STUDIES ON PREVALENCE, RISK FACTORS AND ANTIBIOTIC RESISTANCE OF BACTERIA ISOLATED FROM DIARRHOEIC DOGS WITH SPECIAL EMPHASIS ON *Escherichia coli*

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Science University in partial fulfillment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY MICROBIOLOGY
(Minor Subject: Animal Biotechnology)

By
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CERTIFICATE – I

This is to certify that the thesis entitled, **"STUDIES ON PREVALENCE, RISK FACTORS AND ANTIBIOTIC RESISTANCE OF BACTERIA ISOLATED FROM DIARRHOEIC DOGS WITH SPECIAL EMPHASIS ON *Escherichia coli*"** submitted for the degree of **M.V.Sc.**, in the subject of **Veterinary Microbiology** (Minor Subject: **Animal Biotechnology**) of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Eshan Kaushal (L-2018-V-61-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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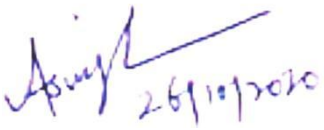

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ABSTRACT

Escherichia coli is a Gram negative bacterium belonging to family *Enterobacteriacea.* is a bacterial commensal of the intestinal microflora of a variety of animals, including humans. Not all *E. coli* strains are harmless, as some are able to cause disease in humans as well as in mammals and birds. In the present study a total of 100 faecal samples from healthy and diarrhoeic dogs were processed for isolation of various bacteria including *E.coli.* Out of 100 samples, 67 isolates of *E.coli* were obtained and all the isolates were identified by cultural, morphological and various biochemical tests. The isolates were further confirmed as *E.coli* by genus specific PCR and an amplification band of 232 bp was observed in isolates as well as the positive control. Out of 100 samples, other bacteria isolated were *Staphylococcus* spp (14),*Klebsiella* spp (13) & *Bacillus* spp (6). Prevalance of various bacteria isolated viz *E.coli*,*Staphylococcus*,*Klebsiella* was 67%,14%,13% and 6%.All the PCR confirmed *E. coli* isolates were tested for resistance against 12 different antibiotics *viz.* Nalidixic acid(5mcg) Tetracycline(30mcg), Ampicillin /Sulbactum (10/10mcg), Trimethoprim (5mcg), Streptomycin (10mcg), Colistin (10mcg) , Ciprofloxacin (5mcg), Amikacin (30mcg) , Co-trimoxazole (25mcg) Furazolidone (10mcg), Gentamicin (10mcg) Doxycycline (30mcg).Culture Sensitivity Test of *E.coli* , revealed highest resistance to ampicillin/ Sulbactam (100%), Nalidixic acid (91.7%), Amikacin (81%).The prevalence of various antibiotic resistance genes viz *bla TEM*, *tetA*, *tetB* and *aadA* in all *E. coli* isolates was determined by PCR. Out of 67 *E. coli* isolates, 43 isolates were positive for the presence of *blaTEM* gene, 23 for *tetB* , 36 for *tetA* and 11 for *aadA.*

Keywords: Dogs, *E.coli*, *bla* TEM, *tetA*, *tetB* and *aadA*

Signature of Major Advisor **Signature of the Student**

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LIST OF ABBREVATIONS

%	:	Percent
µg	:	Microgram
µl	:	Microlitre
°C	:	Degree Cellists
Avg.	:	Average
bp	:	Base pair
CNF	:	cytotoxic necrotizing factors
STEC	:	Shiga toxin-producing *Escherichia coli*
VGs	:	Virulence genes
COT	:	Co-trimoxazole
g	:	Gram
AMC	:	Amoxicillin Clavulanic acid
EMB	:	Eosin methylene blue agar
EtBr	:	Ethidium bromide
etal	:	Et alia (Latin otherworkers)
Fig.	:	Figure
GADVASU	:	Guru Angad Dev Veterinary and Animal Sciences University
GEN	:	Gentamicin
mA	:	Milli ampere
mg	:	Milligram
min	:	Minute
ml	:	Milliliter
mM	:	Milli molar
OD	:	Optical Density
PCR	:	Polymerase chain reaction
Taq	:	*Thermus aquaticus*
hr	:	Hour
i.e.	:	That is
1U	:	International Unit

MCT	:	Microcentrifuge tube
MDR	:	Multiple drug
resistant mg	:	Milli gram
MHA	:	Muller-Hinton
agar ml	:	Milli litre
MLA	:	McConkey's lactose
agar mm	:	millimeter
MR	:	Methyl red test
VP	:	Voges- Proskauer's test

CHAPTER I

INTROD.UCTION

"There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring 'fastness' [resistance]

