EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE PATTERN OF SALMONELLA, STAPHYLOCOCCUS AND ENTEROCOCCUS SPECIES IN FREE RANGING RHESUS MACAQUE AT HIGH RISK WILDLIFE-HUMAN INTERFACES IN BANGLADESH

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Roll no.: 0116/07

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A thesis submitted in the partial fulfillment of the requirements for the degree of Masters of Science in Epidemiology

Department of Medicine and Surgery
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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made.

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Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Elaboration
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Test
BGA	Brilliant Green Agar
C.	Campylobacter
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
CLSI	Clinical and Laboratory Standards Institute
E.	Enterococcus
EDTA	Ethylenediaminetetraacetic Acid
ESBL	Extended-spectrum Beta-lactamase
GDP	Gross Domestic Product
I	Intermediate
ICDDR, B	International Centre for Diarrhoeal Disease Research, Bangladesh
IUCN	International Union for Conservation of Nature
KAA	Kanamycin Aesculin Azide
MRSA	Methicillin-resistant *Staphylococcus aureus*
NHP	Non-human Primate
OR	Odds Ratio
PCR	Polymerase Chain Reaction
R	Resistant
ROC	Receiver Operating characteristic Curve
S	Sensitive
S.	Staphylococcus
TB	Tuberculosis
USDA	United States Department of Agriculture
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant Enterococcus
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

Symbols

Symbols	Stands for
>	Greater than
<	Less than
°C	Degree centigrade
≥	Greater than equal
≤	Less than equal
%	Percentage
µg	Microgram
$\chi 2$	Chi-square
w/v	Weight/volume

Abstract

Antimicrobial resistance (AMR) is a growing global health threat both for human and animal owing to the indiscriminate use of antimicrobials. Environmental pollution of antimicrobials from human and animal waste has been linked to AMR within wildlife populations, including rhesus macaques. This study aims to better describe the epidemiology of AMR in *Salmonella*, *Staphylococcus* and *Enterococcus* Species from rhesus macaques. Total 399 fecal samples were collected noninvasively from the macaques during January to June 2017 and observations of human-livestock-macaque interaction were recorded daily for 4 hours from each site for 3 days. Samples were cultured and an antimicrobial susceptibility test (AST) for 12 antimicrobials for each organism was conducted using the Kirby-Bauer Disc diffusion method on selective media. Isolates were confirmed by biochemical characteristics and PCR. The overall prevalence of *Salmonella* spp., *Staphylococcus* spp. and *Enterococcus* spp. in rhesus macaque was 5%, 16% and 70%, respectively. Results yielded 5% (18/399; 95% CI: 3-7) of fecal samples were positive for resistant *Salmonella* spp., 15% (61/399; 95% CI: 12-19) for resistant *Staphylococcus* spp. and 61% (66/109; 95% CI: 51-70) sample for resistant *Enterococcus* spp. In case of *Enterococcus* spp.; 36% (39/109; 95% CI: 27-46) and 33% (36/109; 95% CI: 24-42) of the fecal sample were positive for *Enterococcus faecalis* and *Enterococcus faecium*, respectively. The odds of antimicrobial resistance of *Salmonella* spp and *Staphylococcus* spp. ware significantly higher in peri-urban habitat (OR=6.6; CI: 1-46, *P*=0.05) and (OR=5.6; CI: 1-26, *P*=0.02), respectively than other habitats (rural and urban). In case of age, *Enterococcus* spp. was significantly higher in adult (OR=3.8; CI: 1-12, P=0.01) than the juvenile macaque. Among the antimicrobials, *Salmonella* spp. detected resistance to tetracycline (89%), azithromycin (83%), sulfamethoxazole-trimethoprim (50%), and nalidixic acid (44%). In case of *Staphylococcus* spp.; Ampicillin (93%) was highly resistant and less resistant to methicillin (31%), clindamycin (26%), rifampicin (18%). *Enterococcus* spp. were resistant to streptomycin (96%) followed by tetracycline 63%, erythromycin 61%, linezolid 30%, ampicillin 29% and ciprofloxacin 25%. AST profiling of. Direct contact (within 15-20 min and <20m) with Macaque-Human and Macaque-Livestock interactions and sharing same resources for feeding/watering was revealed as one of the main reasons for higher AMR in macaque against *Enterococcus* spp. and *Salmonella* spp. Resistant bacteria were found in macaques which may be the greater risk for future. Those bacteria refer to the interaction among human-livestock-macaque for feeding and drinking practices might be the possible source of AMR in macaques. The study suggests the virulent genetic analysis and proper disposal of wastages to prevent the spread of resistant organisms in the environment.

Keywords: Rhesus macaque, AMR, Salmonella, Staphylococcus, Enterococcus.

Chapter-1: Introduction

1.1 Background of the study

Antimicrobial resistance (AMR) is an emerging global public health threat. Today clinically important bacteria are not only single drug resistance but also multiple drug resistance. Human and animal health are now in great danger (Levy and Marshall, 2004). Today it is a global problem rather than a local, as AMR can spread country to countries and continent to continent. In the time of globalization, due to massive increases in travel leads to rapid spread of resistant pathogens. Antimicrobial resistance may vary by region or country but it is clear that Asia is an epicenter of AMR, especially in south-east Asian countries e.g. Bangladesh, India, Pakistan due to their high-density populations (Kang and Song, 2013). Mostly antibiotics use in livestock and poultry to treat the infectious diseases but now extensively used as growth promoters (Akond et al., 2009). In addition, Poultry and fish farming is developing day by day and uses antimicrobials very commonly led to a rising number of resistant bacteria (Faruk et al., 2008). Livestock owners are not concern about using different antibiotics with the suggestions of authorized veterinary doctors, most of the time they treat their animals by themselves and local doctors or quack. Antibiotics are commonly used for therapeutic and prophylactic applications and as well as growth promoter thus increase antimicrobial drug resistance (Roess et al., 2013). Antimicrobial resistance is a One Health issue, it has clear links to people, animals, and environment. The contribution of animal production, both terrestrial livestock and aquaculture, to the global AMR crises is questioned by some on the grounds that animal-associated infections in humans due to many antibiotics are used in animal production, in sub-therapeutic doses and with long exposure periods. Multidrug-resistance genes are now highly prevalent in many important and common pathogens like *Escherichia coli, Klebsiella pneumoniae, Salmonella, Enterococcus* and *Staphylococcus aureus* (Robinson et al., 2016).

Animal agriculture is the dominant source of AMR, application of manure fertilization to agricultural soil led to a bloom in AMR even though the animals that produced the manure had not been treated with antibiotics. The reason is the manure fertilization allowed for the enrichment of resident soil bacteria that harbored AMR elements (Agga et al., 2015). The spread of resistance genes in natural ecosystems can challenge the

population dynamics and physiology of natural microbial populations. Several reports indicate that the resistance genes currently present in human or animal associated microbiota are found in environments without antibiotic pollution. Furthermore, antibiotic pollutions in the environment will affect the human being, livestock, poultry as well as wildlife (Martinez, 2009).

Among the wildlife, non-human primate has the major part and plays an important role in the maintenance and functioning of tropical ecosystems. Non-human primates are free-ranging wild species have a close genetic relationship with humans and the organisms causing disease are easily swapped between them. Non-human primates can be divided into several groups; old world monkeys, new world monkeys and others (great apes, lesser apes, and lower primates). The species Rhesus macaque (*Macaca mulatta*) is an important member of old world monkeys under the family *Cercopithecidae.* In this family also included cynomolgus macaques (*M. fascicularis*), baboons (*Papio hamadryas*), African green monkeys (*Chlorocebus aethiops*), pigtailed (*M. nemestrina*), bonnet (*M. radiata*) (Rogers et al., 2006). Old World monkeys especially rhesus monkey is closely related to humans in the subject of genetic, ecological, behavioral, and biological aspects. They are sharing a common ancestor approximately 25 million years ago (Stewart and Disotell, 1998). The IUCN Red List is listed Rhesus macaque as least concern species due to its wide distribution, presumed large population, and its tolerance of a broad range of habitats. They are widely distributed in the south, southeast and east Asia eastern Afghanistan, Bangladesh, Bhutan, as far east as the Brahmaputra Valley in peninsular India, Nepal, and northern Pakistan (Timmins et al., 2008). This species is diurnal and omnivorous, and alternatively arboreal and terrestrial. It resides in a range of habitats, including temperate coniferous, moist and dry deciduous, bamboo, and mixed forests, mangroves, scrub, rainforest, and around human habitations and developments, including cultivated areas, temples, and roadsides (Srivastava et al., 2001). Five species of macaques occurred in Bangladesh (IUCN 2000). Except for Rhesus Macaque all other macaques (*Macaca nemestrina, M. fascicularis, M. arctoides* and *M. assamensis*) are restricted only to the northeastern and southeastern hill areas of Bangladesh (Hasan et al., 2013).

1.2 Justification of the study

Rhesus macaques are synanthropic, thriving in human-altered environments which help them to be among the most widely distributed and successful primates in the world. There are limited data of the population and distribution of macaque in Bangladesh. Primate populations are being reduced or eliminated in many parts of the world due to habitat destruction, competition for food and space, bushmeat hunting, biomedical research, and the pet trade (Hasan et al., 2013). More than 54% of the world's non-human primate species and subspecies with known conservation status are classified as threatened with extinction on the IUCN Red List of Threatened Species. The Rhesus macaques are distributed throughout the country from urban including temples and shrines to deep forests. (Feeroz, 2001) reported that, 17 primate habitats in the northeastern and southeastern part of the country. A total 1528 into 37 groups of rhesus macaques were identified among 16 urban populations, besides the overall population size was 5313 and group size was varied from 10 to 78 (Hasan et al., 2013).

Non-human primates (NHPs) are the source of transmitting different diseases, one of the most serious zoonotic disease that humans can get from old world macaque is caused by *Herpesvirus simiae* virus (B virus, Cercopithecine herpesvirus). Most of the adult macaque up to 90% can be the carrier of the B virus without any symptom, some may have localized oral lesions. Free-ranging macaque is the common source of rabies. In 1957, the raccoon rabies outbreak occurred in Florida and then up to 1974 more than 640 NHPs were tested for rabies. Macaque is highly susceptible to tuberculosis (TB) and can get an infection from humans and other animals. The area where human TB is predominant has a greater risk of TB (Conly and Johnston, 2008).

Though there are ethical restrictions humans as experimental subjects because it has a great risk with direct research on human especially in clinical trials, different animal models used for biomedical research year by year. Due to genetic similarity to human monkeys are used in research and other less developed animals such as mice, fruit flies also use. NHPs are phylogenetically close to humans, with many similarities in terms of physiology, anatomy, immunology, as well as neurology, all of which make them excellent experimental models for biomedical research (Zhang et al., 2014). Non-human primates (NHPs) have been widely used in lab settings since 1950s. There has been a corresponding increase in the prominence and importance of using NHP models

that have been widely used as subjects in infectious diseases, mental and neurological disorders (Perretta, 2009), cardio-cerebrovascular diseases and endocrine diseases (Pound et al., 2014).

Animals kept in captivity or bred in semi-free-range outdoor areas may become infected with entero-pathogens in their enclosures**.** The intensification of human activities within habitats of previously isolated wild animals is a key factor in the emergence of infectious diseases (Lloyd-Smith et al., 2009). Antimicrobial resistance in both medicine and agriculture is recognized by the World Health Organization (WHO), along with other various national authorities, as a major emerging public health concern. It represents a significant challenge of global dimensions to human and veterinary medicines with the prospect of therapeutic failure for life-saving treatments now a reality**.** Recently, there has been increasing interest in resistant bacteria and resistance genes isolated from wildlife and the environment. Bacteria resistant to antimicrobials have been detected in a variety of wildlife species and wildlife have been implicated as potential reservoirs of resistant bacteria and resistance genes (Sayah et al., 2005). Wild animals are not expected to be exposed directly to antimicrobials, and the source of AMR in the bacteria of wild animals is not clear (Rolland et al., 1985). However, resistant bacteria have been found at high prevalence in the intestinal bacteria of wild rodents living in proximity to livestock with (Kozak et al., 2009) reporting 54% resistance. Antimicrobial resistance *Eschericha coli* and *Enterococcus* spp. isolates from wildlife were first reported in Japanese wild birds (Sato et al., 1978).

Some previous studies showed the presence of different microorganisms in non-human primates and showed antimicrobial resistance as well; Infection of 19%, 13%, and 6% respectively *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp. were found in free-ranging human-habituated mountain gorillas of Uganda (Nizeyi et al., 2001). Research from China, Cambodia, and Indonesia with respective isolation rates of 15%, 36%, and 67% *Campylobacter* spp. and resistance of tetracycline, ciprofloxacin, and erythromycin was common (≥80%) in China, (≥75%) in Cambodia, tetracycline, and ciprofloxacin (100%) in Indonesia (Koga et al., 2017). *Staphylococcus aureus* (75%) were resistant to methicillin/oxacillin in rhesus macaques (*Macaca mulatta*) (Taylor and Grady, 1998). Infections with *Staphylococcus* spp. to the non-human primate are common; 61% in Africa (Schaumburg et al., 2012), 39% in Netherland (Van Den Berg et al., 2011), 23.6% in Wisconsin, USA (Kokan and Bergdoll, 1987).

Intra-species interactions is one of the major cause of transmission of resistant microorganisms, due to direct and indirect contact with various species. Colonization or infection by a resistant bacterial strain can occur as the result of consumption of antimicrobials or antimicrobial residues or by direct transfer of resistant bacteria or resistance genes through direct contact with resistant bacteria or from consumption of contaminated food and water (Sayah et al., 2005).

Conflicts between humans or livestock and non-human primates are very common and recognized as foremost issues. Conflict causes various negative results, including damage to crops and property, habitat destruction, injuries and death of people and wildlife, and livestock depredation. Rhesus macaque lives very close to the human habitat in different districts of Bangladesh and many people have been scratched and injured in Bormi (Gazipur), Dhamrai (Dhaka), Charmuguria (Madaripur), Monohardi (Narsingdi), Chandpur and Chashnipeer-er-Mazar (Ahsan and Uddin, 2014). NHP's has great interaction with human and livestock, the study showed, highest activity followed by 33.75% in feeding, 11.73% in grooming, 4.87% in moving (Alam et al., 2015).

Resistant microorganisms in wildlife are found more, those are abundant closer to human and livestock settlement. In this sense, differences in diet and activity among host species may play an important role in determining antibiotic resistance in wildlife, as some species come into more frequent contact with humans, human landscapes, or domestic animals than others (Cristóbal-Azkarate et al., 2014). The study by Rolland et al., (1985) showed that baboons feeding on human garbage and in contact with other forms of human detritus maintained significantly greater levels of antibiotic-resistant gut bacteria than did their wild counterparts. Another study shows, resistant bacteria were detected more frequently in baboons feeding on human refuse than in animals living in more remote areas with no human contact. *Escherichia coli* exchanges between humans, domestic animals, and great apes have been reported in densely human-populated areas of western Uganda. Within Uganda, habituated groups of wild apes are visited daily by researchers and tourists (e.g., chimpanzees) (Goldberg et al., 2007) and mountain gorillas at Bwindi Impenetrable National Park (Rwego et al., 2008). In particular, the presence of antibiotic-resistant strains in untreated wild animals has been suggested to reflect bacterial exchange with humans or domestic animals and vice versa, in which treatment with antibiotics actively selects antibiotic-resistant strains. Evaluation of the current prevalence of three microorganisms (*Salmonella* spp.,

Staphylococcus spp. and *Enterococcus* spp.) in the rhesus macaque in different districts within different habitats is the first step and then evaluating antimicrobial resistance levels in those microorganisms that pose threat for rhesus monkey.

1.3 Research question

i. What is the prevalence of resistant *Salmonella* spp., *Staphylococcus* spp. and *Enterococcus* spp. in rhesus macaque in Bangladesh?
ii. What are the commonly used antimicrobials to which these 3 organisms are resistant?

1.4 Objectives of the study

i. To estimate the prevalence of resistant *Salmonella* spp., *Staphylococcus* spp. and *Enterococcus* spp. in rhesus macaque in Bangladesh.
ii. To determine the antimicrobial resistance patterns of *Salmonella* spp., *Staphylococcus* spp. and *Enterococcus* spp. isolated from rhesus macaque.
iii. To determine interspecies interaction and transmission of resistant organisms.

Chapter-2: Literature Review

Relevant literature on antimicrobials, antimicrobial resistance, prevalence, and diagnostic techniques have thoroughly been reviewed in this chapter. The main purpose of this chapter is to provide up-to-date scientific information based on past studies and accordingly identify gaps and justify the present epidemiological MS research on Antimicrobial resistance in rhesus macaque. The review findings of relevant published articles have been presented under the following headings as below.

2.1 History and scope of Antimicrobial resistance

After the invention of new antibiotics got resistance immediately within few years due to improper use of those antibiotics. There is a lot of evidence of the presence of resistant microorganisms in nature but those microorganisms are not present in human (Hughes and Datta, 1983). However, in the recent years, the microorganisms which are resistant to antibiotics are alarmingly high in human as well as animals. By the side of the discovery of new antibiotics, researcher began to find out microorganisms that are resistant to new drugs. Surprisingly by the year 1909, Ehrlich discovered resistant trypanosomes when he started to work with dyes and arsenicals. After the invention of penicillin, it became much popular to use as treatment and a research showed *Staphylococcus aureus* resistance in hospitals was 14% in 1946 to 38% in 1947 and today about 90% resistance in hospital cases. All over the world, penicillin and ampicillin together found resistance to *Staphylococcus aureus* is about 80% (O'Brien and 2, 1987). After the end of world war (ii), sulfonamides were used very commonly for the treatment of *Shigella* infections in Japan but it was resistant to about 80% by the year 1952. After that Japanese started to shift to streptomycin, tetracycline, and chloramphenicol as a results *Shigella* became multi-drugs resistant quickly (Falkow, 1975). Sulfonamides was a successful drug for the treatment of meningococcal disease within 30 years of its discovery, but recently it became resistance to common used antibiotics (O'Brien and 2, 1987). Some researcher and clinicians already predict a crisis stage of antibiotics and we may go to face some destructive diseases which will not be cured with our antimicrobials (Lipsitch et al., 2002). We found that resistance has been observed in microorganisms commonly but some microorganisms are remarkably concern. The resistance organisms are increasing significantly due to tremendously

frequency of travel worldwide, highly increase of population in both developed and developing countries.

