than in a laboratory culture medium (Bergdoll, 1983). Besides these, enterotoxins producing *Staphylococcus* spp. are most dangerous and harmful for the human health. About 50% strains of this organism are able to produce enterotoxins associated with food poisoning (Payne and Wood, 1974).

2.8. *Staphylococcus aureus*

S. aureus can be recovered from both dogs and cats and in either species it is the most common Staphylococcal strain. *Staphylococcus aureus* is a microorganism that is present as a commensal on the skin, the nose and mucous membranes of healthy humans and animals. Reports of methicillin resistant *Staphylococcus aureus* infections in animals were rare but have increased in recent years; methicillin resistant *Staphylococcus aureus* (MRSA) has been reported in almost all domesticated species, including dogs, cats, horses, cattle and sheep (Boag et al., 2004; Goni et al., 2004)

2.9. Use of antibiotic in management of cat diseases

Antibiotics are frequently used in cat and dog therapy are: Penicillins, Cephalosporins, Macrolides, Lincosamides, Fusidic acid, Tetracyclines, Chloramphenicol, potentiated Sulphonamides, Aminoglycosides and Fluoroquinolones (Watson and Rosin, 2000). In case of upper respiratory tract infection β-lactams, potentiated β-lactams, Fluoroquinolones and Tetracyclines are often selected as antimicrobial treatment for cats(Ruch-Gallie et al., 2008). Recently antimicrobial susceptibility testing were reported for bacteria grown from the nasal discharges or airways of cats housed in Germany or north-central Colorado (Stein and Lappin, 2001; Schulz et al., 2006). Amoxycillin is commonly used for the treatment of cats with suspected bacterial URTD because it is inexpensive and has a good spectrum against bacterial flora. In addition, Amoxycillin is effective for the treatment of many anaerobic bacteria species; this group of organisms has been suggested to be associated with chronic rhinitis (Johnson et al., 2005). In case of acute meningoencephalitis, Trimethoprim–Sulfamethoxazole administered over several weeks was the main antibiotic (Byun et al., 2009). The Working Group believes that Doxycycline is a good first choice because it is well tolerated by cats. Most *Bordetella bronchiseptica* isolates from cats are susceptible to Doxycycline in vitro (by unapproved standards for testing), despite resistance to other agents such as Beta-lactams and Sulfonamides (Schwarz et al., 2007; Egberink et al., 2009).

2.10. Common mechanism of antimicrobial resistance

2.10.1. Penicillin

In earliest classes of antibiotic, penicillin is one of them and used in treat large and small animals for a variety of disease conditions (Harvey and Hunter, 1999). Penicillin is used more effectively to treat pyoderma in dogs and cats. Intrinsic resistance to Penicillin caused by the production of beta-lactamases is very wide spread among canine staphylococci (Kruse et al., 1996). In animal medicine there have been reported cases of isolation of Methicillin-resistant staphylococci (MRS) in cats and dogs (Box et al., 2003; Kania et al., 2004). Coagulase-negative MRS such as *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus haemolyticus*, *Staphylococcus xylosus*, *Staphylococcus felis* and *Staphylococcus simulans* have also

been isolated from cats and dogs and there are recent reports of detection of the mecA gene in *Staphylococcus schleiferi* and *Staphylococcus aureus* isolated from the ear canals and skin pyodermas of dogs and cats (Yamashita et al., 2005). Methicillin resistance is related to the production of a modified penicillin-binding protein, referred to as PBP2A or PBP2 (Hartman and Tomasz, 1984). The PBP2A is a low-affinity binding protein encoded by mecA gene. Resistance is associated with the acquisition of a large DNA element that ranges from 20 to more than 100 kb termed the staphylococcal cassette chromosome mec (SSCmec) (Katayama et al., 2000) which is integrated into the chromosome of *S. aureus*. Beta-lactam resistance is rare (Clark et al., 2008) despite widespread use of beta-lactam antimicrobials for streptococcal infections over many years.

2.10.2. Aminoglycosides

In veterinary treatment Aminoglycosides have wide applications and have also developed resistance to Streptomycin, Neomycin and Kanamycin. Aminoglycosides resistance is a common phenomenon in animal pathogens (Viola and DeVincent, 2006). A large variety of genes encoding for aminoglycosides resistance by Acetyltransferases, Nucleotidyltransferases and Phosphotransferases have been described in Staphylococci. Aminoglycosides resistance genes aaDE, sat4 and aphA-3 have been identified in canine *Staphylococcus intermedius* (Boerlin et al., 2001). Alternatively, Streptococci are intrinsically resistant to low antibiotic concentrations (MICs ranging from 4 to 64 μg/ml) because of their limited drug uptake. High-level resistance to aminoglycosides (MICs >2,000 μg/ml) that entirely abolishes synergistic bactericidal activity is often due to the enzymatic inactivation mediated by aminoglycoside-modifying enzymes (AMEs), while a less common mechanism corresponds to ribosomal alterations (Jana and Deb, 2006).

2.10.3. Macrolids

Resistance to Macrolides is mainly due to erythromycin-resistance methylases, which cause target-site modification. Active efflux and enzymatic inactivation has also been reported (Schwarz and Noble, 1999). Target modification is the most common mechanism and it involves the demethylation of adenine residues in the 23S rRNA (Werckenthin et al., 2001). Specifically, erythromycin resistance is associated with

the increased cell invasiveness of *S.* spp. Indeed, this association could be due to the presence of the *prtF1* gene, which is more frequently found among Macrolid resistant strains. The *prtF1* gene encodes the fibronectin-binding protein F1, an adhesion that allows *S.* Spp. to be efficiently internalized by and survive within human respiratory cells (Podbielski et al., 2001).

2.10.4. Fluoroquinolones

Fluoroquinolones are a group of antibiotics with a wide spectrum of activity amongst Gram positive and negative bacteria where they act by inhibiting the DNA gyrase, an enzyme responsible for packaging DNA within the cell (Lloyd et al. 1999). The targets of FQ molecules are the type II topoisomerases (namely, DNA gyrase and DNA topoismerase IV) that are both heterotetramers that consist of two subunits, GyrA2B2 and ParC2E2 (Hawkey, 2003). In Gram-positive cocci, FQ resistance results from targ*et* alterations due to point mutations that occur primarily in 120-bp conserved fragments, the so called quinolone resistance determining regions (QRDRs), of both *parC* and *gyrA*genes (Ince and Hooper, 2003). Active efflux of hydrophilic FQ molecules is also possible (Poole and Fruci, 2016).

2.10.5. Tetracyclines

Tetracyclines have been used widely for therapy and prevention of bacterial infections in humans, animals and plants**.** They inhibit protein synthesis by binding to the bacterial 30S ribosomal subunit and blocking entry of the amino-acyl tRNA into the A site of the ribosome (Chopra and Roberts, 2001). Four different tetracycline resistance (tet) genes assigned to classes K, L, M and O have been detected in staphylococci of animal origin (Kim et al., 2005).

2.10.6. Sulfonamides and trimethoprim

This group has broad spectrum activity. Resistance to sulfonamide is believed to emanate from the overproduction of p-amino benzoic acid probably due to chromosomal DNA mutation (Werckenthin et al., 2001). Chromosomal point mutations in the *dhps*(*fol*P) gene coding for the natural dihydropteroate synthase (DHPS), or the acquisition of plasmid-borne *sul*genes coding for resistant DHPS enzymes are also responsible for development of resistance (Huovinen et al., 1995).