— Alexander Fleming, 1946

Antibiotic resistance can be simply defined as the ability of microorganisms to survive and reproduce in the presence of antibiotic doses that were previously thought effective against them. In infectious disease clinical practice, antibiotic resistance that results in clinical failure is governed by the bioavailability of the antibiotics (Wright, 2007). Failures of antimicrobial chemotherapy due to resistance were observed soon after the introduction of penicillin in clinical practice and, thereafter, the emergence of resistance has always followed the release of new antibiotics. However, these "miracle drugs" are losing their efficacy due to the emergence of antimicrobial resistance. Releasing large quantities of antibiotics into the environment during manufacturing process of pharmaceutical products in conjugation with inadequate waste water treatment increases the risk of development of antibiotic-resistant strains (Larsson *et al* 2009)**.**

Some of the bacterial pathogens have become resistant to many antibiotics by exhibiting the phenomenon called multi-drug resistance (MDR). A bacterial isolate is considered to be MDR, when it exhibits resistance against three or more antibiotics. Indeed, some strains have become resistant to practically all of the commonly available agents, irrespective of the chemical class or molecular target of the drug (Martinez *et al* 2009). In contrast to that, the pace of new antibiotic drug development has slowed during the last decade. The pessimistic mood of the professional community has been recently expressed in a report by the Infectious Diseases Society of America (IDSA) entitled "Bad bugs, no drugs" (Boucher *et al,* 2009)**.** In other words, the increasing emergence of bacterial pathogens that are resistant to most or all antibiotics currently available for clinical use has plunged the phenomenon of antibiotic resistance into a major crisis, which threatens to turn the clock back to the pre-antibiotic era.

The emergence of antibiotic resistance is not confined to the particular geographical boundaries, countries or continents. It may happen anywhere and

anytime (Srivastava *et al*, 2011)**.** Resistance to the current library of antibacterial drugs is a serious problem in all parts of the world including the Asia-Pacific region, Latin America, Europe, and North America. The overall rate of resistance is often found to be larger in developing than in developed countries. In developing countries, the increasing rate of antibiotic resistance, largely due to widespread and irrational use of antimicrobial agents in hospital and community is a cause of great concern (Lakshmi, 2008)**.** In addition, overcrowding, poor hygiene, poorly staffed and poorly equipped hospitals allow for a rapid spread, slow detection and grim outcome of multi-resistant infections much more often in developing countries than developed countries. (Amabile-Cuevas, 2010).

Multidrug resistance in *Escherichia coli* has become a worrying issue that is increasingly observed not only in human but also in veterinary medicine worldwide. *E. coli* is intrinsically susceptible to almost all clinically relevant antimicrobial agents, but this bacterial species has a great capacity to accumulate resistance genes, mostly through horizontal gene transfer. *E. coli* of animal origin often show resistance to most of the older-antimicrobial agents, including tetracycline, phenicols, sulfonamides, trimethoprim, and fosfomycin. Plasmids, especially multiresistance plasmids, but also other mobile genetic elements, such as transposons and gene cassettes in class 1 and class 2 integrons, seem to play a major role in the dissemination of resistance genes (Poirel *et al*, 2018).

E.coli is a Gram-negative, facultative anaerobe and nonsporulating bacterium. Cells are typically rod-shaped, and are about 2.0 μm long and 0.25–1.0 μm in diameter, with a cell volume of 0.6–0.7 μm^3 (Lee *et al* 2009). *Escherichia coli* is a bacterial commensal of the intestinal microflora of a variety of animals, including humans. Not all *E. coli* strains are harmless, as some are able to cause disease in humans as well as in mammals and birds (Dho-Moulin *et al*, 1999). Pathogenic *E. coli* strains fall into two categories: those that cause intestinal pathologies and those that cause extraintestinal pathologies. Intestinal pathologies mostly consist of more or less severe diarrhoea caused by different *E. coli* pathotypes such as enterotoxinogenic, enteropathogenic or enterohaemorragic *E. coli* (ETEC, EPEC and EHEC, respectively), potentially evolving into a haemolytic uremic syndrome (HUS) in the case of EHEC infections (Kaper *et al*, 2005).

Resistance to antimicrobials is a very complex problem involving different bacterial organisms, mechanisms of resistance, mechanisms of transfer and reservoirs. Several studies have shown that the use of antimicrobials in animals leads to antimicrobial resistance development and poses risks to humans due to the transmission of resistant zoonotic bacteria via the food chain and the indirect transfer of resistance genes from animals to humans (Kruse 1999)

Pathogenic forms of *E. coli* can cause healthy organisms to develop a gastrointestinal disease (Feng *et al* 2002). *E.coli* are colonized within a few hours of birth in the gastrointestinal tract. Commensal *E.coli* normally communicates in a mutually beneficial way with the host. But some strains of *E.coli* develop virulence characteristics that can induce a wide range of illnesses.

There are different classes of pathogenic *E. coli* strains which are responsible for outbreaks of diarrhea. These include Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enetroaggregative *E. coli* (EAEC), Enteroinvasive *E.coli* and diffusely adherent *E. coli* (DAEC) (Orlando *et al* 2006). Control of *Escherichia coli* infections in veterinary medicine has become especially problematic due to the emergence of multiple-antibiotic-resistant *E. coli* in companion animals (Bass *et al* 1999).