2.2 *Salmonella* spp: zoonotic significance and resistance pattern

Salmonella is a rod-shaped gram-negative, non-spore forming, motile bacteria which have peritrichous flagella. Most of the serotypes inhabit the intestines of mammals, birds, and reptiles. They are intracellular pathogens, can cause diseases and transfer from animal to human and human to human. *Salmonella* spp. have the ability to survive in water, food, and soil for a long time (Angulo et al., 2000). There are so many diseases occurred by *Salmonella* spp. infections but two major diseases are typhoid fever and salmonellosis. *Salmonella typhi* causes typhoid fever which has typical characteristics with high fever of two weeks incubation period the diarrhea and headache. *S. typhi* multiply in the intestinal epithelium and it can also attack the phagocytes and extend to whole body. Organisms can invade the intestinal wall and high mortality may happen if peritonitis and septicemia occur (Everest et al., 2001). Typhoid fever is a deadly disease, many peoples have died in early ages. Present days 16.6 million cases found a year all over the world, in developed countries the incidence decreased remarkably but in developing countries the huge number of deaths around 0.6 million (Shanahan et al., 2000). Asia is the main hotspot of *S. typhi*, a research said that about 30-40% blood culture from the hospital is *S. typhi* in the part of Asia (McCormick, 1998). Most cases in the USA are acquired may be due to the transmission from the international traveler. Species other than *S. typhi* causes salmonellosis, not so extreme like typhoid fever. Salmonellosis is a less severe disease, often self-limited with diarrhea, fever and abdominal cramps. Insufficient cooking of meats and eggs are the most common reason for *Salmonella* infection and most of the cases we were unaware also not reported. There was estimation about 1.4 million salmonellosis cases found in the USA (Angulo et al., 2000). Although according to the nature of the disease antibiotics are not important for treatment but salmonellae can spread into the bloodstream and can make serious illness so that antibiotics are necessary. History expressed chloramphenicol and ampicillin have been used for the treatment of Salmonellosis (Shanahan et al., 2000). By the end of 1960's, there found multiple drug resistance in salmonellosis and after that resistance increased dramatically. From 1988, ciprofloxacin was the most important drug for the treatment of salmonellosis, gradually it became resistance. Transmission of multidrug resistant *S. typhimurium* enhanced the several outbreaks of

it along with cattle and poultry population (Threlfall et al., 2000). Multidrug resistance *S. typhimurium* strain, DT104 first isolated in England in 1988. Last ten years research revealed above 90% human were found multidrug-resistant to *S. typhimurium* strain, DT104 with multiple antibiotics such as ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. The strain also spread all over the world and got more resistance to the antimicrobials. After 1997, DT104 strain became resistance to trimethoprim and fluoroquinolones (Khachatourians, 1998). Before 1986, *S. typhi* was resistant to chloramphenicol in Mexico, later on, it became resistant to ampicillin, streptomycin, sulfonamides, and tetracycline. In some developed countries fluoroquinolone resistance *S. typhi* has been published (Scuderi et al., 2000). Very unfortunate, after showing resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline by various DT104 strain and now become resistant to fluoroquinolones (Angulo et al., 2000). Since 1989 outbreak of *S. typhi* resistance occurred in developing counties of south-east Asia especially Pakistan and India. Strains of *S. typhi* were mainly resistant to chloramphenicol, ampicillin, and trimethoprim; also other antibiotics such as streptomycin, sulfonamides, and tetracyclines (Rowe et al., 1997). In between 1990 to 1992, 236 isolates of *S. typhi* were identified in Bangladesh and they were resistant to ampicillin (66.5%), co-trimoxazole (72.9%), chloramphenicol (78.8%), tetracycline (58.5%) and nalidixic acid (14%) and MIC were >512, >1025, >512, >128 and >16 mg/L, respectively (Saha and Saha, 1994). A longitudinal study was done in captive wildlife including non-human primates in Thailand, got 7% yielded 24 *Salmonella* serotypes with 29% resistance to tetracycline (Gopee et al., 2000). In 1999, free-ranging mountain gorillas (*Gorilla gorilla beringei*) were tested positive for *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp., with an overall prevalence of infection, was 19%, 13%, and 6%, respectively in Bwindi and Mgahinga National Parks, Uganda (Nizeyi et al., 2001). *Salmonella* was identified 3% in national center for primate biology, University of California, Davis (Good et al., 1969). In Nigeria, 6 bacterial pathogens were identified in non-human primates *Escherichia coli* (100.0%), *Salmonella paratyphi* A 31(93.9%), *Proteus mirabilis* 14(42.4%), *Campylobacter* species 6(18.2%), *Citrobacter ferundii* 7(21.2%), and *Yersinia enterocolitica* 3(9.1%) (Okwori et al., 2014). From the year 2012 to 2015, AMR research was done in nonhuman primate and found 4 enteric bacteria: *Shigella flexneri, Yersinia enterocolitica, Y. pseudotuberculosis*, and *Campylobacter jejuni*. *S. flexneri* isolates were resistant to erythromycin (87.5%), doxycycline (73.7%), and

tetracycline (38.3%); *Y. enterocolitica* isolates to ampicillin (100%) and cefazolin (93.6%); and *C. jejuni* isolates to methicillin (99.5%) and cephalothin (97.5%) (Kim et al., 2017a).

2.3 *Staphylococcus* spp.: zoonotic significance and resistance pattern

Staphylococcus is gram-positive cocci, normal flora of the body which are frequently found on the nose and respiratory tract and associated with nosocomial infections. At present days, *Staphylococcus* spp. is resistance to several antimicrobials. In the clinical perspective, *Staphylococcus* are classified into two groups: coagulase-negative and coagulase positive. The most common important opportunistic microorganisms on the skin are from coagulase-negative group staphylococci is *Staphylococcus epidermidis.* On the other hand, important coagulase-positive group Staphylococci are *Staphylococcus aureus* which is commonly found in the nasal cavity of human and animals. *S. aureus* is always opportunistic can cause various complications such as pimples, impetigo, furuncles, folliculitis, abscesses and life-threatening diseases such as pneumonia, osteomyelitis, endocarditis, septicemia, and meningitis etc. (Le Loir et al., 2003)**.** There are some virulence factors that indicate the ability to cause diseases of *S. aureus.* It can cause food poisoning and toxic shock syndrome. Antimicrobial resistance in staphylococci is very usual. History told that AMR in staphylococci started at the beginning of the antibiotic era. In 1948, when penicillin started to use, all *Staphylococcus* isolates were resistant to penicillin and other N-lactam antibiotics also including ampicillin in the hospital. That time 59% of *S. aureus* were resistant to penicillin, all were from hospital patients. Around 1950, most of the strain of *Staphylococcus* group were penicillin resistant in most of the hospital all over the world (Garrod and O'grady, 1971). Due to nosocomial infection of penicillin-resistant *Staphylococcus aureus*, pathogens transferred to the community very rapidly. Nowadays, the percentage of resistance of *Staphylococcus* increased many times, now more than 90% *S. aureus* are resistance and coagulase-negative *Staphylococcus* are resistance about 50% to 70% (O'Brien, 1987). A recent study from Portugal with healthy young volunteers revealed that *S. aureus* was highly resistant (94%) to penicillin or penicillin and erythromycin (Sá-Leão et al., 2001). Other antibiotics are in the same situation after introduction became resistance in a short time. *Staphylococcus* resistance to other antibiotics such as streptomycin, tetracycline, chloramphenicol, and novobiocin was reported in 1953. A pandemic emerged by a notorious penicillin-

resistant strain of *S. aureus* (phage type 80/81) in 1950's and spread all over the world, but it was under control after the invention of penicillinase-resistant β lactams (Robinson et al., 2005). The resistance was recorded in independent strain but sometimes together in a single. The *S. aureus* was resistant to new drugs as for example fluoroquinolones and quinupristin (Srinivasan et al., 2002). In 1960, when *S. aureus* became resistant to penicillin, penicillinase-resistant β lactams such as methicillin began to use the patient in the hospitals (Garrod and O'grady, 1971). But, unfortunately, it was not safe, it became resistant and Methicillin-resistant *S. aureus* (MRSA) distributed all over the world very quickly. At present, MRSA is one of the most important nosocomial organism. In the United States from intensive care unit 47% *Staphylococcus aureus* were isolated that were methicillin resistant (Srinivasan et al., 2002). In 2000, 48% *S. aureus* isolates were methicillin resistant in Portugal. Most of the research presented that, until now MRSA is a hospital-based problem, on the other hand, some publications reported some community-based problem in some countries. MRSA in day-care centers, among the children with some cases of death has been reported (Sá-Leão et al., 2001). Along with the hospital, community-based MRSA is very low (1-2) % that are increasing gradually. *Staphylococcus* spp. were resistant not only to methicillin but also other antibiotics except glycopeptides. At present best treatment for MRSA infections is vancomycin, but some countries showed the resistance to vancomycin. In 1997, vancomycin-resistant *S. aureus* (VRSA) was first identified in Japan (McCormick, 1998). After that VRSA isolated in the USA including other countries. After research, the background of VRSA come to us, the vancomycin resistance gene *vanA* from *Enterococcus faecalis* could be transferred to *S. aureus* by in vitro (Noble et al., 1992). After 1997, no record was found of connection to VRE but in 2004, from patients of U.S., VRSA containing *vanA* gene been isolated (Witte, 2004). In case of identification of VRSA, a large number of strains isolation didn't follow CLSI standards so they know as vancomycin-intermediate *S. aureus* (VISA). If VISA strains failed to meet the principle of resistance in vitro, patients will not respond to vancomycin properly (Sakoulas et al., 2004). Data from 26 European countries from 1999 to 2002 examined and there was <1% MRSA prevalence found in northern Europe and >40% in southern and western Europe and MRSA significantly increased in Belgium, Germany, Ireland, the Netherlands, and the United Kingdom, and decreased in Slovenia (Tiemersma et al., 2004). In the Asian region, 74 MRSA strains were identified from 12 countries and all MRSA strains were resistant to penicillin and

gentamycin. Other antibiotics were resistance in very high level, amoxicillin-clavulanic acid (96%), cefuroxime (85%), clarithromycin (85%), ciprofloxacin (84%), trimethoprim-sulfamethoxazole (50%) (Ko et al., 2005). A study of antibiotic susceptibility testing in 2006 to 2007 in Mymensingh, Bangladesh presented that *Staphylococcus aureus* of MRSA strains were 100% resistant to penicillin, oxacillin, cloxacillin and amoxicillin. Wild animal research presented diversified microorganisms, 61% *Staphylococcus* spp. isolated from non-human primates in Africa (Schaumburg et al., 2012). Rhesus macaque were infected 39% with *Staphylococcus aureus* in Netherland. Wild animals were also susceptible to Methicillin-Resistant *Staphylococcus aureus*, A study of cynomolgus macaques (*Macaca fascicularis*) showed that, 22% of monkey were positive to MRSA (Kim et al., 2017b).

2.4 *Enterococcus* spp.: zoonotic significance and resistance pattern

Enterococci are gram-positive bacteria which are normally present as flora in both humans and some animals (Van den Bogaard et al., 2002). The characteristics feature of *Enterococcus* spp. is gram-positive cocci, generally not considered as virulent. *Enterococcus* is an important opportunistic pathogen, commonly causes nosocomial infections. They are intrinsic resistance to antibiotics such as penicillin, cephalosporins, and aminoglycosides. In the USA about 12% of nosocomial infections caused by 2 species of enterococci, one is *Enterococcus faecium* and another is *Enterococcus faecalis* (Van den Bogaard et al., 2002). It is very difficult to treat infections caused by *Enterococcus* due to resistance. *Enterococcus* might be an opportunistic bacteria but it can cause urinary tract infections, endocarditis and bacteremia frequently. Bacteremia due to *Enterococcus* infection is very high, the mortality rate may reach 70% (Melhus and Tjernberg, 1996). Combination of an aminoglycoside and ampicillin or a glycopeptide are the conventional method of treatment. At very early by the 1970's, in most of the cases only ampicillin and vancomycin were a more effective treatment (Frieden et al., 1993). Research of 2000's revealed that only vancomycin left as the last treatment because of resistance of ampicillin and aminoglycosides (Jeljaszewicz et al., 2000). There was a report in 1998 that about 20 to 40 percent of nosocomial infection were VRE (Khachatourians, 1998). After that when the frequency of VRE infections greatly increased, it became major health issues. In the USA about 0.4% Enterococcal glycopeptide resistance was present in intensive care unit in 1989 and it became 16% in 1997. Not only human are multidrug resistance to *Enterococcus* spp. but also the

animals, a report of 1998 in eastern seaboard of the United States showed that Commercial Poultry Production Environments infected with *Enterococcus faecalis* were resistance to lincosamide, macrolide, and tetracycline, on the other hand, *Enterococcus faecium* was resistance to fluoroquinolones and penicillins (Hayes et al., 2004). After 1980's rapidly increased the prevalence of VRE in Asia. The study showed that, clinical isolates about 12-21% in Korea, 3.9% in 2003 to 18.9% in 2010 in Taiwanese hospital (Kang and Song, 2013**).** The prevalence of VRE increased in the hospital of China from 0 in 2005 to 4.9% in 2010 (Zhao et al., 2012**).** In North India, there were about 105 Enterococcus species identified during 2004 those were resistant to antimicrobials from these 42.90% *E. faecium* and 40% *E. faecali* (Mohanty et al., 2005). The wildlife is also susceptible to VRE, a study of Brazil in 2010 showed that captive nonhuman primate (Capuchin monkeys and Common marmoset) were 12.3% resistant to VRE (Xavier et al., 2010). In Dhaka city, Bangladesh 4.31% *Enterococcus* spp. were isolated and resistance to Cotrimoxazole (100%), Tetracycline (60%), Ciprofloxacin (66%), Cephalexin (22%) and some other antimicrobials in 2012 (Dutta et al., 2013). The environment is getting polluted with the resistance microorganisms, a study on pond water for fish farming in Bangladesh showed that 16.67% *Enterococcus* spp. were resistance to antimicrobials (Neela et al., 2014). *Enterococcus* spp. were identified 73 isolates from the fecal samples from captive baboons (*Papio anubis*) at the Institute of Primate Research (Nairobi, Kenya) and resistant pattern were observed where 35.6% isolates were resistance to erythromycin and 2.7% isolates were resistance to doxycycline and tetracycline (Mwova, 2016).

2.5 Mechanisms of antimicrobial resistance

Based on the mode of action of different antimicrobials, antibiotics can be classified as several major groups. Antimicrobial resistance in different microorganisms can be caused by variety of mechanisms, such as (i) the presence of an enzyme that inactivates the antimicrobial agent; (ii) the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent; (iii) a mutation in the antimicrobial agent's target, which reduces the binding of the antimicrobial agent; (iv) post-transcriptional or post- translational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent; (v) reduced uptake of the antimicrobial agent; (vi) active efflux of the antimicrobial agent; and (vii) overproduction of the target of the antimicrobial agent. In addition, resistance may be caused by a previously unrecognized

mechanism. On the other hand, a gene which is not expressed in vitro may be expressed in vivo (Fluit et al., 2001).

Genetically encoded resistances can vary from mutations in endogenous genes to horizontally acquired foreign resistance genes carried by mobile genetic elements like plasmids (Frye and Jackson, 2013). Point mutations in a promoter or operator can result in the overexpression of endogenous genes such as an antimicrobial inactivation enzyme like the AmpC β-lactamase gene, or an efflux system like the MAR locus. Point mutations in genes encoding antimicrobial targets can result in a resistant target, such as mutations to the gyrase gene leading to the expression of a fluoroquinolone-resistant gyrase enzyme (Hopkins et al., 2005). Exogenous resistance genes encoded on plasmids, integrons, phage, and transposons can be horizontally transmitted by transformation, conjugation, or transduction and these foreign genes can encode all three mechanisms of resistance. This includes genes encoding enzymes that inactivate the antimicrobial, such as β-lactamases that cleave the four-membered ring in β-lactams, genes which encode efflux systems like tet (A), genes encoding a modified version of the enzyme that is the target of the antimicrobial, such as dfrA, or genes encoding an enzyme that modifies the antimicrobial target like a ribosomal RNA methylase, such as *erm* (B) (Ajiboye et al., 2009). Analysis of these resistance mechanisms can then be used to determine the genetic relationship between resistance found in isolates from animals and humans. Because of the diversity of genetic elements that lead to an antimicrobial resistance, it may be possible to determine if resistances seen in bacterial isolates from human infections are closely related to those found in animal isolates, thus identifying animal sources of resistant bacteria in human infections that can be targeted in order to reduce human disease (Frye and Jackson, 2013).

2.6 Sources of resistance in the environment

Concern over resistance was originally confined to the acquisition of resistance by microorganisms which cause epidemic disease and was an issue only with respect to clinically isolated strains. However, in recent years, antibiotic-resistant bacteria have been isolated from virtually every environment on earth. This came as a surprise to many clinicians because resistance was found in regions never exposed to human impacts. Even as awareness of environmental resistance has increased, many investigators have continued to restrict their concern to only those pathogens that

survive in the environment. It was believed that they posed a danger to humans only if the disease they caused involve resistance to antibiotics. For many years, the focus of research on resistance in the environment reflected this viewpoint. However, we now know that resistance genes can be spread far wider than once believed and a pool of resistance is developing in non-pathogenic organisms found in humans, animals, and the environment. These non-pathogenic organisms serve as a source from which pathogens can acquire genes conferring resistance, and in turn, they can become resistant by acquiring genes from pathogens discharged into the environment, e.g. via sewage or agricultural runoff. Thus, dissemination of resistant bacteria is not only a problem of the resistant pathogens themselves but also the availability of resistance genes to pathogens via gene transfer. Although resistant organisms can be found naturally in the environment, most resistance is associated with man-made impacts of some type, either agricultural or direct human impact. Antibiotic use in humans can lead to resistance in the environment via discharge of domestic sewage, hospital wastewater, and/or industrial pollution. In addition, to using in humans, antibiotics are added to animal feed to treat infections, as prophylactics, and in sub-therapeutic doses as growth promoters. Although no definitive numbers are available, some authors have published estimates and, by 1980, almost half of the antimicrobial agents used in the United States were used in animal feed (DuPont and Steele, 1987). In Denmark in 1994, a total of only 24 kg of vancomycin was used to treat infections in humans versus 24,000 kg for animals (Witte, 1998). According to Levy (2001), in 1998 in the U.S., half of the 50 million pounds of antibiotics produced were used for agricultural applications. There are a variety of positive effects from using antibiotics in animal feed, namely, inhibition of harmful gut flora which leads to increased growth rates and decreased mortality. This has allowed more concentrated farming and an estimated $3.5 billion savings in production costs per year in the United States alone (Dupont and Steele, 1987). However, the practice has resulted in the selection of antibiotic-resistant organisms in the guts of food animals. From there, these organisms enter the human food chain via contamination during slaughtering or the environment via waste discharge. Resistance has been found to follow closely the use of any given antibiotic (Aarestrup, 1999). Although some investigators dispute any danger being posed by selection of resistant flora within the guts of animals, there is no doubt that such antibiotic use leads to higher concentrations of resistant pathogens and non-pathogens, as well as resistance genes, throughout the farm environment and nearby environments

affected via runoff from farms. As will be discussed later, once resistant organisms are spread into the environment, they pose a health risk if they colonize or spread resistance genes to bacteria that colonize humans.