2.10.7. Development of antimicrobial resistance

Cats could acquire resistance determinants from their surroundings via food and contaminated bedding and feces (Patel et al., 1999). Resistance to antibiotics seen in staphylococci of animal origin shows both similarities and differences to that in human strains. On the other hand, use of specific antibiotic compounds in both humans and animals is followed by an increase of the prevalence of resistant strains to that antibiotic. On the other hand, the most common resistance seen in cat and dog isolates is to the Penicillin, Ampicillin, Tetracyclines and erythromycin (Prescott et al., 2002). Penicillin resistance, associated with beta-lactamase production, is very common amongst clinical companion animal staphylococcal isolates, with resistance rates of up to 74% (Hoekstra and Paulton, 2002). Macrolide resistance is common and typically mediated by erm(C) (Guardabassi et al., 2004; Rich et al., 2005; Bagcigil et al., 2007).

Chapter-4 Materials and Methods

4.1. Study area and study population

The study was conducted in S. A. Quaderi Teaching Veterinary Hospital (SAQTVH), Chattogram Veterinary and Animal Sciences University (CVASU) (Figure-1) which is one of the renowned veterinary hospitals in Chattogram. At least 50 patients come to the hospital daily for the purpose of treatment, vaccination and general health checkup. Most of them are dogs, cats and pet birds. Small ruminant are also admitted to this hospital. The samples were collected from infected or diseased cats brought to this hospital.

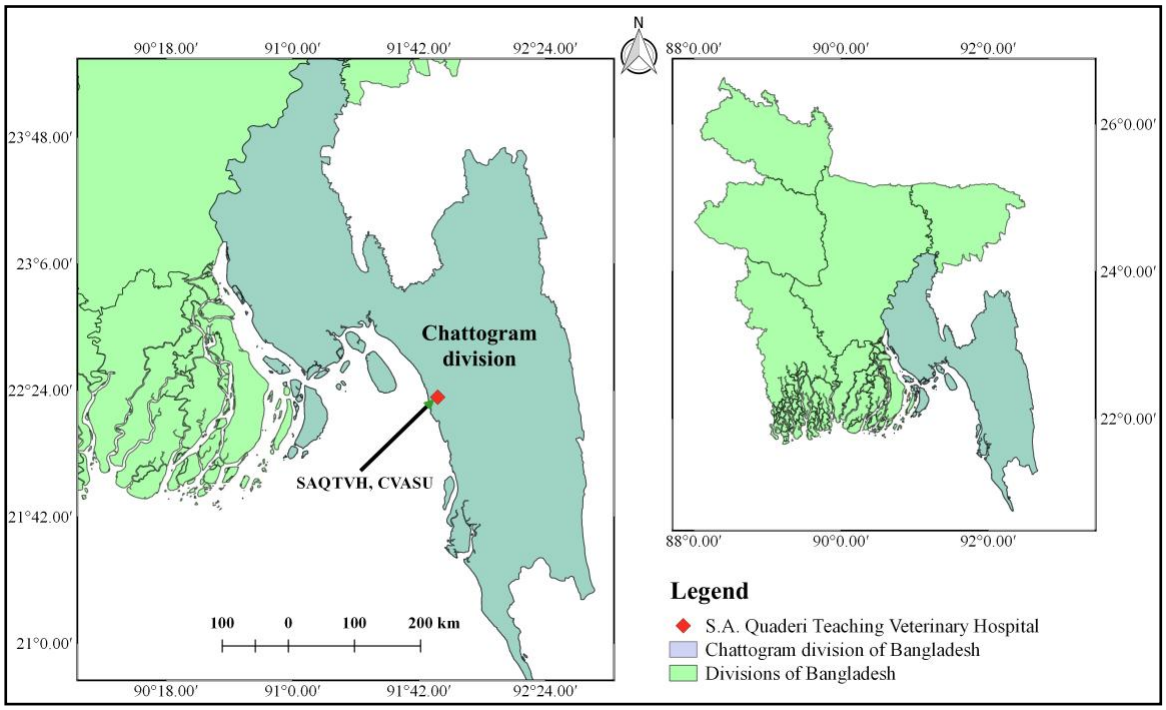

Figure 1: Geographical locations of the study area (SAQTVH, CVASU) (QGIS map)

4.2. Study period

The study was conducted during the period from March 2019 to March 2020.

4.3. Sample collection

One hundred diseased cat (n=100) were sampled having respiratory manifestation during the study period. The samples were collected by inserting a sterile swab into nostril of cats having respiratory disease related problem. The collected swab was

directly inoculated in 5% bovine blood agar (Oxoid, Basingstoke, Hampshire, UK), and shipped to medicinal Laboratory, CVASU for further analysis. A well-structured questionnaire was developed to collect demographic and epidemiological information of sampled animals. Both samples and questionnaire data were collected upon a verbal consent from the cats owner.

4.4. Bacteriological investigation

Different bacteria (*Streptococcus* and *Staphylococcus*) were isolated and identified based on their cultural characteristics and biochemical tests (Catalase and coagulase tests) according to the procedures describes elsewhere (Kateete et al., 2010). Different bacteria and methods of their isolation and identification are described below.

4.5. Isolation and identification of *Streptococcus* spp.

The collected swabs were plated onto blood agar with 5% bovine blood and incubated for 24-48 hours. The suspected colonies (medium-sized, smooth, pigmented or non-pigmented, raised and hemolytic) were then sub-cultured on 5% bovine blood agar and incubated for 24 hours to obtain a pure culture. *Streptococcus* spp. *was* identified according to aforementioned colonial morphology; Gram's staining hemolytic properties and conventional biochemical tests. *Streptococcus* spp. was gram positive cocci arranged in short or long chains. The organisms produced beta hemolysis on blood agar and white to transparent dew drop like colonies and catalase negative.

4.6. Isolation and identification of *Staphylococcus* spp.

To isolate staphylococci, clinical specimens were grown on bovine blood agar. Any colonies producing beta-haemolysis on blood agar were initially considered for the growth of *Staphylococcus.* The colonies were then sub-cultured onto Mannitol salt agar and incubated aerobically at 37ºC for 24 hours. After incubation, the bacterial growth was observed. Any colonies yielding golden yellow colour were suspected for *Staphylococcus aureus.* Finally the bacteria were confirmed by Grams staining, catalase and coagulase test.

4.7. Coagulase test

All the positive samples were subjected to coagulase tests for biochemical confirmation of *Staphylococcus* spp. as previously described (Monica, 1991). For this,

few colonies were picked up and transferred to a 10 ml test tube containing 5 mL of BHIB which was prepared according to the instructions of manufacturer (Oxoid ltd, Basingstoke, Hampshire, UK), incubated at 37 °C for 6 h. On the other hand, whole blood from horse was collected into commercially available sterile tubes containing Ethylene Diamine Tetra Acetic Acid (EDTA). Blood was then centrifuged at 2600 rpm for 10 minutes at 4°C. Resulting supernatant, the plasma was immediately transferred to the sterile 1.5 ml Eppendorf tube using sterile tip and stored at -20ºC for further analysis.