EHEC serotype strains O157:H7 are a established zoonotic pathogen. It is well known that farm animals namely cattle, pigs and goats(O157:H7) constitute an EHEC reservoir, (Manning *et al*., 2008). PCR assay identified the AMR genes for betalactamase {(bla(CMY), bla(SHV), bla(TEM)}, tetracycline (tet(A), tet(B), and tet C)}, sulphonamide (sul1, sul2 and sul3) and aminoglycoside (strA and strB). Genes of resistance can spread on mobile genetic elements such as plasmids, transposons and integrons (Schwarz and Chaslus-Dancla 2001)

The size of the global population is uncertain but is positively associated with that of humans. Depending on different reports, with 700 million dogs, it may be as large as one-tenth of the world population. People's attitudes towards dogs differ widely, ranging from being helpful to humans only to being significant threats to public health. A range of studies have shown that the human-dog relationship has a positive effect on human well being, child growth and quality of life. The use of dogs as service animals, and the "medicine" of companion animals, is increasingly

recognized as important areas leading to improving human health. However, dog related issues exist; they lead to zoonotic diseases and filthy pavements in public parks. Dogs also cause harm to public health through bites; pet harassment; livestock and wildlife; their constant barking particularly at night: rubbish sites; and their involvement in road accidents (Macpherson *et al*,.2013)

The population of Indian dogs is estimated at a million and can be classified into four categories: pets (limited and supervised); family dogs (partially limited, wholly dependent); neighbourhood dogs (unrestricted, partially dependent); and feral dogs (unrestricted, independent) (Menezes,2008). Large pet populations can be responsible for the harm to public health.

In the developed world, dogs and cats live in close contact with human beings and dog numbers have increased. Mainly, the presence of dog faeces in urban areas due to dog owner's habit of not removing dog faeces from the street may present a hygienic and public health issue. Bacteria that are pathogenic to the intestinal tract and cause diarrhoea include *Campylobacter, Samonella, Yersina* and *E. coli* (Aleksic *et al*, 1987; Beutin, 1999; Lefebver et at, 2008; Chaban *et al*,2010).

The appearance of verotoxigenic *E.coli* by the exposure of foodstuffs of animal origin as human pathogens has been a significant public health issue. Infected cattle and sheep are considered VTEC's main reservoir, with spread in humans and animals associated with the ingestion of infected beef products (Griffin and Tauxe 1991) or direct contact with animals and animal feces on the farm. Approximately 29 strains of *E.coli* were identified from sheep and 16 strains from goats. Owing to farming practices, veterinary and human commensal microbiota, especially intestinal microbiata, are subject to numerous antimicrobial pressures. They have been suggested to play a significant role in the spread of bacterial resistance (Kang *et al* 2005).

Tetracycline, alone or in conjunction with other antibiotics, has been an effective antibiotic for prophylaxis or treatment. For most bacteria, tetracycline resistance is due to the development of new genes, often connected to mobile elements. Typically these genes are associated with plasmids and/or transposons, and also are conjugative. Currently there are 38 different *tet* and *otr* genes identified including 23 genes that code for energy-dependent efflux proteins, 11 genes that code

for ribosomal proteins and three genes that code for an inactivating enzyme and one gene which has an unknown resistance mechanism (Roberts 2005). A number of new genera of bacteria have been identified with *tet (A), tet (B), tet (C), tet(D), tet(G), tet (H), tet (K), tet (L),*effux genes and/or *tet (M), tet (O), tet (S), tet(Q), tet(W),*ribosomal defensive genes. Some of these genes were present in Gram-positive genera as well as Gram negative genera (Speer *et at* 1992). Tetracycline resistance in *Enterobacteriaceae* is usually the duty of the *tet(A)* to *tet (E)* determinants, depending on the species (Fluit and Schmitz 2004)**.** *Escherichia coli* can gain high penicillin resistance in two separate ways, either as a result of multiple consecutive alterations affecting at least two chromosome genes (Boman *et at* 1967), or as a result of the addition of an extra-chromosomal R factor. Both forms of resistance were due to cell-bound penicillinase production. It isolated the enzyme made form a chromosomal resistance gene (ampA) and the corresponding wild-type protein (Lindstrom *et at* 1967).

Before the 1980s, plasmid-mediated L-lactamases, such as TEM-1, TEM-2 and SHV-1, triggered the most common problem associated with L-lactamases of Enterobacteriaceae. Later on the development of β-lactams resistant to hydrolysis and the use of combinations of β-lactams and β-lactams inhibitors are the two key strategies that have been pursued to overcome this problem. The introduction of these compounds into therapy was followed rapidly by the emergence of multiple β-lactamases. These enzymes capable of hydrolysing β-lactams oxyimino were isolated in the mid-1980s and are variants of well-established penicillinases TEM and SHV (Thomson *et al* 2000)**.**

Since scientific knowledge on antimicrobial resistance and resistance genes in *E. coli* isolated from dogs faecal sample is scarce in the region of study, therefore, the present study was carried out with the following objectives:

1. Isolation and identification of different bacteria from faecal samples of diarrhoeic dogs and assessment of various associated risk factors.
2. To study the culture sensitivity patterns of isolated bacterial species.
3. Molecular detection of antibiotic resistance genes in *E.coli* isolated from faecal samples of dogs.