2.7 Reasons for getting resistant organisms by rhesus macaque

Wildlife is rarely treated with antibiotics but they have the resistant organisms and also transmitted to others. The main reasons behind the antimicrobial resistance in rhesus macaque is a human-animal interface, which in particular emphasize the transmission of resistant organisms from human to animal or vice versa. Some impacts are responsible for that transmission such as human impacts, livestock and poultry impacts and aquaculture impacts.

2.7.1 Impacts of human on spreading resistant organisms

There is significant impact by the human on the occurrence of antibiotic resistance in the environment which ultimately transmitted to the rhesus macaque. Antibiotic-resistant organisms from the human gastrointestinal tract used to dust, as well as unabsorbed antibiotics can enter the environment. Domestic wastewater has the great impact on resistance on the other hand hospital wastewater has a higher impact.

The antibiotic residues and resistant organisms are excreted and go through the sewage system. Overflow or leakage of untreated sewage mixed with groundwater and contaminated the water which macaque drink. Raw sewage, open drain contains a high number of bacteria, often counting antibiotic-resistant bacteria. The study showed that 80.5% human feces contained microorganisms are resistant (Reinthaler et al., 2003). In USA Vancomycin-Resistant Enterococci (VRE) is high in hospital waste on the other hand VRE is high on community in Europe (Spain 90%, Sweden 60%, U.K 52%, Germany 16%) (Schwartz et al., 2003). Although proper sewage treatment process reduces the load of the bacteria but 3rd world country like Bangladesh, India, Nepal has poor sewage treatment process so, there is increased a number of bacteria in wastewater and the effluent contained large numbers of both resistant and susceptible bacteria. (Schwartz et al., 2003) described VRE 16% in untreated wastewater and a huge number of resistant coliform; a strain resistant to erythromycin (100%), b strain erythromycin (26%), c strain ampicillin (62%) and ciprofloxacin (2.5%) in treatment plant effluents. Even in 1983, during the time of comparing isolates of downstream treatment plant

from upstream, enterococci resistant to tetracycline, erythromycin, streptomycin, ampicillin, and penicillin (Bayne et al., 1983). The treatment plant in the Tama river in Japan has significantly increased coliforms resistance to ampicillin and tetracycline (Iwane et al., 2001). In many counties, treatment plant effluent showed a high amount of resistant as for example in Spain 50% to 90% Aeromonas isolated from treatment plant effluent and 30% to 50% enterobacteria (Goñi-Urriza et al., 2000). In hospital wastewater contained normally heavy amount of resistant organisms because of massive use of antibiotics to the patients. Vancomycin-resistant *E. faecium* was isolated from the hospital wastewater (Harwood et al., 2001). 25% of enterococci were vancomycin resistant in the hospital wastewater and most of them were multi-drug resistant in Germany (Schwartz et al., 2003).

Hospital and agriculture wastewater are a most common source of resistant bacteria for causing environmental pollution, another proof is the study from Chittagong Medical College Hospital (CMCH), Bangladesh; the study revealed that most frequently prescribed antibiotic, fluoroquinolone is 72% resistant on *Escherichia coli* (Akter et al., 2012). Hospital and slaughterhouse is a place of resistance microorganisms, a study occurred in six medical hospitals, five veterinary hospitals and five slaughterhouse and identified resistant *Staphylococcus aureus* that was 100% resistance to Amoxicillin, Cefradin, Colistin, Cefalexin, Oxytetracycline and Pefloxacin and Enrofloxacin were 80%, 50% and 75% respectively; Gentamicin were 40%, 50% and 50% (Ahaduzzaman et al., 2014).

Since 1989, *Salmonella typhi* are resistant to ampicillin, chloramphenicol, trimethoprim, streptomycin, sulfonamides and tetracycline in many developing countries especially India, Pakistan and Bangladesh (Rowe et al., 1997). A report of ICDDR,B showed that multidrug-resistant *Salmonella* spp., *Shigella* spp., *E. coli* causes diarrheal diseases in Dhaka city (Sack et al., 1997).

Inappropriate use of antibiotics by the humans are the main causes of antimicrobial resistance. Including Bangladesh, many developing countries allow buying antibiotics without any prescriptions. One survey occurs in Rajbari district and which showed that 100000 doses of antibiotics have been dispensed without any prescriptions in one month and about 92% patients took antibiotics without prescription (Faiz and Basher,

2011). About more than 2.4 billion dollars was expended annually for the antibiotics in USA (Frieden and Mangi, 1990).

2.7.2 Impacts of Poultry production on spreading resistant organisms

In all of the livestock industries, the poultry industry is the one of biggest industry all over the world and it is growing day by day. The production level increases gradually, in 2008 annual egg production in Bangladesh was 5653. 2 million and broiler meat production was 48.1 million Kg in 2010. Nowadays, farmers using antibiotics heavily in poultry production of their farms. About 95 poultry feed companies use antimicrobials in their feed as growth promoter and the antimicrobials are tetracycline, gentamicin, streptomycin, and trimethoprim-sulfamethoxazole, flavomycin, zinc bacitracin (Hasan et al., 2011). European union used antimicrobials in the poultry industry as a growth promoter during the last 50 years which leads antimicrobial resistance in the poultry (Castanon, 2007). An early report showed that about 80% poultry fed antimicrobials during farming in the U.S. Though many antibiotics used in poultry, fluoroquinolones, ciprofloxacin, vancomycin, enrofloxacin, avoparcin, and virginiamycin are a major concern. Enrofloxacin is used to the chicks in their 1st week of age to remove infection during vaccination and in 3rd & 4th week to check respiratory diseases (Jacobs-Reitsma et al., 1994). Those antibiotics used during the life of a poultry are resulting resistant organisms who remain in the environment for many years, many resistant organisms such as *Streptococcus, Staphylococcus Clostridium, Pseudomonas, and Aeromonas* have been isolated (Kelley et al., 1998). Many studies have been done to detect the prevalence of resistance in organisms. Frequently isolated organisms from the live chickens are *E. coli*, *Salmonella* spp., *Campylobacter* spp., *Enterococcus* spp. etc; E. coli was multiple resistance to tetracycline, streptomycin, sulfonamides, gentamicin, fluoroquinolones and other antibiotics (Bass et al., 1999). *Campylobacter* spp. are another concern for poultry, in Denmark a study occurred on two farms and found prevalence 56% and 91% (Jacobs-Reitsma, 1997). For the treatment of campylobacteriosis the most often used drug is fluoroquinolones so, last 20 years *Campylobacter* spp. are tremendously increased resistance to quinolones. 50% of *Campylobacter* spp. were resistance to ciprofloxacin in Austrian poultry farm (Hein et al., 2003). In the Netherlands, the study suggested 29% of *Campylobacter* spp. were quinolone resistance in live poultry in the year of 1997 (Jacobs-Reitsma, 1997).

Salmonella spp. are the particular concern due to its zoonotic importance found in all ages of poultry. In Spain, the high prevalence of multiple resistance of antibiotics observed 65.4% and individually sulfadiazine (96.2%), neomycin (53.4%), tetracycline (21.8%), and streptomycin (11.3%). Results also found 23 different patterns of *Salmonella* Enteritidis (Carraminana et al., 2004). Even the processed poultry carcass in mid-Atlantic region thirteen serotypes of *Salmonella* spp. were identified, which were resistant to tetracycline (73.4%), ampicillin (52.9%), amoxicillin-clavulanic acid (52%), ceftiofur (51.7%), streptomycin (35.2%), and sulfisoxazole (21.8%) (Parveen et al., 2007).

In some previous studies in Bangladesh, found resistant *Salmonella* spp. in the poultry sector. In Savar, 35% isolates of *Salmonella* spp. showed the resistance to 5-10 antimicrobials (Mahmud et al., 2011). In a study of India showed that poultry eggs (3.3%), poultry feed (2.5%), poultry water (3.3%), poultry fecal (2.5%) and cloacal swabs (4.4%) contain *Salmonella* spp. and all isolates are resistant to clindamycin, oxacillin, penicillin, and vancomycin (Singh et al., 2013).

At recent, resistant *Enterococcus* spp. is the threat in poultry sectors. Vancomycin has been greatly used throughout the world by the farmers and now we confirm about the resistance of vancomycin. In this context, a large amount of avoparcin use banned in some countries. As a result, VRE in chicken declined significantly, Vancomycin-resistant *Enterococcus* spp. declined from 80% to 5% in Denmark (Bager et al., 1999). Recently, quinupristin used for the treatment of human VRE but resistant observed on broiler carcasses although it was restricted to poultry use. The reason might be the use of quinupristin along with the virginiamycin as a growth promoter in the environment. In Germany, 46% of the carcasses from turkey and broiler were positive for quinupristin resistance and other antibiotics erythromycin 100%, oxytetracycline 90% (Kraushaar et al., 2017).

2.7.3 Impacts of Livestock rearing on spreading resistant organisms

To increase the prevalence of antimicrobial resistance livestock has a huge impact, throughout the environment of farm and farm animal, horizontal transmission of the resistant gene leads the infection of farm animal as well as the environment. The production of cattle and goat increasing day by day, Bangladesh Bureau of Statistics 2012 reported, 25.8 million cattle and buffalo, 17.3 million goats and sheep and 135.1

million poultry which contributed 2309.0 million US dollar. In another report showed that dairy, meat, hides and skin and others shared GDP 18.6, 56.3, 2.68 and 2.64% respectively (Huque and Sarker, 2014). Livestock is a strength for poor people, who suffer from poverty and nutrition. In case of disease conditions generally, the farmer used to treat their animals with different antimicrobials. Most of the time they receive treatment from unlicensed village doctors and administer antimicrobial without any concerns (Roess et al., 2013). In case of livestock, cattle production include milk production and beef production and sheep and goat production include meat production. Antimicrobials are administered by group or individual to the animals to prevent diseases as well as a growth promoter. In many countries antibiotics used as a growth promoter, in 1999, at least one antibiotic in food and water was distributed at 83% feedlots, the antibiotics included tetracycline, tylosin, virginiamycin and neomycin (McEwen and Fedorka-Cray, 2002). Dairy calves placed in a group in the herd are usually administered antibiotics, including cephalosporin, penicillin, tetracycline and erythromycin. A study was conducted with clinical and subclinical mastitis affected goat in Joypurhat and Mymensingh district in 2010 which showed moderate to the higher sensitivity of *Staphylococcus* spp to gentamicin, ciprofloxacin, chloramphenicol, enrofloxacin and oxytetracycline (Sarker and Samad, 2013).

2.7.4 Impacts of Aquaculture on spreading resistant organisms

Fish farming has become the biggest industry all over the world. In Bangladesh, fish farming is a grown sector. Different types of fishes such as silver carp, catla, rohu, mrigal, grass carp, sor puti, tilapia, and shrimp. The freshwater farms are established in almost all parts of Bangladesh and saltwater farms are established in the south parts near the districts of the bay of Bengal. Both fresh and saltwater farms are very common and antibiotics have generally used those farms. Though there have restrictions to use antibiotics as growth promoter like USA (Sarker and Samad, 2013) but most of the countries antibiotics used as a growth promoter in the fishes. In case of USA Ormetoprim-sulfadiazine and oxytetracycline are used for treatment purpose and in Denmark oxolinic acid, sulfadiazine-trimethoprim, amoxicillin, oxytetracycline, and florfenicol. Most of the countries, as well as Bangladesh treatment with antibiotics, occur regularly. Treatment with antibiotics in fishes in aquaculture mainly done with treated feeds. Those feed mixed with the water and uneaten feeds are placed beneath the water as the sediment, this procedure has a direct impact on the environment of

aquatic. In this process, antibiotics consumed by the fishes and also move directly to the environment. One study reported that antibiotics introduced in the fish farm end up 70 to 80% in the environment (Halling-Sørensen et al., 1998). There are so many pathogens in the aquatic environment, and it is very difficult to determine the true effects of the use of antibiotics because the aquatic environment is very complex. Some studies show the effects, most of the farms and the effluent of fish farms have the higher level of resistance to antibiotics including multi-drug resistance. In catfish farm 58%-83% oxytetracycline resistance microorganisms found by DePaola et al. (1988). In Denmark fish farms, 69% aeromonads isolated that were oxytetracycline resistant (Schmidt et al., 2001). Another Danish study showed that 43% microorganisms were sulfadiazine-trimethoprim resistance and 20% were oxolinic acid resistance and 100% oxolinic acid, 36% amoxicillin resistance to flavobacterium (Schmidt et al., 2000). In Italy, higher tetracycline, ampicillin, streptomycin resistance was found in the sediment in fish farms (Chelossi et al., 2003).

So there found a high level of resistance from the sediment of the water with *Acinetobacter* isolates (Petersen et al., 2002). Another study of *Enterococcus* isolates from fish farm indicted that increased resistance of *Enterococcus* spp. (Petersen and Dalsgaard, 2003). A study of the department of aquaculture from BAU, Mymensingh delivered that higher resistance of antibiotics such as erythromycin, oxytetracycline and moderate resistance to chloramphenicol, sulphamethoxazole and streptomycin and lowest to oxolinic acid (Chowdhury, 1998). A study from Pabna and Sylhet district of diseased carp fishes showed that 78.9% of the isolates were resistant to oxytetracycline and chloramphenicol, cephradine and sulphamethoxazole resistant respectively 5%, 11% and 16%. About 21% isolated carp fishes were multi-drug resistant (Rahman and Hossain, 2013)

2.8 Human-livestock-macaque and Environment interface

A study conducted in human and veterinary medicine sectors, the doses of antibiotics used in rationally can select the genes encoding resistance and those gene strains can easily transmit to surrounding humans and animals. The reservoir of those strains in human and livestock indicates a potential risk for the transmission to rhesus macaque. Antimicrobial resistant strains can easily spread to macaque through food and water sources, direct contact and environmental routes (Lhermie et al., 2017). Many scientists

show the relationship between antimicrobial usage and occurrence of AMR strains in animal and human close contact. Those direct or indirect contacts may lead to zoonotic transmission of resistant organisms and resistant genes. Macaques always roam around 2 kilometers from their roosting area for their foods. During the roaming, they got direct and indirect contact with the livestock and humans. Already different studies proved that human origin strain transmitted in livestock as for example, genome sequencing and phylogenetic studies of methicillin-resistant *Staphylococcus aureus* (MRSA) in livestock developed from a human with methicillin-susceptible S. aureus strains (Founou et al., 2016). Another study showed that ESBL-producing E. coli transmission from food animals to human and produce risk via foods (Köck et al., 2017). The problem is more protruding in developing countries like Bangladesh, because of no recognized guidelines to follow, and high population rate with a high burden of infectious diseases. The uses of different antibiotics lead to increased resistance in humans and animals and as well as environments. Resistance genes have the ability to spread via horizontal transfer in every environment (soil, air and water). There are various evidence that resistant genes spread from environment to animal and to human and vice versa. Most of the evidence was established by the presence of similar gene sequences in the isolated bacteria from human and animal and also from the environment. In a study, tetracycline resistance gene (tetQ) were isolated in *Bacteroides* and *Prevotella* from human in different areas and this organism normally found in the rumen of livestock. CDC investigated different outbreaks of salmonellosis from 1971 to 1983; results found that human infected with salmonellosis was the source of food animals and 69% AMR strains were identified (Holmberg et al., 1984). Environment is a great source of transmitting resistant organisms; a study was conducted in Dhaka, the capital of Bangladesh to know the possible source of spreading the organisms; where 71% New Delhi metallo-β-lactamase-1 (NDM-1) infections were isolated from the hospital wastewater with resistance genes including $bla_{\text{CTX-M-1}}$ (80%), $bla_{\text{CTX-M-15}}$(63%), bla_{TEM} (76%), bla_{SHV} (33%), $bla_{\text{CMY-2}}$ (16%), $bla_{\text{OXA-48-like}}$ (2%), $bla_{\text{OXA-1}}$ (53%), and $bla_{\text{OXA-47-like}}$ (60%). Other organisms such as *Escherichia coli* (29%), *Acinetobacter* spp. (15%), and *Enterobacter* spp. (9%) also identified (Islam et al., 2017). The occurrence, distribution, ecological and resistance risks of antibiotics in the surface water of freshwater finfish and brackish water shellfish aquaculture in Bangladesh reported that, sulfamethoxazole (73%), trimethoprim (60%), tylosin (60%), sulfadiazine (SDZ) (53%), sulfamethazine (33%), sulfamethizole (40%) and

penicillin G (7%) resistance detected and transmitted through water (Hossain et al., 2017).

The country like Bangladesh, animal husbandry practices has a great impact on emerging zoonotic disease and antibiotic resistance. A study was conducted by ICDDR, B with the survey of 700 households where 70.6% respondents treated by own self and village doctors or quacks, only 9.7% treated with government animal healthcare provider. Animal husbandry practices that could stimulate the transmission of organisms from animals to humans; 50.1% of households reported that the chickens slept in the bedroom, 78.3% shared water for human and animal bathing and 60.9% livestock waste used as fertilizer (Roess et al., 2015).

Rhesus macaque got their food in the agriculture land, different fruits trees and often stolen foods from the houses. They roam in the field along with the livestock and poultry. Often conflict occurs between macaque and dog, this type of direct contact promoted AMR in the macaque. Macaques have the ability to adopt a variety of habitats such as rural, urban and peri-urban. Macaque from the rural group has the longer feeding time and on the other hand, urban macaque did other activities such as grooming and object manipulation/play. Seasonal variation is another factor, macaques spent more times in monsoon and summer seasons (Jaman and Huffman, 2013).

Interactions between different species may lead to the transmission of resistant microorganisms due to direct and indirect contact. Colonization or infection by a resistant bacterial strain can occur as the result of consumption of antimicrobials or antimicrobial residues or by direct transfer of resistant bacteria or resistance genes through direct contact with resistant bacteria or from consumption of contaminated food and water (Sayah et al., 2005).

Conflicts between humans or livestock and non-human primates are very common and recognized as foremost issues. Conflict causes various negative results, including damage to crops and property, habitat destruction, injuries and death of people and wildlife, and livestock depredation. Rhesus macaque lives very close to the human habitat in different districts of Bangladesh and many people have been scratched and injured in Bormi (Gazipur), Dhamrai (Dhaka), Charmuguria (Madaripur), Monohardi (Narsingdi) Chandpur and Chashnipeer-er-Mazar (Ahsan and Uddin, 2014). Non-human primates has great interaction with human and livestock, the study showed,

highest activity followed by 33.75% in feeding, 11.73% in grooming, 4.87% in moving which leads to transmission of resistant microorganisms and vice versa (Alam et al., 2015).