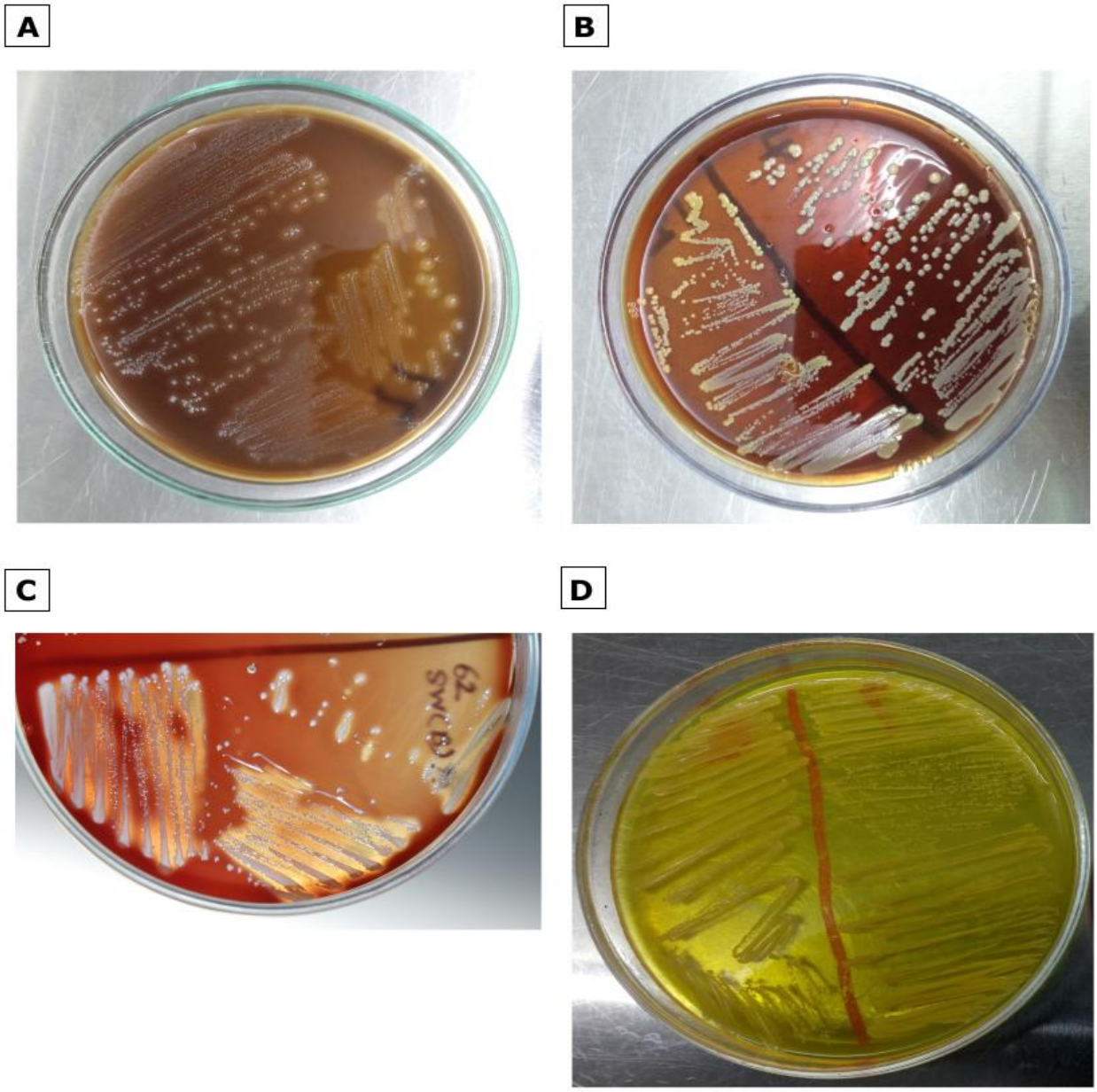

Figure 2: Growing of Bacteria at different agar: A. Small, whitish color colony with hemolysis on Blood agar (*Streptococcus* spp.), B. whitish and yellow color colony with Hemolysis *Staphylococcus* spp. on Blood agar, C. Clear hemolysis on blood agar by *Staphylococcus aureus*, D. Yellowish to pinkish colony on MSA for *Staphylococcus* spp.

4.9. Tube coagulase test

Fifty micro liters of cultivated samples containing BHIB was transferred to the sterile tubes containing 50 µL of horse plasma and incubated at 37ºC for 6 hours. The presence of coagulates were considered when large organized coagulation of all the contents of the tube occurred which do not come off when inverted (Brasil, 2003). A control tube without horse plasma also was placed to validate the result.

4.10. Antimicrobial resistance profile testing of bacteria

All positive samples from biochemical tests for *Streptococcus* spp. and *Staphylococcus* spp. were investigated for their diversity in antimicrobial susceptibility profiles by disk diffusion method on Mueller-Hinton agar (MH) (Oxoid ltd, Basingstoke, Hampshire, UK) according to the Clinical Laboratory Standards Institute (CLSI, 2018). In case of *Streptococcus* spp. test we use 5% blood with Muller-Hinton agar. Bauer-Kirby disk diffusion procedure was used on MH agar according to the method described previously (Bauer, 1966). Briefly, a sterile swab was dipped into the inoculums, prepared for antimicrobial susceptibility test, and rotated against the side of the tube with firm pressure. A bacterial turbidity equivalent of 0.5 McFarland standards was used for each isolate. A 0.5 McFarland standard was prepared by adding 0.5 ml of 1% (11.75g/L) $BaCl_2.2H_2O$ to 99.5 ml of 1% (0.36N)H_2SO_4 (Carter et al., 1990). After removing the excess fluid, the dried surface of MH agar was inoculated by streaking the swab three times over the entire surface rotating the plates approximately at 60 degrees for each time to ensure an even distribution of the inoculums. The antimicrobial disks were then placed on the surface of the inoculated agar. A separate forceps was always used to dispense each of the antimicrobial disks. The disks (Penicillin, Ampicillin, Cephradine, Ceftriaxone. Erythromycin, Azithromycin, Gentamycin, Oxytetracycline, Nalidixic acid, Sulphamethoxazole-trimethoprim) were placed carefully on the surface of the agar with a gentle pressure to make a complete contact. After dispensing all of the disks, the agar plate was incubated at 37oC for 18 hours. At the end of incubation the size of zone of inhibition around a micro-disk was measured with a cm scale and the result was recorded according to CLSI (2018). The panel of antibiotics used for two

bacterial species along with the sizes of zone of inhibition of them to be considered as resistant (R), intermediately resistant (I) and sensitive (S) against the tested isolates.

