

**EPIDEMIOLOGY OF COLISTIN RESISTANCE
IN ENTEROBACTERIACEAE ISOLATES FROM
PERIANAL REGION OF PET DOGS**

Thesis

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**IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF**

**Master of Veterinary Science
(Epidemiology)**

2017



Dedicated to...

My Beloved Family



भा.कृ.अनु.प.-भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)

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Certificate

*This is to be certified that the research work embodied in this thesis entitled **“Epidemiology of Colistin Resistance in Enterobacteriaceae Isolates from Perianal Region of Pet Dogs”** submitted by **Dr. Archana Saraf, Roll No. M-5596**, for the award of **Master of Veterinary Science Degree in Epidemiology** at ICAR-Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.*

*It is further certified that **Dr. Archana Saraf, Roll No. M-5596**, has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the **Master of Veterinary Science Degree** in this Deemed University, as required under the relevant ordinance.*


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ABBREVIATIONS

%	: Percentage
°C	: Degree Celsius
AE	: Elution Buffer
AL	: Lysis Buffer
AMR	: Antimicrobial Resistance
AT	: Aztreonam
ATL	: Tissue Lysis Buffer
B	: Regression coefficient
<i>bla</i> IMP	: Imipenemase
<i>bla</i> KPC	: <i>Klebsiella pneumonia</i> carbapenemase
<i>bla</i> NDM	: New Delhi Metallo-β-lactamase
<i>bla</i> OXA	: Oxacillin hydrolyzing
BLAST	: Basic Local Alignment Search Tool
bp	: Base Pair
BPL	: Biomedical Refrigerator
BPW	: Buffered peptone water
CDT	: Combined Disk Test
CI	: Confidence Intervals
CLSI	: Clinical and Laboratory Standards Institute
CR	: Colistin Resistant
CRE	: Carbapenem Resistant Enterobacteriaceae
DDST	: Double Disk Synergy Test
DNA	: Deoxyribonucleic acid
<i>E. coli</i>	: <i>Escherichia coli</i>
EDTA	: Ethylene diamine tetra acetic acid
EMB	: Eosin Methylene Blue Agar
ESBL	: Extended-Spectrum-β-Lactamase
<i>et al</i>	: <i>et alii</i>
EtBr	: Ethidium Bromide
ETP	: Ertapenem
EUCAST	: European Committee on Antimicrobial Susceptibility Testing
Fig.	: Figure
GNBs	: Gram negative Bacteria's
h	: Hour (s)
H ₂ S	: Hydrogen sulfide
<i>i.e.</i>	: id test (that is)

IMViC	: Indole, Methyl-Red, Voges-Proskauer, Citrate Test
IPM	: Imipenem
IVRI	: Indian Veterinary Research Institute
KH	: Key Hole
KOH	: Potassium Hydroxide
L	: Litre
LB	: Luria-Bertani
MBL	: Metallo- β -Lactamase
<i>Mcr</i>	: Mobilized colistin resistance
MDR	: Multi Drug Resistance
MHA	: Mueller-Hinton agar
MHDR	: Multi Herbal Drug Resistance
MHT	: Modified Hodge Test
MIC	: Minimum Inhibitory Concentration
MIL	: Motility-Indole-Lysine
min	: Minutes
ml	: Milliliter
MLST	: Multilocus Sequence Typing
MOX	: Moxalactam
m-PCR	: Multiplex-PCR
MR	: Methyl Red
MRP	: Meropenem
n	: Number
NDM	: New Delhi metallo- β -lactamase
OIE	: Office international des epizooties
OR	: Odds Ratio
p	: Probability
PCR	: Polymerase Chain Reaction
pH	: Potential Hydrogen Concentration
QIAamp	: DNA Stool Mini Kit
rpm	: Revolution per minute
SAS	: Statistical Analysis System
sec	: Seconds
ST	: Sequence Type
TBE	: Tris borate EDTA buffer
TSA	: Trypticase Soy Agar
TSB	: Tryptose Soya Broth
TSI	: Triple Sugar Iron Agar
UK	: United Kingdom

USA	:	United State of America
UV	:	Ultra Violet
V	:	Volt
<i>viz.</i>	:	Videlicet (namely)
VP	:	Voges-Proskauer
w/v	:	Weight per unit volume
WHO	:	World Health Organization
XDR	:	Extensively Drug Resistant
β	:	Beta
μ	:	Micron
μg	:	Microgram (s)
μL	:	Microliter

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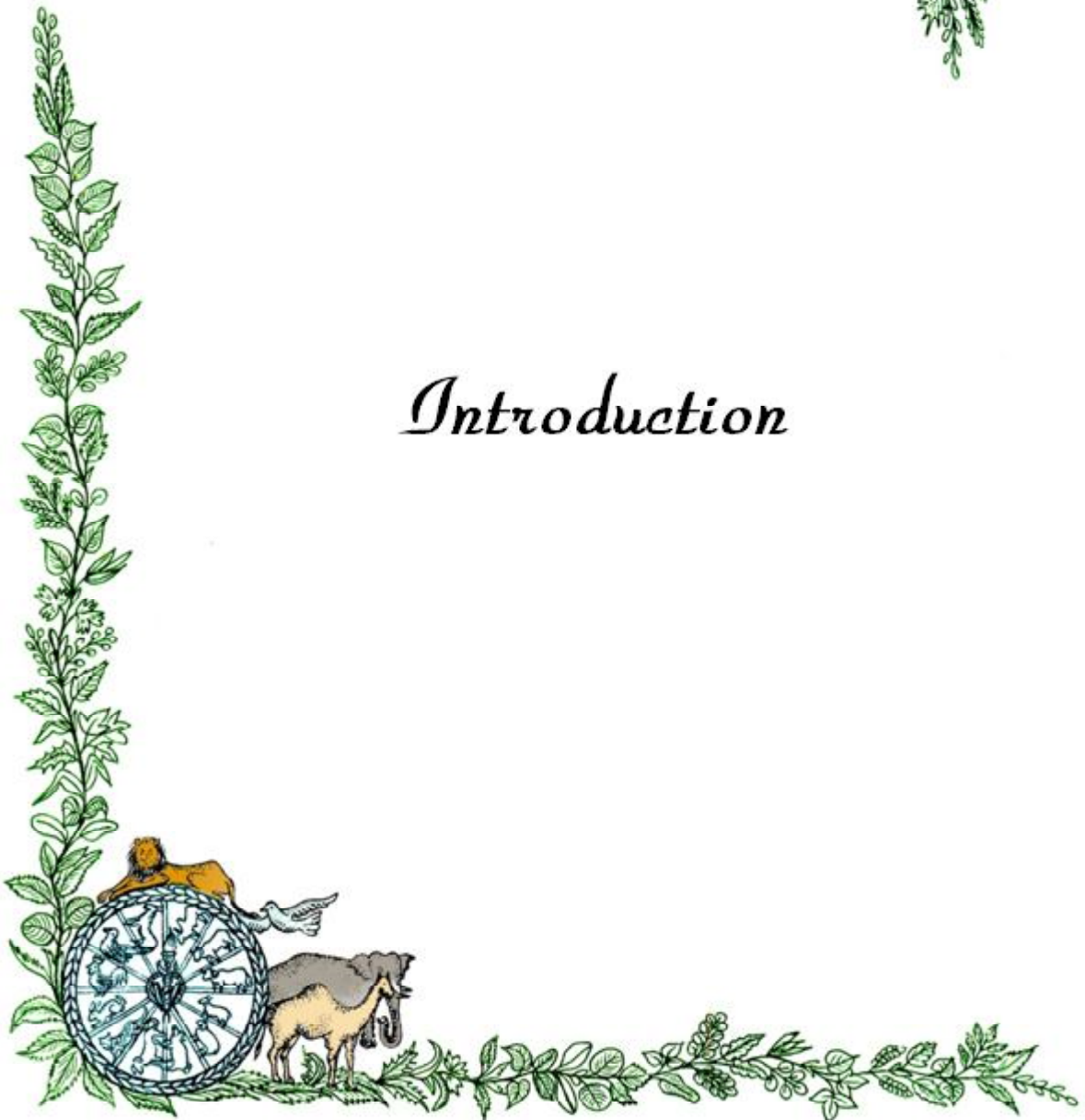
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Introduction



Antibiotics are antimicrobial drugs used in the treatment and prevention of the infections caused by bacteria. Antibiotics revolutionized medicine since 1928, when Alexander Fleming identified penicillin, the first biological compound with antibiotic properties. Their effectiveness has led to their excessive and indiscriminate use leading bacteria to develop resistance. Gram-negative bacteria, which include *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter* spp., are an especially dangerous group of pathogens, because they are difficult to treat and have become increasingly resistant to currently available antimicrobial drugs (NAID, 2012).

Antibiotic resistance is rising to dangerously high levels in all parts of the world. In India, antibiotic-resistant neonatal infections cause deaths of nearly 60,000 new-born each year (The Economic Times, 2016). In 2010, India was the world's largest consumer of antibiotics for human health (Laxminarayan and Chaudhury, 2016). Hence, emergence and spread of antimicrobial drug resistance (AMR) have a high socio-economic impact in India.

Bacteria have the natural ability to multiply rapidly and mutate their genes to adapt rapidly to changing environment. Once the desired character is acquired by a bacterium it rapidly spread in the bacterial community through two main mechanisms called “vertical” and “horizontal” gene transfer. In vertical transmission, mutations are passed on to subsequent generations leading to a population of resistant bacteria. In horizontal transmission, resistant genes are transferred from one microbe to another through various means of genetic exchange (Kumar and Singh, 2013).

The important means of expression of AMR includes , a) Creation of impermeable barrier i.e., modifications in bacterial cell membrane leading to the development of an impermeable barrier which blocks antibiotics to reach the targets, b) Target modification which includes modification of components of the bacteria which are targeted by the antibiotic, meaning the antibiotic can no longer bind properly to its target (ligands) to destroy the bacteria, c) Antibiotic modification i.e., the bacterial cell produces substances (usually proteins called “enzymes”) that inactivate the antibiotic before it can harm the bacteria, d) Efflux pumps, the mechanism by which the antibiotic is actively pumped out of the bacterial cell so that it cannot harm the bacteria (Kumar and Singh, 2013). Whenever a person takes antibiotic the sensitive bacteria are killed but the resistant bacteria survive and are left to grow and multiply leading to infection with AMR strains. Repeated use with improper knowledge of antibiotics is the major cause of the increase in drug-resistant bacteria (Kumar and Singh, 2013).

The Enterobacteriaceae is a large family of ubiquitously distributed Gram-negative bacteria that includes several opportunistic and nasty pathogens as *Salmonella* spp., *Escherichia coli*, *Yersinia pestis*, *Klebsiella* spp., *Shigella* spp., *Proteus* spp., *Enterobacter* spp., *Serratia* spp., and *Citrobacter* spp. etc. The emergence and spread of AMR in Enterobacteriaceae members has complicated the treatment of serious nosocomial infections often caused by multiple-drug-resistant (MDR) or pan-drug-resistant strains (Paterson, 2006).

Colistin (Polymyxin E) is an antibiotic produced by certain strains of *Paenibacillus polymyxa*. It is a mixture of the cyclic polypeptides colistin A and B. Colistin was first isolated in Japan from *Bacillus polymyxa* var. *colistinus* by the Japanese scientist Koyama in 1949 and became available for clinical use in 1959. Colistin is effective against most of the Gram-negative bacilli. Colistin is often administered orally in poultry and pigs to control colibacillosis (Kempf *et al.*, 2013). The increasing prevalence of infections caused by multi-drug resistant (MDR) Gram-negative bacteria (GNBs) has led to increase in the interest in colistin as a last-line therapy with the inevitable risk of emerging resistance. Hence there is a need to screen colistin resistance even in patients without a history of colistin usage for the timely detection to prevent clonal transmission.

Colistin, a mixture of polycationic peptides has both hydrophilic and lipophilic moieties. These cationic regions interact with the bacterial outer membrane, by displacing magnesium and calcium cations in the lipopolysaccharide. Hydrophobic/hydrophilic regions interact with the bacterial cytoplasmic membrane to solubilize it in an aqueous environment leading to its bactericidal action.

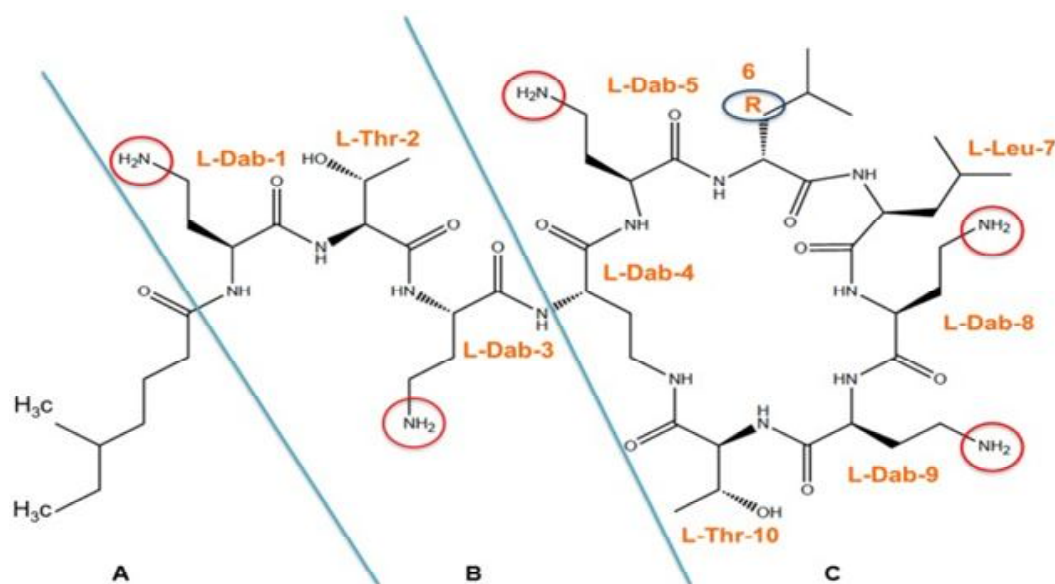


Fig 1: Chemical structure of colistin.

It is composed of three parts: (A) hydrophobic acyl tail, (B) linear tripeptide segment, (C) hydrophilic, heptapeptide ring. Arabic numerals indicate the position of amino acids on the structure and the reactive amino groups are encircled. R6: D- phenylalanine in polymyxin B or D- leucine in (colistin) polymyxin E (Rhouma *et al.*, 2016).

The discovery of plasmid mediated colistin resistance in *E. coli* is of serious concern among scientists, physicians and veterinarians (Rhouma *et al.*, 2016). The emergence of colistin resistance evoked more attention because of the use of colistin as the last resort drug against MDR, carbapenemase and extended spectrum β -lactamase (ESBL) producer strains of *P. aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Li *et al.*, 2005; Kumarasamy *et al.*, 2010; Biswas *et al.*, 2012).

Infections with MDR GNBs specifically those are carbapenem-resistant and acquired *mcr* gene (plasmid-borne gene responsible for colistin resistance) leave hardly any scope for therapeutic management of the patients. Enterobacteriaceae strains are often fast in acquiring AMR genes. If acquired by a member of Enterobacteriaceae the *mcr* gene might be quite dangerous due to their commensal habitat, food and waterborne nature, zoonotic ability and ability to cause fatal infections.

The dog is the most beloved pet of children and elderly people and we often forget or compromise the rule of general hygiene. Dogs may carry several enteropathogens on their perianal area (Singh *et al.*, 1994). Handling and hugging dogs by the owners may lead to the transfer of potential pathogens along with their AMR to their owners and *vice-versa*. Therefore, this study has been planned with the following objectives:

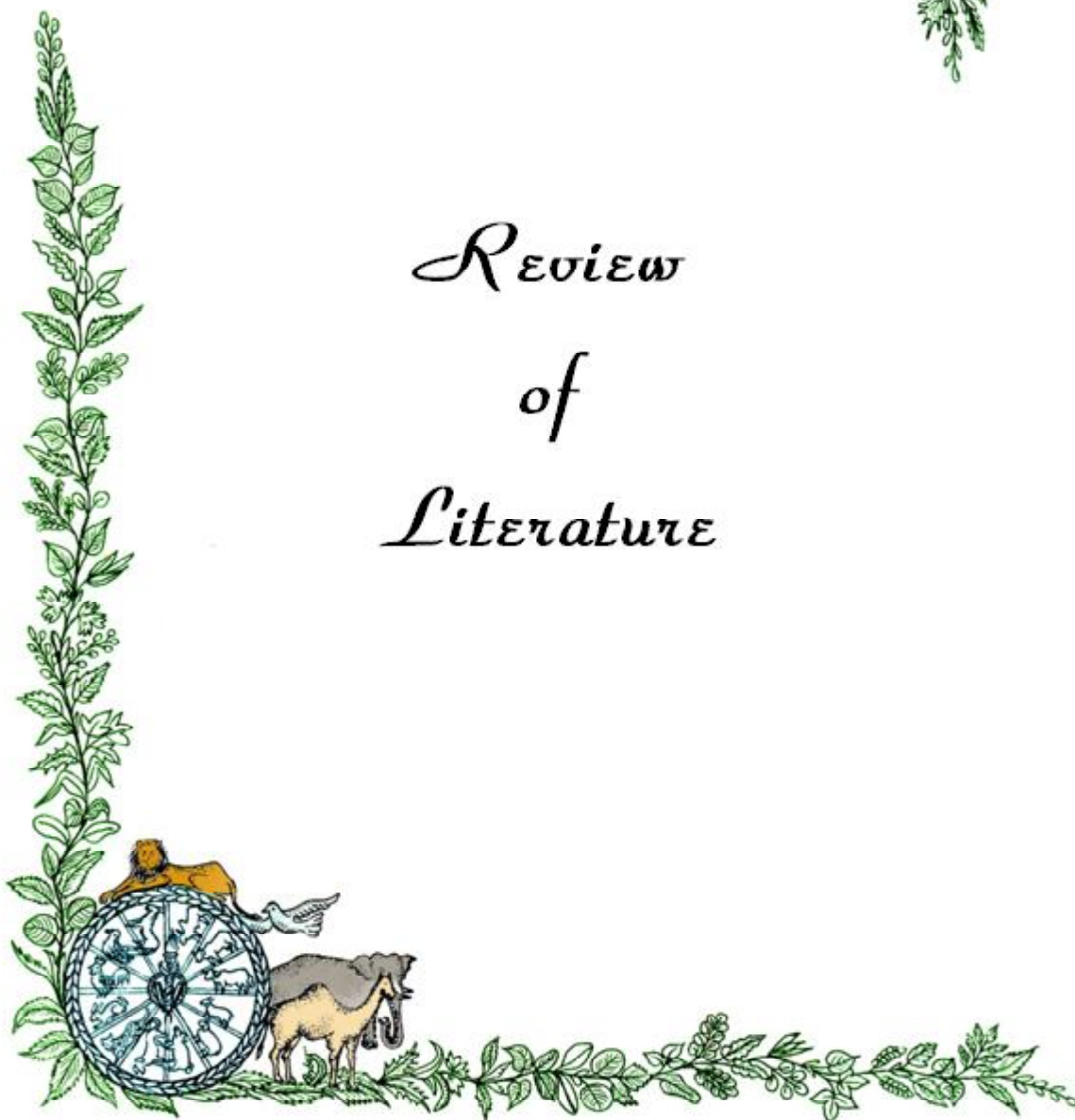
Objectives

- **To determine the occurrence of colistin resistance in isolates of Enterobacteriaceae members from perianal area of pet dogs.**
- **To determine epidemiological factors associated with carriage of colistin resistant bacteria by pet dogs.**





*Review
of
Literature*



2.1 Prevalence of Colistin resistance

To define colistin efficacy on bacteria, Clinical and Laboratory Standards Institute (CLSI) established minimum inhibitory concentration (MIC) breakpoints to differentiate between susceptible ($\text{MIC} \leq 2 \text{ mg/L}$), intermediate sensitive ($\text{MIC} = 2\text{-}8 \text{ mg/L}$) and resistant ($\text{MIC} \geq 8 \text{ mg/L}$) strains about a decade ago. Though the existence of colistin-resistant bacteria is known for >50 years, emergence of Pan-drug resistance amongst GNB made colistin as one of the drug of choice in therapeutics about a decade ago due to its activity on nosocomial bacteria. The first case in series from India using colistin based combination therapy was reported only a few years ago (Ghafur *et al.*, 2014).