CHAPTER II

REVIEW OF LITERATURE

2.1 An overview of antimicrobial resistance

The quest for appropriate and preventive therapeutic regimens started in the 19th century when infectious agent was discovered, however successful treatment only came in the 20th century with the advent and introduction of antibiotics; an organic molecule that inhibits or kills microbes by specific interactions with the bacterial targets without any consideration of the source of the particular compound or class (Davies and Davies, 2010). Early successes marking the beginning of chemotherapy against bacterial infectious disease was the use of Salvarsan as an anti-syphilitic drug during the World War I and the use of sulfonamide. However the real breakthrough into the world of antibiotics occurred in late 1930s and early 1940s when Penicillin and Streptomycin were discovered by Alexander Fleming and Selman Waksman respectively. This achievement was the greatest in the history of medicine and their use in clinical practice saved countless lives (Davies and Davies, 2010). Nevertheless, this success was short lived as emergence and spread of antimicrobial resistance was observed among the bacterial population.

The first reported case of antibiotic resistance was documented after the introduction of sulfonamides, since then, development of specific mechanism of resistance has continued to plague the therapeutic use of successive antimicrobial agents discovered (White *et al.,* 2005). After the discovery of penicillin, and before its successful introduction as a therapeutic agent, bacterial penicillin's was identified by two members of the penicillin discovery team, and on introduction of this antibiotic into clinical practice, resistant strains capable of inactivating the drug became prevalent (Abraham and Chain, 1940). Synthetic studies were then undertaken to modify penicillin chemically to prevent cleavage by penicillinases, but soon after its introduction as therapy, it was again greeted by resistance. As other antibiotics were discovered and introduced into clinical practice, a similar course of event ensued (Davies and Davies, 2010). Even though antimicrobial resistance exists even before the discovery of antibiotics , the clinical development of generations of antibiotic resistant microbes and their distribution are results of indiscriminate application of antibiotics such as under use, overuse and misuse. This is not a natural process, but it is a

situation created by man which has affected the nature to an extent. There is perhaps no better illustration of Darwinian notion of selection and survival than this (Davis and Davis 2010; Kozhevin *et al*, 2013). Although evolution of resistance was recognized since the dawn of antibiotic era, this phenomenon became a significant threat to global public health only in the last two decades, due to unprecedented emergence of newer resistance mechanisms and enzymes, which confer resistance to almost all available antibiotics (WHO, 2014).

2.2 An Overview On *E.coli*

E. coli is a Gram-negative, flagellated, rod-shaped, oxidase negative, motile, facultative anaerobe. *E. coli* is genetically classified as the most versatile bacteria and is major source of many plasmid and phage mediated genes (Buxton and Fraser, 1977). *E. coli* is the member of Enterobacteriaceae family and accounts for75-90% of urinary tract infections in humans (Nicolle, 2001).The emergence of antibiotic resistance in *E. coli* has become an important clinical factor. *E. coli* is one of the major organism which is most commonly isolated from various clinical cases of diarrhea and others (Tobih , 2006). Pathogenic strains of *E. coli* cause intestinal and extra intestinal infections which comprise gastroenteritis, urinary tract infection, meningitis and septicemia (Von and Marre, 2005; Sodha *et al*., 2011). Recent data shows that resistance in case of *E. coli* strains is generally high for antimicrobial agents which are used in human and animal medicines. (US Food and Drug Administration, 2012).

2.3 Clinical importance of *E.coli*

E. coli conventionally exists in the intestine of humans and animals. *E. coli* is the vital cause of nosocomial infections in humans and major cause of hospital acquired and community acquired infections in humans. *E. coli* is a familiar inhabitant of human and animals and is also contemplate as a measure of fecal contamination in food. It is cosmopolitan in nature and has ability to commence, establish and causes discrete types of infections in humans and animals. Urinary tract infections caused by *E. coli* are vital type of infections in humans (Steadman and Topley, 1998). Most common infections caused by *E. coli* are bloody diarrhea, anemia, urinary tract infections, kidney failure, food poisoning, hemorrhagic colitis and hemolytic uraemic syndrome infections. Infections caused by *E. coli* can be patently transmitted from