Table 1: Panel of antimicrobials used with their concentrations and zone diameter interpretative standards for *Streptococcus* spp. and *Staphylococcus* spp. *(*CLSI, 2018)

Antimicrobial agent	Disc Code	Disc concentration	Diffusion zone breakpoint (mm) For *Streptococcuss* spp.			Diffusion zone breakpoint (mm) For *Staphylococcus* spp.		
			S	I	R	S	I	R
Beta-lactams								
Penicillin	P	10 units	≥24	-	-	≥29	-	≥28
Ampicillin	AMP	10 µg	≥24	-	-	≥29	-	≥28
Amoxicillin	AML	10 µg	≥24	-	-	≥29	27- 28	≤26
Quinolones and fluoroquinolones								
Ciprofloxacin	CIP	5 µg	≥21	16-20	≤15	≥21	16-20	≤15
Nalidixic acid	NA	30 µg	≥19	14- 18	≤13	≥19	14-18	≤13
Aminoglycosides								
Gentamicin	CN	10 µg	≥15	13-14	≤12	≥15	13-14	≤12
Tetracyclines								
Oxytetracycline	TE	30 µg	≥28	25-27	≤24	≥15	12-14	≤11
Doxycycline	DO	30 µg	≥28	25-27	≤24	≥16	13-15	≤12
Cephalosporins								
Ceftriaxone	CRO	30 µg	≥24	-	-	≥23	20-22	≤19
Cephradine	CH	30 µg	≥26	-	-	≥24	21-23	≤20
Cefotaxime	CTX	30 µg	≥24	-	-			
Macrolids								
Erythromycin	E	5 µg	≥21	16-20	≤15	≥23	14-22	≤13
Azithromycin	AZN	15 µg	≥18	14-17	≤13	≥18	14-17	≤13

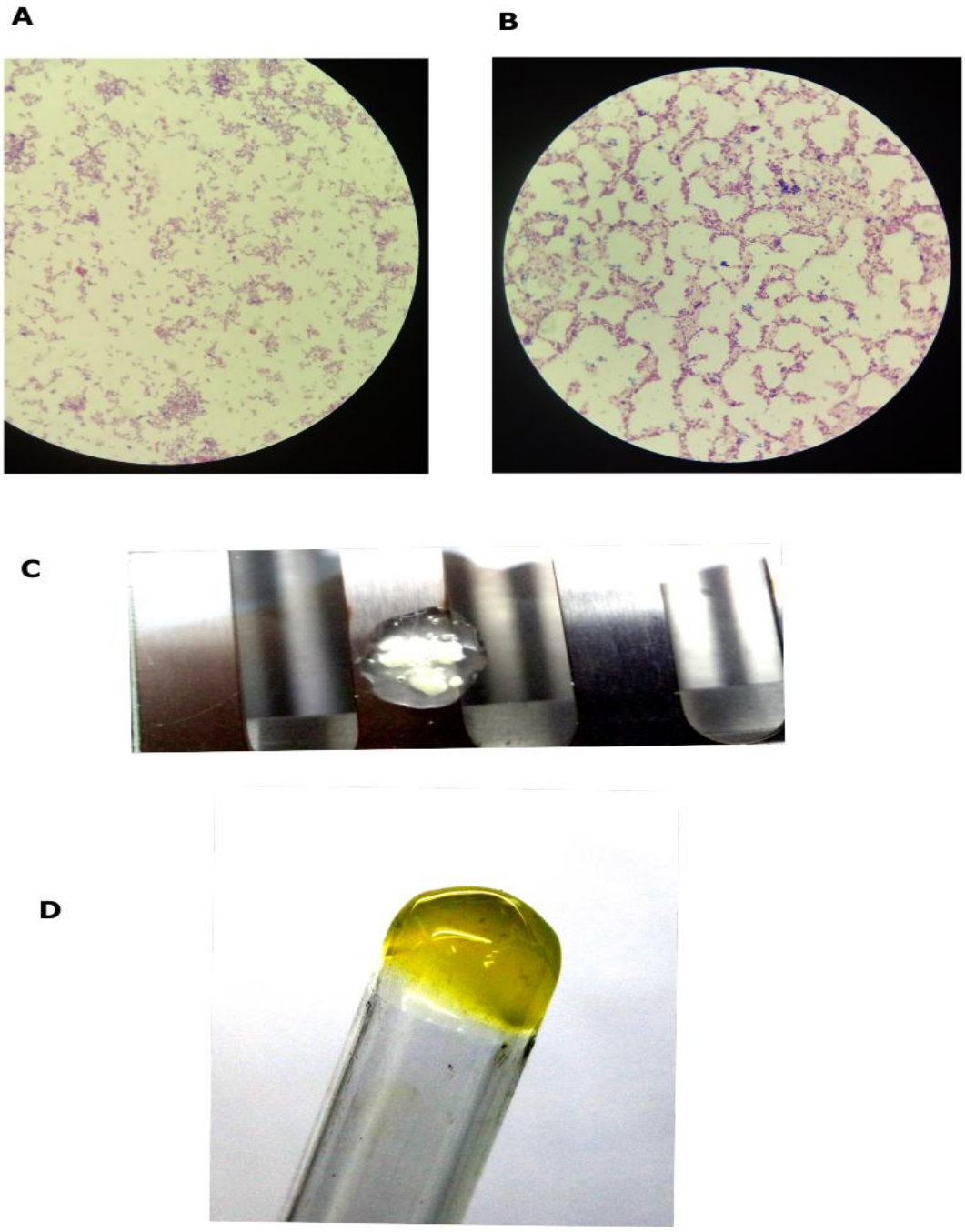

Figure 3: Gram's Staining of Bacteria: A. Grams staining of *Streptococcus* spp. B. Grams staining of *Staphylococcus* spp. C. Catalase test for Staphylococcus spp. (catalase positive)D. Coagulase test

4.10. Sample preservation

All positive isolates were inoculated into brain heart infusion (BHI) broth from blood agar, incubated overnight at 37 and then preserved at -80C with 50% glycerol (v/v) in 1.5ml Eppendorf tubes (700µl broth culture and 300µl 50% glycerol) for future investigation

4.11. Detection of species through polymerase chain reaction

4.11.1 Extraction of DNA from positive samples

Bacterial DNA was extracted by boiling method (Ahmed et al., 2014). A loop full fresh colonies (about 3-4) was picked from blood agar and transferred to 1.5 ml Eppendorf tube containing 200µl ultrapure water. The tubes were then vortexed to make a homogenous cell suspension. A ventilation hole was made on the top of each tube so that excess vapors were removed during boiling. Then the tubes were placed into 99ºC for 15 minutes in a hot water bath. Immediately after boiling, the tubes were placed into -20ºC for 5 minutes. After freezing, the tubes were again placed into a hot water bath at 99ºC for 10 minutes and the boiled tubes were placed into -20ºC for 5 minutes. The process of repeated boiling at high temperature followed by immediate freezing allowed the cell wall to break down to release DNA from the bacterial cell. Finally, the tubes with the suspension were centrifuged at 13000 rpm for 5 minutes. The 100 µl of supernatant containing bacterial DNA from each tube was collected and preserved at -20ºC until used.

4.11.2 Polymerase chain reaction

PCR assays were performed using primers described by (Sasaki et al., 2010) for *Staphylococcus aureus* and (Lysková et al., 2007) for *Streptococcus canis* detection. The primer sequences used for the PCR are shown in Table 2.