The *mcr-1* gene, a plasmid-mediated one, confers resistance to colistin. Colistin is a polymyxin and is one of the numbers of last-resort antibiotics (Liu *et al.*, 2016). The strain first discovered with *mcr-1* gene was *E. coli* SHP45 isolated from a pig in China in November 2015. The *mcr-1* is the first known polymyxin resistance gene capable of horizontal transfer (Liu *et al.*, 2016).

In recent years colistin resistance has been reported worldwide (Fig. 2) in bacterial isolates from human, animals and environment (Haenni *et al.*, 2016; Liu *et al.*, 2016; Quesada *et al.*, 2016; Teo *et al.*, 2016). In food animals, bacteria harboring *mcr* genes have been reported from many countries (Fig. 3) including pigs in China (Liu *et al.*, 2016), poultry and swine in Spain (Quesada *et al.*, 2016).

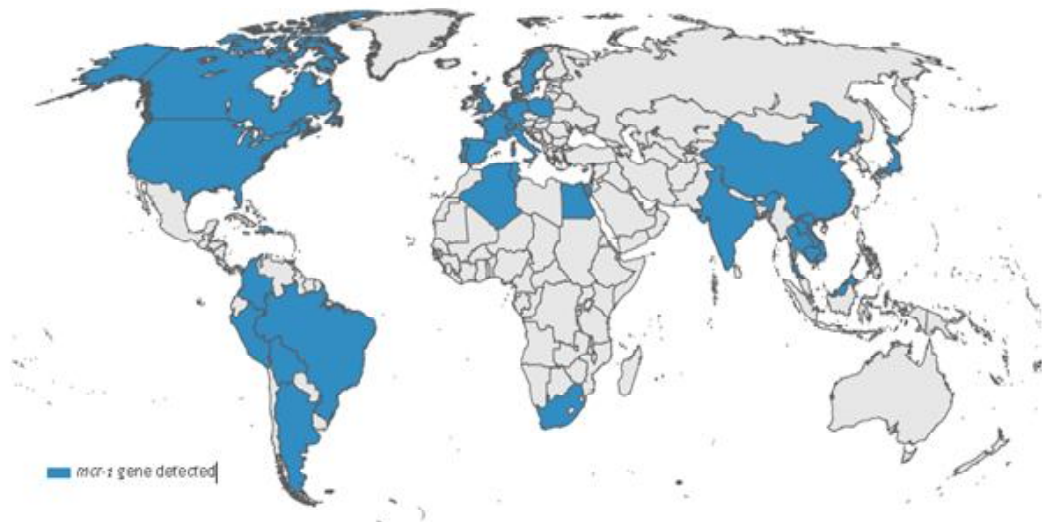


Fig 2: Countries reporting *mcr-1* gene in samples of animal, environmental or human origin (Xavier *et al.*, 2016)



Fig 3: Countries reporting *mcr-1* gene in food-producing animal (Skov and Monnet, 2016)

Transmissible colistin resistance encoded by *mcr-1* has been detected in clinical isolates of Enterobacteriaceae in Singapore (Teo *et al.*, 2016). The *mcr-1* gene was detected in 0.4% clinical isolates of Enterobacteriaceae in Hong Kong and in 1% isolates from China (Liu *et al.*, 2016). The reason behind detection of *mcr* gene in bacteria from human clinical cases has been assigned to be the high prevalence of colistin resistant bacteria in livestock and meat in China. The prevalence of colistin-resistant isolates is reported up to 25.4% and 28.0% in pigs

and retail chicken meat, respectively in China (Wong *et al.*, 2016). There are reports from Denmark where plasmid-mediated colistin-resistance gene, *mcr-1*, was detected in an *E. coli* isolate from a Danish patient with bloodstream infection and in five *E. coli* isolates from imported chicken meat (Hasman *et al.*, 2015).

Resistance to colistin in human gut microbiota is conferred by the diversified *mcr-1*-harbouring plasmid reservoirs (Ye *et al.*, 2016). Besides, colistin and tigecycline resistance in carbapenemase-producing Gram-negative bacteria has been shown to be associated with mutations in the ribosomal 16S rRNA operon *rrnBC* and through target site modifications (Sekyere *et al.*, 2016). There is also a report (Chen *et al.*, 2011) of independent emergence of colistin resistance in *Klebsiella pneumoniae* carbapenemase (KPC)-producing carbapenem-resistant Enterobacteriaceae (CRE) isolates. There was no clinical treatment history with colistin and it is stated to be necessary to evaluate the efficacy of combination therapy with colistin. It is also important to conduct surveillance of emergence of resistant strains and evolution of resistance mechanism investigation in order to avoid the emergence of more colistin-resistant (CRE) isolates in health care-associated facilities.

In June 2016 identification of a novel plasmid-mediated colistin resistance gene, *mcr-2*, in *E. coli* in Belgium (Xavier *et al.*, 2016) has indicated fast emergence and evolution of colistin resistance in Enterobacteriaceae.

A study conducted in Zurich, Switzerland evaluated the occurrence of *mcr-1* and *mcr-2* in colistin-resistant Enterobacteriaceae isolated from slaughtered livestock from faecal samples of 325 randomly selected fattening pigs and 241 randomly selected calves as well as from 100 poultry flocks. The assessed data revealed no *mcr-1/mcr-2* producing Enterobacteriaceae detected in Swiss livestock (Buess *et al.*, 2016).

Among Enterobacteriaceae members *Proteus*, *Providentia*, *Morganella* and *Serratia marcescens* are intrinsically resistant to colistin and polymyxin (Shimizu *et al.*, 1977; Olaitan *et al.*, 2014; Erfanimanesh and Hashemi, 2016). In early 1970, Sud and Feingold reported that polymyxin resistance in *Proteus mirabilis* is due to the cell envelope, preventing access of the drug to the susceptible lipid target sites. Shimizu and co-workers (1977) reported rise in

the level of colistin resistance by prior treatment. However the development of resistance to colistin in multidrug-resistant *Pseudomonas aeruginosa* was not observed under similar circumstances (Li *et al.*, 2006).

In recent years there has been renewed interest in colistin for the treatment of infections with MDR GNBs, causing concern that increasing use may be accompanied with the emergence of resistance. There are reports not only in animals but also in humans. One of them is the emergence of plasmid-mediated colistin resistance gene *mcr-1* in a clinical *E. coli* isolate from Egypt. The PCR and DNA sequencing have been used to screen for the presence of *mcr-1* in 241 non-duplicate Gram-negative clinical bacterial isolates. Analysis showed that *mcr-1* was present only in one *E. coli* isolate (SP-1), with MIC of 16 mg/L for both colistin and polymyxin B, which was from the sputum of a patient with bacteremia (Elnahriry *et al.*, 2016). Few other reports are there for colistin- and carbapenem-resistant *E. coli* harboring *mcr-1* and *bla*_{ndm-5}, causing a complicated urinary tract infection in a patient from the United States. The isolate exhibited resistance to both colistin and carbapenems, but was susceptible to amikacin, aztreonam, gentamicin, nitrofurantoin, tigecycline and trimethoprim-sulfamethoxazole (Mediavilla *et al.*, 2016).

In retrospective cohort study on colonization and infection with colistin-resistant GNBs in critically ill 150 patients in Greece (Kontopidou *et al.*, 2011) 78 (52%) were colonized by colistin-resistant GNBs. Out of the colonizing pathogens, 30 (20%) were *Klebsiella pneumoniae* isolates and 51 (34%) were intrinsically colistin resistant Enterobacteriaceae. Their finding indicated that resistance emerged through selection pressure due to extensive colistin use rather than spread by horizontal transmission. In addition, superinfection with pathogens intrinsically resistant to colistin, including *Proteus*, *Providencia*, *Serratia* and *Morganella* species, may also be a matter of concern (Kontopidou *et al.*, 2011). This observation further supports the rational use of colistin as a main factor in avoiding emergence of resistance.

Apart from animals there are reports from human patients infected with colistin resistant bacteria. A prospective study done in Switzerland on 2049 non-duplicate enterobacterial isolates

identified six strains resistant to colistin. The colistin resistance was determined with according to the rapid polymyxin Nordmann/Poirel (NP) test as well as through minimum inhibitory concentration (MIC) test in 0.29% bacteria, including two *E. coli*, two *K. pneumoniae*, one *H. alvei* and one *Salmonella* isolate. However the polymerase chain reactions (PCR) targeting the *mcr*-like genes performed on the colistin-resistant isolates remained negative. In the same study on testing of colistin-susceptible isolates, a single *E. coli* strain (C1624) was positive for the *mcr-1* gene with the MIC value of 0.125 mg/l (European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint tables for the interpretation of MICs and zone diameters, version 2.0, 2014). The overall prevalence rate of MCR-producing enterobacterial strains from human UTIs in Western Europe remains very low (0.05%)(Liassine *et al.*, 2016). Similarly, in Spain, 53 colistin resistant *E. coli* isolates were detected on testing 10011 clinical isolates from humans were found (Prim *et al.*, 2016).

Study on *mcr-1* among *E. coli* clinical isolates collected worldwide as part of the SENTRY Antimicrobial Surveillance Program in 2014 and 2015 reported the gene from bacteria from Belgium (1), Brazil (1), Germany (5), Hong Kong (1), Italy (4), Malaysia (1), Poland (1), Russia (1), Spain (3), and the United States (1). Isolates positive for *mcr-1* were associated with bloodstream infections (8), skin and skin structure infections (5), urinary tract infections (3), respiratory tract infections (2), and intra-abdominal infections (1). Prevalence of 0.1% was observed for the overall population surveyed under the SENTRY Program related to *mcr-1* among *E. coli* isolates (Castanheira *et al.*, 2016).

2.2 Colistin resistance status in India

About 11,942 tons of colistin worth \$187.2 million, was used worldwide in year 2015 mostly on swine and poultry farms. While antibiotics are helping to sustain intensive food production, their uncontrolled use on farms is turning animals into reservoirs of hard-to-kill bacteria that can spread rapidly and globally (Pearson and Limaye, 2016).

Out of the 10-largest colistin producing firms, one is Indian, one Danish, and eight are Chinese (Pearson and Narayan, 2016). Sales of colistin for human use in India are more than doubled since 2013 (Pearson and Narayan, 2016).

Hospitals in India are now recording cases of infections resistant to colistin, the last antibiotic available in the world, which was brought back from a 40-years exile in 2005 to treat increasing number of infections resistant to other high-end antibiotics (Srivastava, 2014). About 100 miles from the poultry farm in Telangana state, medical college microbiologists studying a bacterium spreading mostly in hospitals reported that 6.7 percent of the specimens contained bacteria resistant to all antibiotics (Pearson and Limaye, 2016).

In one of the important study, Laxminarayan and colleagues (2016) estimated that more than 58,000 newborns died nationwide in 2013 from bacterial infections capable of evading most of the antibiotics. Another communication has revealed the emergence of plasmid-mediated colistin resistance and further indicated that it heralds the breach of the last group of antibiotics, polymyxins (Kumar *et al.*, 2016). There are case reports in India about the patients affected with complicated urinary tract infections revealing tigecycline and colistin resistant *Acinetobacter baumannii* (Taneja *et al.*, 2011).

The Department of Animal Husbandry, Dairying and Fisheries, under the Ministry of Agriculture (Govt. of India) issued a notice in late 2014 to advise all state animal husbandry departments to restrict the use of antibiotics as growth promoters in the food animal production but no strict actions are taken till date to curb the practice (Nagaraju, 2016).

Colistin resistance is being increasingly reported in Indian human hospitals also. In Chennai, twenty four colistin resistant isolates from patients of mean age of 58.33 were recorded. The patients were mostly with the use of invasive device 22 (91.67%). Urine was found to be the commonest source of isolation 8 (33%), followed by blood 6 (25%), respiratory tract infections 5 (20.8%), pus 4 (16.67%) and others (CSF) 1 (4.17%). The most common organism isolated was *Klebsiella* spp. 21 (87.5%) (Arjun *et al.*, 2016). Similarly, from Indian hospitals colistin resistant strain in urine samples were also found. On PCR screening, plasmid-borne *mcr-1* gene was detected in *E.coli* (Kumar *et al.*, 2016). However, several *E.coli* (292) isolates have also been reported without *mcr-1* gene from clinical cases (Purohit *et al.*, 2017).

2.3 Prevalence of MDR Enterobacteriaceae strains in pets and their zoonotic importance

Cats and dogs are considered to be the potent source for the spread of antimicrobial resistant microbes due to the extensive use of antimicrobials in pets and also of their close contact with other animals as well as humans (Jayalakshmi, 2015).

In a study on important enteropathogens of zoonotic significance on perianal area of pet dogs reported *E. coli* and *K. pneumoniae* (Singh *et al.*, 1994) to be the most common enteropathogen. Role of zoonotic pathogens in emergence of AMR has been evidenced (Wieler *et al.*, 2011) and household pets are considered as a source of infection from MDR bacteria (Damborg *et al.*, 2016).

Out of 39 *Pseudomonas aeruginosa*, 29 *Proteus* spp. and 449 *Escherichia coli* strain tested, >5% colistin-resistant strains of *E. coli*, *Pseudomonas aeruginosa* and *Proteus* strains were confirmed in diagnostic sample from dogs (Pedersen *et al.*, 2007). Tests, done on 39 dogs and 14 cats in the pet shop in China, revealed presence of *mcr-1* in isolates from four dogs and two cats (Zhang *et al.*, 2016). Transmission of *mcr-1*-harbouring *E. coli* between companion animals and humans has recently been proposed and confirmed (Woodmansey, 2016).

The use of colistin (as therapeutic agent or growth enhancer) in animals is said to be the originator of the emergence of resistant (Richez *et al.*, 2016). Besides, observations revealed that meat from pigs and chickens (Liu *et al.*, 2016; Skov and Monnet, 2016) might be a serious risk and is of urgent public health concern (Skov and Monnet, 2016).

Investigation was done for the colistin resistance genes *mcr-1* and *mcr-2* in clinical Enterobacteriaceae isolates from cats and dogs in Switzerland. A total of 347 isolates (231 from dogs, 116 from cats) were analyzed. Out of the total, 274 (79%) were from urine and 73 (21%) from surgical sites, abscesses and other sources but none of the isolates tested positive by PCR for the *mcr-1* or the *mcr-2* gene even though broth microdilution tests revealed MICs of 2mg/L–64 mg/L of colistin for four isolates (two *Enterobacter* spp., one *Klebsiella* spp. and one *E. coli*) from dogs (one from skin exudate, one from a surgical site and two

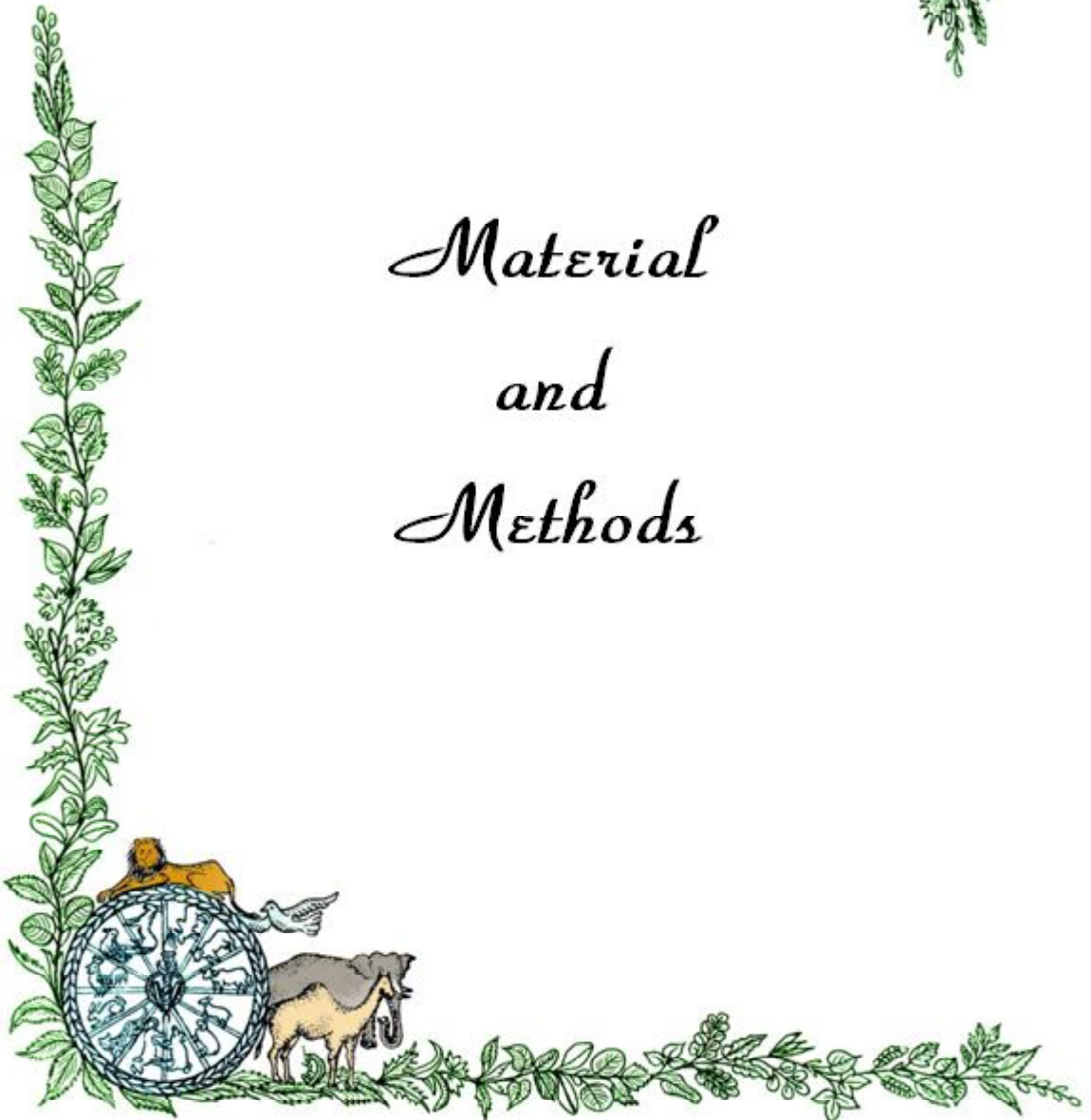
isolates from urine). This study documents that so far, companion animals in Switzerland do not represent a reservoir for plasmid-mediated colistin resistant Enterobacteriaceae (Simmen *et al.*, 2016).