2.9 Problems associated with antimicrobial resistance

It is an alarming issue and major concern about the problem of Antimicrobial resistance in the whole world. World Health Organization (WHO) is much concern about the AMR and increased anxiety about the role of antimicrobials used in animal husbandry. Many meetings and conferences occurred to prevent and control the emergence and spread of antimicrobial resistant micro-organisms. Now it is impossible to return the pre-antibiotic era so we have to concern about the antimicrobial resistance.

AMR is a global threat to both human and animals and day by day it is increasingly growing and poses a huge health risk to the human, animals, and environment. Antimicrobial resistance has the direct and indirect effects on the health. When the levels of antimicrobials are high, then it can be toxic to the human or animals. Most of the antibiotics have the direct effect as for example Penicillin causes hypersensitivity reactions and produces allergy. In USA, self-reported penicillin allergy was reported about 80% to 90% of the individuals. The report also suggested that unnecessarily exposed to broader-spectrum antibiotics leads to developing of antimicrobial resistant microorganisms (Pongdee and Li, 2018). Some antimicrobials cause endocrine disruption such as oxytetracycline, tetracycline and sulfamethoxazole and some causes nervous effects (cefuroxime, neomycin) (Lee et al., 2001).

The main problem of AMR is growing the resistance to the specific antibiotics that wouldn't work further. Improper and inappropriate use of antibiotics leads to develop the resistance. Most antibiotics are used in two disciplines: treatment of humans and growth promotion and prophylaxis in animals. Data shared a book related issues and options of AMR, suggested that about 75% of antibiotic use with questionable therapeutic uses (Lederberg and Harrison, 1998). In recent years increasing of broad-spectrum agents to the patients and crowd of people in the nursing home and hospitals are other major causes of transferring resistant microorganisms.

AMR is accompanied with high mortality rates, it provokes hindrance of treatment of the diseases with the spreading of resistant pathogens, resulting in a persistent time of

infection to the patient. The cost of the treatment increased due to the resistant pathogens and in most of the cases commercially available drugs didn't work for patients. So they had to buy uncommon antibiotics at a high price.

2.10 Present status of antimicrobial resistance in non-human primate

Non-human primate is an important animal model due to its genetic resemblance with human. Antimicrobial resistance is now a great problem for human for the inconsistency and improper uses of antibiotics. It is now a major concern in case of common livestock (cattle, sheep, goat and buffalo). In spite of a number of research on AMR in humans and livestock are most common but in non-human primates are rare. In Spain, from 25 macaques identified with 72 bacterial isolates; most common are *Staphylococcus aureus* (n = 20), *Enterococcus faecalis* (n = 15) and *Proteus mirabilis* (n = 6). The organism *Enterococcus faecalis* represented multi-drug resistance characteristics, with resistance to ciprofloxacin, enrofloxacin, trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, bacitracin, erythromycin and aminoglycosides. AMR genes were *aac(6')-aph(2''), aph(3')-III, str, ant(6)-Ia, tetM, tetS, tetL, ermB, bcrABR, cat, dfrG,* and polymorphisms in *parC (S80I)* and *gyrA* (S83I) (Woods et al., 2017). In captive condition a research occurred in Nigeria and the objective was to identify *Salmonella paratyphi* among the non-human primates. Research finding was dangerous, 93.9% resistant *Salmonella paratyphi*-A was identified from those non-human primates (Okwori et al., 2014). In 2011, 74 newly weaned rhesus macaque suffered from acute diarrhea, fever, apastia, bloody stool, and bacteremia. Four monkeys died, samples were collected from 74 macaques and found 9.5% were infected with *Proteus mirabilis.* Sequence analysis showed that strain was closely related to *Proteus mirabilis* strain HI4320. In vitro susceptibility tests proved that organism was resistant to ampicillin, amikacin, norfloxacin, chloramphenicol, gentamicin, sulfamethoxazole/trimethoprim, and sulfamethylisoxazole (Yu et al., 2015). 47 cynomolgus monkeys (*Macaca fascicularis*) were collected from China, Cambodia, and Indonesia for isolation of *Campylobacter* spp. and got respectively 15%, 36%, and 67% isolation rate. Macaque from China had erythromycin, tetracycline and ciprofloxacin resistant *C. coli,* from Cambodia had ciprofloxacin-resistant *C. coli* and amoxicillin and ciprofloxacin-resistant *C. jejuni* on the other hand macaques in Indonesia had ciprofloxacin resistant *C. coli* and Tetracycline and ciprofloxacin resistant *C. jejuni* (Koga et al., 2017). Mwova (2016) represented a study where

Enterococcus spp. was 35.6% resistant to erythromycin and 2.7% resistant to doxycycline and tetracycline in captive baboon in Kenya.

2.11 Management and remedies of AMR

Global collaborative efforts are necessary for the management and prevention of AMR and it should be at individual, community, regional, national and international level. Strategies should develop the appropriate use of antibiotics, reduce interaction between microorganisms and antibiotics. The WHO global Action Plan emphasizes on increased awareness and understanding on antimicrobial use and associated AMR; build up knowledge regarding AMR through proper surveillance and research; optimal and rational use of antibiotics; lowering the incidence of infectious diseases; and on organizing resources, research, and development for proper integrated prevention and containment of antibiotic resistance (WHO, 2015). Management of AMR in both human and veterinary sectors needs ideal action plans for the development of newer antimicrobials, possible intervention measures. Drugs should only be prescribed by the professionals and drugs should be taken by proper prescription. Patients should complete treatment course of antibiotics, stopping of medication in the middle, generate resistant organisms. Self-medication by the patients and livestock should be avoided. Uses and sharing of leftover drugs should not be done and not to be saved for next time of illness. There is a great role of the scientists and policymakers. The researcher should develop novel drugs for effective treatment. Awareness programs should build for suitable use of drugs and increase cooperation and information networking among stakeholders. Proper law enforcement should be done to limit the sale of un-prescribed drugs.

Chapter-3: Materials and Methods

3.1 Description of study areas

Dhaka division is an administrative division of Bangladesh, lies between 24˚47', 22˚51' N and 90˚04', 89˚57' E with an area of 20,508.8 km². The capital, Dhaka situated at the center of Dhaka division with about 17 million peoples. Dhaka is one of the top densely populated cities in the world. From the early civilization temples and shrines were established in Dhaka and around of it and those areas were particularly occupied by rhesus macaque. Though Dhaka division is highly populated, human-macaque interaction occurs very frequently. Rhesus macaque is synanthropic, thriving in human-created environments (rural, urban and peri-urban) and play a major part in the traditions and cultures of some societies. Macaque population in Bangladesh are divided into two major categories: i) close to human settlements ii) living in forested habitats.

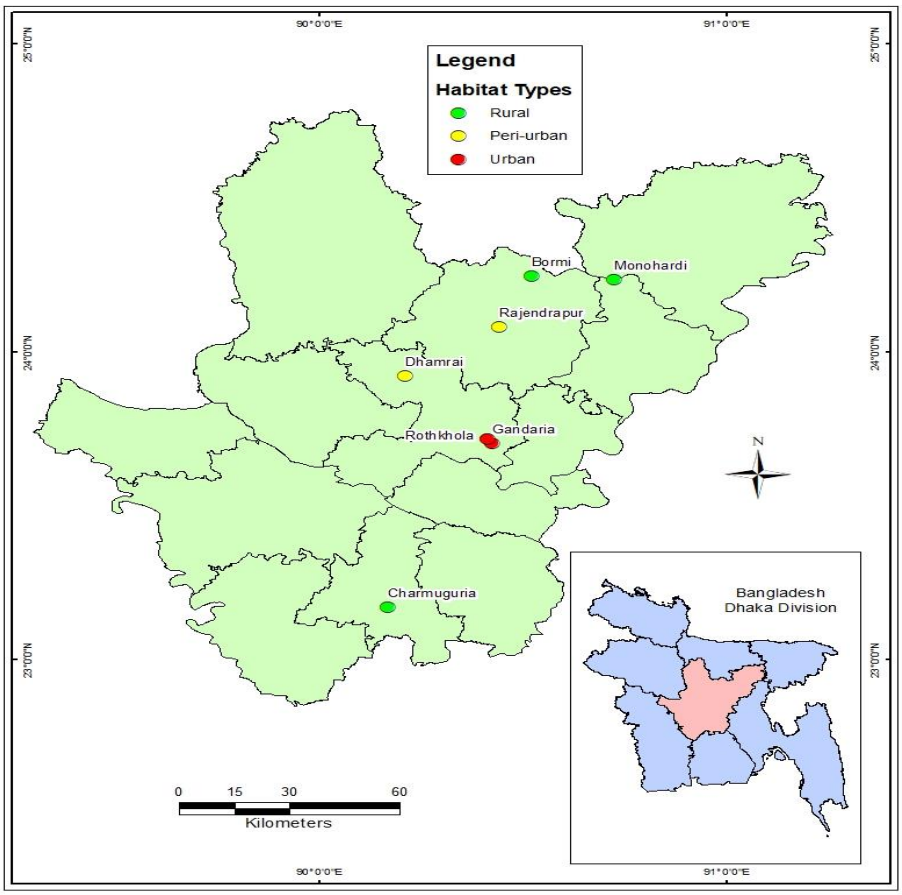


Figure 1: Map of study area

Close to human settlement many groups were found around Dhaka city: old Dhaka, Dhaka cantonment and Dhamrai. Districts around Dhaka are also predominant to large group of macaque, the districts are Madaripur, Narshingdi and Gazipur (Hasan et al., 2013).

So, a study was conducted to evaluate the epidemiology of antimicrobial resistance of *Salmonella* spp., *Staphylococcus* spp. and *Enterococcus* spp. in rhesus macaque in Dhaka city and around selective districts. The locations are Dhaka (Gendaria, Rothkhola and Dhamrai), Madaripur (Charmuguria), Gazipur (Rajendrapur and Bormi) and Narshingdi (Monohardi). (**Figure 1**).

3.2 Ethical approval

Ethical approval was taken from Chittagong Veterinary and Animal Sciences University-Animal Experimentation Ethics Committee (AEEC), Chittagong, Bangladesh (**AEEC approval number: CVASU/Dir (R&E) AEEC/2015/751**) before starting the main study. With the help of different protocol, we had assured the animal ethics and animal safety as well as the safety of working personnel in both field and laboratory throughout the whole study period.

3.3 Sample size calculation

The sample size was calculated by the formula by Daniel and Cross (1995)

n= $\frac{Z^2P(1-P)}{d^2}$ Where n=sample size,

Z= Z statistic for a level of confidence,

P = expected prevalence or proportion,

d = precision

For the level of confidence of 95%, Z value is 1.96. There is no record of the prevalence of antimicrobial resistance in rhesus macaque in Bangladesh as well as neighboring countries, so to yield maximum sample size set P equal to 0.5 (Lwanga et al., 1991). In case of precision, for 95% confidence interval the precision d=0.05. After calculation the sample size is 385. The sample was collected by proportional allocation of sample sites. A total of 399 samples were collected by adding 2 sample from each site.

3.4 Study design

A cross-sectional study was done in seven locations from four districts of Dhaka division to investigate the epidemiology of antimicrobial resistance of *Salmonella*, *Staphylococcus* and *Enterococcus* species in free ranging rhesus macaque in human, livestock and wildlife interface in Bangladesh.

3.5 Study period

The fecal samples of macaques were collected through the period of January to June 2017. Collecting samples and laboratory testing were performed simultaneously. Rest of laboratory test such as PCR and disc diffusion method was done up to October 2017.

3.6 Sample collection

Freshly voided fecal sample from free-ranging rhesus macaques collected from seven locations (Gandaria, Rothkhola, Dhamrai, Charmuguria, Rajendrapur, Bormi and Narshingdi). Convenient sample was collected randomly from the sites. Prior to collection of samples from the monkeys, they were accumulated by feeding bread and banana. To prevent repeat sampling, all sample were collected within 30 minutes. Sample collection was successfully done with the help of four trained persons so that proper identification of sex and age was done of individual sample. Confusing sample with the age was confirmed by the fecal lobe diameter (Nizeyi et al., 2001). The freshly voided fecal sample was collected by sterile swab (**Figure-11**). The collected swabs were placed in the falcon tube (15ml) containing Buffered Peptone Water (Oxoid, Basingstoke, Hampshire, UK) for *Salmonella* spp. (Putturu et al., 2013) and Mueller-Hinton broth for both *Staphylococcus* spp. and *Enterococcus* spp. as their transport media (Kateete et al., 2010). After collecting the samples in falcon tube placed in a cool box and maintained the proper cool chain. Within 24 hours, samples sent to the Bangladesh Livestock Research Institute (BLRI), Savar for laboratory analysis.

3.7 Data collection

A questionnaire was developed and administered to the local people at the time of sample collection. The questions were aimed at collecting ecological data on the rhesus macaque populations; the age of the macaque with three categories (adult, sub-adult, and baby) and sex of the animal. Geographical location's data was collected such as

GPS coordinate, type of habitat of the animal (rural, urban and peri-urban), water resources, season (winter and summer) etc.

3.8 Conceptual framework of the study

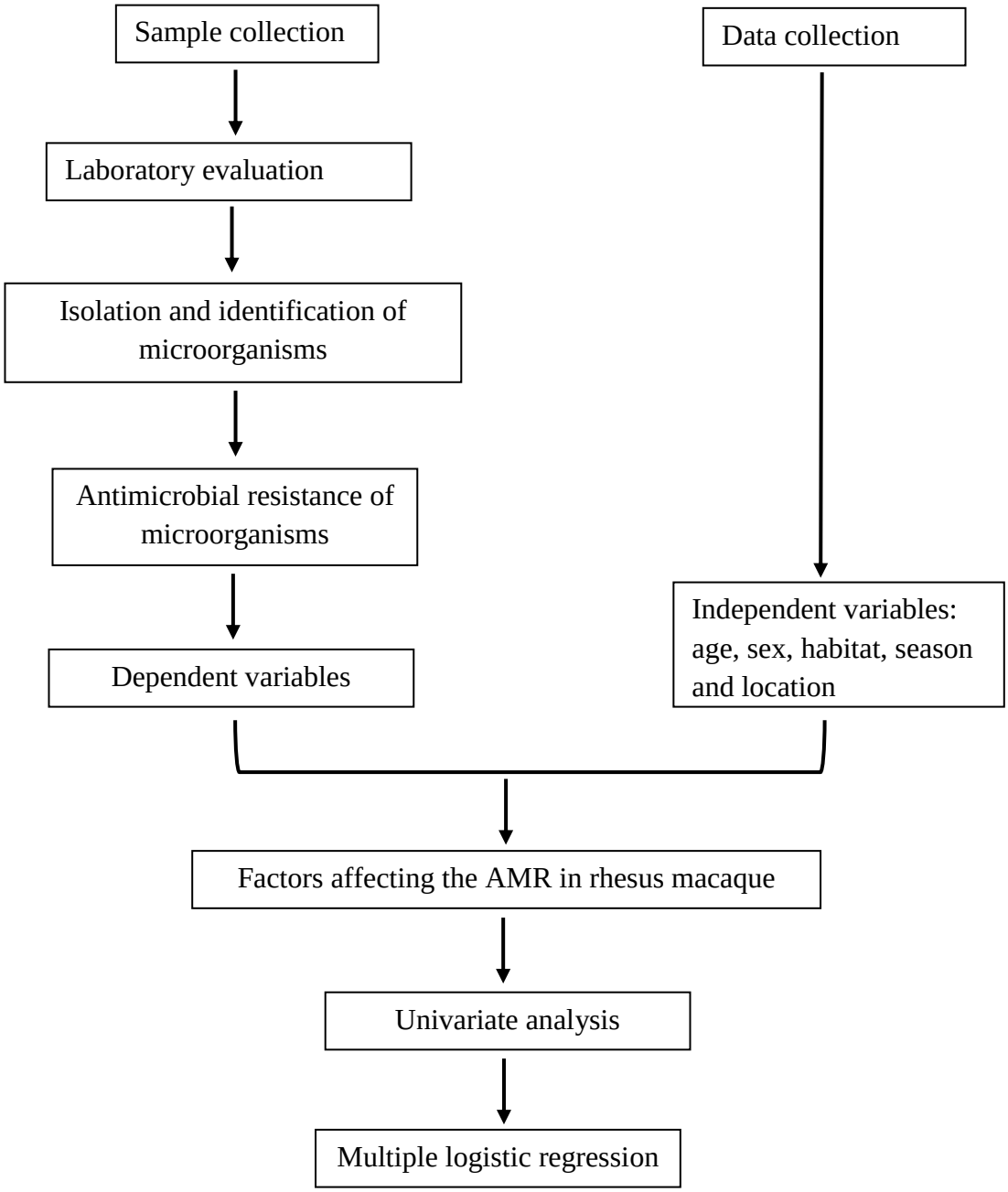


Figure 2: Conceptual framework of the study

3.9 Observation checklist

To observe interactions and distribution between rhesus macaque, livestock and human a questionnaire was made to observe the contact and distributions. Morning (2 hours) and evening (2 hours) observations per area were done for 3 days. Two categories of contact information (direct contact and indirect contact) were collected from Macaque-Human, Macaque-Cattle, Macaque-Goat, Macaque-Dog and Macaque-Cat. Direct contact was defined as direct contact or touch and indirect contacts defined as occurring contacts within 15min and <20m (Sayah et al., 2005). (**Figure-8, 9**)

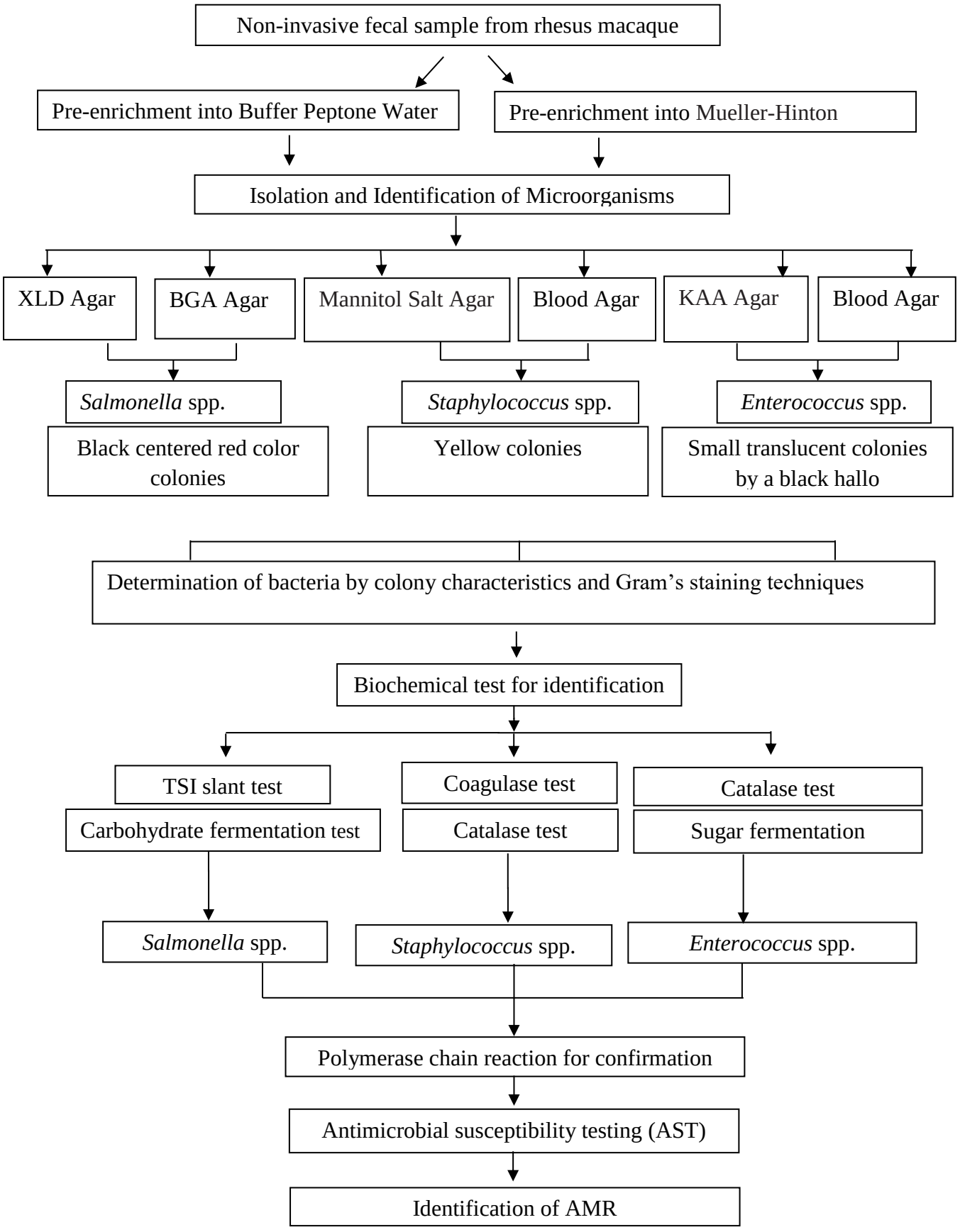
3.10 Sample processing

Freshly voided fecal samples were collected per Macaque and every sample pre-enriched on Buffered Peptone Water and Mueller-Hinton broth separately in the cool box. All the samples were transferred to the laboratory with individual labeling. The samples were stored in the laboratory on -4˚C for further processing.