Table 2: Oligonucleotide primer sequences for *Staphylococcus aureus* and *Streptococcus canis* confirmation genes

Species	Gene	Primer	Sequence (5-3)	Annealing temperature	Amplicn size (bp)	References
S. aureus	nuc	au-F3	TCGCTTGCTAG ATTGTGG	56ºC	359	Sasaki et al., 2010
		au-nucR	GCCAATGTTC A CCATAGC			
S. canis	16S-23S rDNA interge c spacer	C-I	TAAACCGAAA CGCTGTAAGT TTA	61ºC	215	Lyskova et ai., 2007
		C-II	ACCATTAGTT A GTGGGTTCCC C			

PCR reaction was conducted with a 25 µl reaction volume. Proportion of different reagents used for PCR for two different species of bacteria are given in Table 3. Negative and positive controls were used in each reaction. Nuclease-free water was used as negative control and one previously identified *Staphylococcus aureus* used as a positive control. No positive control used for *Streptococcus* spp.

Table 3: Contents of PCR reaction mixture for the nuc and 16S-23S rDNA intergenic spacer genes

SL. NO	Contents	volume
1	Thermo scientific dream taq PCR master mix (2x) ready to use	12.5µl
2	Forward primer	0.5 µl
3	Reverse primer	0.5 µl
4	Nuclease- free water	9.5 µl
5	DNA template	2 µl
	Total	25 µl

PCR amplification was performed in a thermo cycler (Applied Biosystem 2720 thermal cycler. Singapore). The cycling conditions for *Staphylococcus aureus* are shown in Table 4. A total of 30 cycles were run and the cycling conditions for *Streptococcus canis* are shown in Table 5.

Table 4: Cycling conditions used for PCR detection of *Staphylococcus aureus*

SL. NO	Steps	Temperature and time
1	initial denaturation	95ºC for 2 minutes
2	Final denaturation	95ºC for 30 seconds
3	Annealing	56ºC for 30 seconds
4	Initial extension	72ºC for 30 seconds
5	Final extension	72ºC for 2 minutes
6	Final holding	4ºC for infinity

Table 5: Cycling conditions used for PCR for the detection of *Streptococcus canis*

SL. NO	Steps	Temperature and time
1	initial denaturation	94ºC for 4 minutes
2	Final denaturation	94ºC for 10 seconds
3	Annealing	61ºC for 30 seconds
4	Initial extension	72ºC for 10 seconds
5	Final extension	72ºC for 10 minutes
6	Final holding	4ºC for infinity

4.11.3. Visualization of amplified PCR products by agar gel electrophoresis

A gel tray was assembled with setting proper teeth sized gel comb in the tray. Then 1% agarose gel was prepared for electrophoresis of PCR- amplified products by mixing 0.5 gm of molecular grade agarose powder (SeaKem LE agarose-lonza) with50 ml of 1X TAE buffer in a conical flask. Then the solution was boiled in a microwave oven for 2 minutes. The melted agarose was cooled at 40-50ºC in a water bath, having added with 5µl ethidium bromide at a concentration of 5pg per ml. Finally, the melted agarose was poured into the gel tray and allowed about twenty minutes to stand for solidification of the gel. The gel was placed in an electrophoresis tank, already filled in with 50ml of 1X TAE buffer. Then 5µl of each of the PCR products was loaded to gel-holes. One hole was loaded with DNA marker (thermo scientific O'Gene ruler 1kb plus) to compare the amplicon size of the gene product. Negative and positive controls were used in each electrophoresis run. Electrophoresis was run at 100 volts and 80mA for 35 minutes. After completion of electrophoresis the gel was placed in a water bath for rinsing for a while. Finally the gel was examined under an UV trans illuminator (BDA digital, biometra GmbH, Germany). Gel electrophoresis was repeated twice with the same PCR products.

4.11.4 Statistical Analysis for determination of significant difference between the data

Field and laboratory data obtained were entered into MS Excel-2010 spread sheets. Data were analyzed using STATA IC-13 (Statecrop, 4905, Lakeway Drive, College Station, Texas 77845, USA). Statistical significance was determined as $p<0.05$

Chapter-5 Results

5.1. Confirmation of *Streptococcus* spp. by cultural characteristics and biochemical analysis and then PCR for the conformation of species

A total of 100 samples of nasal swab were collected, of which 40 samples were found as positive for *Streptococcus* spp. based on microscopic, growth and biochemical properties. Characteristics the growth of *Streptococcus* species on blood agar, typical Gram's staining characteristics; result of catalase test are displayed in Figures (2, 3) respectively.

Then 40 samples subjected to the PCR for the identification of *Streptococcus canis* of which none was found positive.

5.2. Confirmation of *Staphylococcus* spp. by cultural characteristics and biochemical analysis and confirm *Staphylococcus aureus* through PCR

At first 100 nasal swab of cat suffering from upper respiratory problem were examined by cultural characteristics and biochemical analysis for *Staphylococcus* spp. Circular, small, smooth raised colonies with grey in color were initially identified as *Staphylococcus* spp. Then *Staphylococcus* spp. was identified by microscopy with Gram's staining where it was Gram positive coccoid shaped. Then *Staphylococcus* spp. was further confirmed in Mannitol salt agar (MSA) with the production of yellowish colonies.

For further conformation positive isolates were subjected to catalase and coagulase tests and were found to be positive in the catalase and tube coagulase tests. Isolates which showed heavy coagulation of all the contents of the tube and did not come off even after inverting the tube upside down were recorded as positive for coagulase test. Among them, a total of 18 samples were found as positive for *Staphylococcus* spp. which was 18% of total samples. All coagulase positive samples were subjected to PCR for identification of *Staphylococcus aureus*. Among them 9 samples were positive for *Staphylococcus aureus* which was 9% of total sample.

Table 6: Prevalence based on bacterial infection confirmed by cultural properties

Variable name	Prevalence (%)	95% confidence interval
Streptococcus infection	24	16.5-33.4
Streptococcus with *staphylococcus* infection(mixed)	17	10.7-25.8
Staphylococcus aureus	9	4.1-16.3

5.3. Prevalence of bacterial infection at different age group

In the data sheet of 100 cats, the prevalence of *Streptococcus* infection in cat was 15% in less than 1.5 years of old, 62.5% in 1.5-2 years of age and 22.5% in more than 2 years of age. On the other hand, the prevalence of Staphylococcal infection was 50% in less than 1.5 years of old 38.88% in 1.5-2 years of age and 11.11% in more than 2 years of age in this study. *Staphylococcus aureus* infection occurs in below the 18 months of age was 66.6%.

5.4. Prevalence of bacterial infection at different breed

The local breeds were more susceptible to infection than exotic breed in the study. A total of 88.88% of the local breeds were infected by *Staphylococcus* spp. where 11.11% infection were found in exotic breeds while 77.5% of original breed of cats were infected by *Streptococcous* spp. Similarly, *Staphylococcus aureus* infections were also more commonly associated with local breed than that of the exotic breeds.