Increases in infections due to MDR GNBs, particularly those resistant to carbapenems, are likely to lead to greater reliance on colistin for treatment until new antibiotics with activity against these bacteria become available. In terms of assessing the future risk, a number of factors need to be considered, many of which will require active surveillance and research. Lei and co-workers (2016) investigated the prevalence of the emerging colistin resistance gene *mcr-1* in Enterobacteriaceae among companion animals and examined 566 isolates collected from cats and dogs in Beijing, China, during 2012–2016. Of these isolates, 49 (8.7%) were *mcr-1*–positive and suggested that because of frequent and close contact between humans and companion animals opportunities exist for transmission of colistin-resistant Enterobacteriaceae to and from the both groups. Thus classical surveillance activities are also needed to identify risk factors in both human and animal populations to inform policy and intervention strategies (Schwarz and Johnson, 2016). This current study has been conducted to fill the gap of knowledge on colistin resistant microbes in pet animals.





*Material
and
Methods*



3.1 MATERIALS

3.1.1 Glassware and plastic wares

During the course of study, sterilized glassware (Corning/Borosil grade) and plastic wares from Tarson (India), Hi-Media (Mumbai, India) and Axygen (USA) attuned for molecular biology work were used.

3.1.2 Media, Stains, Chemicals, Buffers, Reagents etc.

The details of media, stains, chemicals, buffers and molecular biology reagents used in the study are given in the appendix. General chemicals used were of analytical grade, procured from Sigma (USA) and Merck (India). The media and antibiotic discs used in study were procured from BD Scientific, India. The PCR master mix was procured from Thermo Scientific, USA. The PCR primers were custom synthesized from Eurofin Pvt. Ltd., Bengaluru, India. All media and reagents for molecular biology work were prepared in triple glass distilled water. For isolation and culture of bacteria all media was prepared using single glass distilled water.

3.1.3 Equipments

Equipments used in the study were autoclave (Scientific Equipments Works, New Delhi, India), biomedical refrigerator (BPL), bio-safety cabinet (Kartos International, Noida, India), cooling centrifuge (REMI Instruments Ltd., Mumbai, India), deep freeze -20°C (Vestfrost), electronic balance (Mettler Toledo, Swizerland), gel documentation system (Alpha Innotech Co., USA), incubator (Khera Instruments Pvt. Ltd., New Delhi, India), variable volume micro pipettes (Eppendorf), water bath (Narang Scientific Works Pvt. Ltd., New

Delhi, India), PCR thermal cycler (MJ Research PTC-200, Peltier Thermal Cycler, USA), Gel doc (Alphaimage), hot air oven (Scientific Equipments Works, New Delhi, India) and nanodrop (Thermofisher scientific, USA).

3.2 METHOD

3.2.1 Sample collection

A suitable proforma was prepared for collection of relevant epidemiological data viz., animal number, age, sex, breed, type of clinical sample collected, clinical condition, antibiotics previously used with confirmation of veterinary officer etc (Annexure 1).

A total of 216 perianal swabs of dogs and 58 hand swabs from willing owners were collected taking strict aseptic precautions and transported on ice or in transport medium depending on the distance of place of sampling from the laboratory.

3.2.2 Isolation of individual bacteria

Sample was kept at 37°C for 4-6 h in buffer peptone water for pre-enrichment. The broth culture was then swabbed for inoculum and streaked to get individual colonies on MacConkey agar and Hektoen Enteric Agar. The plates were kept for 37°C for 24h. The individual colonies were picked and re-streaked on MacConkey agar and HEA to get pure culture. The plates were kept again at 37°C for 24h.

The isolates were then biochemically characterized.

3.2.3 Biochemical characterization of Enterobacteriaceae isolates

Bacterial isolates were identified using standard protocol (Singh, 2009) for catalase, oxidase, gelatinase and urease production, methyl red (MR) and Voges Proskauer (VP) test, malonate and citrate utilization as carbon source, nitrate reduction, lysine decarboxylation, H₂S production, motility, phenyl alanine deaminase activity and growth on triple sugar iron agar slants.

3.2.4 Assay for antimicrobial drug resistance of the Enterobacteriaceae isolates

The bacterial isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method (Singh, 2013) on Mueller-Hinton agar (BD, India) plates. Isolates were tested against

antibiotics viz., amoxicillin and clavulanic acid (AMC, 30µg), ampicillin (AMP, 10µg), aztreonam (AT, 30µg), cefepime (FEP, 30µg), cefotaxime (CTX, 10µg), cefotaxime and clavulanic acid (CTX-CLA, 30/10µg), ceftazidime (CAZ, 30µg), ceftriaxone (CRO, 30µg), cephalexin (CN, 30µg), chloramphenicol (C, 30µg), ciprofloxacin (CIP, 5µg), colistin (CL, 10µg), cotrimoxazole (SXT, 23.75/1.25µg), erythromycin (E, 10µg), gentamicin (GM, 10µg), imipenem (IPM, 10µg), meropenem (MRP, 10µg), moxalactam (MOX, 30µg), nalidixic acid (NA, 30µg), nitrofurantoin (N/F, 300µg), piperacillin (PIP, 100µg), piperacillin and tazobactam (TZP, 110µg), streptomycin (S, 10µg), tetracycline (TE, 30µg), tigecycline (TGC, 15µg), vancomycin (VA, 5µg), ajowan oil (2µL), carvacrol (2µL), cinnamon oil (2µL), guggal oil (2µL), lemon grass oil (2µL), patchouli oil (2µL) and thymol (2µL). The EDTA disk (~1900 µg) was put close to carbapenem disk to look for synergy as an indicator of metallo-β-lactamase production. Isolates were grown in LB broth overnight and lawn cultures were prepared on MHA plates. The swab inoculated plates were allowed to dry. Antibiotic discs were placed on agar surface at about two cm apart. The plates were incubated at 37°C overnight and diameter of the zones of growth inhibition was measured in mm. The measurements were compared with zone size interpretative chart furnished by the manufacturer and the isolates were graded as sensitive, intermediate and resistant as per CLSI (2015). After overnight incubation the presence of any synergistic inhibition or sensitivity zone was interpreted for ESBL and carbapenemase production as per CLSI (2015).

3.2.5 Phenotypic confirmation of colistin resistance

The positive isolates from initial screening for colistin resistance were subjected for determination of minimum inhibitory concentration (MIC) by using colistin E-test strip (Biomérieux, India). Pure culture isolates were grown in LB broth overnight at 37°C and then swabbed on MHA plates. MIC strip was placed in the middle of the plate and again kept for 24h at 37°C to determine the minimum inhibitory concentration as per guideline (CLSI, 2015).

3.2.6 Genotypic detection and confirmation of colistin resistance producing *mcr-1* gene

3.2.6.1 Isolation of genomic DNA from bacterial suspension cultures

The genomic DNA of isolated culture was extracted by QIAamp DNA Mini Kit (Qiagen India Pvt. Ltd., New Delhi, India).

Steps for isolation of genomic DNA from bacterial suspension cultures

(As per Manufacturer Instruction)

1. The culture was prepared by inoculating the isolate in Luria-Bertani broth (LB-Broth; Hi-media, India) and incubating at 37°C for 24h.
2. 1 ml of bacterial culture was pipetted into a 1.5 ml micro centrifuge tube, and centrifuged for 5 min at 7500 rpm
3. Buffer ATL (supplied in the QIAamp DNA Mini Kit) was added to a total volume of 180 µl.
4. 20µl proteinase K was added, mixed by vortexing, and incubated at 56°C until the cells were completely lysed. Vortexed occasionally during incubation to disperse the sample, or placed in a shaking water bath or on a rocking platform.
5. Briefly the 1.5 ml micro centrifuge tube was centrifuged to remove drops from the inside of the lid.
6. 200 µl buffer AL was added to the sample, mix by pulse-vortexing for 15 s, and incubated at 70°C for 10 min. briefly centrifuged the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
7. 200 µl of ethanol (96-100%) was added to the sample, and mixed by pulse-vortexing for 15 s. After mixing, briefly the 1.5 ml microcentrifuge tube was centrifuged to remove drops from inside the lid.
8. Carefully applied the mixture from step 7 (including the precipitate) to the QIAamp Minispin column (in a 2 ml collection tube) without wetting the rim. Closed the cap, and centrifuged at 8000 rpm for 1 min. The QIAamp Mini spin column was place in a clean 2 ml collection tube, and discarded the tube containing the filtrate.
9. The QIAamp Mini spin column was carefully opened and 500 µl Buffer AW1 was added without wetting the rim. The cap was closed, and centrifuged at 8000 rpm for 1 min. Place the QIAamp filtrate.
10. The QIAamp Mini spin column was opened carefully and 500 µl Buffer AW2 was added without wetting the rim. The cap was closed, and centrifuged at full speed (14,000 rpm) for 3 min.

11. Centrifuged at full speed for 1 min additionally to eliminate the chance of possible BufferAW2 carry over.
12. The QIAamp Mini spin column was placed in a clean 1.5 ml micro centrifuge tube, and the collection tube containing the filtrate was discarded.
13. The QIAamp Mini spin column was carefully opened and 200 μ l Buffer AE was added. Incubated at room temperature for 1 min, and then centrifuged at 8000 rpm for 1 min.
14. The bacterial genomic DNA was stored at -20°C till further use.

3.2.6.2 Isolation of plasmid DNA from bacterial suspension cultures

The plasmid DNA of isolated culture was extracted by Thermo Scientific GeneJET Plasmid Miniprep Kit.

Steps for isolation of plasmid DNA from bacterial suspension cultures

1. A single colony was picked from a freshly streaked selective plate to inoculate 1-5 mL of LB medium supplemented with the appropriate selection antibiotic. Incubated for 12-16 hours at 37°C while shaking at 200-250 rpm. Use a tube or flask with a volume of at least 4 times the culture volume.
2. The bacterial culture was harvested by centrifugation at 8,000 rpm ($6,800 \times g$) in a microcentrifuge for 2 min at room temperature. Decant the supernatant and remove all remaining medium.
3. All centrifugations were carried out in a table-top microcentrifuge at $>12,000 \times g$ (10,000-14,000 rpm, depending on the rotor type).
4. The pelleted cells were resuspended in 250 μ L of the Resuspension Solution. The cell suspension was transferred to a microcentrifuge tube. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain. Note. It was ensured that RNase A was added to the Resuspension Solution initially.
5. 250 μ L of the Lysis Solution was added and mix thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly clear. Note. Do not vortex to avoid

- shearing of chromosomal DNA. Do not incubate for more than 5 min to avoid denaturation of supercoiled plasmid DNA.
6. 350 μ L of the Neutralization Solution was added and mix immediately and thoroughly by inverting the tube 4-6 times. Note. It is important to mix thoroughly and gently after the addition of the Neutralization Solution to avoid localized precipitation of bacterial cell debris. The neutralized bacterial lysate should become cloudy.
 7. Centrifuged for 5 min to pellet cell debris and chromosomal DNA.
 8. The supernatant was transferred to the supplied GeneJET spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate. Note. Close the bag with GeneJET Spin Columns tightly after each use!
 9. Centrifuged for 1 min. flow-through was discard and the column was placed back into the same collection tube. Note. Do not add bleach to the flow-through
 10. (For EndA+ strains only) Optional: use this preliminary washing step only if EndA+ strains which have high level of nuclease activity are used. Wash the GeneJET spin column by adding 500 μ L of Wash Solution I (#R1611, diluted with isopropanol) and centrifuge for 30-60 sec. Discard the flow-through. Note. This step is essential to remove trace nuclease activity.
 11. 500 μ L of the Wash Solution (diluted with ethanol prior to first use) was added to the GeneJET spin column. Centrifuged for 30-60 seconds and discard the flow-through. The column was placed back into the same collection tube.
 12. The wash procedure was repeated using 500 μ L of the Wash Solution.
 13. The flow-through was discarded and centrifuged for an additional 1 min to remove residual Wash Solution. This step is essential to avoid residual ethanol in plasmid preps.
 14. The GeneJET spin column was transferred into a fresh 1.5 ml microcentrifuge tube. 50 μ L of the Elution Buffer was added to the center of GeneJET spin column membrane to elute the plasmid DNA. All care was taken with the pipette tip that it did not touch the membrane. Incubated for 2 min at room temperature and centrifuged for 2 min. Note. An additional elution step (optional) with Elution Buffer or water can recover

residual DNA from the membrane and increase the overall yield by 10-20%. For elution of plasmids >20 kb, Elution Buffer was prewarmed to 70°C before applying to silica membrane.

15. The column was discarded and the purified plasmid DNA was stored at -20°C

3.2.6.3 PCR based detection of colistin resistance (*mcr-1* gene)

PCR was carried out in a final reaction volume of 25 µl using 0.2 ml thin wall PCR tube (Tarsons, Mumbai). A master-mix for minimum of 10 samples was prepared and aliquoted in 22µl quantities in each PCR tube. 3µl sample of DNA was added in each tube to make the final volume of 25µl. Details regarding oligonucleotide primers and PCR conditions used are illustrated below.

Conditions for the PCR: 94°C 15 min + 25X (94°C 30 sec + 58°C 90 sec + 72°C 60 sec) + 72°C 10min.

Primers

Target gene	Primer sequences	Reference
<i>mcr-1</i> (35-343)	CLR F 5'CGGTCAGTCCGTTTGTTC'3 CLR R 5'CTTGGTTCGGTCTGTAGGG'3	Liu <i>et al.</i> , 2015

Positive control for *mcr-1* gene: The DNA of a positive strain for *mcr-1* gene was kindly provided by Prof. Alberto Quesada Molina, Departamento de Bioquímica, Biología Molecular y Genética, Facultad de Veterinaria, Universidad de Extremadura Av. de la Universidad s/n, 10003-Cáceres, España. It was used as positive template after 10 fold dilution in nucleus free water.

Agarose gel electrophoresis

The following reagents were used for agarose gel electrophoresis

- Agarose
- Tris borate EDTA (TBE) buffer, pH 8.3 (5X)
- Ethidium bromide (1%)

To confirm the targeted PCR amplification, 10 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading buffer from each tube and electrophoresed on 1.0 percent agarose gel (stained with ethidium bromide, 1 percent solution at the rate of 5 µl) along with 100 bp DNA ladder (GeneRuler 100 bp plus, Thermo Scientific) at constant 80 V for 45 min 0.5X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system. The PCR was performed two times to ensure the repeatability of the technique and to make sure that isolates were correctly assigned to respective patterns.

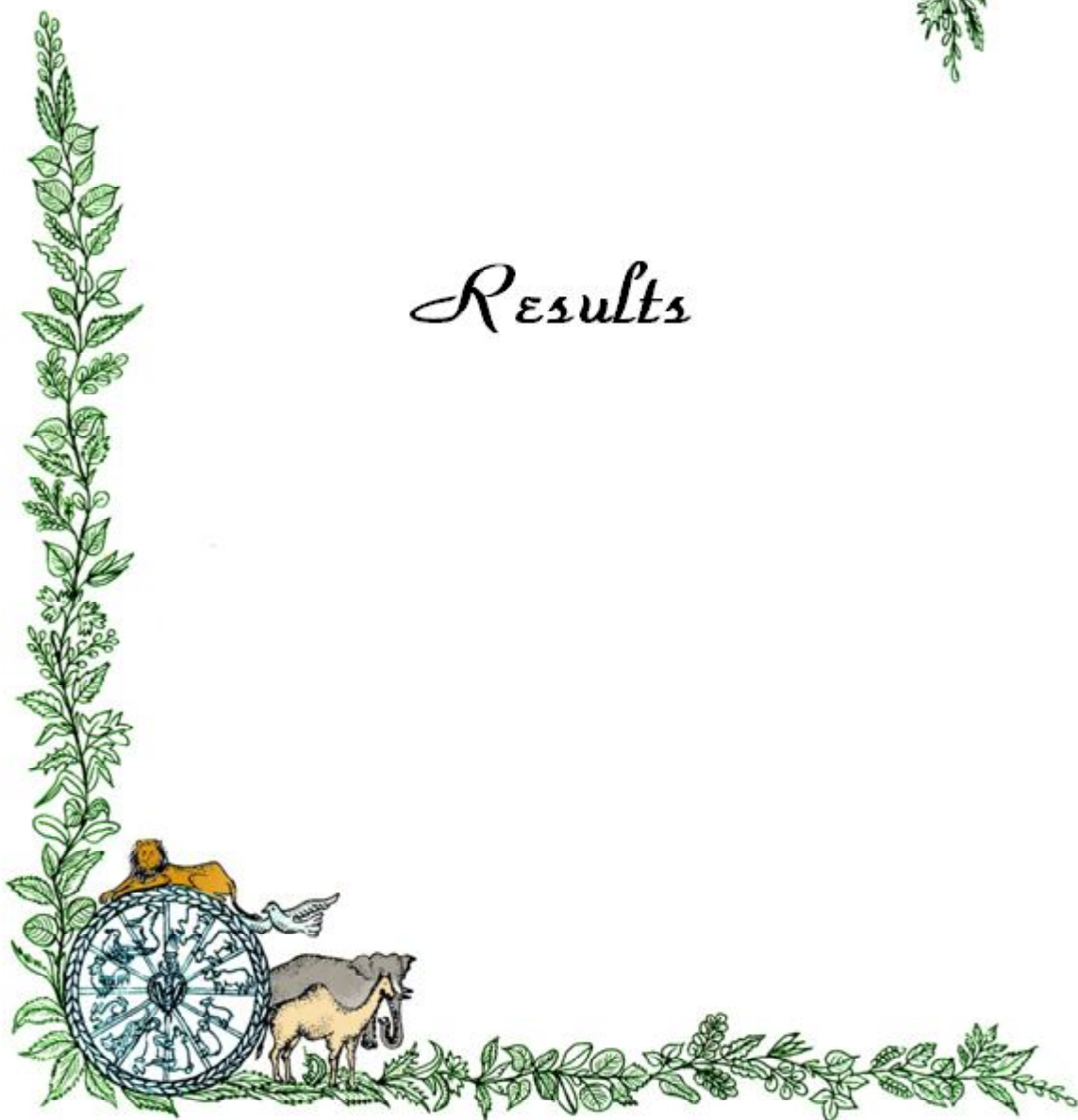
3.2.7 Statistical analysis

Statistical analysis was performed by using Microsoft Excel 2007. Data was tabulated by calculating odds ratio and chi-square analysis (Snedecor and Cochran, 1968).