one person to other through direct contact. 75-90% of *E. coli* infections are urinary tract infections which are reported in patients. Most of infections caused by *E.coli* take place in children. Uropathogenic isolates of *E. coli* causes 90% of urinary tract infections. Enterotoxigenic *Escherichia coli* strains are involved in production of two type's enterotoxins (Smith and Gyles, 1970). First type of enterotoxin is characterized having larger molecular weight, heat-labile and posesses immunogenic quality. The second type of enterotoxin has smaller molecular Weight & is a heat stable toxin (Smith and Gyles, 1970). *E. coli* is a common colonizer of human gastrointestinal tract and a major cause of bacteremia (Diekema *et al*., 1997; Weinstein *et al*., 1997; Diekema *et al*., 1999). Recent studies have made several attempts to be attentive on antimicrobial resistance of *E. coli* to antibiotic ciprofloxacin (Kern *et al*., 1994; Lautenbach *et al*., 2001; Garau *et al*., 1999; Pena *et al*., 1995). The commensal *E. coli* strains rarely cause infections except in case of immune compromised hosts. The niche of commensal *E. coli* is the mucous membrane of the mammalian colon where the bacterium is a highly successful competitor . It comprises of the most abundant facultative anaerobe of the human intestinal micro flora (Sweeney *et al.*, 1996).

2.4 Antimicrobial resistance in *Escherichia coli*

The emergence of antibiotic resistance in *E. coli* has become an important clinical factor. Antibiotic-resistant *E. coli* strains are prevalent around the world; antibiotic resistance varies according to the geographical distributions and regional locations. Multiple-antibiotic-resistance (Mar) mutants of *Escherichia coli* continuously express elevated levels of resistance against range of antibiotics (Morgan *et al*., 2009.), and the resistance to tetracycline and fluoroquinolones, has been reported to result from increased levels of active efflux (Morgan *et al*., 2009). In India, *Escherichia coli* isolates show resistance to ampicillin (75%), Nalidixic acid (73%), and co-trimaxazole 59% (Holloway *et al*., 2009). Resistance in *E. coli* to third generation cephalosporin's increased from 70% to 83%(Holloway *et al*., 2009), and resistance to fluoroquinolone increased from 78% to 85%. 10% of *E. coli* isolates were resistant to carbapenems in 2008, increasing to 13% in 2014 (CDDEP, 2015). Out of 40 putative efflux transporters in *Escherichia coli*, only AcrAB-TolC, MdFA, and NorE affects the MIC of fluoroquinolone when expressed with promoters under laboratory growth conditions (Sulavik *et al*., 2001; Yang *et al*., 2005).

Diarrhoea is one of the main causes of morbidity and mortality and a large proportion is caused by diarrhoeagenic *E.coli* (Clarke, 2001). Till the late 1950s, *E.coli* was considered a non-pathogenic normal cohabitant of all warm-blooded animals. Still, certain strains are now identified to cause different diarrhoeal diseases such as Enterotoxigenic *E.coli*, Enteropathogenic *E.coli*, Enteroinvasive *E.coli* and Enterohemorrhagic *E.coli* (Greene,1998). Food originating from warm blooded animals may contain *E.coli.* Several outbreaks have been associated with consumption of meat and meat products. Food could be a route for spreading pathogenic organism to human beings. Human beings differ in their risk of *E.coli* infection. Important factors that affect risk are the immunological and nutritional status of the host. *E.coli* strains have been implicated in disease in persons with AIDS (Olsvik *et al.*,1991).

Healthy domestic animals can benefit as *E. coli* reservoir. General *E. coli* was containedin stable cattle faecal samples (21.1%). Sheep (66.6%), goats (56.1%), swine (7.5%), cats (13.8%) and dogs (4.8%) (Beutin *et al*., 1993). In addition, haemolytic *E. coli* was isolated from healthy cats (Blanco *et al*., 1993) and 21 percent from healthy slot feaces (Chen *et al*., 2003). *E.coli* shiga toxin (six1 and six2) was present in 3% and 36% of non-diarrhoeal greyhounds, respectively (Staats *et al*.,2003). Fecal swabs from 52 Healthy dogs in a kennel of Midwestern study have been tested for *E.coli.* Enterotoxin-producing *E.coli* were isolated and belonged to O42, O170 and O-negative serogroups (Holland *et al*., 1999) VTEC can be found in the intestinal tract of a large range of domestic and wild animals and ruminants (sheep, goats, cattle and buffalo), the main source for *E. coli* and other VTEC is known to be healthy cattle. In general, they are asymptomatic carriers of bacteria that live in the colon and travel through the faeces (Ingrid *et al*., 2000). The key animal carriers are healthy domesticated ruminants, mostly cattle (Gyles, 2007) and to a lower degree, sheep and possible goats (La-Ragione et at., 2009).

Isogai *et al* (1989) examined that *Escherichia coli* bacteremia and endotoxemia was observed in three adult mongrel dogs, which had been prediagnosed as canine parvovirus disease. The endotoxin level was 46.5pg/ml in the plasma of clinical cases, while 2.3pg/ml in healthy controls. The microflora of the feces was confused in the clinical cases. The percentage of *E. coli* was major in the feces. Serologically, similar strains were isolated from the blood. These strains did not produce enterotoxins such as heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT).