3.11 Laboratory study design

The laboratory study design is schematically presented in (**Figure 3).** The entire study was conducted into four major steps: firstly, collection of fecal samples from the macaque, pre-enrichment of samples and transportation to the laboratory. Secondly, isolation and identification of bacterial pathogens by gram staining. Thirdly, characterization of microorganisms by different biochemical test and PCR. Finally, antibiotic sensitivity testing was performed.

Figure 1: Laboratory study design



3.12 Laboratory investigation

3.12.1 Isolation and identification of *Enterococcus* spp.

Due to the limitation of time and resources some representative samples (according to the systematic random sampling; every third sample selected for the AMR of *Enterococcus* sp. Samples were collected in MH broth (Oxoid Ltd, Basingstoke, Hampshire, UK) in falcon tube and stored in a cool box for transportation in the lab. Samples were pre-enriched in Brain heart infusion (BHI) broth (Biotec, Dorset, UK) and streaked on Kanamycin aesculin azide (KAA) agar (Oxoid Ltd, Basingstoke, Hampshire, UK), incubated at 37°C for 24 hours in 5% CO_2 environment by CO_2 incubator (Binder CB-150 CO_2 Incubator, Chelmsford, UK). Small translucent colonies by a black halo in KAA agar was sub-cultured in Nutrient agar (NA) media (Oxoid Ltd, Basingstoke, Hampshire, UK) for pure culture isolation. The culture from KAA was streaked on blood agar medium (Oxoid Ltd, Basingstoke, Hampshire, UK) addition of 5% sheep blood, 10µg/ml colistin sulfate and 5µg/ml nalidixic acid and incubated at 37°C for 24 hours. To isolate pure culture α-hemolysis colonies were selected and sub-cultured in Blood agar (Stalker et al., 2010). Bacteria were cultured in BHI broth (Biotec, Dorset, UK) at 37°C under 5% CO2 environment and after 24 hours incubation culture stored as a pure culture. Gram staining was done and observed under the microscope Gram-positive cocci in short chain was found.

3.12.1.1 Catalase test

A single colony was placed and smeared in a sterile glass slide and one drop of hydrogen peroxide (H_2O_2) was added. In case of positive catalase activity, H_2O_2 was degraded, on the other hand in negative catalase activity, H_2O_2 was not degraded. Presence of bubble in the glass slide indicated the degradation.

3.12.1.2 Esculin hydrolysis

To inhibit Gram-positive bacteria other than enterococci, agar slants was prepared by Bile-esculin agar (HiMedia, Mumbai, India) medium where esculin and peptone provide nutrition and bile prevent other Gram-positive bacteria except enterococci. As a color

indicator Ferric citrate was added. In Bile-esculin agar, inoculum from a pure culture streaked along the slant and incubated at 37˚C for 24 hours under 5% CO_2. Chocolate brown coloration indicated the positive growth on the slant.

3.12.1.3 Sugar fermentation

Buffered peptone water (100 ml) was prepared by adding 10 ml of stock phenol red solution. To prepare final fermentation broth added 1gm of desired sugar. After that media was sterilized at 115˚Cfor 15 minutes. Media with sterile fermentation tube was inoculated with pure culture and incubated at 37˚Cfor 18-24 hours. Sugar fermentation indicates by red to yellow coloration in the broth.

Isolates with positive Esculin hydrolysis, Ribose, Raffinose and Lactose fermentation and Negative Catalase and Arabinose fermentation confirmed *Enterococcus faecalis*, on the other hand, isolates with Esculin hydrolysis, Ribose, Raffinose, Arabinose and Lactose fermentation and Negative Catalase indicate *Enterococcus faecium* (Manero and Blanch, 1999).

3.12.2 Isolation and identification of *Staphylococcus* spp.

Fecal samples (n=399) from transport media were placed into sterile Mueller-Hinton (MH) broth (Oxoid Ltd, Basingstoke, Hampshire, UK) and enriched for 24 hours at 37˚C. Both Mannitol salt agar (MSA) medium and Blood agar base were prepared according to the instructions of the manufacturer (Oxoid Ltd, Basingstoke, Hampshire, UK). Blood agar was prepared by adding 5% citrated-bovine blood in the blood agar base. A loopful of inoculum from enrichment broth were streaked onto blood agar (Oxoid Ltd, Basingstoke, Hampshire, UK) and incubated at 37˚C for 24 hours for detection of hemolysis. The growth of yellow colonies on MSA (Oxoid Ltd, Basingstoke, Hampshire, UK) surrounded by yellow zones as a result of fermentation of mannitol after 24 hours of incubation at 37°C indicated a positive result (Kateete et al., 2010). The smear was prepared from the isolated colony on clean grease free microscope glass slide and stained with Gram`s method of staining. All the positive samples were subjected to coagulase and catalase tests for biochemical confirmation of *Staphylococcus* spp. as described by Monica (1991). After the five cross-sectional colonies were picked up and moved to a 10 ml test tube which

containing 5ml of brain heart infusion broth (BHIB), were prepared according to the guidelines of the manufacturer (Oxoid Ltd, Basingstoke, Hampshire, UK), incubated at 37°C for 6 hours.

3.12.2.1 Coagulase test

Whole blood from a horse was collected into commercially available sterile tubes containing EDTA to perform the test. Then using a centrifuge device (refrigerated), the blood was centrifuged at 2600 rpm for 10 minutes. The follow-on supernatant, the plasma, was instantly transferred by sterile tips to a sterile 1.5 ml Eppendorf tube and kept at -20°C for future use.

3.12.2.2 Tube coagulase test

From each tube cultivated in BHIB, 50µL was transferred to sterile tubes containing 50µL of horse plasma. The incubation was done at a temperature of 37°C for 6 hours. The presence of coagulates was justified, considering large organized coagulation and coagulation of all the contents of the tube which do not come off when inverted (Graham et al., 2006). A control tube also was placed to validate the result.

3.12.2.3 Slide coagulase test

Staphylococcus spp. (which were confirmed by tube coagulase test) were further confirmed by slide coagulase test. One drop of the horse plasma was placed on a clean grease free glass slide. A loopful of suspected culture were mixed with plasma separately and checked for agglutination. The cultures showing agglutination were recorded as positive for coagulase test and thus were confirmed as *Staphylococcus* spp.

3.12.2.4 Tube Catalase test

Nutrient agar slant was prepared according to the instructions of the manufacturer (Oxoid Ltd, Basingstoke, Hampshire, UK). Suspected bacterial colonies inoculated into agar slant and incubated at 37°C for 24 hours. After that 1ml of 3%, H_2O_2 was added and rapid bubbling of gas considered as the positive reaction of *Staphylococcus* spp. (Hogan et al., 1999).

3.12.2.5 Slide Catalase test

A small amount of colony was placed on a fresh, clean and grease free slide. One drop of 3% H_2O_2 poured on to the colony, a coverslip was placed and bubble formation was indicated as positive (Hogan et al., 1999).

3.12.2.6 Preservation of the culture

Samples those were positive for biochemical test inoculated into BHIB (Oxoid Ltd, Basingstoke, Hampshire, UK), incubated overnight at 37˚C and then preserved at -80$^{\circ C}$with 50% glycerol in 1.5ml Eppendorf tubes for future investigation.

3.12.3 Isolation and identification of *Salmonella* spp.

Freshly voided fecal sample was collected by sterile swab stick and pre-enriched in BPW (Oxoid Ltd, Basingstoke, Hampshire, UK), incubated at 37˚C for 16 hours. After pre-enrichment 1 ml of inoculum was transferred into Selenite-cysteine broth (Oxoid Ltd, Basingstoke, Hampshire, UK) (Putturu et al., 2013). For the growth of *Salmonella* a loopful of inoculums plated onto Xylose Lysine Deoxycholate (XLD) (Oxoid Ltd, Basingstoke, Hampshire, UK) medium and incubated at 37˚C for 24 hours. Black centered colony in XLD agar is the characteristic sign then again inoculated in Brilliant Green Agar (BGA) (Oxoid Ltd, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 hours and colonies with red color in BGA indicated as positive for *Salmonella* spp (Nesa et al., 2012)**.** Inoculum from XLD agar was done gram staining technique and observed under a microscope for characteristics sign as rod-shaped gram-negative bacteria.

3.12.3.1 Reaction in TSI agar slant

To identify sucrose, lactose and dextrose fermentation, the TSI agar slant was used. It also helped to determine the ability of the microorganisms to produce H_2S. Minimum of three black centered or black colonies were inoculated into triple sugar iron (Oxoid Ltd, Basingstoke, Hampshire, UK) slant and incubated at 37˚C for 24 hours. Isolates with positive reaction (pinkish slant and yellow butt or black slant and yellow butt) are considered as positive *Salmonella* sp (Pao et al., 2005). If there was blackening in the butt

that indicates H_2S reaction and bubble of air in the butt showed gas production in slants (White et al., 1997).

3.12.3.2 Carbohydrate fermentation test

Tube with different sugar media (dextrose, lactose, sucrose, maltose and mannitol) were inoculated by a loopful of nutrient broth culture of the microorganisms and incubated at 37°C for 24 hours. The isolates which were unable to ferment lactose and sucrose remain red and gas production was noted by the accumulations of a bubble in the inverted Durham's tube and thus *Salmonella* spp. was suspected describe by Hossain et al. (2006).

3.12.3.3 Slide agglutination test

Slide agglutination test was done mainly for the serotyping of *Salmonella,* here *Salmonella* 'O' antiserum (Sand A Reagents Lab Ltd., Bangkok, Thailand) was used. In the slide agglutination test, a drop of serum and a drop of saline were placed on the slide and for the control, a clean grease free slide was taken. A loopful of the colony from nutrient agar placed on the test slide and mixed properly tilting the glass slide and after one to two minutes agglutinations occurred which indicate positive *salmonella.* The poly 'O' antiserum gave positive agglutination reactions with any serovars of *Salmonella.*

3.12.3.4 Preservation of the culture

Those samples were positive in the biochemical test were inoculated into BHIB (Oxoid Ltd, Basingstoke, Hampshire, UK), incubated overnight at 37°C and preserved at -80°C with 50% glycerol in 1.5ml Eppendorf for further testing.

3.13 Polymerase Chain Reaction (PCR)

Positive samples after different biochemical tests were performed polymerase chain reaction for confirmation of microorganisms. For *Salmonella* and *Staphylococcus* were confirmed up to genus level and for *Enterococcus* two species were identified: *Enterococcus faecalis* and *Enterococcus faecium.*

3.14 Antimicrobial susceptibility testing (*Staphylococcus* spp., *Salmonella* spp. and *Enterococcus* spp.)

All positive bacterial isolates (*Staphylococcus* spp., *Salmonella* spp., *Enterococcus* spp.) were inspected for their diversity of antimicrobial susceptibility profiles. The test was carried out by disk diffusion method on Mueller-Hinton agar (Oxoid Ltd, Basingstoke, Hampshire, UK) according to the Bauer-Kirby disk diffusion procedure (Bauer et al., 1966) (**Figure 15**).A bacterial turbidity equivalent of 0.5 McFarland standard was prepared adding 0.5ml of 1.175% (w/v) barium chloride dehydrate ($BaCl_2.2H_2O$) solution to 99.5ml of 0.18 mol/L (1% v/v) sulfuric acid (H_2SO_4). The tubes of McFarland standard were sealed with parafilm to stop vaporization and stored in the dark at room temperature (22˚C to 25°C) (Wiegand et al., 2008). 3-5 well-isolated colonies were selected from the agar plate culture and growth was transferred with a loop into a 4ml tube with Tryptic soy broth (TSB) (Oxoid Ltd, Basingstoke, Hampshire, UK).

The surface of Mueller-Hinton agar (Oxoid Ltd, Basingstoke, Hampshire, UK) plate was inoculated by streaking the swab, this procedure was repeated by streaking two more times and rotated the plate approximately 60°C for even distribution of inoculation. Each disc were pressed down to the agar surface to ensure complete contact. The disc was placed not more than 24 mm from each other and a total 6 disc in 150mm plate. The plate was incubated at 37˚C under 5% CO_2 incubator.

The antibiotics used for three bacterial species against the tested isolates along with the size of the zone of inhibition of them to be considered as resistant (R), intermediate (I) and sensitive (S) (**Table 1**) (CLSI, 2007).

Table 3.1: Panel of antibiotics used, their concentrations and zone diameter interpretative standards for *Salmonella* spp., *Staphylococcus* spp. and *Enterococcus* spp. (CLSI, 2007)

Antimicrobial agents	Disk Contents	Zone Diameter, nearest whole (mm)								
		Salmonella spp.			*Staphylococcus* spp.			*Enterococcus* spp.		
		R	I	S	R	I	S	R	I	S
Amoxicillin	10µg	≤13	14-17	≥18						

Ampicillin					≤28	-	≥29	≤16	-	≥17
Azithromycin	15µg	≤12	-	≥13						
Cefixime	5µg	≤15	16-18	≥19						
Cefotaxime	30µg	≤22	23-25	≥26						
Ceftriaxone	30µg	≤19	20-22	≥23				≤19	20-22	≥23
Chloramphenicol	30µg	≤12	13-17	≥18	≤12	13-17	≥18	≤12	13-17	≥18
Ciprofloxacin	5 µg	≤15	16-20	≥21				≤15	16-20	≥21
Clindamycin	2 µg	-	-		≤14	15-20	≥21			
Erythromycin								≤13	14-22	≥23
Gentamicin	10µg	≤12	13-14	≥15	≤12	13-14	≥15	≤12	13-14	≥15
Imipenem	10µg	≤13	14-15	≥16						
Linezolid	30µg				≤20	-	≥21	≤20	21-22	≥23
Methicillin	5 µg				≤9	10-13	≥14			
Nalidixic Acid	30µg	≤13	14-18	≥19						
Oxacilline	1 µg				≤10	11-12	≥13			
Rifampicin	5 µg				≤16	17-19	≥20			
Streptomycin	10 µg				≤11	12-14	≥15	≤11	12-14	≥15
Sulfamethoxazole-Trimethoprim	23.75/1.25µg	≤10	11-15	≥16	≤10	11-15	≥16	≤10	11-15	≥16
Tetracycline	30µg	≤11	12-14	≥15	≤14	15-18	≥19	≤14	15-18	≥19
Tigecyclin	15 µg				≤25	-	≥26			
Vancomycin								≤14	15-16	≥17

3.15 Statistical evaluation

Field and laboratory data were entered into Microsoft Office Excel 2013 and then exported to STATA/IC13 (StataCorp 4905, Lakeway Drive, College Station, Texas 77845, USA) for epidemiological analysis.

3.15.1 Descriptive analysis

Distribution of rhesus macaque was presented according to the locations and quantities of the group, population size, sample size, age and sex variables. Prevalence of three microorganisms (*Salmonella* spp., *Staphylococcus* spp. and *Enterococcus* spp.) was calculated using positive samples divided by the total number of samples tested and the results were expressed as a percentage with 95% confidence interval (CI). Then location wise prevalence of microorganisms were calculated with 95% CI. In case of positive samples, antimicrobial susceptibility testing was done and the percentage of susceptibility was done according to the resistance, intermediate and sensitive antimicrobials. Percentage of different antimicrobials were presented by bar diagram.

3.15.2 Risk factor analysis

Based on data collection, rhesus macaque samples were grouped according to the locations they were collected from, district, habitat type (urban, peri-urban and rural), age (adult, sub-adult and juvenile), sex (male and female), season (winter and summer). Chi-square test was done to identify significant risk factors for the presence of AMR in three organisms.

Four variables- district, habitat, season and age were found significant ($p<0.2$) by chi-square test for the prevalence of AMR in *Salmonella* spp. and *Staphylococcus* spp. on the other hand 3 variables- district, habitat and age were found significant for *Enterococcus* spp.

3.15.3 Random effect model

The initial risk factors were identified by Chi-square test for the presence of antimicrobial resistance in Macaque samples. The significant risk factors ($p \leq 0.2$) were promoted to the random effect model. But random effect model was not fitted. So the data was forwarded to run a logistic regression model to observe odds ratio.