Results



4.1 Preliminary screening and isolation

Samples were collected from Teaching Veterinary Clinical Complex (TVCC), Referral Veterinary Polyclinic, Indian Veterinary Research Institute and local areas of Bareilly city. On analysis of collected samples i.e., peri-anal swabs of dogs and hand swabs of owners on MacConkey agar plates and Hektoen Enteric agar and then on biochemical characterization, 336 *E. coli*, 14 *Enterobacter agglomerans*, 56 *P. mirabilis*, 4 *P. penneri*, 33 *K. pneumoniae* and 1 *C. freundii* strain were identified (Table 1). Besides, 34 *P. aeruginosa* strains were also isolated. (Fig. 4 -14).

4.2 Phenotypic identification of isolates

Antibiotic sensitivity (ABST) by disk diffusion assay revealed that 64 isolates were resistant to colistin (Table 2).

MIC of Colistin resistant isolates: Data of MIC indicated that 61 isolates were resistant to Colistin (MIC >2µg/ mL). Of the 61 colistin resistant (CR) isolates 60 belonged to *Proteus* spp. and 1 was of *Citrobacter freundii* (Table 3).

Table 3: MIC result for different bacteria

Type of bacteria	MIC of colistin µL
<i>Proteus</i> spp.	≥256
<i>Citrobacter</i> spp.	≥256
<i>E.coli</i>	≤0.125
<i>Pseudomonas</i> spp.	≤1

4.3 Polymerase Chain Reaction (PCR):

Resistant isolates' DNA as well as plasmid preparations were tested separately for the presence of *mcr-1* gene. However, none of the preparations tested positive for the gene. Although the positive control always yielded the desired product (309 kb) indicating the sensitivity of the test.

4.4 Statistical analysis:

Antimicrobial drug sensitivity of bacterial isolates was compared in relation to source, dog breed, age (pup, 0-2 month; young, 2-6 month; adult, 6-72 month; old, more than 72 month), health status and also the genus of the bacteria.

4.4.1 Bacterial isolates from dog-owner versus bacterial isolates from dogs:

Escherichia coli could be isolated both from dogs and their owners respectively, in 67 pairs, similarly *P. aeruginosa* from 3 pairs and *K. pneumoniae* from 2 pairs. Although *P. aeruginosa*, *K. pneumoniae* and *E. coli* strains isolated from dogs and their owners were apparently similar but could be differentiated on the basis of their antibiograms and there was no similarity between the paired isolates of a dog and its owner with respect to their antibiotic sensitivity pattern. Results (Table 4) revealed that the bacterial isolates from dogs were significantly ($p=0.001$) more often (15.63%) resistant to colistin than those from dog owners (1.33%). For imipenem ($p=0.02$) and tigecycline ($p=0.02$), bacteria isolated from dog owners were less resistant than bacteria isolated from dogs. Bacteria isolated from dogs (87.10%) were significantly ($p=0.01$) less often multi (up to 3) herbal drug resistant than bacteria isolated from dog-owners (97.33%). However, bacteria isolated from dogs (12.66%) were significantly ($p=0.004$) more often multi (up to 7) herbal drug resistant than bacteria isolated from dog owners (1.33%). Resistance to thymol was significantly ($p=0.03$) less common in bacteria from dog owners (1.33%) than in bacteria from dogs (8.68%).

Table 1: Biochemical tests performed for identification of bacteria

Tests	<i>E. coli</i>	<i>Proteus</i> spp.	<i>Klebsiella</i> <i>pneumoniae</i>	<i>Enterobacter</i> <i>agglomerans</i>	<i>Citrobacter</i> <i>freundii</i>	<i>Pseudomonas</i> <i>aeruginosa</i>
MIL						
Lysine	+	-	+	-	-	-
Indole	+	+				
		(<i>P. vulgaris</i>)/ - (<i>P. mirabilis</i> and <i>P. paneeri</i>)	-	-	-	-
Motility	-	+	-	-	+	+
Deaminase	-	+	-	-	-	-
Slant	+	- (<i>P. vulgaris</i> and <i>P. mirabilis</i>)/ + (<i>P. penneri</i>)	-	+	-	-
But	+	- (<i>P. vulgaris</i> and <i>P. mirabilis</i>)/ + (<i>P. penneri</i>)	-	+	-	-
H ₂ S	-	+	-	-	+	-
Gas	+	+	+	+	+	-
MR	+	+	-	-	+	-
VP	-	-	+	+	-	-
Citrate	-	-	+	+	+	+
Nitrate	+	+	+	+	+	+
Urea	-	+	+	+	+	-
Malonate	-	-	+	+	+	+
Phenylalanine	-	+	-	-	-	-
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	-	-	+
Gelatinase	-	+	-	-	+	+
KOH test	-	-	-	-	-	-
(Grams)						

Table 2: Isolates grouped according to different source

Type of Bacteria	Source				Total	Colistin resistant according to MIC	
	Peri-anal swab		Owner's hand swab				
	No. of isolates tested	Colistin resistant (by ABST)	No. of isolates tested	Colistin resistant (by ABST)			
<i>E.coli</i>	285	05	51	0	336	05	0
<i>Proteus mirabilis</i>	56	52	0	0	56	52	56
<i>Proteus penneri</i>	04	04	0	0	04	04	04
<i>Klebsiella pneumoniae</i>	23	0	10	0	33	0	0
<i>Enterobacter agglomerans</i>	07	0	07	0	14	0	0
<i>Pseudomonas aeruginosa</i>	27	01	07	01	34	02	0
<i>Citrobacter freundii</i>	01	01	0	0	01	01	01

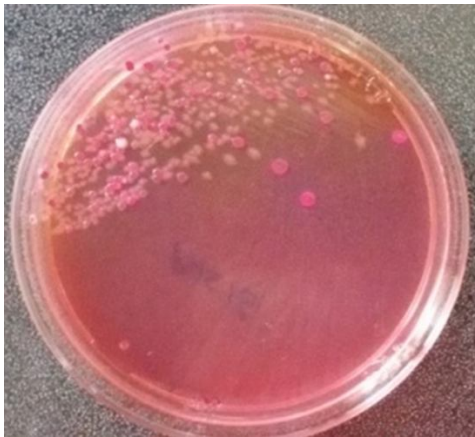


Fig. 4.a: Enterobacteriaceae isolates in perianal sample on MacConkey agar (pink colonies of putative *E. coli*, whitish colonies of *Proteus* spp.)



Fig. 4.b: Enterobacteriaceae in perianal sample on Hektoen Enteric agar (orange colonies of putative *E. coli*, black colonies of *Proteus* species)



Fig. 5: Pink colonies of *E. coli* on MacConkey agar



Fig. 6: Metallic sheen of *Escherichia coli* on Eosin Methylene Blue agar



Fig. 7: Various biochemical tests results for *Escherichia coli*
From left: 1.Nitrate 2.MIL 3.Malonate 4.M.R. 5.Citrate 6.TSI 7.Gelatinase 8.Urease



Fig. 8: Pure culture of *Klebsiella* spp. on MacConkey agar



Fig. 9: Pure culture of *Proteus* spp. on Hektoen Enteric agar

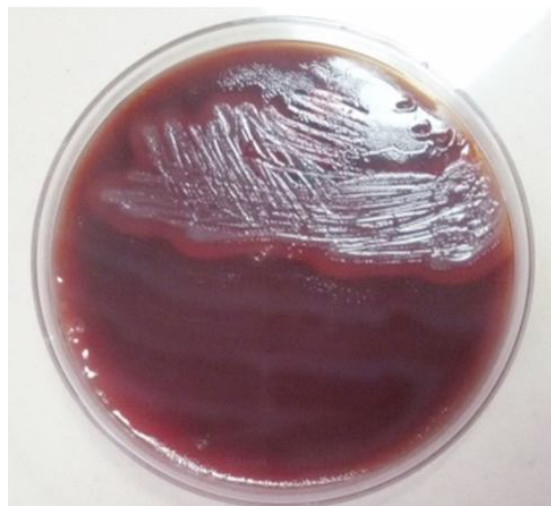


Fig. 10: Swarming growth of *Proteus* spp. on Blood agar

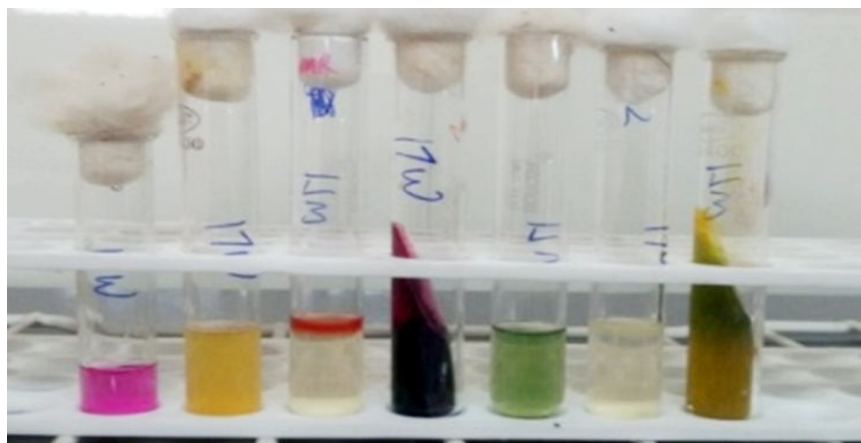


Fig. 11: Various biochemical tests results of *Proteus* spp.

From left: 1.Urease 2.Nitrate 3.M.R 4.TSI 5.Malonate 6.Gelatinase 7.Citrate

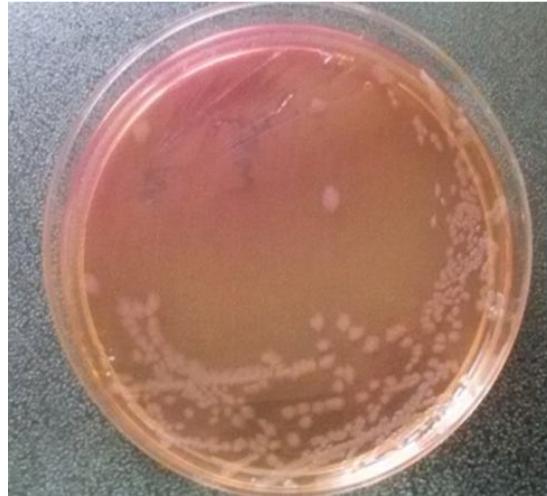


Fig. 12: Pure culture of *Pseudomonas* spp. on MacConkey agar



Fig. 13: Pure culture of *Pseudomonas* spp. on Hektoen Enteric agar

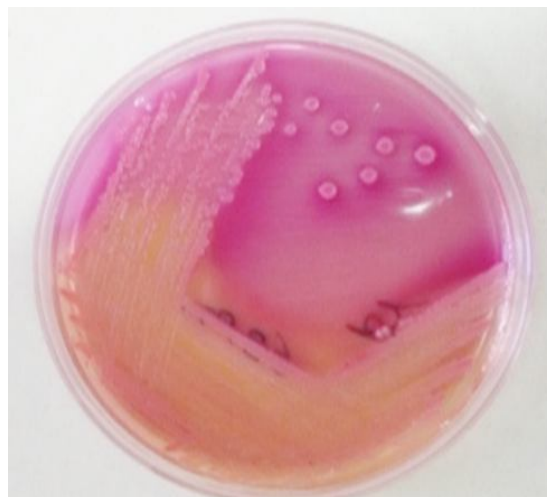


Fig. 14: Pure culture of *Enterobacter* spp. on MacConkey agar

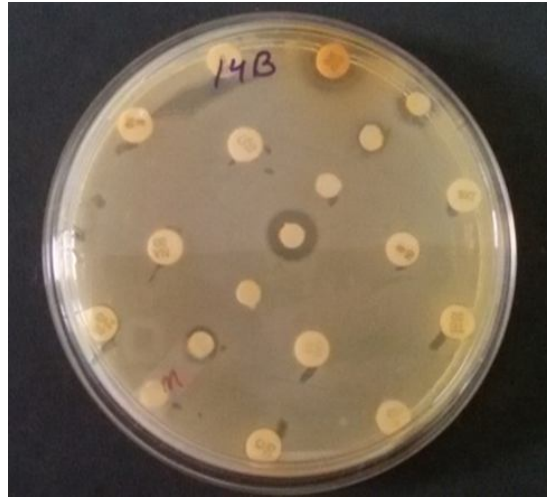


Fig. 15: Colistin Resistance seen in *Proteus* spp isolate



Fig. 16: Antibiotic sensitivity for *Proteus* spp. isolate



Fig. 17: a) Isolate showing D-effect between Piperacillin and Tazobactam combination, Piperacillin and Ceftriaxone with Imipenem individually. b) Key hole between Meropenem and Ampicillin

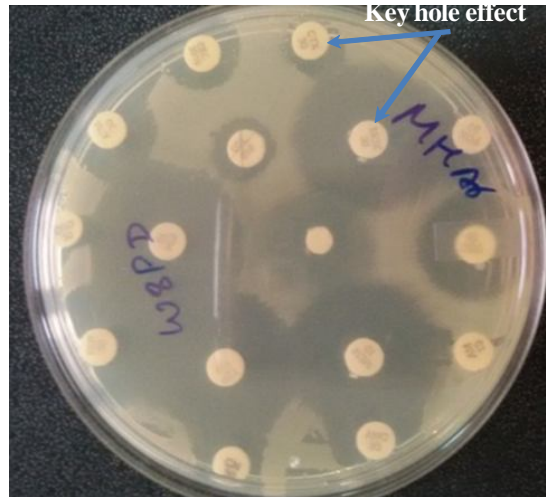


Fig. 18: Key hole effect between Ceftriaxone and Moxalactam in *E.coli* isolate

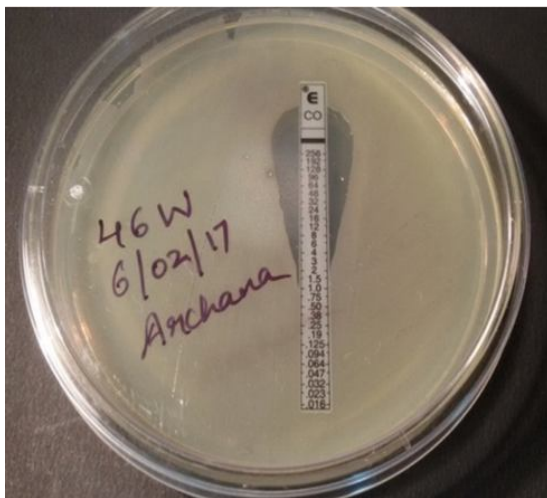


Fig. 19: MIC of Colistin showing sensitivity in *Pseudomonas* spp. isolate (MIC > 1 μ L)

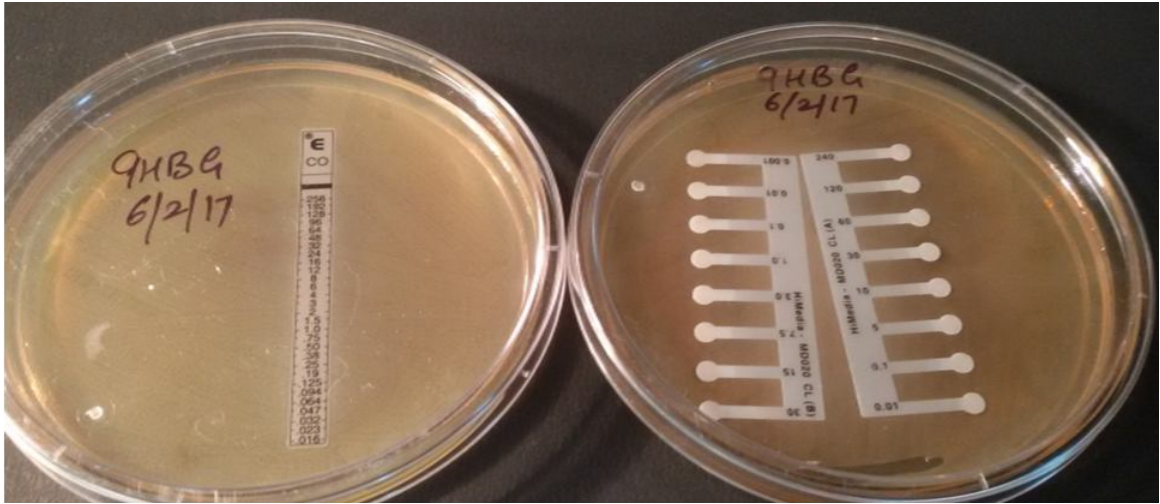


Fig.20: MIC result showing resistance in *Proteus* spp. isolate



Fig. 21: MIC result showing sensitivity in *E.coli* isolate (MIC>0.125 μ L)