Sandvang *et al* (2000) investigated 78 gentamicin-resistant *Escherichia coli* strains from swine (27) and cattle (51) and they were characterized by phenotypic resistance, presence of selected aminoglycoside resistance genes, class 1 integrons and gene cassettes, and pulse-field gel electrophoresis. Gentamicin resistance was mainly encoded by the *ant(2'')-I* gene that was found in 76% of all the strains investigated, whereas the *aac(3)-IIa* gene was found in 14% of strains. The *ant(2'')-I* gene was predominant in strains from cattle, whereas the porcine strains contained both *ant(2'')-I*, *aac(3)-IIa*, and the *aac(3)-IVa* genes. Among the *E. coli* that were investigated, 20% contained class 1 integrons. Genes encoding resistance to trimethoprim (*dhfrI*, *dhfrIb* and *dhfrVII*), gentamicin, tobramycin, and kanamycin (*ant(2'')-Ia*, streptomycin and spectinomycin (*ant(3'')-Ia*) and streptothricin (*sat1*) were identified as gene cassettes. The most prevalent gene cassettes were *ant(3'')-Ia* (11 isolates) and the *dhfrI* (nine isolates).

Lanz *et al* (2003) examined a total of 581 clinical *Escherichia coli* isolates from diarrhoeic and from acute mastitis in dairy cattle, from urinary tract infections in dogs and cat, and from septicemia in laying hen. Among the 16 antimicrobial agents tested, resistance was most for sulfonamides, tetracycline and streptomycin. Isolates from swine presented significantly more resistance than those isolated from the other animal species. The distribution of the resistance determinants for sulfonamides, tetracycline, and streptomycin was assessed by hybridization and PCR in resistant isolates.

Nijsten *et al* (1996) examined the degree of antibiotic resistance of *E.coli* in 266 faecal samples of pig farmers was significantly lower than that observed in 285 samples obtained from their pigs. Moreover, porcine *E.coli* were significantly more resistant to chloramphenicol, nitrofurantoin, oxytetracycline, streptomycin and sulphamethoxazolethan.

Sanchez *et al* (2002) evaluated that Multidrug-resistant opportunistic pathogens were endemic to the veterinary hospital environment. Review of 21 retrospective and prospective hospital-acquired *E. coli* infections revealed that the isolates had similar antibiotic resistance profiles, characterized by resistance to most cephalosporins, β-lactams and the β-lactamase inhibitor clavulanic acid as well as

resistance to tetracycline, spectinomycin, sulfonamides, chloramphenicol and gentamicin.

Rzewuska *et al* (2015) determined the antimicrobial susceptibility of *E.coli* isolates associated with various types of infections in dogs and cats . The studied isolates were most frequently susceptible to fluoroquinolones and the extended-spectrum cephalosporins (ESCs); antimicrobials commonly used in treatment of infections in companion animals. The frequency of multidrug-resistant (MDR) *E. coli* isolation (66.8% of isolates) was alarming. These results indicate that continuous monitoring of canine and feline *E.coli* antimicrobial susceptibility was required.

Younis *et al* (2015) reported dogs and cats as a possible reservoir of virulent *E.coli* strains that may cause enteric and extra-intestinal infections in humans, diarrheagenic *Escherichia coli* (DEC) in dogs and cats and their antibiotic resistant pattern(s). These animals suffered from diarrhea and other symptoms as fever, nausea, vomiting, chills, loss of appetite, muscle aches and bloating. 41 *E. coli* positive samples were detected by culturing and biochemical tests, and were subjected to antimicrobial disc diffusion susceptibility test by using 10 different antibiotic discs.

Qekwana *et al* (2018) investigated the burden and predictors and antimicrobial resistance of canine *E. coli* and among dogs presented at a veterinary teaching hospital in South Africa. There were high levels of AMR to penicillin-G (99%), clindamycin (100%), tylosine (95%), cephalothin (84%) but relatively low levels of resistance to enrofloxacin (16%), orbifloxacin (21%). Almost all, 98%,(164/167) of the isolates exhibited multidrug resistance (MDR).

Cid *et al* (2001) studied and characterized attaching and effacing *Escherichia coli* (AEEC) strains isolated from diarrhoeic lambs and goat kids for intimin (eae) and EspB (espB) gene subtypes by PCR and sequencing. Fifty (23 ovine and 27 caprine) AEEC strains of 398 (246 ovine and 152 caprine) analysed were detected by colony blot hybridization. Ovine AEEC strains belonged to serogroups O2, O4, O26, O80, O91 or were untypable, and caprine strains belonged to serogroups O3, O153 and O163. Two intimin subtypes were detected among the ovine and caprine strains studied.