3.15.4 Logistic regression model

For *Salmonella* spp. and *Staphylococcus* spp., variables- district, habitat, season and age ($p<0.3$) were forwarded to logistic regression model after chi-square test. In case of *Enterococcus*, season were dropped and sex was added. The district was omitted for *Salmonella* spp. due to collinearity. After adjusting the factor with each other; *Salmonella* spp. (habitat and age), *Staphylococcus* spp. (district, habitat and age) and *Enterococcus* spp. (district, age) were found to be a significant risk factor. Confounder was checked by observing the variation in the coefficient. If the variation was greater than 10%, then the factor was considered as a confounder. The validity of the model was checked. The model was valid by Receiver Operating Characteristic curve (ROC) and goodness of fit test (lfit) (Dohoo et al., 2003). The results were expressed as OR, 95% CI and P value.

Chapter-4: Results

4.1 Distribution of rhesus macaque in Bangladesh

To accomplish the goal of the study four districts were chosen where rhesus macaque are available. According to different habitat (rural, urban and peri-urban) within 4 districts, seven sites were selected. From Dhaka and Gazipur 2 sites were selected in each. In Dhaka, two sites are old Dhaka and Dhamrai. In old Dhaka, 2 places named Gendaria and Rothkhola were selected for collecting the sample. **(Table 4.1).**

Table 4.1: Distribution of rhesus macaque in Bangladesh

District	**Locations**	**Groups**	**Population size**	**Sample size**	**Age –n (%)**			**Sex –n (%)**	
					Adult	**Sub-adult**	**Juvenile**	**Male**	**Female**
Dhaka	Gendaria	2	105-110	70	36 (51%)	19 (27%)	15 (22%)	38 (54%)	32 (46%)
	Rothkhola	2	105-110	70	31 (44%)	24 (34%)	15 (22%)	31 (44%)	39 (56%)
	Dhamrai	2	100-105	66	26 (40%)	24 (36%)	16 (24%)	20 (30%)	46 (70%)
Gazipur	Bormi Bazar	2	90-95	51	24 (47%)	14 (27%)	13 (26%)	22 (43%)	29 (57%)
	Rajendropur	1	70-75	44	19 (43%)	7 (16%)	18 (41%)	21 (48%)	23 (52%)
Madaripur	Charmuguria	1	70-75	45	20 (44%)	9 (20%)	16 (36%)	18 (40%)	27 (60%)
Narshingdi	Rampur	2	90-95	53	25 (47%)	16 (30%)	12 (23%)	24 (45%)	29 (55%)

Gendaria, Rothkhola, Dhamrai, Bormi Bazar and Rampur had 2 groups of monkey. On the other hand, Rajendrapur and Charmuguria had 1 group in each.

The population size in each group of monkey varied from 100-110 in Dhaka district. On the other hand, the groups of Bormi Bazar and Rampur had 90-95 monkeys in each group whereas Rajendrapur and Charmuguria have 70-75 monkeys in each group.

In the present study, we collected 70, 66 and 53 samples from old Dhaka, Dhamrai and Rampur. 51, 45 and 44 samples collected from Bormi Bazar, Charmuguria, Rajendrapur, respectively. In case of age, the number of adult monkey was higher than sub-adult and juvenile in all locations. Female macaque was higher in all location than male except Gendaria, Dhaka. **(Table 4.1).**

Table 4.2: Overall prevalence of microorganisms

The prevalence of *Salmonella* spp., *Staphylococcus* spp. and *Enterococcus* spp. was 5%, 16% and 64%, respectively (**Table 4.2**)

Microorganism	**n (N)**	**Percentage (%)**	**95% CI**
Salmonella spp.	18 (399)	5	2.7-7.1
Staphylococcus spp.	64 (399)	16	12.6-20
Enterococcus spp.	70 (109)	64	54.5-73.2

Univariate and multivariate association between three microorganisms and selected variables were performed and shown in **Appendix I.**

Table 1.3: Prevalence of resistant microorganisms

Resistant microorganism	**n (N)**	**Percentage (%)**	**95% CI**
Salmonella spp.	18 (399)	5	2.7-7.1
Staphylococcus spp.	61 (399)	15	11.9-19.2
Enterococcus spp.	66 (109)	61	50.7-69.8

If any microorganism became resistant to single antimicrobial then it considered as a resistant microorganism. The prevalence of resistant *Enterococcus* spp. in rhesus macaque was 61% with 95% CI (50.7-69.8) which was the highest. The prevalence of resistant

Staphylococcus spp. and *Salmonella* spp. were 15% (95% CI: 11.9-19.2) and 5% (95% CI: 2.7-7.1) respectively **(Table 4.3).**

4.2 AMR of *Salmonella* spp.

4.2.1 Location wise prevalence of resistant *Salmonella* spp.

The prevalence of *Salmonella* spp. in macaque was the highest in Rothkhola and the percentage was 8.6% with 95% CI (3.2-17.7). The prevalence of Gendaria, Dhamrai and Narshingdi were 7.1%, 6.1%, 1.9%, respectively. The same prevalence was found in Rajendrapur and Madaripur which is 2.3% (95% CI: 0.06-12.1) **(Table 4.4).**

Table 4.4: Prevalence of resistant *Salmonella* spp. by location

Name of the location	***Salmonella* spp. (n)**	**Total sample (N)**	**%**	**95% CI**
Gendaria, Old Dhaka	5	70	7.1	2.3-15.9
Rothkhola, Old Dhaka	6	70	8.6	3.2-17.7
Dhamrai	4	66	6.1	1.7-14.8
Bormi, Gazipur,	0	51	0	0-6.9
Rajendrapur, Gazipur	1	43	2.3	0.06-12.3
Madaripur	1	44	2.3	0.06-12.1
Narshingdi	1	52	1.9	0.05-10.3

4.2.2 Univariate and multivariate association between AMR of *Salmonella* spp. and selected variables

The prevalence of *Salmonella* spp. was 7.3% which was significantly the highest in Dhaka district (P≤0.05), whereas the significantly the lowest prevalence found among the macaque of Gazipur and the percentage was 1.1%. Salmonella was significantly more prevalent (7.9%) among the macaque with urban habitat (P=0.03) than that of rural and peri-urban. The prevalence of *Salmonella* spp. among macaque in peri-urban and rural were 4.6% and 1.3%, respectively. In summer, the prevalence of *Salmonella* spp. was significantly higher than winter (P≤0.05), the percentage was 7.1%. Though there was no

significant difference but *Salmonella* was more prevalent among adult macaque which was 7.2% and female was more prevalent than male (4.9%) **(Table 4.5).**

The significant variables (district, habitat, seasons and age; $p \leq 0.3$) identified through univariate chi-square analysis were forwarded to the logistic regression model. After adjustment of the factors with each other through the model, the odds of antimicrobial resistance of *Salmonella* spp. was significantly higher in peri-urban habitat (OR=6.6; CI: 1-46.4, P=0.05) and urban habitat (OR=4.4; CI: 1-21.3) than that of rural. In case of seasons, the odds of AMR was 3.1 times higher in summer than winter season. On the other hand, the odds of AMR was higher in adult (OR=3.9; CI: 0.8-17.8) and sub-adult (OR=1.2; CI: 00.2-7.7) than that of juvenile macaque. **(Table 4.5)**

Table 4.5: Frequency distribution of AMR of *Salmonella* spp. in rhesus macaque of Bangladesh

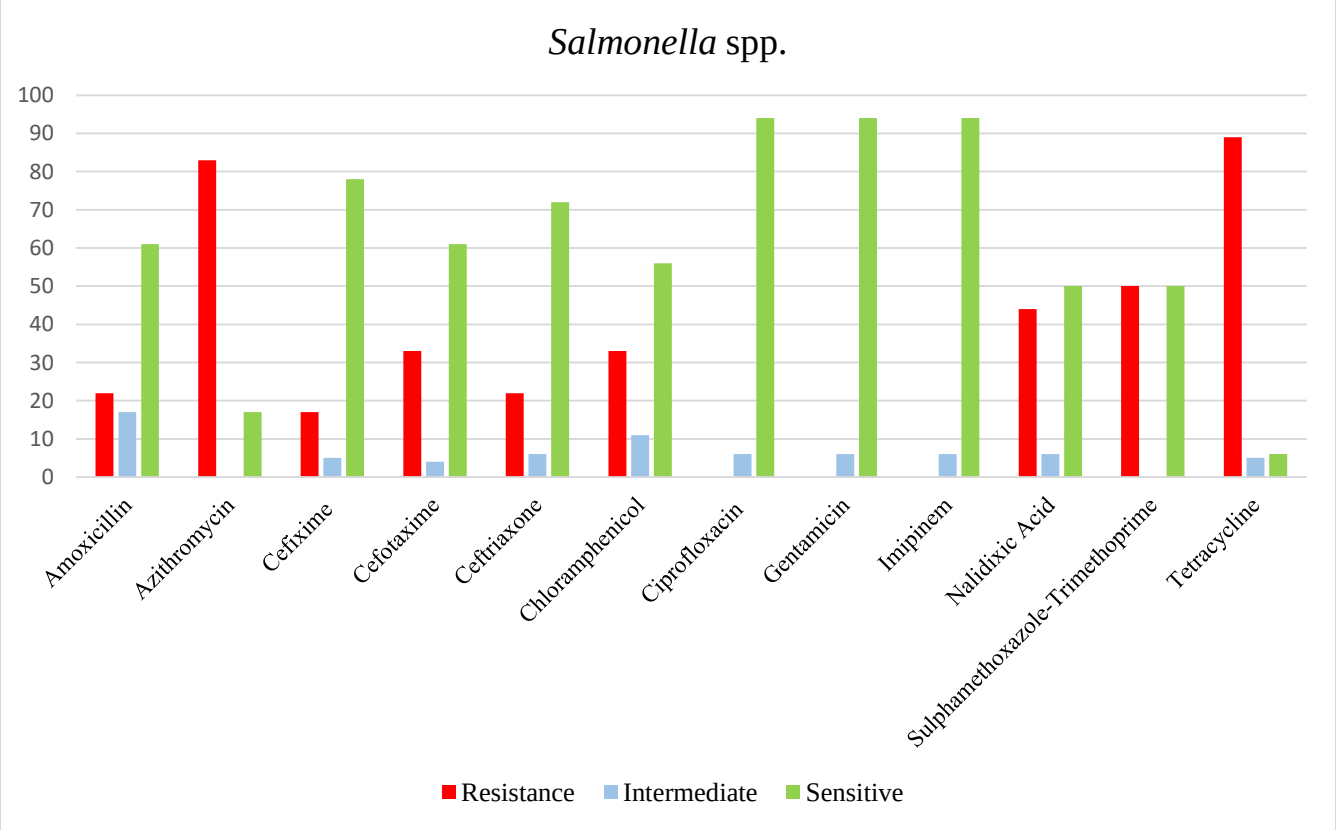
Variables	**Categories**	**AMR of *Salmonella* spp.**			**Multiple logistic regression**		
		n (%)	**95% CI**	***P* (χ^2-test)**	**OR**	**95% CI**	***P***
District	Dhaka (206)	15 (7.3)	4.1-11.7	0.05			
	Gazipur (95)	1 (1.1)	0.03-5.7				
	Madaripur (45)	1 (2.2)	0.06-11.8				
	Narshingdi (53)	1 (1.9)	0.05-10.1				
Habitat	Rural (149)	2 (1.3)	0.16-4.7	0.03	1		
	Urban (140)	11 (7.9)	3.9-13.6		4.4	1-21.3	0.06
	Peri-urban (110)	5 (4.6)	1.5-10.3		6.6	1-46.4	0.05
Seasons	Winter (242)	7 (2.9)	1.2-5.9	0.05	1		
	Summer (157)	11 (7.1)	3.6-12.2		3.1	0.6-15.3	0.16
Age	Juvenile (105)	2 (1.9)	0.2-6.7	0.06	1		
	Sub-adult (113)	3 (2.7)	0.6-7.6		1.2	0.2-7.7	0.81
	Adult (181)	13 (7.2)	3.9-11.9		3.9	1-17.8	0.08
Sex	Male (174)	7 (4.1)	1.6-8.1	0.60			
	Female (225)	11 (4.9)	2.5-8.6				

4.2.3 Antimicrobial resistance pattern of *Salmonella* spp.

To observe the antimicrobial resistance pattern, cultural sensitivity test was done against 12 different antimicrobials. Tetracycline (89%) followed by Azithromycin (83%) were highly resistant and Cefixime (17%) showed the lowest resistance against *Salmonella* spp. in macaque within the study areas. On the other hand, Ciprofloxacin, Gentamicin and Imipinem didn't show any resistance.

In case of the sensitivity of antimicrobials, Ciprofloxacin (94%), Gentamicin (94%), Imipinem (94%) and Cefixime (78%) were the highest sensitive among all drugs, whereas tetracycline was the lowest sensitive just 6% against *Salmonella* spp. Combination of Sulphamethoxazole and Trimethoprim and Nalidixic acid showed the equal sensitivity and resistance amongst all drugs **(Figure 4).**

Figure 2: Antimicrobial resistance pattern of *Salmonella* spp.



4.3 AMR of *Staphylococcus* spp.

4.3.1 Location wise prevalence of resistant *Staphylococcus* spp.

The prevalence of *Staphylococcus* spp. in macaque was the highest in Madaripur and the percentage was 20.5% with 95% CI (9.8-35.3). In Dhamrai, the prevalence was the lowest and the percentage was 10.6% with 95% CI (4.4-20.6). The prevalence of *Staphylococcus* in Narshingdi, Rajendrapur and Bormi were 19.2%, 13.9% and 13.7%, respectively. 15.7% prevalence with 95% CI (8.1-26.4) was observed in both Gendaria and Rothkhola **(Table 4.6).**

Table 4.6: Prevalence of resistant *Staphylococcus* spp. by location

Name of the location	***Staphylococcus* spp. (n)**	**Total sample (N)**	**Prevalence (%)**	**95% CI**
Gendaria, Old Dhaka	11	70	15.7%	8.1-26.4
Rothkhola, Old Dhaka	11	70	15.7%	8.1-26.4
Dhamrai, Dhaka	7	66	10.6%	4.4-20.6
Bormi, Gazipur	7	51	13.7%	5.7-26.3
Rajendrapur, Gazipur	6	43	13.9%	5.3-27.9
Madaripur	9	44	20.5%	9.8-35.3
Narshingdi	10	52	19.2%	9.6-32.5

4.3.2 Univariate and multivariate association between AMR of *Staphylococcus* spp. and selected variables

In districts wise prevalence there was no significant difference but the prevalence in Gazipur was higher (20%). Within 3 habitats, AMR of *Staphylococcus* spp. was significantly high in the peri-urban area (23.6%, 95% CI: 16.1-32.7, *P*=0.01) than that of urban and rural areas. *Staphylococcus* spp. was most prevalent in winter season (19.1%

with CI: 14.3-24.5, *P*=0.01) sample in winter was positive which was higher than that of summer (9.6% with 05.4-15.3). There was no significant difference in age and sex but a higher percentage of positive in sub-adult (21.2%) than others and in male (16.1%)

Having adjusted the factors (district, habitat, season and age) with each other through the Logistic regression Model, the odds of antimicrobial resistance of *Staphylococcus* spp. was significantly higher in Dhaka (OR=10.8, CI: 1.1-104.1, *P*=0.03), Gazipur (OR=9.1, CI: 1.1-71.2, *P*=0.03) and Madaripur (OR=8.3, CI: 1.1-67.4, *P*=0.04). In case of habitat, peri-urban area (OR=5.6, CI: 1.2-25.9, *P*=0.02) was significantly higher on AMR of *Staphylococcus* spp. than the urban area. Sub-adult and juvenile macaque were 2.1 and 1.2 times respectively higher infected with resistant *Staphylococcus* spp. than adult macaque. **(Table 4.7)**

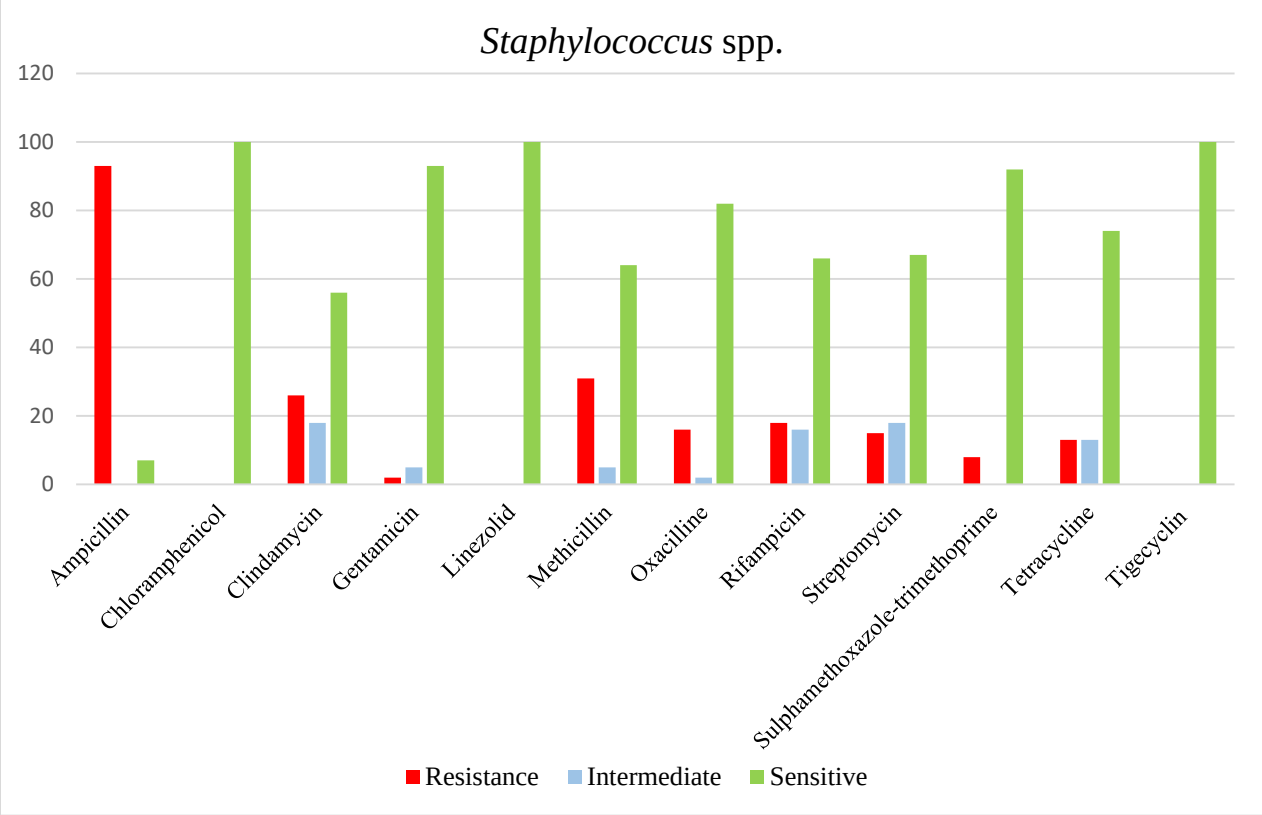
Table 4.7: Frequency distribution of AMR of *Staphylococcus* spp. in rhesus macaque of Bangladesh

Variables	**Categories**	**AMR of *Staphylococcus* spp.**		**Multiple logistic regression**			
		n (%)	**95% CI**	***P*** **(χ^2-test)**	**OR**	**95% CI**	***P***
District	Narshingdi (53)	3 (5.7%)	01.2-15.7	0.13	1		
	Madaripur (45)	8 (17.8%)	08-32.1		8.3	1.1-67.4	**0.04**
	Gazipur (95)	19 (20%)	12.5-29.4		9.1	1.1-71.2	**0.03**
	Dhaka (206)	31 (15.1%)	10.5-20.7		10.8	1.1-104.1	**0.03**
Habitat	Urban (140)	14 (10%)	05.6-16.2	0.01	1		
	Rural (149)	21 (14.1%)	08.9-20.7		4.9	0.7-31.5	0.08
	Peri-urban (110)	26 (23.6%)	16.1-32.7		5.6	1.2-25.9	**0.02**
Seasons	Summer (157)	15 (9.6%)	05.4-15.3	0.01	1		
	Winter (242)	46 (19.1%)	14.3-24.5		0.4	0.09-2.1	0.33
Age	Adult (181)	21 (11.6%)	07.3-17.2	0.08	1		
	Juvenile (105)	16 (15.2%)	08.9-23.6		1.2	0.6-2.5	0.55
	Sub-adult (113)	24 (21.2%)	14.1-29.9		2.1	1.1-4.1	**0.03**
Sex	Male (174)	28 (16.1%)	10.9-22.4	0.69			
	Female (225)	33 (14.7%)	10.3-19.9				

4.3.3 Antimicrobial resistance pattern of *Staphylococcus* spp.

Chloramphenicol, Linezolid and Tetracycline were the highest sensitive (100% of each) among all drugs, whereas Ampicillin was the lowest sensitive (7%) against *Staphylococcus* spp. Only Ampicillin showed the highest resistance (93%) amongst all drugs, whereas Gentamicin showed the lowest resistance (2%). Methicillin showed moderate resistance against *Staphylococcus* spp. 31% followed by Clindamycin (26%), Rifampicin (18%), Oxacillin (16%), Streptomycin (15%), Tetracycline (13%) and Sulphamethoxazole-trimethoprim (8%). **(Figure 5**).