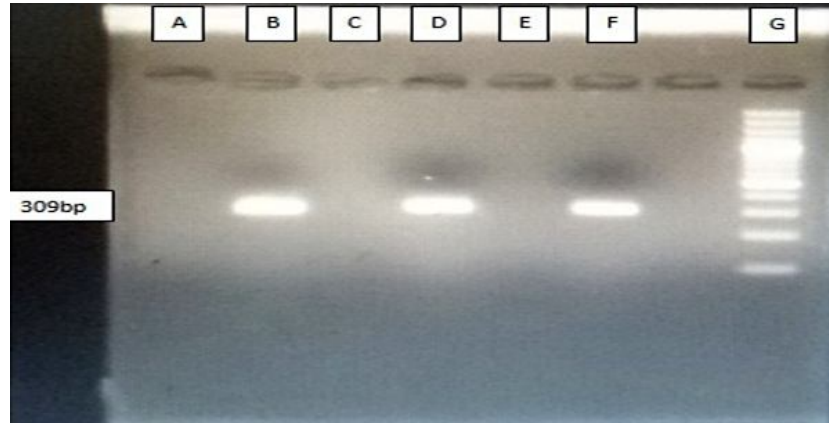


Fig 22: PCR result of different isolates and positive control

Lane A : Sample No. 5BH plasmid (negative result)

Lane B : 1:2 dilution (positive control)

Lane C : Sample No. 36B plasmid (negative result)

Lane D : 1:4 dilution (positive control)

Lane E : Sample No. 38B plasmid (negative result)

Lane F : 1:8 dilution (positive control)

Lane G : 100 bp ladder

PCR product size 309bp

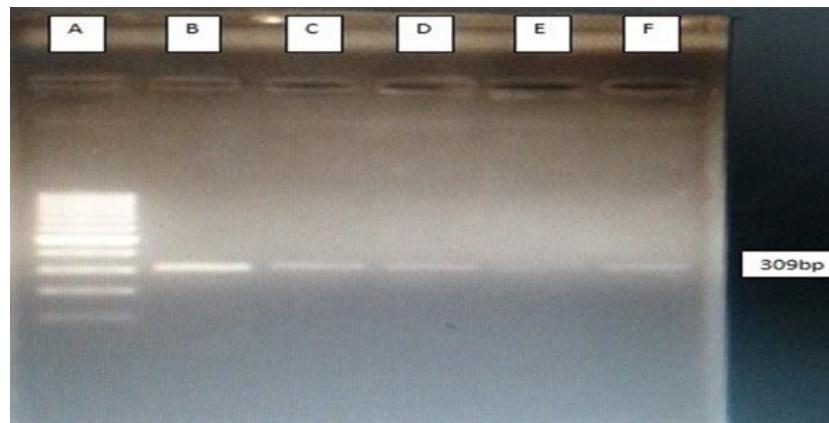


Fig 23: PCR result of positive control at different dilutions

Lane A : 100 bp ladder

Lane B : 1:10 dilution (positive control)

Lane C : 1:50 dilution (positive control)

Lane D : 1:100 dilution (positive control)

Lane E : 1:200 dilution (positive control)

Lane F : 1:400 dilution (positive control)

PCR product 309bp

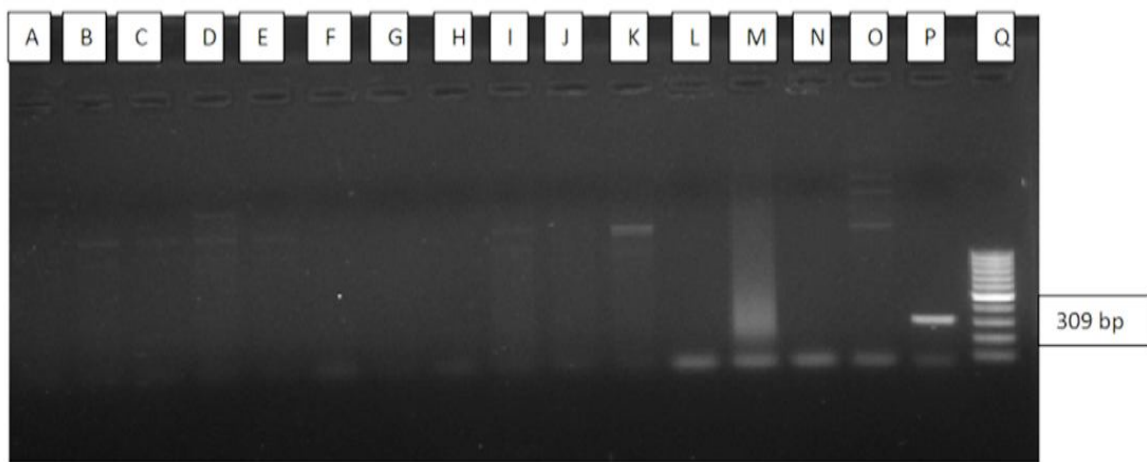


Fig 24: PCR results of different isolate (DNA template)

Lanes A to O : Negative result of different samples tested

Lane P: Positive control

Lane Q : 100 bp ladder

PCR product size 309 bp

Table 4: Multinomial logistic regression for colistin resistance in dogs and dog-owners' bacterial isolates

	Isolates from dog	Isolates from dog owner
Total isolate tested	403	75
No. of colistin resistant isolates	63	1
% Resistance	15.63	1.33
Odds Ratio	13.71	
p-value	0.001	

4.4.2 Effect of Breed of dog on colistin susceptibility of the bacterial isolates

On comparing the isolates from dogs (Table 5) of different breeds results indicated that bacteria from Indian Spitz (1.59%) were more often colistin sensitive than isolates from Great Dane (21.43%; $p=0.002$), Labrador (17.42%; $p=0.002$), German Shepherd (14.29%; $p=0.01$ for 95%), Pug (14.29%; $p=0.02$), Mongrel (13.77%; $p=0.01$) and Rottweiler (13.04%; $p=0.03$) (Graph 1).

Table 5: Multinomial logistic regression for colistin resistance in isolates from different breeds of dog as compared to isolates from Indian Spitz 63 (1.59%)

Different breeds compared to Indian Spitz	Isolate tested	Isolate resistant (%)	p-value
Great Dane	14	21.43	0.002
Labrador	132	17.42	0.002
German Shepherd	56	14.29	0.01
Pug	21	14.29	0.02
Mongrel	138	13.77	0.01
Rottweiler	23	13.04	0.03

Besides, isolates from Labrador dogs were significantly more resistant to chloramphenicol ($p=0.003$) and ciprofloxacin ($p=0.04$) than isolates from Indian Spitz. Similarly, isolates from Rottweiler breed (91.30%) were significantly ($p=0.002$) more resistant

to cefoxitin as compared to bacterial isolates from Indian Spitz (55.56%). Bacterial isolates from Pug breed dogs (57.14%) were significantly ($p=0.04$) more resistant to imipenem than isolates from Indian Spitz (31.75%). Bacterial isolates from Indian Spitz were also more often sensitive to cefoxitin ($p=0.02$), chloramphenicol ($p=0.004$) and ceftazidime ($p=0.05$) than isolates from German Shepherd. However, bacterial isolates from Mongrel were significantly more resistant to streptomycin ($p=0.04$), cefoxitin ($p=0.04$) and chloramphenicol ($p=0.05$) than isolates from Indian Spitz. Similarly, bacterial isolates from Great Dane were more often resistant to chloramphenicol ($p=0.01$) and guggal oil ($p=0.03$) than isolates from Indian Spitz. On the other hand, bacteria from Great Dane dogs were more commonly sensitive to amoxicillin+clavulanic acid ($p=0.04$), ampicillin ($p=0.0002$) and erythromycin ($p=0.01$) than isolates from Indian Spitz.

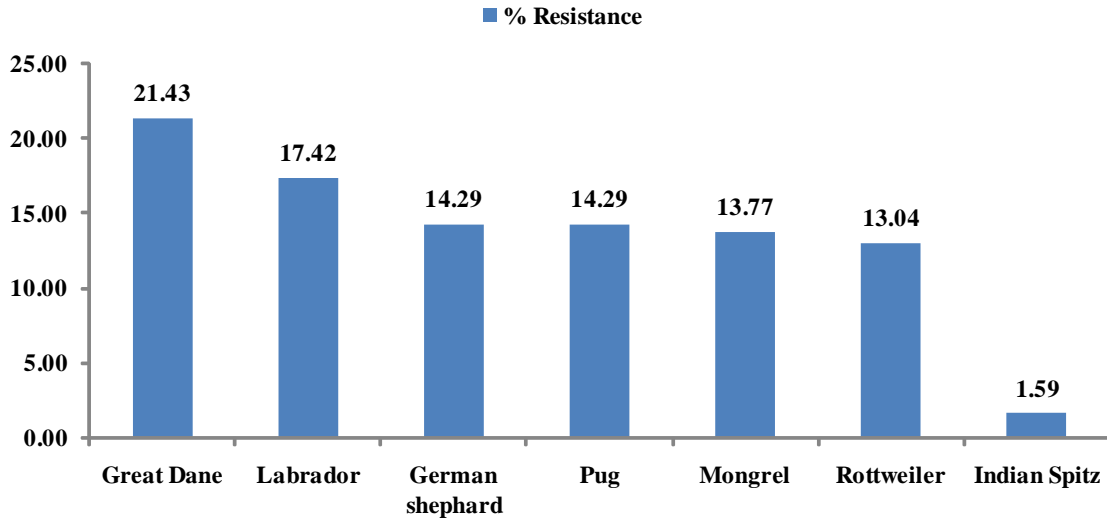
4.4.3 Effect of age of dog on antimicrobial sensitivity of bacteria isolated:

As per age sampled dogs were classified into four groups viz., pups (0-2month), young's (2-6month), adults (6-72month) and aged (>72month). More of the bacteria isolated from young dogs (66.35%) were significantly ($p=0.04$) resistance to gentamicin than bacteria from puppies (48.98%). Bacterial isolates from aged dogs were often resistance to moxalactam, piperacillin and erythromycin than isolates from puppies (Table 6). Multi (up to 6) drug resistance (MDR_6) was significantly ($p=0.02$) more common in bacteria from puppies (67.35%) than from aged dogs (47.37%).

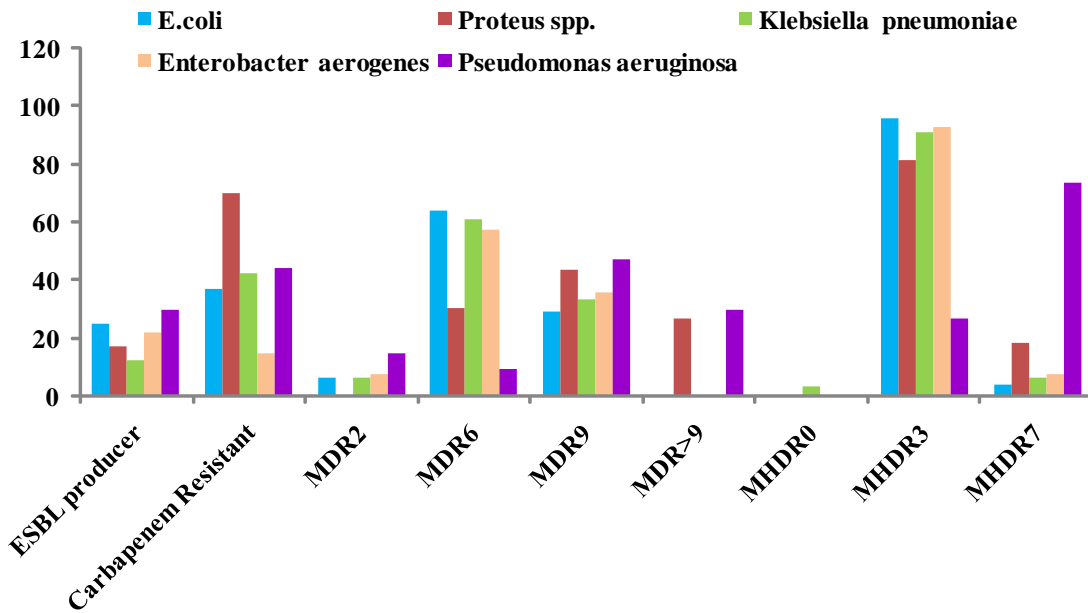
Bacterial isolates from young dogs (23.08%) were significantly more often ($p=0.01$) sensitive to imipenem than bacteria from adult dogs (37.39%). However, the isolates were more often resistant to aztreonam ($p=0.006$) and gentamicin ($p=0.01$) than bacteria from adult dogs. For imipenem ($p=0.05$), moxalactam ($p=0.02$), piperacillin ($p=0.05$), ciprofloxacin ($p=0.05$) and erythromycin ($p=0.01$) bacterial isolates from young dogs were more often sensitive than isolates from aged dogs (Table 7). Bacterial isolates from young dogs (62.50%) were significantly ($p=0.03$) more MDR_6 type than those from aged dogs (47.37%). However, bacterial isolates from old dogs (16.84%) were significantly ($p=0.05$) more multi (up to 7) herbal drug resistant ($MHDR_7$) than bacterial isolates from young dogs (7.69%). For nalidixic

Table 7: Multinomial logistic regression for different antibiotic resistance in isolates of young dogs and isolates of old dogs.

	MDR6	MHDR7	Imipenem	Moxalactam	Piperacillin	Ciprofloxacin	Erythromycin
Young dogs (% resistance)	62.50	7.69	23.08	14.42	44.23	45.19	90.38
Old dogs (% resistance)	47.37	16.84	35.79	27.37	57.89	58.95	98.95
O.R	1.85	0.41	0.54	0.45	0.58	0.57	0.10
Chi-T	0.03	0.05	0.05	0.02	0.05	0.05	0.01



Graph 1: Percent resistance to colistin seen in different breeds of dog



Graph 2: Percent of MDR, MHDR, Carbapenem resistant and ESBL producing bacteria's

acid ($p=0.02$) and erythromycin ($p=0.02$) bacterial isolates from old dogs were more often resistant than isolates from adult dogs.

Table 6: Multinomial logistic regression for different antibiotic resistance in isolates of puppies and isolates of old dogs

	Moxalactam	Piperacillin	Erythromycin	MDR>6
Puppies (% resistance)	10.20	38.78	89.80	67.35
Old dogs (% resistance)	27.37	57.89	98.95	47.37
Odds Ratio	0.30	0.46	0.09	2.29
P value	0.02	0.03	0.01	0.02

4.4.4 Antimicrobial sensitivity of bacterial isolates related to sex of dogs

Bacterial isolates from male dogs are significantly more commonly resistance to herbal drugs like ajowan oil ($p=0.01$), carvacrol ($p=0.01$), patchouli oil ($p=0.003$) and thymol ($p=0.01$) than bacteria isolated from female dogs. However, bacteria isolated from male dogs (79.12 %) were significantly ($p=0.01$) more often resistant to cefepime than those isolated from bitches (67.96 %). However, there was no significant ($p>0.05$) difference in sensitivity for other antimicrobials.

Table 8: Multinomial logistic regression for different antibiotic resistance in bacterial isolates of male and female dogs

	Ajowan Oil	Carvacrol	Patchouli Oil	Thymol Oil
% Male Dog resistance	10.77	9.43	97.31	10.10
% Female Dog resistance	3.87	3.31	91.16	3.31
Odds Ratio	3.00	3.04	3.50	3.28
Chi-T	0.01	0.01	0.003	0.01

4.4.5 Antibiotic resistance in bacterial isolated from clinically sick and healthy dogs

Tigecycline ($p=0.02$), ajowan oil ($p=0.004$), thymol ($p=0.02$) and carvacrol ($p=0.01$) resistance was more often seen in bacteria isolated from healthy dogs than from sick

dogs. Similarly, bacterial isolates from health dog were more often MHDR₇ (p= 0.04) and carbapenem resistant (p= 0.03) than bacteria from sick dogs. However, bacteria from sick dog were more commonly ESBL producers (p= 0.02) and MHDR₃ (p= 0.05) than bacteria from healthy dogs. There was no significant (p >0.05) difference in sensitivity of bacteria for other antimicrobials with respect to health status of the source dogs. Out of the total 34 *P. aeruginosa* isolates (85.29%) were from sick dogs.

Table 9: Multinomial logistic regression for different antibiotic resistance in bacterial isolates of healthy and sick dogs

	Healthy	Sick	p-value
ESBL producer	1(3.85%)	110(24.34%)	0.02
Carbapenem resistance	16(61.54%)	181(40.04%)	0.03
MHDR 3	20(76.92%)	404(89.38%)	0.05
MHDR 7	6(23.08%)	46(10.18%)	0.04
Tigecycline	5(19.23%)	32(7.08%)	0.02
Ajowan oil	6(23.08%)	33(7.30%)	0.004
Carvacrol	5(19.23%)	29(6.42%)	0.01
Thymol oil	5(19.23%)	31(6.86%)	0.02

4.4.6 Bacterial isolate of different species and their antimicrobial susceptibility pattern

Among different bacteria, most (93.33%) of the *Proteus* isolates and only a few of *E. coli*, *C. freundii* and *P. aeruginosa* isolates were colistin resistant (Table 10).

Table 10: Multinomial logistic regression for colistin resistance in different genera of bacterial isolate

	% Resistance	p-value
<i>E. coli</i>	1.49	1.20E-73
<i>Proteus</i> spp.	93.33	
<i>Pseudomonas</i> spp.	5.88	

Resistance pattern of bacteria isolated from dogs and dog owners revealed that none of the antimicrobial drug or herbal antimicrobials was able to inhibit growth of all the 478 isolates (Table 11). Among the herbal antimicrobials carvacrol (92.9%) was the most effective followed by thymol (92.5%), ajowan oil (91.8%) and cinnamon (90.6%). Among the antibiotics tigecycline was the most effective (92.3%) followed by colistin (86.6%) and moxalactam (81.8%).

Escherichia coli isolates were more commonly patchouli oil resistant ($p= 0.0003$) but more often sensitive to cinnamon oil ($p= 0.03$) than *K. pneumoniae* isolates. However, there was no significant ($p >0.05$) difference in sensitivity of the two bacteria for other antimicrobials.

Proportionately more *E. coli* isolates were sensitive to gentamicin ($p= 0.05$), nitrofurantoin ($p= 0.03$), ceftazidime ($p= 0.05$) and cinnamon oil ($p= 1.32E-07$), but more often resistant to patchouli oil ($p= 6.39E-10$), ceftriaxone ($p= 0.05$), cefotaxime and cefotaxime+clavulanic acid ($p= 0.03$) than *E. agglomerans* isolates.