Guerra *et al* (2003) carried out phenotypic and genotypic characterization for the antimicrobial resistance of *E. coli* strains isolated from cattle, swine and poultry. Isolates (n=317) were tested for their resistance to 17 antimicrobial agents by broth microdilution. Resistant strains were screened by molecular methods for resistance genes, integrons and mutations in quinolone-resistance determining regions.Resistance was found in 40% and multi drug-resistance in 32% of the strains. The resistance was significantly higher in isolates from poultry (61%) and swine (60%) than from cattle (25%) ($P<0.01$). The most prevalent resistance observed was to sulfamethoxazole, tetracycline, streptomycin, ampicillin and spectinomycin (15–30%)

Blanco *et al* (1996) isolated 144 *E.coli* strains from 144 diarrhoeic lambs (5 to 21 days old) from 38 flocks in Spain. The isolates were serotyped and investigated for production of enterotoxins (LT and STa), verotoxins (VT1 and VT2), cytotoxic necrotizing factors (CNFI and CNF2), haemolysin (Hly) and enterohaemolysin (EntHly), for necrotic and lethal activities and for antibiotic resistance. In total, 10 (7%) toxigenic strains were detected: two LT+, two VTl+ EntHly+, four VTl+EntHly-, one CNFl +Hly + and one CNF2+. Highest percentages of antibiotic resistance was seen for tetracycline, streptomycin, sulphadiazine, ampicillin, kanamycin, neomycin, chloramphenicol, trimethoprim and cotrimoxazole.

Saenz *et al* (2004) identified 17 multiple-antibiotic-resistant nonpathogenic *E.coli* strains of human, animal and food origin. Many of them carried class 1 and class 2 integrons. Amino acid changes in MarR and mutations in *marO* were identified for 15 and 14 *E. coli* strains, respectively.

Beutin *et al* (1993) examinedthe fecal samples of 720 healthy domestic animals representing seven different species (cattle, cows, goats, pigs, poultry, dogs and cats) for verotoxin (VT [shiga like toxin]) producing *Escherichia coli* (VTEC). VTEC was isolated from 208 animals (28.9%), mainly from cows (66.6% VTEC carriers), goats (56.1%) and cattle (21.1%). VTEC was less often isolated from pigs (7.5%), cats (13. 8%), dogs (4.8%) and was not present in chicken (< 0.7%)

Mora *et al* (2005) determined antimicrobial resistance profiles of 722 Shiga toxin-producing *Escherichia coli* (STEC) isolates recovered from humans, cattle,

ovine and their association with serotypes, phage types and virulence genes. Most common antimicrobial resistance was found to Sulfisoxazole (36%), followed by tetracycline (32%), streptomycin (29%), ampicillin (10%), trimethoprim (8%), cotrimoxazole (8%), chloramphenicol (7%), kanamycin (7%), piperacillin (6%), and neomycin (5%). The multiple resistance pattern most often observed was that to streptomycin, sulfisoxazole, and tetracycline.

Sayah *et al* (2005) conducted a repeated cross-sectional study to determine the patterns of antimicrobial resistance in 1,286 *E.coli* strains isolated from human septage, wildlife, domestic animals, farm environments, and surface water in the Red Cedar watershed in Michigan. In general, *E. coli* isolates from domestic species showed resistance to the largest number of antimicrobial agents compared to isolates from human septage, wildlife, and surface water. The agents to which resistance was demonstrated most frequently were tetracycline, cephalothin, sulfisoxazole, and streptomycin. Multidrug resistance was seen in a variety of sources, and the highest levels of multidrug-resistant *E. coli* were observed from swine fecal sample.

Rajput (2013) studied multiple drug resistance in VTEC isolated from Fecal, milk and milk products from certain areas of the Mathura and Vrindavan region. VTEC was found to be extremely susceptible to Ciprofloxacin (100%) followed by Nalidix acid (90%) after an antibiogram test againt 13 antibiotics. Cefotaxime (90%), Ceftriaxone (85%), norfloxacin (80%), erythromycin (80%), gentamycin (80%), respectively. Antibiotics such as contrimoxazole (80%) demonstrated highest resistance followed by Pencillin-G (45%), Amoxicillin (35%), Cefuroxine (35%), Amikacin (30%) and Ampicillin (25%).

Guler *et al* (2008) tested 120 *E. coli* isolates, 75 isolated from diarrhoeic or septicemic calves and 45 from clinically healthy calves aged between one day and two months for antimicrobial resistance. Antimicrobial susceptibility of 75 isolates from diseased calves was determined by agar disk diffusion method for 14 antimicrobial agents. In 77.3% of the isolates, multiple resistance was detected. Higher resistance rates were detected for cephalothin (72%), tetracycline (69.3%), kanamycin (69.3%), ampicillin (65.3%), nalidixic acid (53.3%), trimethoprim-sulphamethoxazole (52%) and enrofloxacin (41.3%), respectively. No resistance was found for ceftiofur and cefoxitin.

Lee (2009) investigated antimicrobial resistance profiles of *Escherichia coli* O26 and O111 from cattle and characterized the virulence genes of the resistant isolates. Among 37 *E. coli* O26 and 25 *E. coli* O111 isolates from the fecal specimens obtained from cattle, 26 (70%) and 15 (60%) were resistant to at least one antibiotic respectively. Forty (98%) of the 41 resistant isolates showed resistance to two or more antibiotics. Among the 22 antibiotics tested in this study, ampicillin was the most common antibiotic that the isolates were resistant to, followed by tetracycline and streptomycin.