Table 7: Univariate analysis of risk factors for the presence of *Staphylococcus* spp. in cats

Risk factor	Categories	Proportional % of sampled animal (no/total)	Odds ratio	95% confidence interval	P value
Age	(>2 years)	11.11(2/18)	Re	-	-
	(1.5-2 years)	38.88(7/18)	3.705882	0.6732585- 20.39865	0.132
	(<1.5 years)	50(9/18)	1.723404	0.3391413- 8.757773	0.512
Breed	Exotic	11.11(2/18)	Re	-	-
	Local	88.88(16/18)	2.933333	0.6232058- 13.80675	0.173
Sex	Male	66.66(12/18)	Re	-	-
	Female	33.33(6/18)	1.018519	0.3449125- 3.007662	0.974
Deworming	Dewormed	44.44(8/18)	Re	-	-
	Not-dewormed	55.55(10/18)	1.52027	0.5446991- 4.243116	0.424

Table 8: Univariate analysis of risk factors for the presence of *Staphylococcus aureus* in cats

Risk factor	Categories	Proportional % of sampled animal (no/total)	Odds ratio	95% confidence interval	P value
Age					
	(>2 years)	0	Re	-	-
	(1.5-2 years)	33.33(3/9)	1	1	
	(<1.5 years)	66.66(6/9)	1.190	0.271- 5.2121	0.817
Breed					
	Exotic	11.11(1/9)	Re	-	-
	Local	88.88(8/9)	2.70583	0.320937- 22.8137	0.360
Sex					
	Male	55.5(5/9)	Re		
	Female	44.4(4/9)	1.710345	0.427391- 6.844508	0.360

5.5. Prevalence of bacterial infection at different sex of cats

Male cats were found more susceptible to Staphylococcal infection which was 66.66% while no biological significant relation with sex was found associated with *Streptococcus* infection. About 55.5% *Staphylococcus aureus* infection occured in males where about 44.4% female infected by *Staphylococcus aureus*.

Table 9: Univariate analysis of risk factors for the presence of *Streptococcus* spp. in cats

Risk factor	Categories	Proportional % of sampled animal (no/total)	Odds ratio	95% confidence interval	P value
Age					
	(1.5-2 years)	62.5(25/40)	Re	-	-
	(>2 years)	22.5(9/40)	2.4545	0.6847789-8.79815	0.168
	(<1.5 years)	22.5(9/40)	2.41935	0.835191-7.00830	0.104
Breed					
	Exotic	22.5(9/40)	Re	-	-
	Local	77.5(31/40)	1.14814	0.4464542-2.952696	0.774

5.6. Antimicrobial resistance pattern of S*treptococcus.* spp. *and Staphylococcus* spp.

The results of antimicrobial sensitivity testing of *Streptococcus* and *Staphylococcus* isolates are displayed in Table 10 and Figure 4. The highest resistance in *Streptococcus* spp. was observed against penicillin (62.5%) and nalidixic acid (62.5%) followed by sulphamethoxazole-trimethoprim (52.5%). Resistance against ampicillin and cephradine was detected in 35% isolates. All *Streptococcus* positive isolates were sensitive to gentamicin and ciprofloxacin.

About 67% *Staphylococcus* isolates displayed resistance to nalidixic acid and 50% isolates showed resistance against cephradine and sulphamethoxazole-trimethoprim. No resistance was found against doxycycline and ciprofloxacin.

Table 10: Univariate analysis of prevalence of different antibiotics resistant to *Streptococcus* spp. and *Staphylococcus* spp. in cat (Penicillin, Ampicillin, Cephradine, Ceftriaxone. Erythromycin, Azithromycin, Gentamycin, Oxytetracycline, Nalidixic acid, Sulphamethoxazole-trimethoprim)

Name of antibiotic	Bacteria		P value
	***Streptococcus* spp. % of Resistance (R)**	***Staphylococcus* spp. % of Resistance(R)**	
P	62.5 (25/40)	38.89 (7/18)	0.011
AMP	35 (14/40)	44.44 (8/18)	0.002
CH	22.5 (9/40)	33.33 (6/18)	0.683
CRO	35 (14/40)	50 (9/18)	0.553
E	25 (10/40)	22.22 (4/18)	0.639
AZM	10 (4/40)	11.11 (2/18)	0.921
CN	0	5.56 (1/18)	0.156
OT	10 (4/40)	16.67 (3/18)	0.547
DO	5 (2/40)	0	0.124
CIP	0	0	
NA	62.5 (25/40)	66.67 (12/18)	0.750
SXT	52.5 (21/40)	50 (9/18)	0.785

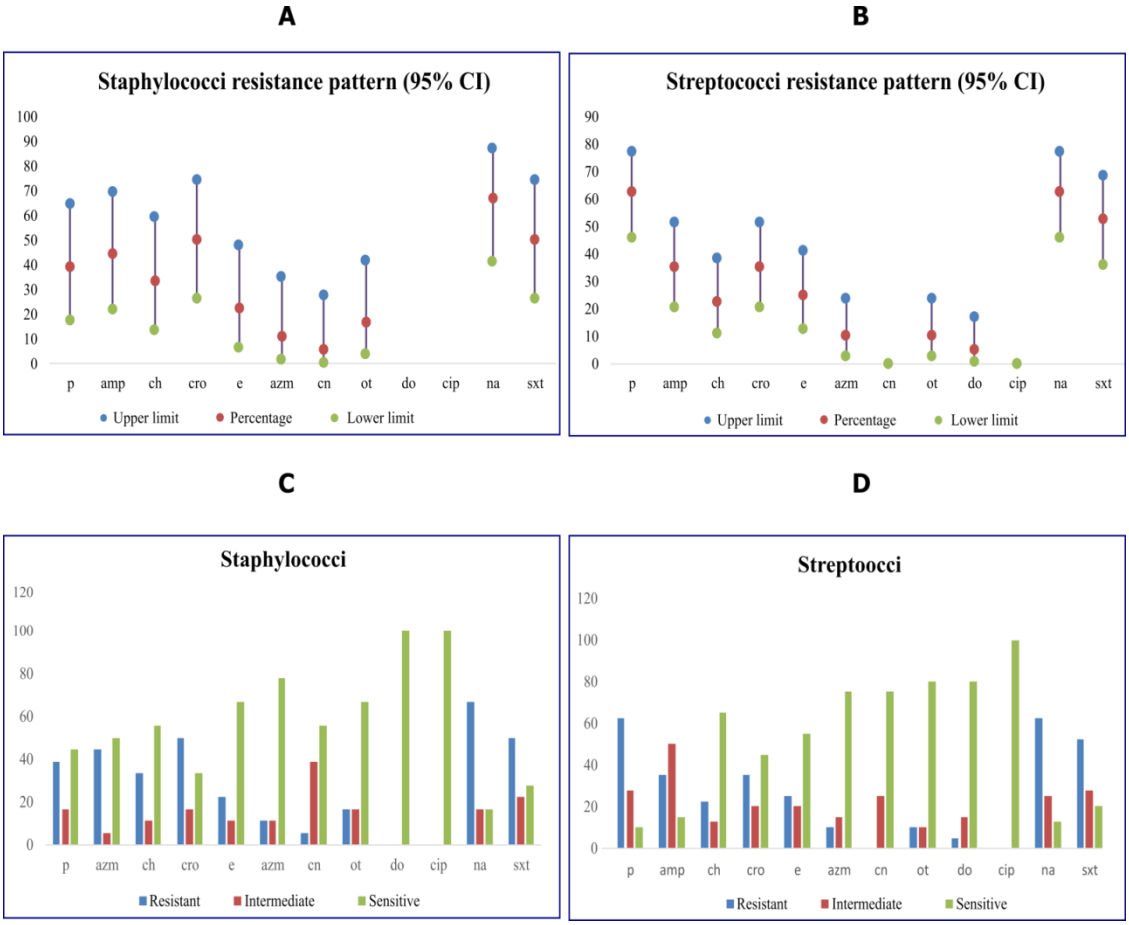

Figure 4:Frequencies of *Staphylococcus* spp. (C) and *Streptococcus* spp. (D) isolates showing resistant (R), intermediately resistant (I) and sensitive (S) to the antimicrobials tested. (p, Penicillin; amp, Ampicillin; ch, Cephradine; cro, Ceftriaxone; azm, Azithromycin; cn, Gentamycin; ot, Oxytetracycline; do, Doxycycline; cip, Ciprofloxacin; na, Nalidixic acid; sxt, Sulphamethoxazole-Trimethoprim.)