Proteus spp. isolates were more commonly resistant than *E. coli* isolates to ciprofloxacin ($p= 7.69E-04$), chloramphenicol ($p= 6.26E-07$), nitrofurantoin ($p= 7.10E-12$), tigecycline ($p= 6.05E-14$) and carbapenems (imipenem and meropenem, $p= 1.70E-06$). Similarly *Proteus* spp. isolates were significantly ($p= 1.52E-05$) more MHDR₇ than *E. coli* isolates. However, *E. coli* isolates were more often MHDR₃ ($p= 3.23E-05$) than *Proteus* spp. isolates.

More of the *E. coli* isolates ($p= 8.32E-07$) were MDR₆ type than *Proteus* spp. isolates. However, for MDR₉ ($p= 8.32E-07$) and MDR₉ ($p= 4.24E-19$) was more common among *Proteus* spp. isolates than *E. coli* isolates.

Escherichia coli isolates were significantly more MDR₆ ($p= 4.650E-10$) and MHDR₃ ($p= 3.908E-36$) types than *P. aeruginosa* isolates. *Pseudomonas aeruginosa* isolates were more MDR₉ ($p= 0.03$), MDR₉ ($p= 1.581E-19$) and MHDR₇ ($p= 3.088E-37$) than *E. coli* isolates.

Enterobacter agglomerans isolates (85.71%) were significantly ($p= 0.04$) more often resistant to ceftazidime as compared to *K. pneumoniae* (54.55%).

Proteus spp. isolates were more often resistant to chloramphenicol ($p= 0.03$), ciprofloxacin ($p= 0.04$), nalidixic acid ($p= 0.04$), nitrofurantoin ($p= 7.365E-07$), tetracycline ($p= 0.01$), patchouli oil ($p= 0.04$) and carbapenem ($p= 0.01$) than *K. pneumoniae* isolates. However, *K. pneumoniae* isolates were significantly ($p= 0.004$) more MDR₆ type than *Proteus* spp. isolates.

Klebsiella pneumoniae isolates were more MHDR₃ ($p= 8.968E-08$) and MDR₆ ($p= 8.08E-06$) type than *P. aeruginosa* isolates. However, *P. aeruginosa* isolates were more MHDR₇ ($p= 1.813E-08$) and resistant to ceftazidime ($p= 0.002$ for), chloramphenicol ($p= 0.002$) and nitrofurantoin ($p= 0.002$) than *K. pneumoniae* isolates.

Proteus spp. isolates were more often resistant to carbapenems ($p= 1.32E-04$), patchouli oil ($p= 0.0002$) and tetracycline ($p= 0.01$) than *E. agglomerans* isolates but more sensitive to ceftazidime ($p= 0.03$) and cinnamon oil ($p= 0.03$) than the latter.

Pseudomonas aeruginosa isolates were significantly more often resistant to carbapenems ($p= 0.05$), ceftazidime ($p= 0.0004$), chloramphenicol ($p= 0.004$) and had MHDR₇ than *E. agglomerans* isolates. However, *E. agglomerans* isolates were more MDR₆ ($p= 0.0003$) and MHDR₃ ($p= 2.72E-05$) type and more often resistant to ceftazidime ($p= 0.05$) than *P. aeruginosa* isolates.

Proteus spp. isolates were more often carbapenem resistant ($p= 0.01$), MDR₆ ($p= 0.01$) and MHDR₃ ($p= 1.22591E-07$) types than *P. aeruginosa* isolates. However, more of the *P. aeruginosa* isolates were resistant to tigecycline ($p= 1.226E-07$), moxalactam ($p= 4.64E-05$), ampicillin ($p= 0.03$), ceftazidime ($p= 0.002$) and chloramphenicol ($p= 0.05$) and were more MHDR₇ ($p= 1.226E-07$) types than *Proteus* spp. isolates.

4.4.7 Correlation between sensitivity of bacteria to colistin and other antimicrobials

Positive correlation between sensitivity of bacteria to colistin was evident for tigecycline ($p= 0.0005$), imipenem ($p= 0.005$), nalidixic acid ($p= 0.025$), gentamicin ($p= 0.05$), chloramphenicol ($p= 0.005$), nitrofurantoin ($p= 0.0005$), tetracycline ($p= 0.025$), erythromycin ($p= 0.0005$) and patchouli oil ($p= 0.05$) but negative correlation to sensitivity of bacteria to

Table 11: Number of resistant isolates for different antimicrobials

Antimicrobial drug tested	Number of resistant isolates							Total, n, 478
	<i>E. coli</i> n, 336	<i>P. mirabilis</i> n, 56	<i>P. penneri</i> n, 4	<i>K. pneumoniae</i> n, 33	<i>E. agglomerans</i> n, 14	<i>P. aeruginosa</i> n, 34	<i>C. freundii</i> n, 1	
Amoxicillin+								
clavulanic acid	222	35	0	23	10	26	0	316(66.1%)
Ampicillin	303	42	1	31	12	31	1	421(88.1%)
Aztreonam	266	34	1	27	10	25	1	364(76.2%)
Cefepime	262	38	2	25	8	72	1	408(85.4%)
Cefotaxime	333	54	4	32	13	31	1	468(97.9%)
Cefotaxime+	333	54	4	32	13	30	1	467(97.7%)
clavulanic acid								
Cefoxitin	228	38	1	21	7	32	1	328(68.6%)
Ceftazidime	202	32	1	18	12	19	1	285(59.6%)
Ceftriaxone	283	42	1	25	9	26	1	387(80.9%)
Cephalixin	236	43	3	25	10	29	1	347(72.6%)
Chloramphenicol	75	31	1	10	4	25	1	147(30.7%)
Ciprofloxacin	156	40	2	16	6	20	1	241(50.4%)
Colistin	5	52	4	0	0	2	1	64(13.4%)
Cotrimoxazole	204	37	3	18	9	25	1	297(62.1%)
Erythromycin	307	56	4	32	13	32	1	445(93.1%)
Gentamicin	176	38	1	21	11	18	1	266(55.6%)
Imipenem	89	37	3	10	2	15	0	156(32.6%)
Meropenem	108	26	1	13	2	11	0	161(33.7%)
Moxalactam	57	5	0	9	0	15	1	87(18.2%)
Nalidixic acid	253	49	2	22	13	27	1	367(76.8%)
Nitrofurantoin	141	51	3	14	10	27	1	247(51.7%)
Piperacillin	168	24	0	14	4	18	1	229(47.9%)
Piperacillin+	133	21	0	11	3	14	1	183(38.3%)
tazobactam								
Streptomycin	240	40	3	21	11	22	1	338(70.7%)
Tetracycline	91	54	3	25	10	30	1	214(44.8%)
Tigecycline	1	10	1	0	0	25	0	37(7.7%)
Ajowan oil	5	7	0	1	1	25	0	39(8.2%)
Carvacrol	6	2	0	0	0	26	0	34(7.1%)
Cinnamon oil	8	4	1	3	4	25	0	45(9.4%)
Guggal oil	331	55	4	31	14	33	1	469(98.1%)
Lemon grass oil	323	52	4	32	14	31	1	457(95.6%)
Patchouli oil	327	54	4	28	9	31	1	454(95.0%)
Thymol	6	5	0	0	0	25	0	36(7.5%)

Table 12: Total number of isolates which are MDR, MHDR, Carbapenem resistant and ESBL producers

Type of bacteria	ESBL producer	Carbapenem Resistant		MDR2	MDR6	MDR9	MDR>9	MHDR0	MHDR3	MHDR7								
		Total	Colistin resistant															
<i>E. coli</i>	83	3	124	2	21	0	215	1	98	3	2	1	1	0	32	5	13	0
<i>Proteus penneri</i>	1	1	4	4	0	0	3	3	1	1	0	0	0	0	3	3	1	1
<i>Proteus mirabilis</i>	9	8	38	36	0	0	15	13	25	23	16	16	0	0	46	43	10	9
<i>Klebsiella pneumoniae</i>	4	0	14	0	2	0	20	0	11	0	0	0	1	0	30	0	2	0
<i>Enterobacter agglomerans</i>	3	0	2	0	1	0	8	0	5	0	0	0	0	0	13	0	1	01
<i>Pseudomonas aeruginosa</i>	10	0	15	1	5	0	3	1	16	0	10	1	0	0	9	1	25	1
<i>Citrobacter freundii</i>	1	1	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0

piperacillin (p= 0.05), ceftriaxone (p= 0.005), piperacillin + tazobactam (p= 0.005), and carvacrol (p= 0.01) was observed.

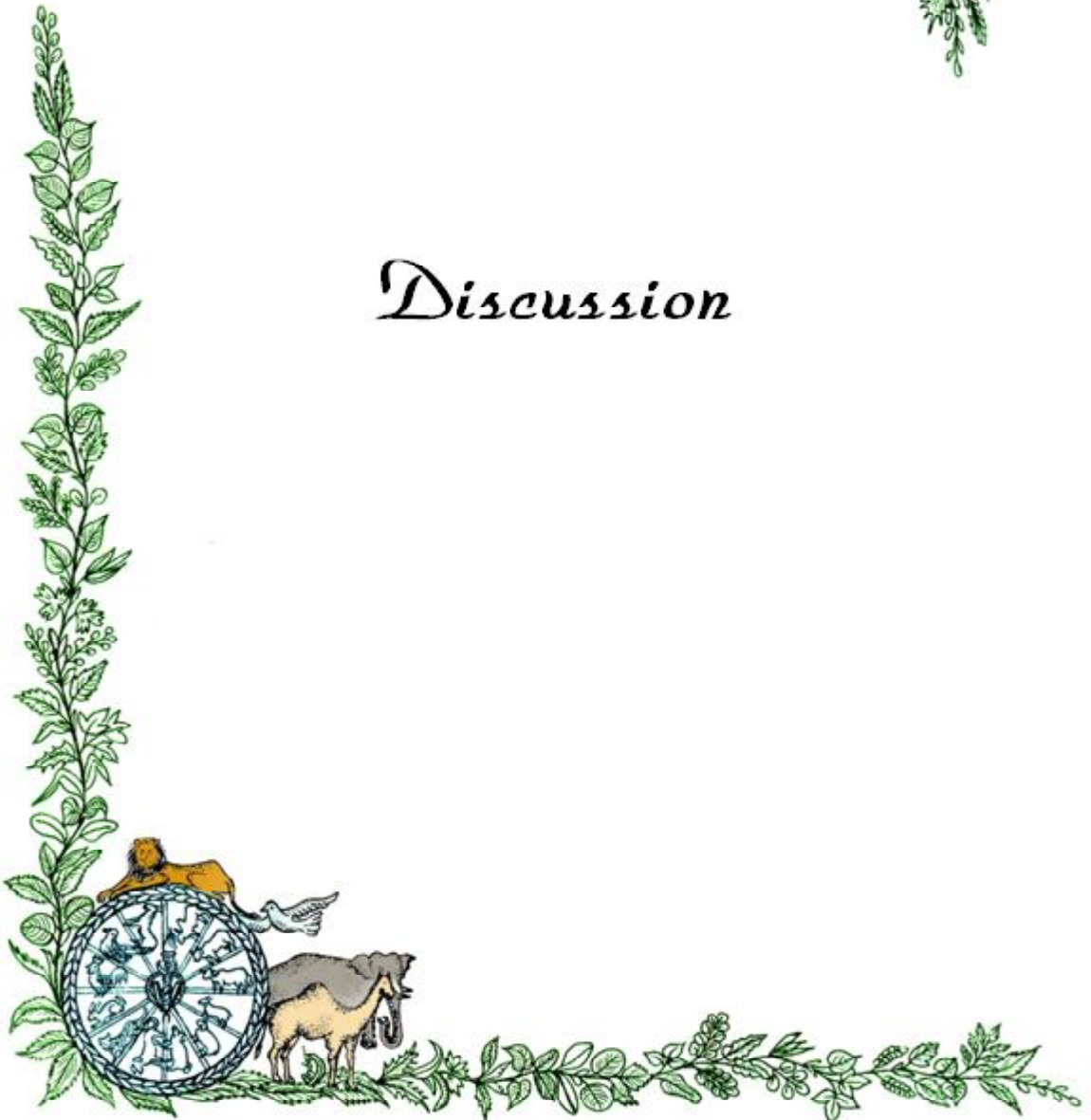
4.4.8 Colistin resistance in bacteria and antibiotics used previously in dogs for therapeutic purpose:

Most of the screened dogs were previously treated with antibiotics and among them most commonly used antibiotic was ceftriaxone and its combinations. In the study of the 478 isolates, 104 isolates were from those dogs which were earlier treated with ceftriaxone and its combinations. Resistance in those 104 isolates to ceftriaxone was 83.65% while among rest of the isolates 80.21% were ceftriaxone resistant. Isolates from dogs earlier treated with ceftriaxone were more often colistin resistant (16.35%) than isolates from dogs not treated with ceftriaxone or its combinations.





Discussion



Alarms sounded in the microbiology community in late 2015 when the first plasmid-mediated mobile gene *mcr-1* for colistin-resistance was identified in China. Soon after, the *mcr-1* gene has been reported in *Enterobacteriaceae* from humans and food-producing animals in various countries and continents (Lei *et al.*, 2016). As colistin is an ultimate line of refuge against fatal infections by multidrug-resistant Gram-negative pathogens (Gao *et al.*, 2016) resistance to colistin or its evolution in commensal or potentially pathogenic bacteria of public health significance is an important health threat. On their first introduction in early sixties polymyxins were not widely used because of their adverse effects like nephrotoxicity and neurotoxicity (Falagas and Kasiakou, 2006). However, with the rise of carbapenem resistant organisms, polymyxins gained attention as a compromise between their toxicity and utility as one of the few agents available for treatment of such MDR or XDR strains (Falagas *et al.*, 2005). However, recently, the emergence of the plasmid-borne transferrable (*mcr-1*) colistin resistance has greatly challenged the use of polymyxins too (Gao *et al.*, 2016).

Cats and dogs are considered to be the potent source for the spread of antimicrobial resistance due to the extensive use of antimicrobials in pets. Further their close contact with other animals as well as humans multiplies the risk many fold (Jayalakshmi, 2015). In dogs, ear infections, pyoderma, wound infections, gastroenteritis and urinary tract infections are the most common bacterial infections treated with antimicrobials (Pedersen *et al.*, 2007). Colistin sulfate may be used to treat intestinal infections, to suppress the flora of colon and also used as topical creams, powders and otic solutions (Biswas *et al.*, 2012). Certain infections in dog can be long-standing and may predispose for development of resistance due to repeated or prolonged antimicrobial treatment (Pedersen *et al.*, 2007).

Though scanty information on colistin resistant microbes in animals including dogs in India is available, reports from other countries indicated that animals might be the reservoir of colistin resistant bacteria. However, in recent studies colistin resistant, no *mcr* gene positive bacteria could be isolated from dogs (Buess *et al.*, 2016). Thus, it is important to confirm the occurrence of colistin resistant bacteria on dogs in different regions. To examine epidemiological factors associated with colistin resistance in Enterobacteriaceae family (a group of commensal, many potentially pathogenic and noscomial pathogens) may be important to formulate strategies to reduce or prevent emergence of colistin resistance in animals especially pet animals living in close proximity of humans. Considering the need and present knowledge, the study was carried out to identify the colistin resistant Enterobacteriaceae members on perianal area of pet dogs and hands of their owners. Bacterial isolates were phenotypically and genotypically characterized for colistin resistance in association with various epidemiological factors.

From 216 perianal swabs of pet dogs and 58 owners' hand swabs collected from different locations of Bareilly, India i.e., TVCC, Referral Veterinary Polyclinic, Indian Veterinary Research Institute and Bareilly local city yielded a total of 478 bacterial isolate. Detailed proforma for history with proper technique and sterilized condition was taken into account. The bacterial isolates were identified belonging to different species included *Escherichia coli* (336), *Enterobacter agglomerans* (14), *Citrobacter freundii* (1), *Proteus mirabilis* (56), *Proteus penneri* (4), *Klebsiella pneumoniae* (33), *Pseudomonas aeruginosa* (34) (not included in Enterobacteriaceae). In earlier studies too, (Singh *et al.*, 1994) similar type of bacteria (*E. coli*, *K. pneumoniae* subsp. *aerogenes*, *Enterobacter* spp., *P. mirabilis*, *Kluyvera* spp. and *Providencia stuartii*) were reported on perianal area of dogs but no *P. aeruginosa* was reported on perianal region of pet dogs in Bareilly. Isolation of *P. aeruginosa* in present study on dog perianal swabs might be due to commensal nature of it on dogs' skin and also being a part of the normal gastrointestinal microflora (Miller *et al.*, 2012). Isolation of *P. aeruginosa* from sick dogs was more frequent (85.29%) than from healthy dogs (14.71%). It might be due to the inclusion of samples from clinical cases under hospital settings, as *P. aeruginosa* has been identified as the most common pathogen causing nosocomial infection (Rosas *et al.*, 2015).

Of the 478 isolates tested, 64 isolates (*E. coli*, 5; *Citrobacter freundii*, 1; *Proteus mirabilis*, 52; *Proteus penneri*, 4 and *Pseudomonas aeruginosa*, 2) were colistin resistant through disk diffusion method. Further analysis of minimum inhibitory concentration (MIC) revealed that 61 isolates (*P. mirabilis*, 56; *P. penneri*, 4 and *Citrobacter freundii* 1) were colistin resistant (CLSI, 2015). Besides, the four colistin sensitive *Proteus* strains (by disc diffusion assay) also tested resistant using MIC method. Similar discrepancies have been reported among various disk diffusion methods and MIC-based methods earlier (Foe *et al.*, 2007). The discrepancy has been reported as one of the main challenge in testing colistin and polymyxin susceptibility of bacteria (Balaji *et al.*, 2011). Polymyxins are large cationic peptide molecules and hence they do not diffuse rapidly through agar well; thus disk diffusion is not a reliable but an indicator method (Pragasam *et al.*, 2017).