Figure 3: Antimicrobial resistance pattern of *Staphylococcus* spp.



4.4 AMR of *Enterococcus* spp

The prevalence of *Enterococcus faecalis* was the highest and the prevalence was 35.8% with 95% CI (26.8-45.5) and the prevalence of *Enterococcus faecium* was 33% with 95% CI (24.3-42.7). On the other hand, both species positive was the lowest and the prevalence was 8.3% with CI (3.8-15.1). (**Table 4.8).**

Table 4.8: Species wise prevalence of resistant *Enterococcus* spp.

Resistant *Enterococcus* spp.	**n (N)**	**Prevalenve**	**95% CI**
Enterococcus faecalis	39 (109)	35.8%	26.8-45.5
Enterococcus faecium	36 (109)	33%	24.3-42.7
Both	9 (109)	8.3%	3.8-15.1

4.4.1 Location wise prevalence of resistant *Enterococcus* spp.

The prevalence of resistant *Enterococcus* spp. in macaque was the highest in Gendaria, Old Dhaka and the percentage was 73.5% with 95% CI (55.6-87.1). In Narshingdi, the prevalence was the lowest and the percentage was 33.3% with 95% CI (11.8-61.6). The prevalence of resistant *Staphylococcus* in Rothkhola, Bormi and Rajendrapur were 66.7%, 46.7% and 60%, respectively (**Table 4.9).**

Table 4.9: Prevalence of resistant *Enterococcus* spp. by location

Name of the location	***Enterococcus* spp. (n)**	**Total sample (N)**	**Prevalence (%)**	**95% CI**
Gendaria, Old Dhaka	25	34	73.5%	55.6-87.1
Rothkhola, Old Dhaka	20	30	66.7%	47.2-82.7
Bormi, Gazipur,	7	15	46.7%	21.3-73.4
Rajendrapur, Gazipur	9	15	60%	32.3-83.7
Narshingdi	5	15	33.3%	11.8-61.6

4.4.2 Univariate and multivariate association between AMR of *Enterococcus* spp. and selected variables

There is a significant difference in the prevalence AMR of *Enterococcus* spp. among districts, the macaque of Dhaka (70.3%; 95% CI: 57.6-81.1) was significantly high prevalent to *Enterococcus* spp. than other districts. The prevalence of *Enterococcus* spp. was significantly higher (P=0.02) in urban (70.3%) macaques than other habitats. In case

of age, adult macaque was significantly higher in adult (76.1%; 95% CI: 61.2-87.4) than sub-adult and juvenile. Although there was no significant difference but male was highly prevalent (66.7%). (**Table 4.10)**

The significant variables from univariate analysis (district, habitat, age and sex) were transferred to the logistic regression model. The factor habitat was omitted because of collinearity. The prevalence of AMR of *Enterococcus* spp. was 6.4 times higher in Dhaka district and 3.5 times higher in Gazipur district than the Narshingdi. The odds of AMR of *Enterococcus* spp. was significantly higher in adult (OR=3.8, CI: 1.2-11.8, *P*=0.01) than the juvenile macaque. **(Table 4.10)**

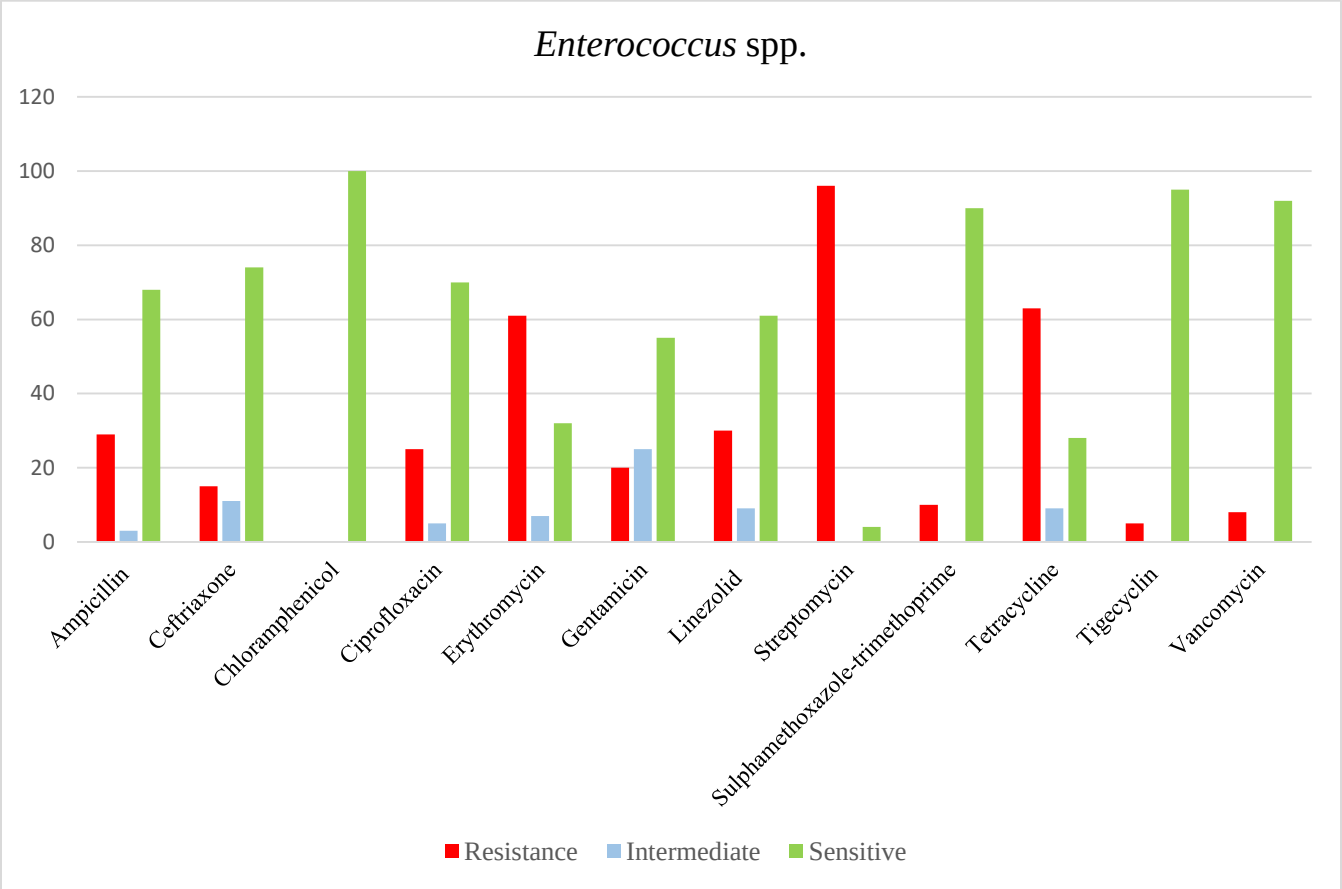
Table 4.10: Frequency distribution of AMR of *Enterococcus* spp. in rhesus macaque of Bangladesh

Variables	**Categories**	**AMR of *Enterococcus* spp.**			**Multiple logistic regression**		
		n (%)	**95% CI**	***P* (χ^2-test)**	**OR**	**95% CI**	***P***
District	Narshingdi (15)	5 (33.3%)	11.8-61.6	0.02	1		
	Gazipur (30)	16 (53.3%)	34.3-71.7		3.5	0.8-14.8	**0.07**
	Dhaka (64)	45 (70.3%)	57.6-81.1		6.4	1.7-23.6	**0.005**
Habitat	Urban (64)	45 (70.3%)	57.6-81.1	0.02			
	Peri-urban (15)	9 (60%)	32.3-83.7				
	Rural (30)	12 (40%)	22.7-59.4				
Seasons	Summer (79)	50 (63.3%)	51.7-73.8	0.34			
	Winter (30)	16 (53.3%)	34.3-71.7				
Age	Juvenile (26)	13 (50%)	29.9-70.1	0.01	1		
	Sub-adult (37)	18 (48.7%)	31.9-65.6		0.9	0.3-2.6	0.85
	Adult (46)	35 (76.1%)	61.2-87.4		3.8	1.2-11.8	**0.01**
Sex	Male (45)	30 (66.7%)	51.1-80	0.27	1		
	Female (64)	36 (56.3%)	43.3-68.6		0.5	0.2-1.4	0.22

4.4.3 Antimicrobial resistance pattern of *Enterococcus* spp.

AMR pattern of *Enterococcus* spp. showed higher resistance in streptomycin (96%), followed by tetracycline (63%), erythromycin (61%), linezolid (30%) and ampicillin (29%). In opposite case, Chloramphenicol (100%), Tigecyclin (95%), Vancomycin (92%), Sulphamethoxazole-trimethoprim (90%), ceftriaxone (74%), ciprofloxacin (70%) showed higher sensitivity. (**Figure 6**)

Figure 4: Antimicrobial resistance pattern of *Enterococcus* spp.



4.5 Inter-species interaction in different locations

Total 8 hours observation was done in Gendaria, Rothkhola and Dhamrai area, on the other hand, four hours was done each in other places. Both direct and indirect human-macaque interaction observed higher than the other inter-species interaction. In Gendaria, Old Dhaka direct Human-Macaque contact was higher (40 times) and indirect was 204 times in 2 days total in 8 hours. In Narshingdi, Human-Macaque contact was the lowest; direct (8 times per 4 hours) and indirect (38 times per 4 hours). There was no direct contact of goat-macaque and cattle-macaque in Gendaria and Rothkhola in Dhaka and no direct and indirect contact of goat, cattle and dog with macaque in Rajendropur **(Table 4.11)**

Table 4.11: Location wise inter-species interaction

Locations	**Direct Contact (times)**				**Indirect Contact (times)**			
	Human-Macaque	Goat-Macaque	Cattle-Macaque	Dog-Macaque	Human-Macaque	Goat-Macaque	Cattle-Macaque	Dog-Macaque
Gendaria, Old Dhaka (8 hrs)	40 (34%)	0	0	7 (14%)	204 (29%)	0	0	27 (20%)
Rothkhola (8 hrs)	13 (11%)	0	0	3 (6%)	157 (23%)	9 (8%)	0	14 (11%)
Dhamrai (8 hrs)	27 (23%)	9 (28%)	3 (23%)	22 (43%)	109 (16%)	35 (31%)	13 (21%)	43 (33%)
Bormi (4 hrs)	9 (7%)	7 (22%)	3 (23%)	11 (21%)	54 (8%)	25 (23%)	19 (31%)	25 (19%)
Rajendropur (4 hrs)	9 (7%)	0	0	0	52 (7%)	0	0	0
Madaripur (4 hrs)	13 (11%)	13 (41%)	5 (39%)	5 (10%)	82 (12%)	25 (23%)	23 (37%)	17 (13%)
Narshingdi (4 hrs)	8 (7%)	3 (9%)	2 (15%)	3 (6%)	38 (5%)	17 (15%)	7 (11%)	6 (4%)
Total	119 (100%)	32 (100%)	13 (100%)	51 (100%)	696 (100%)	111 (100%)	62 (100%)	132 (100%)

4.5.1 Contact rate per hour

In urban habitat, contact rate of a human with macaque was high; 3 direct contacts per hour (hr) and 22 indirect contacts per hour (hr). In rural habitat, Goat and cattle interaction with macaque was higher than another habitat, 4 times/hr (direct) and 5 times/hr (indirect) for goat and 1 time/hr (direct) and 4 times/hr (indirect) for cattle. In case of the dog, peri-urban was high, direct contact 2times/hr and indirect contact 4 times/hr. **(Table 4.12)**

Table 4.12: Direct and Indirect contact rate per hour

Habitat	Inter-species interaction	Direct contact/hour	Indirect contact/hour
Urban	Human-Macaque	3	22
	Goat-Macaque	0	1
	Cattle-Macaque	0	0
	Dog-Macaque	1	3
Peri-urban	Human-Macaque	3	13
	Goat-Macaque	1	4
	Cattle-Macaque	1	2
	Dog-Macaque	2	4
Rural	Human-Macaque	1	15
	Goat-Macaque	4	5
	Cattle-Macaque	1	4
	Dog-Macaque	1	3

Chapter-5: Discussion

Antimicrobial resistance (AMR) in livestock and wildlife is an emerging public health threat in the world, especially in Bangladesh. Rhesus monkey lives in a thriving ecosystem with human and livestock, hance resistant microorganisms can easily transmitted to them. Although it is a serious health concern for rhesus monkey as well as wildlife but unfortunately there are no studies related to AMR in rhesus macaque and few studies have been performed on antimicrobial resistance in wild species in this country. Antimicrobials are widely used in livestock and poultry for treatment and growth promotion and ultimately the resistant organisms spread to the free-ranging rhesus macaque, who are normally habitat adjacent to in urban, peri-urban and rural ecosystems. Therefore, the present study was conducted on rhesus macaque in urban, peri-urban and rural habitat to estimate the status of eco-epidemiology of antimicrobial resistance.

In this study, rhesus macaque was found most commonly around human habitat in the district of Dhaka division which support the previous study of Hasan et al. (2013), where presented largest group of macaque at Charmuguria in the Madaripur district, small group at Rampur in the Nashingdi district and moderate number of macaque at Gendaria, Rothkhola, Dhamrai in Dhaka and at Bormi in Gazipur.

The present study represented the higher amount of female macaques than male in a group which is also supported Hasan et al., (2013) because female macaques are philopatric, they remain in the same group throughout their life but the male may leave their natal group when they become mature. On the other hand, adult macaques were found higher than the immature due to close contact with the provision of local inhabitants and visitors.

Rhesus macaque treated as a commensal in most Asian countries as well as Bangladesh due to their conflicting behaviors. Conflicts between humans or livestock and non-human primates are very common, including damage to crops and property, habitat destruction, injuries and death of people and wildlife, and livestock depredation. This study found that rhesus macaque lives very close to the human habitat and regular conflict happened which lead the transmission of resistant organisms. Study corresponds to the previous findings of Ahsan and Uddin, (2014) where many people have been scratched and injured in Bormi

(Gazipur), Dhamrai (Dhaka), Charmuguria (Madaripur), Monohardi (Narsingdi) Chandpur and Chashnipeer-er-Mazar.

5.1 Prevalence of microorganisms in rhesus macaque

Among three microorganisms, the prevalence of *Salmonella* spp. was lower 5% in rhesus macaque in Bangladesh which corresponds to the study conducted in University of California (Good et al., 1969). *Staphylococcus* spp. was 16% which is not supported by the previous study occurred in Africa where 61% prevalence found in non-human primates (Schaumburg et al., 2012). On the other hand, rhesus macaque was highly prevalent (64%) to *Enterococcus* spp., the somewhat similar result observed in captive baboon in Kenya (Mwova, 2016)

5.2 Prevalence of antimicrobial resistance of *Salmonella* spp. and resistant pattern in rhesus macaque

The overall prevalence of antimicrobial resistance of *Salmonella* spp. was quite low; 5% which corresponds a longitudinal study in captive wildlife including non-human primates in Thailand, got 7% *Salmonella* serotypes (Gopee et al., 2000), 13% *Salmonella* in free ranged mountain gorillas of the Bwindi and Mgahinga in Uganda (Nizeyi et al., 2001) and 3% in national center for primate biology, University of California, Davis (Good et al., 1969). On the other hand, the present study does not correspond to the findings of previous studies of Nigeria. In a study of captive non-human primates in Nigeria found *Salmonella paratyphi* A (93.9%) (Okwori et al., 2014).

Among all the resistant antimicrobials analyzed tetracycline had the highest prevalence which is supported by the previous study where tetracycline was 95% resistant in direct shipment but found less amount of tetracycline (29%) (Gopee et al., 2000) and 38.3% (Kim et al., 2017a). This may be due to the higher amount of tetracycline used more frequently in livestock for treatment and residue transmission to other species.

For *Salmonella* another higher resistant antibiotics were Azithromycin (83%) which is a dangerous result not similar to other studies, a study was done about Azithromycin resistance in *Shigella* spp. in Southeast Asia and found 37.8% (Darton et al., 2018). But the parallel result found in Bangladeshi study, 95% resistant to Azithromycin (Lina et al.,

2007) due to commonly used in human even in small duration fever and common cold thus the resistant organisms may transmit environmental wastage.