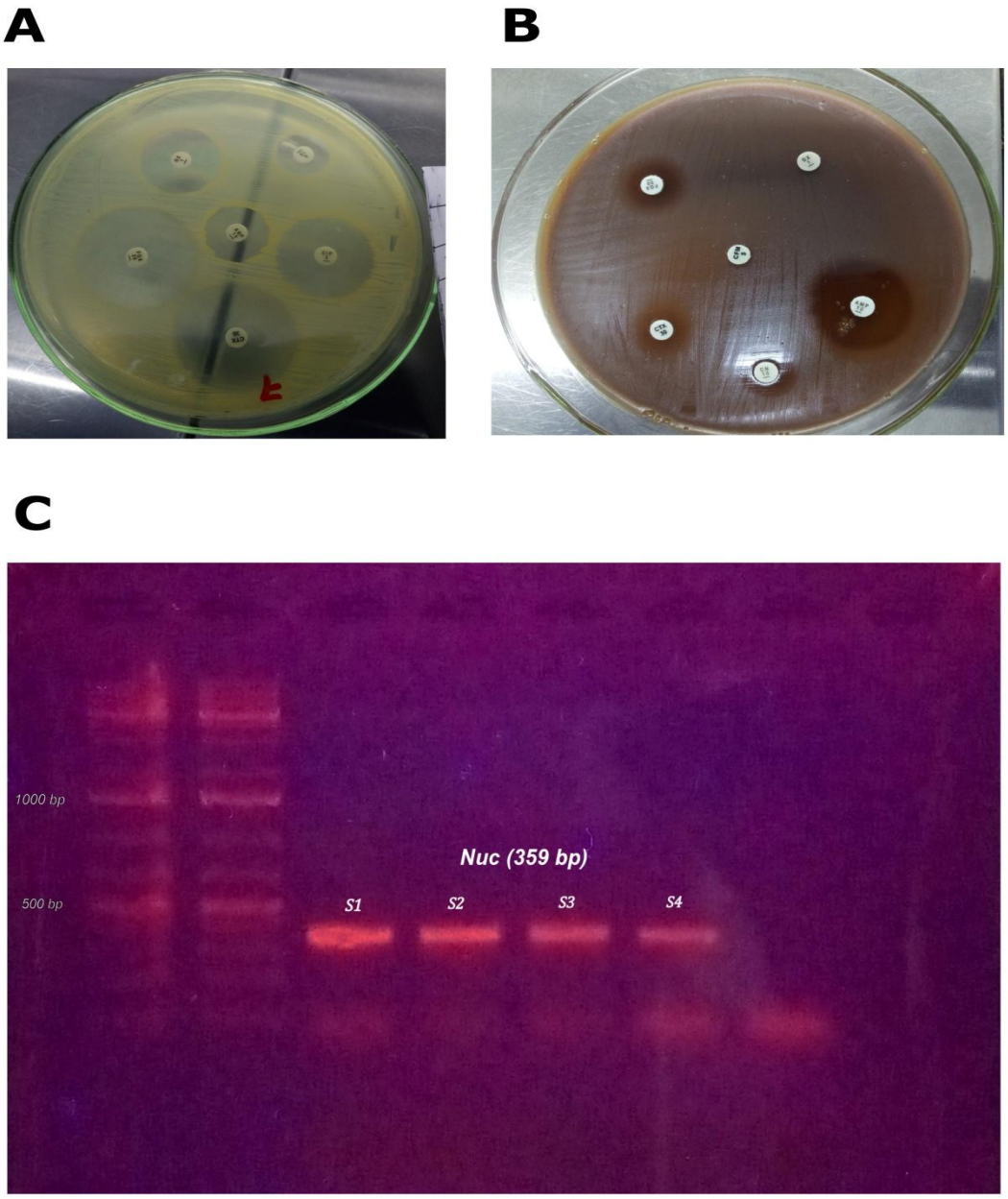

Figure 5: Culture Sensitivity Test: A, B. Antibiotic sensitivity result of *Staphylococcus* spp. and *Streptococcus* spp. C. PCR products amplified using nuc gene specific primers of *Staphylococcus aureus.*

Chapter-6 Discussion

The prime objective of this study was to isolate the *Streptococcous* spp. and *Staphylococcous* spp. Species of *Staphylococcus aureus* which have multifarious effect on respiratory tract in cat also conformed through PCR. Finally antibiotic sensitivity pattern of these bacteria were identified with risk factor analysis from cats admitted at SAQTVH, CVASU.

The isolation percentage of *Streptococcus* and *Staphylococcus* were 40% and 18% respectively which is quite similar with the Schulz et al. (2006) and Dossin et al. (1998). In a previous study 12.9% *Staphylococcus* species isolated from the nasal swab of cat(Ruch-Gallie et al., 2008).

In this study 9% *staphylococcus aureus* were isolated from the nasal cavity of cat. According to the (Bierowiec et al., 2019), 10% to 12% *Staphylococcous aureus* isolated from the domestic cat which also supports the findings of this study. A study conducted in Luisiana, indicated 8.3% *Staphylococcus aureus* isolated (Lane et al., 2018) in cat.

No *Streptococcus canis* found in this study. Similarly after analyzing a total of 42 samples collected from the different anatomical location of cat. It was reported that the proportion of *Streptococcus canis* was null in the nasal mucosa (Lysková et al., 2007).

In this study, 66.66% male cat and 33.33% female cat were infected by *Staphyloclccus* spp. which was similar to other study (Sykes and Greene, 2013). Moreover, clinical case in cat were more reported than the male cat in SAQTVH, CVASU (Yadav et al., 2017)

Staphylococcus infection occurs in less than 1.5 years of age in more than 2 years of age. Similarly, 62.5% *Streptococcus* infection occurs in less than 2 years of age and 22.5% infection occurs in more than 2 years of age. So small cat are more susceptible

to infection which may be due to less development of immunity (Davies, 2011). It was reported earlier that young cats under one years are more susceptible to the respiratory disease (Yadav et al., 2017) and (Pesavento and Murphy, 2014)

Bacterial infection was found more in local breed than the exotic breed in Chattogram area which was previously published (Yadav et al., 2017). About 78% of the local breeds were infected by *Streptococcus* spp. and 88.88% local breed of cat were infected by *Staphylococcus* spp. in this study which was contradictory with the previous study of Mark, 2005.