The present study on comparing bacterial isolates from dog owners and dog revealed that the bacterial isolates from dogs were significantly more often resistant (15.63%) to colistin ($p=0.001$) than those from dog owners (1.33%). *Escherichia coli* could be isolated both from dogs and their owners respectively in case of 67 pairs, similarly *P. aeruginosa* from 3 pairs and *K. pneumoniae* from 2 pairs. Although *P. aeruginosa*, *K. pneumoniae* and *E. coli* strains isolated from dogs and their owners were apparently similar but could be differentiated on the basis of their antibiogram and thus identity could not be established between the paired isolates of from a dogs and their owners. There are no reports from India on companion animals establishing relation between bacterial isolates from dog and its owner but researchers in China have reported that only one *E. coli* isolate from a pet owner was colistin-resistant (*mcr-1*-positive) of the total 25 Enterobacteriaceae isolates from pet owners tested on the other hand they identified 47 *E. coli* and 2 *K. pneumoniae* isolates positive for *mcr-1* out of the total of 566 Enterobacteriaceae strains from the companion animals (Lei *et al.*, 2016). Similarly in Egypt high prevalence of *mcr-1* strains have been reported in animals than in human clinical isolates (Elnahriry *et al.*, 2016). Resistant bacteria in animals might be associated with more use of the antibiotic in the animals. European Centre for Disease Prevention and Control (ECDC, 2016) stated that the use of colistin is much higher in animals than in humans. Apart from showing colistin resistance, the *mcr-1* gene was also identified in bacteria isolated

from pet shop in China where they tested 39 dogs and 14 cats and found four dogs and two cats to be carrier of *mcr-1* positive bacteria (Zhang *et al.*, 2016). Use of hand sanitizers by human may be the reason for less number of Enterobacteriaceae isolates from the human hand swabs. Kampf and Hollingsworth (2008) reported that the hand gel (85% ethanol, w/w) reduced many Gram-positive as well as Gram-negative bacteria including members of Enterobacteriaceae family.

In the present study out of 478 isolates all *Proteus* strains and one *Citrobacter freundii* were resistant to colistin. This may be because of intrinsic resistance of *Proteus* spp. to colistin (Shimizu *et al.*, 1977; Olaitan *et al.*, 2014; Erfanimesh and Hashemi, 2016) as the cell envelope of *Proteus* spp. prevents access of the drug to the susceptible lipid target sites (Sud and Feingold, 1970). Though, in the present study *mcr-1* gene was not detected in *C. freundii* strains resistant to colistin, recently Li and co-workers (2017) reported *mcr-1* in *C. freundii*.

This study could not evidenced occurrence of *mcr-1* in Enterobacteriaceae in dogs as the gene could not be amplified by specific primers used as per standard protocol with high sensitivity while the positive control always yielded the required amplicon. There are no reports of *mcr-1* gene positive bacteria from pets in India. Similarly, in Switzerland bacterial isolates from urine and wounds of pets were found negative for *mcr-1* and *mcr-2* gene in PCR (Simmen *et al.*, 2016). Even the colistin resistant isolates in the study were *mcr-1* negative which might be due to a different mechanism of colistin resistance. Further the mechanistic aspects of the *mcr-1* colistin resistance are still poorly understood as phenotypically colistin sensitive strains has also shown to be *mcr* positive and *vice-versa* too (Gao *et al.*, 2016).

Frequency of isolation of colistin resistant isolates from sick dogs (13.5%) was slightly higher than from healthy dogs (11.5%) without much statistical difference. Isolation of colistin resistant bacteria from both sick and healthy dogs might be due to the fact that most of the colistin resistant bacteria in the study were commensal in nature including *Proteus* and *Citrobacter* strains (Singh *et al.*, 1994). In previous studies too colistin-resistant strains were isolated from healthy individuals (Olaitan *et al.*, 2014).

There is no significant difference in occurrence of colistin resistance among the bacterial isolates from dogs of either sex but isolates from male (12.46%) dogs were slightly less often

resistant to colistin than those isolated from female (14.92%) dogs. Though reports are scanty with respect to colistin resistance, sex-related differences in sensitivity to other antimicrobial has been reported (Hoekstra and Paulton, 2002). Similarly, there is no significant difference in colistin resistance among the bacterial isolates from dogs of different age groups. A slightly lower rate of colistin resistance in bacteria from puppies and young dogs than in isolates from adult and old dogs might be due to the less exposure of young and puppies to the antibiotic as the clinical use of antibiotics in juveniles is limited due to danger of antimicrobial toxicity (Hoekstra and Paulton, 2002).

There was positive correlation between sensitivity of bacteria to colistin with sensitivity to tigecycline ($p=0.0005$), imipenem ($p=0.005$), nalidixic acid ($p=0.025$), gentamicin ($p=0.05$), chloramphenicol ($p=0.005$), nitrofurantoin ($p=0.0005$), tetracycline ($p=0.025$), erythromycin ($p=0.0005$) and patchouli oil ($p=0.05$) but negative correlation to sensitivity of bacteria to piperacillin ($p=0.05$), ceftriaxone ($p=0.005$), piperacillin + tazobactam ($p=0.005$), and carvacrol ($p=0.01$). Reports have mentioned colistin resistance in carbapenem-resistant *K. pneumoniae* (Kalem *et al.*, 2016; Gallet *et al.*, 2017) and *E. coli* harboring *mcr-1* and *bla*_{NDM-5} gene (Mediavilla *et al.*, 2016). Synergy between meropenem and ciprofloxacin with colistin at subinhibitory concentrations (Pankuch *et al.*, 2008) might be cause of correlation between colistin resistance and resistance to other antibiotics. Co-emergence of tigecycline and colistin resistant in *Acinetobacter baumannii* in human patients has also been reported (Taneja *et al.*, 2011).

Probability of ceftriaxone resistance among bacteria isolated from ceftriaxone (the most commonly used antibiotic in dogs) treated dogs (83.65%) was higher than in bacteria isolated from non-antibiotic-treated dogs (80.21%). Observations are in concurrence to earlier reports revealing an increase in resistance to ceftriaxone due to increased prescribing of the drug (Conus and Francioli, 1992; Muller *et al.*, 2004). Moreover, isolates from the dogs treated with ceftriaxone and its combinations were more often colistin resistant (16.35%) than those from non-ceftriaxone treated dogs (12.57%). However, ceftriaxone or colistin resistance in bacteria isolated from ceftriaxone treated and non-ceftriaxone treated dogs had no statistically significant difference. In contrast, when isolates were more sensitive to ceftriaxone they were

less sensitive to colistin and had a strong ($p= 0.005$) negative correlation among zone of inhibition by colistin and ceftriaxone. Either use of ceftriaxone as drug of choice in dogs have or not have association with non-isolation of colistin resistant *E. coli* and other potential pathogens is not lucid from the study and need more targeted studies.

There was significant difference in colistin resistance among bacterial isolates from dogs of various breeds. The highest colistin resistance was seen in the bacteria isolated from Great Dane (21.43%; $p= 0.002$) followed by those from Labrador (17.42%; $p= 0.002$), German Shepherd (14.29%; $p= 0.01$), Pug (14.29%; $p= 0.02$), Mongrels (13.77%; $p= 0.01$), Rottweiler (13.04%; $p= 0.03$) and the least number of colistin resistant isolates were from Indian Spitz (1.59%). The reasons behind the observation are not lucid; however the diet, habits and affinity to owners might be an important factor yet to be evaluated in further studies. No earlier study on this aspect of colistin resistance in bacteria from different breeds of dogs to compare the observations could be cited. Further investigations are needed in respect to colistin resistance and its spread in dogs and other pets in India in light of reports from China suggesting pet foods as an important source of colistin resistant bacteria for companion animals (Lei *et al.*, 2016).

The data obtained from the dogs in the study provide a baseline to understand prevalence of colistin resistance in pets in India. The information may help in understanding the epidemiology of antimicrobial resistance and its spread in dogs.

The study concludes that:

1. In the study only 60 *Proteus* spp. and 1 *Citrobacter freundii* were resistance to colistin phenotypically. The colistin resistance in *Proteus* spp. (93.33%) isolates was significantly higher than in isolates of other members of Enterobacteriaceae family.
2. Colistin resistance was more common in bacterial isolates from dogs (15.63%) than isolates from their owners (1.33%).
3. Isolates from Indian Spitz were the least often resistant to colistin (1.59%) than from dogs of other breeds, highest resistance was in isolates from Great Dane (21.43%; $p= 0.002$) followed by those from Labrador (17.42%; $p= 0.002$), German Shepherd

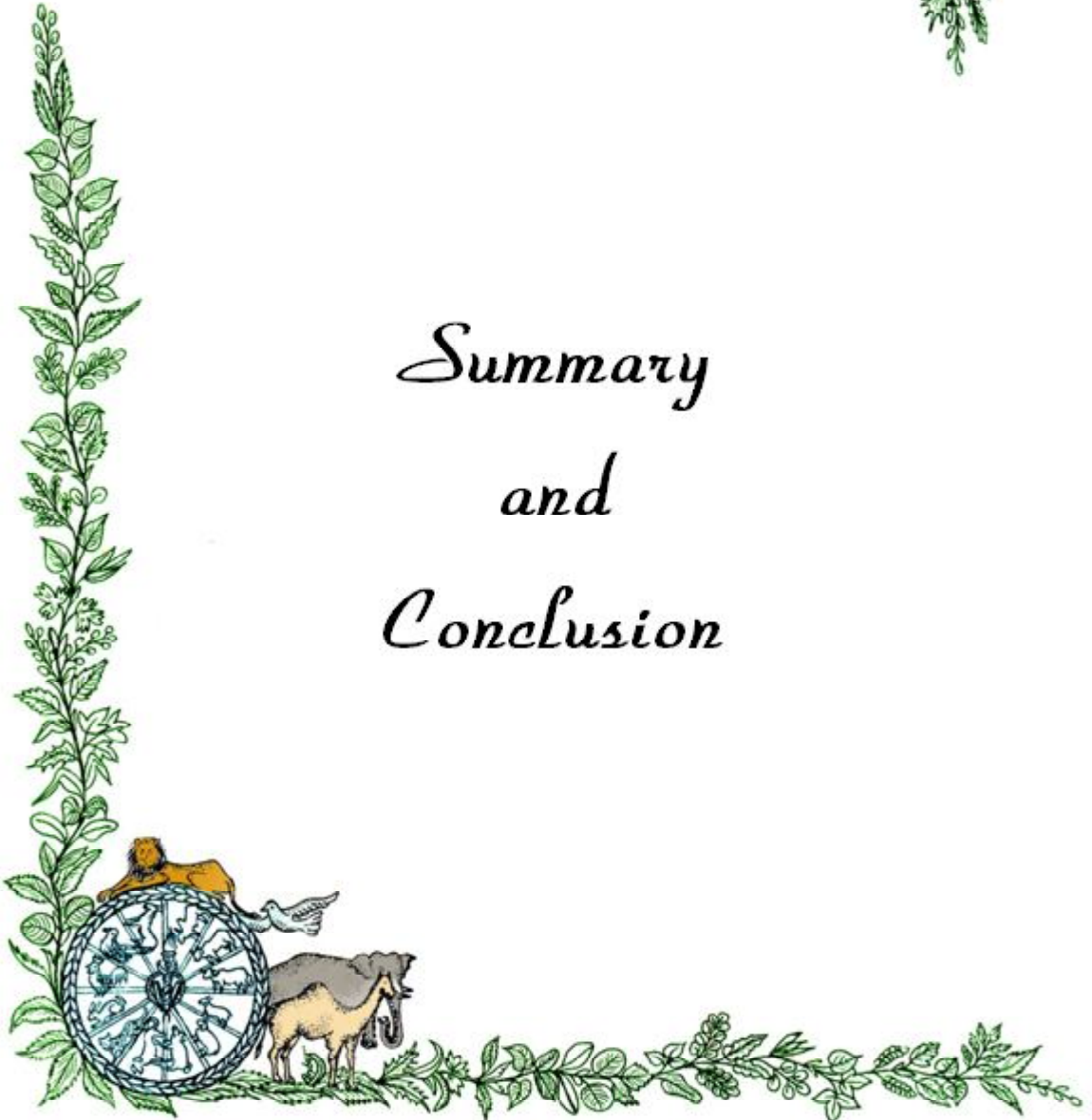
(14.29%; $p= 0.01$), Pug (14.29%; $p= 0.02$), Mongrels (13.77%; $p= 0.01$) and Rottweiler (13.04%; $p= 0.03$).

4. Positive correlation was evident between sensitivity of bacterial isolates to colistin and sensitivity to tigecycline ($p= 0.0005$), imipenem ($p= 0.005$), nalidixic acid ($p= 0.025$), gentamicin ($p= 0.05$), chloramphenicol ($p= 0.005$), nitrofurantoin ($p= 0.0005$), tetracycline ($p= 0.025$), erythromycin ($p= 0.0005$) and patchouli oil ($p= 0.05$) but correlation was negative with sensitivity of bacteria to piperacillin ($p=0.05$), ceftriaxone ($p= 0.005$), piperacillin + tazobactam ($p= 0.005$) and carvacrol ($p= 0.01$).
5. Though insignificant, ceftriaxone resistance in isolates from dogs previously treated with the same drug was more (83.65%) than (80.21%) dogs not-treated ceftriaxone.
6. The study indicated that transferrable colistin resistance is not common in bacteria on dogs and their owners in and around Bareilly.





*Summary
and
Conclusion*



The global rise of MDR gram-negative bacteria is persistently alarming and represents an increasing threat to health of animals as well as humans. Colistin (polymixin E) is a cationic, multicomponent lipopeptide antibacterial agent killing most of the gram-negative bacteria (including *Acinetobacter*, *Pseudomonas*, *Escherichia* and *Klebsiella* species) through disrupting the cell membrane. Because of its nephrotoxicity, use of colistin is restricted for topical infections except in case of extensively drug resistant bacteria causing systemic or deeply located infection. Identification of transferable plasmid-mediated colistin resistance gene (*mcr-1*) in *E. coli* in 2015 rung an alarm about the future of the drug as last line treatment. Besides, plasmid mediated colistin resistance may develop in gram-negative bacteria through chromosomal mutations or adaptive mechanisms. In view of importance of colistin in modern therapeutics and emergence of resistance to it, the present study was conducted to understand the occurrence of colistin resistant Enterobacteria and associated epidemiological factors in dogs.

In the study, 216 perianal swabs of dogs and hand swabs from 58 dog owners were collected along with information of breed, age, sex, health status and antibiotic used in dog. Samples were collected from TVCC, Referral Veterinary Polyclinic, Indian Veterinary Research Institute as well as from Bareilly (Uttar Pradesh) and nearby localities.

For preliminary examination the collected samples were incubated in buffered peptone water (BPW) for 3-4 hours for 37 °C. Then primary isolation of Enterobacteriaceae was done on MacConkey agar and Hektoen Enteric agar with the incubation at 37 °C for 24 h. A total

of 478 bacterial isolates were identified belonging to different species like *Escherichia coli* (336), *Enterobacter agglomerans* (14), *Citrobacter freundii* (1), *Proteus mirabilis* (56), *Proteus penneri* (4), *Klebsiella pneumoniae* (33) and *Pseudomonas aeruginosa* (34) (not included in Enterobacteriaceae).

The bacterial isolates were tested for antibiotic sensitivity by disk diffusion assay against antimicrobials including amoxicillin and clavulanic acid (AMC, 30µg), ampicillin (AMP, 10µg), aztreonam (AT, 30µg), cefepime (FEP, 30µg), cefotaxime (CTX, 10µg), cefotaxime and clavulanic acid (CTX-CLA, 30/10µg), ceftazidime (CAZ, 30µg), ceftriaxone (CRO, 30µg), cephalexin (CN, 30µg), chloramphenicol (C, 30µg), ciprofloxacin (CIP, 5µg), colistin (CL, 10µg), cotrimoxazole (SXT, 23.75/1.25µg), erythromycin (E, 10µg), gentamicin (GM, 10µg), imipenem (IPM, 10µg), meropenem (MRP, 10µg), moxalactam (MOX, 30µg), nalidixic acid (NA, 30µg), nitrofurantoin (N/F, 300µg), piperacillin (PIP, 100µg), piperacillin and tazobactam (TZP, 110µg), streptomycin (S, 10µg), tetracycline (TE, 30µg), tigecycline (TGC, 15µg), vancomycin (VA, 5µg), ajowan oil (2µL), carvacrol (2µL), cinnamon oil (2µL), guggal oil (2µL), lemon grass oil (2µL), patchouli oil (2µL) and thymol (2µL). The EDTA disk (~1900 µg) was put close to carbapenem disk to look for synergy as an indicator of metallo-β-lactamase production. Out of the 478 isolates of bacteria tested, 64 were resistant to colistin (5 *E. coli*; 1 *C. freundii*; 52 *P. mirabilis*; 4 *P. penneri* and 2 *P. aeruginosa*). However, using MIC methods to determine colistin resistance only 61 isolates (56 *P. mirabilis*, 4 *P. penneri* and 1 *C. freundii*) were found to be resistant. The statistical analysis revealed that resistance to colistin was significantly more often in *Proteus* (93.33%) strains than in of *Pseudomonas* ($p= 5.263E-17$; 5.88%) and *E. coli* ($p= 1.20E-73$; 1.49%) isolates. Despite repeated attempts *mcr-1* gene could not be detected either in chromosomal DNA or plasmid DNA preparations of any of the isolates in the study using PCR.

The bacterial isolates from dogs were significantly more often resistant (15.63%) to colistin ($p= 0.001$) than those from their owners (1.33%). *Escherichia coli* could be isolated both from dogs and their owners respectively in case of 67 pairs, similarly *P. aeruginosa* from 3 pairs, and *K. pneumoniae* from 2 pairs. Although *P. aeruginosa*, *K. pneumoniae* and *E. coli* strains isolated from dogs and their owners were apparently similar but could be

differentiated on the basis of their antibiogram. The identity could not be established between the paired isolates of from a dogs and their owner. Distribution of colistin resistant isolates in dogs was independent of their sex or age and health status. However, breed of dog appeared as an important determinant to affect carriage of colistin resistant bacteria by dogs. Colistin resistant bacteria were more commonly isolated from Great Dane (21.43%; $p= 0.002$), Labrador (17.42%; $p= 0.002$), German Shepherd (14.29%; $p= 0.01$), Pug (14.29%; $p= 0.02$), Mongrel (13.77%; $p= 0.01$) and Rottweiler (13.04%; $p= 0.03$) than from Indian Spitz (1.59%).

There was a positive correlation between sensitivity of bacteria to colistin and tigecycline ($p= 0.0005$), imipenem ($p= 0.005$), nalidixic acid ($p= 0.025$), gentamicin ($p= 0.05$), chloramphenicol ($p= 0.005$), nitrofurantoin ($p= 0.0005$), tetracycline ($p= 0.025$), erythromycin ($p= 0.0005$) and patchouli oil ($p= 0.05$) but had negative correlation with sensitivity of bacteria to piperacillin ($p= 0.05$), ceftriaxone ($p= 0.005$), piperacillin + tazobactam ($p= 0.005$), and carvacrol ($p= 0.01$).

Most of the screened dogs were previously treated with antibiotics and ceftriaxone and its combination was the drug of choice. Resistance to ceftriaxone in bacteria isolated from ceftriaxone treated dogs was higher (83.65%) than in bacteria isolated from non-ceftriaxone treated dogs (80.21%).