Sulfamethoxazole-Trimethoprim is an essential drug and widely used, (50%) resistant to *Salmonella* spp. in rhesus macaque. This result is somewhat less than the previous study of AMR in *Salmonella enterica* Serovar Typhi Isolates from Bangladesh and result the was Sulfamethoxazole 68.4% and Trimethoprim 57.9% (Chiou et al., 2014). Nalidixic acid (44%) was less prevalent in the tested samples than tetracycline, azithromycin, and sulfamethoxazole-Trimethoprim but dissimilar (i.e., lower) to the prevalence of 81.6% (Chiou et al., 2014) and (72.73%) (Suman et al., 2017), (92.5%) (Afroj et al., 2012) isolates were resistant reported in Bangladesh.

On the other hand, *Salmonella* spp. was highly sensitive to Ciprofloxacin, Gentamicin, Imipenem and the study supported for Ciprofloxacin (99.5%) (Mijović, 2012) Gentamicin (100%) (Hassan et al., 2014), Imipenem (100%) (Singh and Cariappa, 2016). Along with higher sensitive antibiotics, there had some moderate level of sensitive antimicrobials, which may be due to organisms with lower sensitivity in pond, river and tap waters in Bangladesh (K.H.M.N.H. Nazir et al., 2005).

5.3 Prevalence of antimicrobial resistance of *Staphylococcus* spp. and resistant pattern in rhesus macaque

The prevalence of antimicrobial resistance in *Staphylococcus* spp. was quite high than the *Salmonella* spp. in the present study (15%) which does not corresponds to the findings of a published study (80%) in Spain (Woods et al., 2017), 39% in Netherland (Van Den Berg et al., 2011), 23.6% in Wisconsin, USA (Kokan and Bergdoll, 1987).

Among all the antimicrobials, ampicillin was high resistance (93%) to *Staphylococcus* spp. in rhesus monkey but in the previous study about the divergent *Staphylococcus aureus* isolates from African non-human primates isolated only 2.9% resistance to penicillin (Schaumburg et al., 2012). On the other hand in Bangladesh perspective, 92-100% resistance of ampicillin in *Shigella* isolated from urban Dhaka and rural Matlab (Hossain et al., 1998) and a similar proportion of resistance of ampicillin found in the present study.

The higher amount of ampicillin resistance is due to overuse of antibiotics and transmission of resistance gene.

Most of the antimicrobials were sensitive to *Staphylococcus* spp. in macaque and chloramphenicol, gentamycin, linezolid, oxacillin, Tigecycline and Sulphamethoxazole-trimethoprim were highly sensitive, corresponded the findings of greater than 90% of multidrug resistance were sensitive to trimethoprim-sulfamethoxazole, linezolid, and vancomycin (Styers et al., 2006). Taylor and Grady (1998) reported dissimilar result with 75% resistant to methicillin/oxacillin in vitro. Higher sensitivity was due to only direct or indirect contact with infected species and less transmission with food and water. Another reason may be not getting frequent exposure to chloramphenicol, clindamycin, linezolid, Oxacillin etc. by the human and animals.

5.4 Prevalence of antimicrobial resistance of *Enterococcus* spp. and resistant pattern in rhesus macaque

Enterococcus spp. in rhesus macaque in Bangladesh found 61% from the fecal samples and the similar result found in Nairobi, Kenya, 59.3% *Enterococcus* spp. was isolated from the feces of captive healthy baboons (*Papio anubis*) (Mwova, 2016). In case of species level, the prevalence of *E. faecalis* and *E. faecium* was respectively 35.8% and 33% which is not supported the study occurred recently in Spain where *E. faecalis* was 60% with in rhesus macaque (Woods et al., 2017)

The present study showed that resistance of streptomycin, erythromycin, tetracycline, ampicillin and linezolid of *Enterococcus* spp. in the rhesus monkey, similar types of results found in previous studies but in the lower level of resistance (Rolland et al., 1985; Cristóbal-Azkarate et al., 2014; Mwova, 2016). The higher resistance of antimicrobials in this country due to indiscriminate use of antibiotics in medical, veterinary and agricultural practices and discharges of residue in a various amount in the environment. *Enterococcus* spp. showed sensitivity in most of the antimicrobials such as vancomycin, ceftriaxone, ciprofloxacin, chloramphenicol, Sulphamethoxazole-trimethoprim which corresponds earlier study of Xavier et al. (2010) and Mwova (2016). This may be happened because of less exposure to those antibiotics.

5.5 Risk factors associated with the prevalence

The odds of occurrence of AMR of *Salmonella* spp. in the peri-urban and urban area were 6.6 and 4.4 times respectively higher than the rural area that supports the study of Rolland et al. (1985) due to feeding on human garbage available in urban and near-urban habitat in contact with another form of human detritus where present greater levels of resistant gut bacteria. Adult macaques were 3.9 times more resistance to *Salmonella* and 3.8 times more to *Enterococcus* than the juvenile. The reason behind that, adult is more active than the juvenile, collected their own food and also for the child. Usually, direct contact happens between livestock, dog, and human during the conflict, damage to crops and time of food provided by the human (Ahsan and Uddin, 2014).

In case of locations, the odds of the existence of AMR of *Salmonella* spp. and *Enterococcus* spp. was higher in Dhaka district, OR respectively 10.8 and 6.4. There is available related literature to support the results (Talukdar et al., 2013). This variation may be attributed to the huge population density of Dhaka city and improper management of wastage disposal, household drinking water supply with diversified microorganisms and the chance of getting resistance organisms easily.

The prevalence of *Salmonella* spp. and *Enterococcus* spp. was higher in summer seasons which may be related with the more time spending in summer and monsoon season for feeding, grooming and object manipulation/play (Jaman and Huffman, 2013).

AMR of *Staphylococcus* spp. was higher in rural and peri-urban area than the urban habitat which may be due to direct transfer of resistant bacteria or resistance genes through consumption of contaminated water (Sayah et al., 2005)

5.6 Inter-species interactions

Both direct and indirect per hour contact rate between human and macaque in urban and peri-urban was higher which may one of the important risk factors for transmitting resistance organisms human to macaque and vice versa. Result support the previous study of Hasan et al. (2013), where presented the distribution of macaque in densely populated urban and some temples and shrines are particularly occupied and people provided foods very frequently. In case of livestock-macaque interactions, contact rate in the rural area

was higher due to the presence of huge number of livestock availability and transmission of resistant organisms because, most of the livestock are resistant to available antibiotics due to improper use of antimicrobials (Roess et al., 2013).

Rhesus monkey tried to snatch food and cloth, damaged crops, so people used a Patrolling dog for minimizing the monkey menace (Imam and Ahmad, 2013)**,** similar results found in this study. Direct and indirect contact of a macaque with dogs occurred higher in the peri-urban area for patrolling.

Human-to-monkey transmission of microorganisms appears to have taken place around three decades ago. This appears to be the result of human encroachment into the natural habitat of monkey, started to live nearby to the human habitat and probably resulted from the transfer of human bacteria from hands to food that was then fed to monkeys.

5.7 Limitations of the present study

Small sample size for *Enterococcus* spp.: Due to the time and budget limitation, only 109 sample was tested for *Enterococcus* spp. in the laboratory.

Diagnostic techniques: Disc diffusion method is not as sensitive as other detection methods such as minimum inhibitory concentration.

Species identification: In case of *Salmonella* and *Staphylococcus* species identification was not done due to time and budget limitation.

Virulent gene analysis: Virulent gene analysis was not done in this study.

Chapter-6: Conclusion

Antimicrobial resistance is a global concern and the public health threat due to inappropriate use of antimicrobials. By the side of human and livestock, wildlife is most prevalent in resistant organisms. In rhesus macaque, resistant organisms; *Salmonella* spp., *Staphylococcus* spp. and *Enterococcus* spp. was respectively 5%, 15% and 61%. Both *Salmonella* spp. and *Staphylococcus* spp. was higher in peri-urban habitat but *Enterococcus* spp. was higher in the urban habitat. AMR among adult macaques was higher than the sub-adult and juvenile. Tetracycline (89%) and Azithromycin (83%) were highly resistant to *Salmonella* spp. Ampicillin (93%) was highly resistant among all the antimicrobials in case of *Staphylococcus* spp. On the other hand, streptomycin (96%) was the most prevalent resistant antimicrobial to the *Enterococcus* spp. Interaction of human and livestock with the macaque influenced the resistance of different microorganisms. In urban habitat, human-macaque and in peri-urban and rural area livestock-macaque interaction was higher. Although, rhesus macaque doesn't take antimicrobials directly but this study found the alarming level of resistant organisms with commonly used resistant microorganisms. So, necessary steps should be taken to control the inappropritate use of antimicrobials.

Chapter-7: Recommendations

Human and livestock interaction with the macaque prone to increase the resistance of different antimicrobials, so necessary steps should be taken to reduce the interaction with the macaques. Water and food in the environment are contaminating with various resistant microorganisms due to improper management of waste disposal. So, proper waste management is needed to solve this antimicrobial resistance problem in rhesus macaque. Improper utilization of antimicrobials should be stopped. In terms of treatment both human and animals, antimicrobial susceptibility testing should be done to select proper drugs. Everyone should follow proper withdrawal period and drugs prescribed by registered doctor and veterinarian. Further research should be done to know the source of resistance pattern and identify the risk factors. Public awareness should be increased regarding antimicrobial resistance.

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Appendix I: Univariate and multivariate association between microorganisms and selected variables

Frequency distribution of *Salmonella* spp. in rhesus macaque of Bangladesh

Variables	**Categories**	***Salmonella* spp.**			**Multiple logistic regression**		
		n (%)	**95% CI**	***P* (χ^2-test)**	**OR**	**95% CI**	***P***
District	Dhaka (206)	15 (7.3)	4.1-11.7	0.05			
	Gazipur (95)	1 (1.1)	0.03-5.7				
	Madaripur (45)	1 (2.2)	0.06-11.8				
	Narshingdi (53)	1 (1.9)	0.05-10.1				
Habitat	Rural (149)	2 (1.3)	0.16-4.7	0.03	1		
	Urban (140)	11 (7.9)	3.9-13.6		4.4	0.9-21.3	0.06
	Peri-urban (110)	5 (4.6)	1.5-10.3		6.6	0.9-46.4	0.05
Seasons	Winter (242)	7 (2.9)	1.2-5.9	0.05	1		
	Summer (157)	11 (7.1)	3.6-12.2		3.1	0.6-15.3	0.16
Age	Juvenile (105)	2 (1.9)	0.2-6.7	0.06	1		
	Sub-adult (113)	3 (2.7)	0.6-7.6		1.2	0.2-7.7	0.81
	Adult (181)	13 (7.2)	3.9-11.9		3.9	0.8-17.8	0.08
Sex	Male (174)	7 (4.1)	1.6-8.1	0.60			
	Female (225)	11 (4.9)	2.5-8.6				

Frequency distribution of *Staphylococcus* spp. in rhesus macaque of Bangladesh

Variables	**Categories**	***Staphylococcus* spp.**			**Multiple logistic regression**		
		n (%)	**95% CI**	***P* (χ^2-test)**	**OR**	**95% CI**	***P***
District	Narshingdi (53)	3 (5.7%)	01.2-15.7	0.11	1		
	Madaripur (45)	9 (20%)	9.6-34.6		7.2	1.1-49.5	**0.04**
	Gazipur (95)	19 (20%)	12.5-29.4		6.7	0.9-45.1	**0.05**
	Dhaka (206)	33 (16.1%)	11.3-21.7		8.4	01-71.1	**0.05**
Habitat	Urban (140)	16 (11.4%)	6.7-17.9	0.03	1		
	Rural (149)	22 (14.7%)	9.5-21.5		3.6	0.6-19.2	0.13

	Peri-urban (110)	26 (23.6%)	16.1-32.7		4	1.1-15	**0.04**
Seasons	Summer (157)	16 (10.2%)	05.9-16.1	0.01	1		
	Winter (242)	48 (19.8%)	15-25.4		0.6	0.1-2.4	0.49
Age	Adult (181)	22 (12.2%)	7.8-17.8	0.05	1		
	Juvenile (105)	16 (15.2%)	8.9-23.5		1.2	0.6-2.5	0.56
	Sub-adult (113)	26 (23%)	15.6-31.9		2.12	1.2-4.1	**0.01**
Sex	Male (174)	30 (17.3%)	11.9-23.7	0.56	1		
	Female (225)	34 (15.1%)	10.7-20.5		0.7	0.4-1.3	0.31

Frequency distribution of *Enterococcus* spp. in rhesus macaque of Bangladesh

Variables	**Categories**	***Enterococcus* spp.**			**Multiple logistic regression**		
		n (%)	**95% CI**	***P*** **(χ^2-test)**	**OR**	**95% CI**	***P***
District	Narshingdi (15)	5 (33.3%)	11.8-61.6	0.11	1		
	Gazipur (30)	17 (56.7%)	37.4-74.5		2.2	0.5-8.4	0.25
	Dhaka (64)	46 (71.8%)	57.6-81.1		3.5	1.1-12.1	0.04
Habitat	Urban (64)	46 (71.8%)	59.2-82.4	0.11			
	Peri-urban (15)	9 (60%)	32.3-83.7				
	Rural (30)	15 (50%)	31.3-68.7				
Seasons	Summer (79)	53 (67.1%)	55.6-77.2	0.31			
	Winter (30)	17 (56.7%)	37.4-74.5				
Age	Juvenile (26)	13 (50%)	29.9-70	0.01			
	Sub-adult (37)	21 (56.7%)	39.5-72.9		1.2	0.4-3.6	0.64
	Adult (46)	36 (78.2%)	63.6-89.1		3.9	1.3-11.8	0.01
Sex	Male (45)	31 (68.9%)	53.3-81.8	0.27	1.5	0.6-3.5	0.35
	Female (64)	39 (60.9%)	47.9-72.9		1		

Appendix II: Questionnaire for antimicrobial resistance of microorganisms in free ranging rhesus macaque in human, livestock and wildlife interface in Bangladesh

Date of sample collection:

Animal Id No:

Name of the location:

GPS coordinate: Latitude: Longitude:

Age: Adult/ Sub-adult/ Juvenile

Sex: Male/ Female

Season:

BCS:

Fecal consistency: Solid/ semisolid/ liquid

Odour: Yes/ No

Presence of Dustbin/ waste materials: Yes/ no

Water source: Pond/ River/ Lake

Human-Livestock-Macaque interaction:

Morning: (2 hours) (Counting by tally)

Interface	**Direct contact**	**Indirect contact**
Human-Macaque		
Cattle-Macaque		
Goat-Macaque		
Dog-Macaque		

Evening: (2 hours) (Counting by tally)

Interface	**Direct contact**	**Indirect contact**
Human-Macaque		
Cattle-Macaque		
Goat-Macaque		
Dog-Macaque		

Appendix III: Pictorial presentation

Figure 7: Rhesus macaque in human settlement

Figure 8: Human-macaque interaction in human dwelling community

Figure 9: Livestock-macaque interaction

Figure 10: Macaque drinking habit

Figure 11: Fecal sample collection

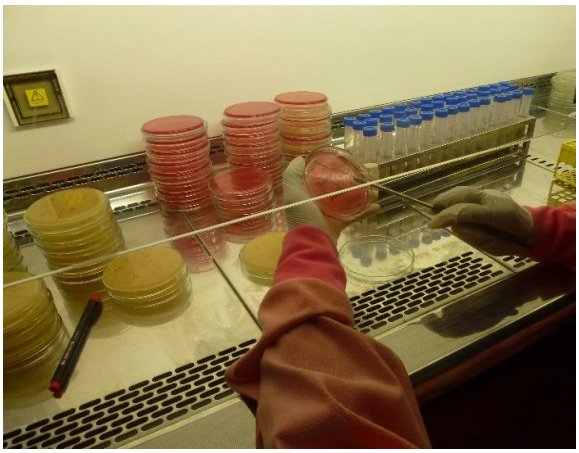

Figure 12: Sample processing in laboratory

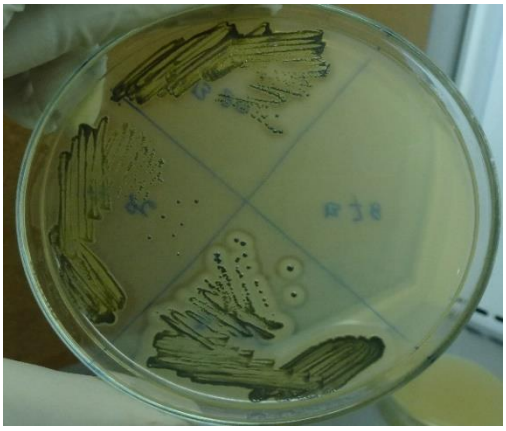

Figure 13: *Salmonella* spp. in XLD agar

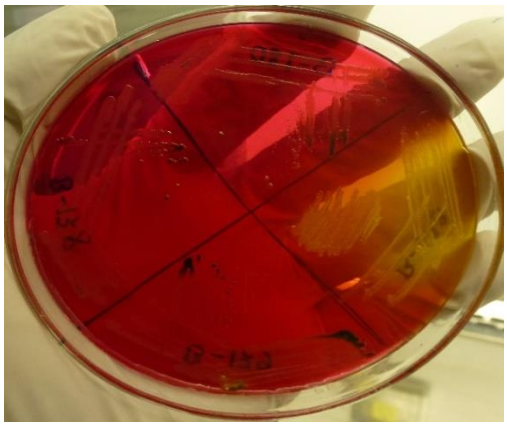

Figure 14: *Staphylococcus* spp. in Blood agar

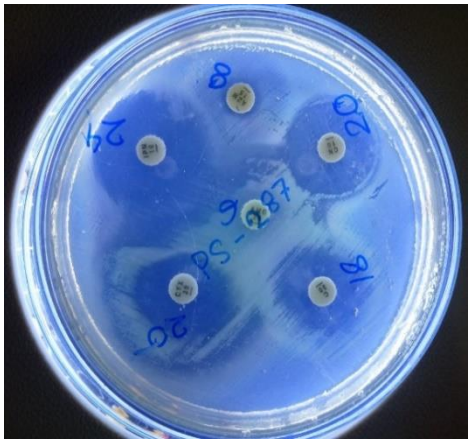

Figure 15: AST by disc diffusion method

Brief biography

Md Kaisar Rahman passed the Secondary School Certificate Examination in 2006 followed by Higher Secondary Certificate Examination in 2008. He obtained his Doctor of Veterinary Medicine Degree in 2014 (held in 2015) from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. Now, he is a candidate for the degree of MS in Epidemiology under the Department of Medicine and Surgery, Faculty of Veterinary Medicine, CVASU. He has immense interest in epidemiological research on wildlife.