Antibiotics are commonly used in the treatment of respiratory infection, pyoderma, ear infections, wound infections, gastroenteritis and urinary tract infections in cats. A broad spectrum of antimicrobials is prescribed for these infections in cats, including almost all major antimicrobial groups, and broad spectrum ones. In recent years certain infections, such as respiratory infections, skin infection, pyoderma and enteritis can be long standing problems that may predispose for development of resistance due to repeated or prolonged antimicrobial treatment.

The isolated *Staphylococcus* species from the nasal swab of cat have shown significant resistant to the penicillin (38.8%) which was almost similar to the study of (Onwubiko and Sadiq, 2011). Only 6% *Staphylococcus* spp. isolates in this study exhibited resistance to gentamicin. A higher resistance to gentamicin also studied in (Jayatilleke and Bandara, 2012). The higher sensitivity to the gentamicin is due to less use of this antibiotic in infectious disease of cat in Chattogram area.

Half of the staphylococcus isolates displayed resistance to the Ceftriaxone. Similar type of results were obtained from the work carried out in a study (Bukhari et al., 2011). The resistance to Ampicillin was 44.44% in this study while another study (Bukhari et al., 2011) reported 100% resistance to Ampicillin through disk diffusion method.

In the present study 22.22% and 11.11% of resistance were observed for erythromycin and azithromycin respectively by the isolates. However it was reported 11% and 8%

resistance to erythromycin and azithromycin (Tirupati, 2016) which were almost similar to the results obtained in this study.

Isolated *Staphylococcus* species resistance to oxytetracycline was 16.67% in this study. Nearer to a similar result was obtained in a study where 31.2% resistance to tetracycline was found. Overall, tetracycline resistant is with the levels of 1% and 20% in 1986–2001 in European country (Rich, 2005). Another study revealed that tetracycline resistant was 23.5% isolates where sample was collected from the respiratory tract of infected cat (Schulz et al., 2006). This result also closely supports the findings of this study.

The resistance of Cephradine in the present study is 33.33% which is near to the resistance observed in a study (Tirupati, 2016) where he observed 25% resistance to Cephradine for *Staphylococcus* spp.. The resistance to Nalidixic acid and Sulphamethaxazole-Trimethoprim were found 66.67% and 50%.

The study also revealed that all the isolates were sensitive to ciprofloxacin. Similar to the results of the present was found in a study (Hoerlle and Brandelli, 2009; Ray et al., 2013). Fluoroquinolones, which were licensed for veterinary use, also show the resistance in Europe during the mid-1990s. The resistance was 0.9% in pet animal (Lloyd et al., 1999). The reason of higher sensitivity is the less use of ciprofloxacin in pet animal.

Streptococcus spp. isolated in this study was highly resistant to Penicillin (62.5%) higher than the results were obtained from the work carried out in a study (Norton et al., 2004). where 40% and Ceftriaxon resistant in this study was 35% which was 21% resistance to the Ceftriaxone observed respectively.

The resistance to Ampicillin, was 35% which was quite similar to the study result (Schulz et al., 2006). The study was done by Schulz et al. 2006, through disk diffusion method where result was 22.5%. These study revealed that *Streptococcus* spp.

resistant to erythromycin is 25% which is quite similar to the result obtained in a study (Kataja et al., 1998) and in case of azithromycin it was 10% in this study.
Resistances against oxytetracycine and doxycycline were found in 10% and 5% of *Streptococcus* isolates, respectively in this study which is similar to the result obtained in a study (Norton et al., 2004). Overall tetracycline resistance from respiratory infected cat was 23% (Schulz et al., 2006). All the isolates were susceptible to Ciprofloxacin and Gentamycin in this study.

The most common use of antibiotic are Penicillin, Ampicillin, Gentamicin, Cephradine and Tetracyclines in different disease of cat. These antibiotics are frequently used in the respiratory infectious disease of cat. The main Gram positive bacteria such as *Streptococcus* and *Staphylococcus* species are tested for their resistance pattern of this antimicrobial agents. The study revealed that studied bacteria achieved significant resistant to the Penicillin and Ampicillin where p value is 0.011 and 0.002 respectively. The Penicillin and Ampicillin are the "Access"group of antibiotics according to the world Health organization (WHO) in 2017. The second group of antibiotics was in the "Watch" list in the WHO bulletin and those were with higher resistance potential, the use of which as first or second choice treatment should be limited to few syndromes or patient groups. From this group, ceftriaxone, ciprofloxacin, azithromycin, erythromycin was tested in the present study and it was found that these bacteria become resistance to these antibiotics. Multidrug resistant bacteria can infect pet owners, handlers and even other people who come to close contact of pets and/or of their excretions. If this happens, it could be very difficult to treat the patients with these antibiotics. Therefore, indiscriminate use of antibiotics should be stopped immediately for the betterment of humankind. Proper legislation protocol should be implementing for the use of antibiotic in different species of animals.

Chapter-7 Limitations

We had following limitations in our study.

1. The sample size in our investigation was not large.
2. We did not use any laboratory diagnostic procedure for the examination of respiratory infection of cat such as cough test, chest X-ray etcetera .
3. We did not use control of bacteria for Streptococcus *spp.* in PCR.
4. We only use disk diffusion technique for the identification of the antibiotic resistant but MIC can be performed for better result.

Chapter-8 Conclusion

This study was performed to justify the antibiotic sensitivity patter of common Gram positive bacterial pathogens and their cultural sensitivity especially *Streptococcus* spp. *Staphylococcus* spp. from the nasal cavity which have multifarious effect on respiratory tract of cat. The study also revealed that the prevalence of *Streptococcus canis* and *Staphylococcus aureus* infection in cat. From nasal swab samples 40% *Streptococcus* spp, 18% *Staphylococcus* spp. were found to be positive for in all cultural and biochemical tests respectively. 9% *Staphylococcus aureus* were positive among the 18% *Staphylococcus* spp. *Streptococcus canis* was not identified by the PCR technique. Antibiotic sensitivity pattern was observed with all the positive isolates of bacteria. Each of the bacteria has shown different pattern of sensitivity to antimicrobials. Penicillin and ampicillin have shown significant p value of 0.011 and 0.002 respectively. Random use of antibiotic is the main reason of development of resistant or it might be cross infection from the environment. Indiscriminate use of antibiotic that developed antibiotic resistant bacteria from pet animals to human has a public health significant. Proper legislative action should be implemented to reduce the frequent use of antibiotic and above all public awareness should be developed.

Chapter-9 Recommendations

9.1. Recommendations:

1. Awareness should be built against random and excessive uses of antimicrobials.

2. Veterinarians should be more careful when approaching to the companion animals.

3. Reserve group of antimicrobials should not be used in treatment of pet animal.

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Annex-I

Study on antibiotic sensitivity pattern of Gram positive bacteria isolated from cats in Chattogram Metropolitan area

Questionnaire for collecting relevant data from infected cats

ID No.: …….

Owner Name: ……..... Address: ……

Mobile No.: ……

Dog's Name: …… Breed: ……

Age: …… Sex: ……

Vaccination status: ……

Previous Disease status: ……

Days of illness: ……

Clinical signs: ……..

Presumptive Diagnosis: ……

Antibiotics Used: ……....

Completion the course of Antibiotics: Yes/No (……..)

Sample: ……..

Any other information we may need to know: ……

Signed: ……. Dated: ……