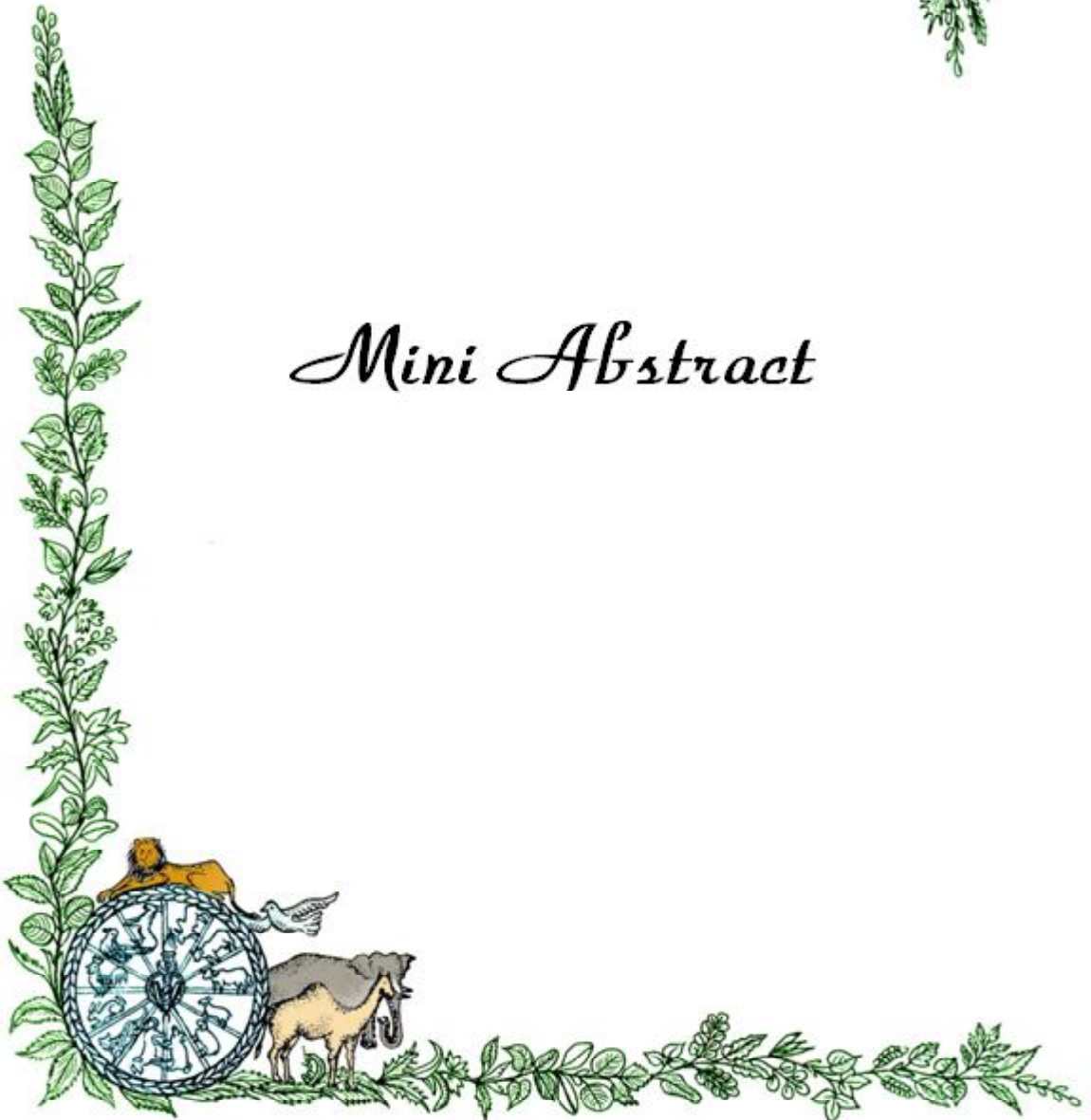
The study revealed that out of all members of Enterobacteriaceae family isolated from dogs and their owner only *Proteus* spp. (93.33%) and *C. freundii* strains had resistance to colistin. Though phenotypically 61 strains were confirmed colistin resistant, *mcr-1* gene responsible for transferable colistin resistance could not be detected in any of the strains. Colistin resistance was more common in bacterial isolates from dogs (15.63%) than from their owners (1.33%). Indian Spitz was identified as the least prone breed of dogs to carry colistin resistant bacteria and Great Dane (21.43%) as the most prone breed for colonization of colistin resistant bacteria on perianal region followed by Labrador (17.42%), German Shepherd (14.29%), Pug (14.29%), Mongrel (13.77%) and Rottweiler (13.04%). Ceftriaxone resistance in isolates from dogs previously treated with the same drug was more common (83.65%) than

in isolates from dogs not-treated with ceftriaxone (80.21%). The study indicated that transferrable colistin resistance is not common in bacteria isolated from perianal region of pet dogs and from hands of their owners in and around Bareilly.





Mini Abstract



A cross-sectional study was conducted to estimate the extent of colistin resistance in Enterobacteriaceae isolates from perianal region of pet dogs. A total of 216 perianal swabs of dogs and 58 swabs of willing pet owners were collected from I.V.R.I polyclinic and local area of Bareilly and analyzed. Of the total 274 swab samples, a total of 478 bacterial isolates were identified. The bacterial isolates were characterized as *E. coli* (336), *E. agglomerans* (14), *C. freundii* (1), *P. penneri* (4), *P. mirabilis* (56), *K. pneumoniae* (33) and *P. aeruginosa* (34) (a non Enterobacteriaceae member). The phenotypic screening of bacteria for antibiotic sensitivity by disk diffusion technique identified 64 (5 *E. coli*, 2 *P. aeruginosa*, 1 *C. freundii*, 52 *P. mirabilis* and 4 *P. penneri*) colistin resistant bacteria. However, minimum inhibitory concentration (MIC) studies for colistin indicated 61 (4 *P. penneri*, 56 *P. mirabilis* and 1 *C. freundii*) isolates being truly resistant to colistin. The colistin MIC for resistant isolates was always ≥ 256 $\mu\text{g/ml}$. *Escherichia coli* could be isolated both from dogs and their owners respectively in case of 67 pairs, similarly *P. aeruginosa* from 3 pairs and *K. pneumoniae* from 2 pairs. Although *P. aeruginosa*, *K. pneumoniae* and *E. coli* strains isolated from dogs and their owners were apparently similar but could be differentiated on the basis of their antibiograms indicating difference in bacteria of the same species isolates from dog and its owner. On PCR screening for *mcr-1* gene none of the colistin resistant isolate was detected positive. Further, the multinomial logistic regression analysis for understanding epidemiology of colistin resistance revealed that bacterial isolates from dogs (15.63%) were significantly ($p= 0.001$) more often colistin resistant than isolates from their owner (1.33%). Colistin resistance was the most common in isolates from Great Dane (21.43%) followed by those from Labrador (17.42%), German Shepherd (14.29%), Pug (14.29%), Mongrel (13.77%) and Rottweiler (13.04%) and Indian Spitz (1.59%). Most of the *Proteus* spp. (93.33%) strains were colistin resistant probably due to their intrinsic property of being colistin resistant. The isolates when analyzed according to age, sex and health status of dogs did not show any significant difference in resistance to colistin. There was positive correlation between sensitivity of bacteria to colistin and tigecycline ($p= 0.0005$), imipenem ($p= 0.005$), nalidixic acid ($p= 0.025$), gentamicin ($p= 0.05$), chloramphenicol ($p= 0.005$), nitrofurantoin ($p= 0.0005$), tetracycline ($p= 0.025$), erythromycin ($p= 0.0005$) and patchouli oil ($p= 0.05$) but correlation was negative with respect to sensitivity to piperacillin ($p= 0.05$), ceftriaxone ($p= 0.005$), piperacillin + tazobactam ($p= 0.005$), and carvacrol ($p= 0.01$). Most of the screened dogs were previously treated with antibiotics and ceftriaxone was the most commonly used antibiotic. Ceftriaxone resistance in bacteria isolated from ceftriaxone treated dogs (83.65%) was slightly higher than dogs not treated with ceftriaxone (80.21%). Isolates from dogs treated with ceftriaxone were more often colistin resistant (16.35%) than those from dogs not treated with ceftriaxone. The study indicated that transferrable colistin resistance is rare in bacteria from perianal area of pet dogs and hands of their owners at Bareilly.



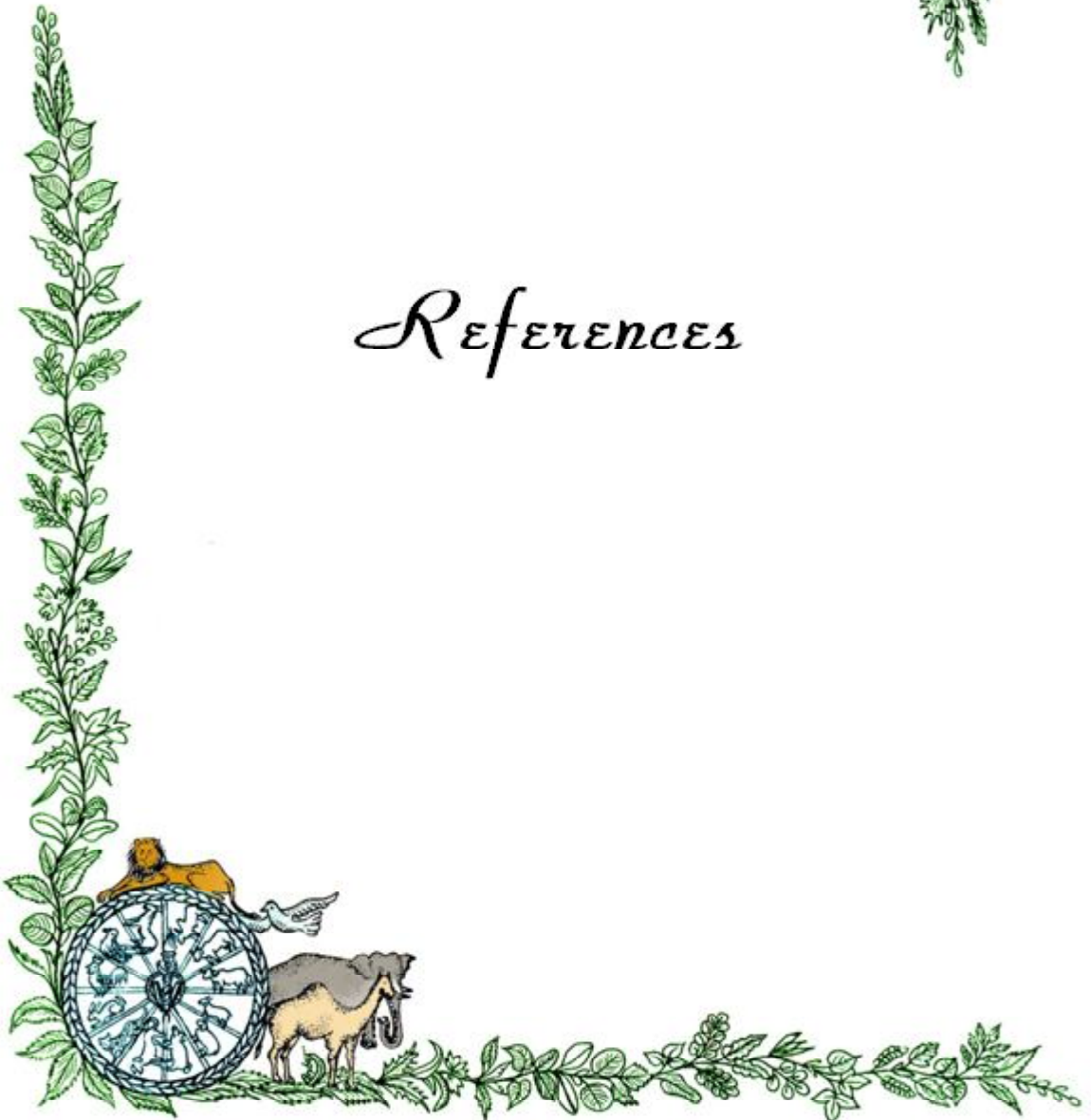
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 dksyflVv & ifrjksk I {ke thok.kq “okuka vls mudsikydkal eaughak; stkr; k cgq de ik; stkrsgA



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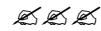
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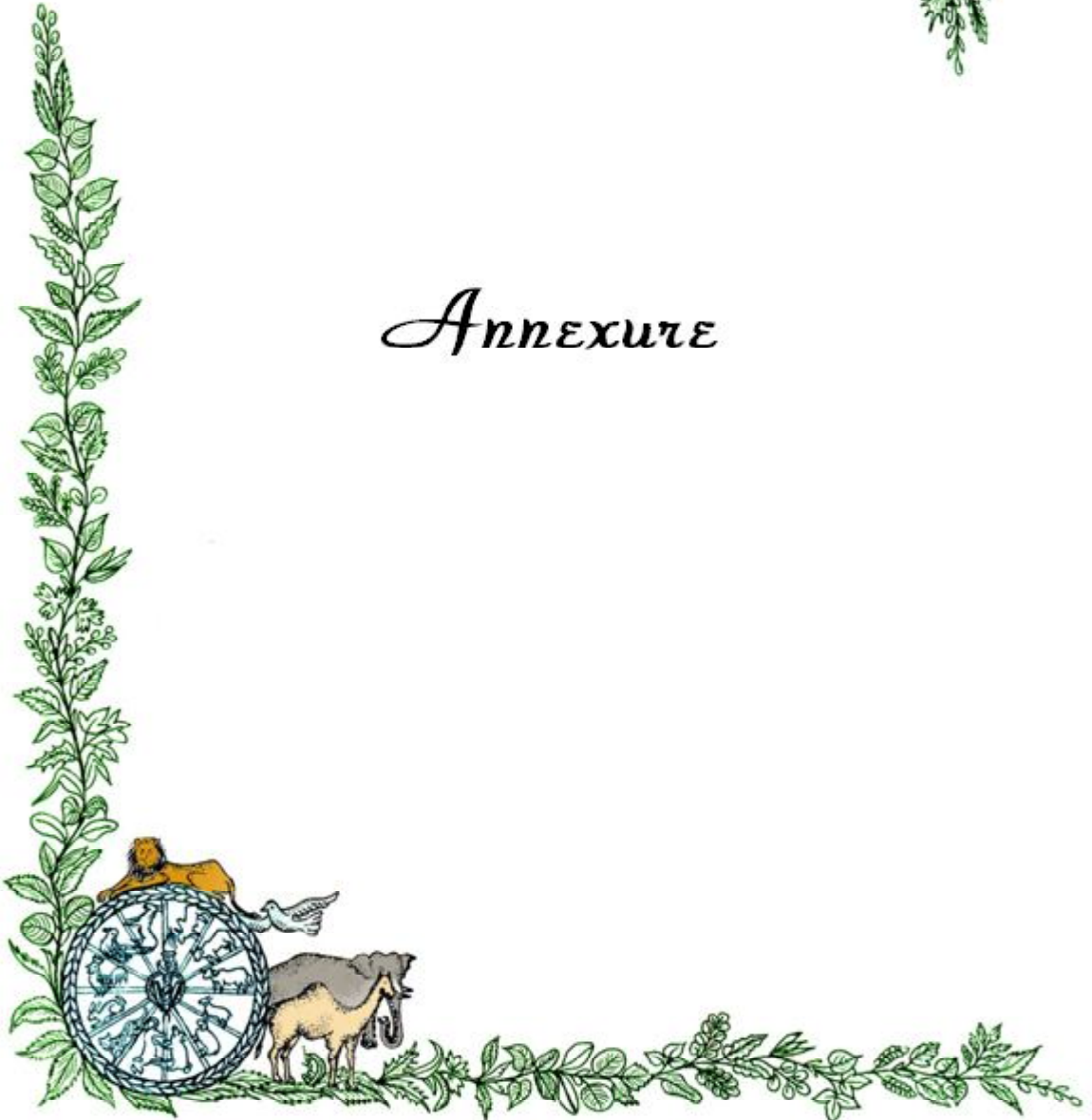
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Annexure



ANNEXURE I

PROFORMA FOR COLLECTION OF CLINICAL SAMPLE DIVISION OF EPIDEMIOLOGY

INDIAN VETERINARY RESEARCH INSTITUTE (IVRI)
IZATNAGAR, BAREILLY- 243 122 (UP)

Date.....

1. Animal No :
2. Breed :
3. Sex :
4. Age :
5. Clinical sample collected :
- (Feces, urine, blood, pus, serum, vaginal swab, placenta, aborted fetus material etc)
6. Type of wound/clinical condition :
7. Antibiotics previously used in treating the animals :
8. Antibiotics supposed to be used now :

Signature of Veterinary Officer

ANNEXURE II

Gram's stain

1 Ammonium oxalate crystal violet

Solution 1: Crystal violet	2.0 gm
Ethyl alcohol (95 percent)	20.0 ml
Solution 2: Ammonium oxalate	0.8 gm
Distilled water	80.0 ml

Solution 1 and 2 were mixed well and then filtered.

2 Lugol's (Gram's) iodine solution

Iodine	1.0 gm
Potassium iodide	2.0 gm

The ingredients were dissolved and then filtered.

3 Acetone or Ethyl alcohol (decolorizer)

4 Safranin (counter stain)

Safranin -O (2.5 percent solution) in 95 percent alcohol	10 ml
Distilled water	100 ml

Solutions for agarose gel electrophoresis

a) 0.5 M EDTA (pH 8.0)

EDTA (Disodium dihydrate salt)	37.4 g
Double distilled water	200 ml

The pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 200 ml.

b) TBE buffer (5 X)

TRIS Base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml

The final volume was made up to 1000 ml with double distilled water.

c) Ethidium bromide stock (10 mg/ml)

Ethidium bromide	10 mg
Double distilled water	1 ml

The ethidium bromide was dissolved in water and stored at 4°C in amber colored tubes.

d) Agarose gel

Agarose	1.50 g
TBE (1 X)	100 ml
Ethidium bromide (1%)	5 µl

Name : **Dr. Archana Saraf**
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Educational Qualifications:

Degree	Year of passing	College/ Institute	OGPA
B.V.Sc. & A.H.	2015	GBPUAT, Pantnagar, Uttarakhand	7.484
M.V.Sc.	2017	Deemed University ICAR-Indian Veterinary Research Institute, Izatnagar	8.230

Membership

- ☞ Lifetime member of Veterinary Council of India
- ☞ Lifetime member of Uttarakhand State Veterinary Council

Fellowship:

- ☞ ICAR-JRF fellowship (M.V.Sc.)