Lanz *et al* (2003) studied antimicrbial susceptibility of 16 antimicrobial agents on 581 clinical *E.coli* isolated from pigs, diary cattle,and from urinary tract infections in dogs and cats in Switzerland, Resistance was most frequently found for sulfonamides, tetracycline and streptomycin.

Chattopadhyay et at (2001) examined the pattern of antibiotic suseceptibility of STEC strains from animal, human and food products and reported that STEC strains were consistently susceptible to comman antibiotics except for Tetracycline, Cephalexin, Dicloxacillin, Erythromycin and Lincomycin. Similary, Khan *et al* (2002) also analysed antimicrobial resistance patterns of 63 STEC isolates from 19 samples of human stools 40 cow stools and four samples of beef in Kolkata using 15 a ntimicrobials. .

Rahimi et al (2011) studied VTEC antibiography pattern and found that resistance to ampicillin and gentamycin was the most common finding (44.4%) followed by resistance to erythromycin(33.3%), amoxicillin (11.1%), tetracycline (11.1%) and nalidixic acid (11.1%) respectively.Both isolates of the *E.coli* O157 were suspectible to chloramphenicol, cefuroxime and streptomycin.

Carlos *et al* (2010) studied *Escherichia coli* strains commonly found in the gut microflora of warm-blooded animals. These strains could be assigned to one of the four main phylogenetic groups, A, B1, B2 and D, which can be divided into seven subgroups (A0, A1, B1, B22, B23, D1 and D2), according to the combination of the three genetic markers chuA, yjaA and DNA fragment TspE4.C2. Distinct studies have demonstrated that these phylo-groups differ in the presence of virulence factors, ecological niches and life-history. They analyzed the distribution of these *E. coli* phylo-groups in 94 human strains, 13 chicken strains, 50 cow strains, 16 goat strains,

39 pig strains and 29 sheep strains and verified the potential of this analysis to investigate the source of fecal contamination. Strains from group B1 were present in all hosts analyzed but were more prevalent in cow, goat and sheep samples. Medina *et al* (2011) tested 226 attaching and effacing *Escherichia coli* (AEEC) strains (20 enterohemorrhagic *E. coli* and 206 atypical enteropathogenic *E. coli*) isolated from calves, lambs, and goat kids with diarrhoea and from healthy cattle, sheep, and goats for their resistance to 10 antimicrobial agents by the disc diffusion method. Resistant and intermediate strains were analyzed by polymerase chain reaction for the presence of the major resistance genes. The overall percentage of resistance to tetracycline, streptomycin, erythromycin, and sulfamethoxazole was very high (65%). A high level of resistance (approximately 30%) to ampicillin, chloramphenicol, trimethoprim and trimethoprim– sulfamethoxazole was also detected.

Wani *et al* (2013) processed 728 faecal samples from 404 calves (286 diarrhoeic, 118 healthy) and 324 lambs (230 diarrhoeic, 94 healthy) in Kashmir, India, for the presence of enterotoxigenic *Escherichia coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and Salmonellae. Antimicrobial sensitivity patterns were also investigated. In total, 23 ETEC isolates were obtained from the diarrhoeic calves and 12 from diarrhoeic lambs. Most (74%) of the isolates from calves harboured the gene encoding heat-labile enterotoxin I, whereas 75% of the isolates from lambs possessed only the gene encoding for heat-stable enterotoximia.

Ferreira *et al* (2015) detected virulence factors in Shiga toxin-producing *Escherichia coli* (STEC) isolates isolated from rectal swab of healthy sheep and investigated the antimicrobial resistance profile. Out of 115 *E. coli* isolates obtained, 78.3% (90/115) were characterized as STEC, of which 52.2% (47/90) carried *Stx1*gene, 33.3% (30/90) *Stx2* and 14.5% (13/90) both genes. According to the analysis of the antimicrobial resistance profile, 83.3% (75/90) were resistant to at least one of the antibiotics tested. In phylogenetic classification, 24.4% (22/90) were grouped in group D (pathogenic), 32.2% (29/90) in group B1 (commensal) and 43.3% (39/90) in group A (commensal)

An overview on Antibiotic resistance Genes

The resistance to penicillin was detected even before the extensive use of penicillin started. Abraham and Chain showed that *E. coli* cell extract could destroy antimicrobial activity of penicillin by an enzymatic action (Abraham and Chain, 1940). Sir Alexander Fleming, also, had warned about the development of antibiotic resistance due to the overuse of antibiotic, in as early as 1945, stating:

"*I would like to sound one note of warning. It is not difficult to make microbes resistant to penicillin in the laboratory, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily under dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant*." Sir Alexander Fleming: *Nobel Lecture*, December 11, 1945.

The third-generation cephalosporin resistance is often mediated by TEM- and SHV-type L-lactamases in Enterobacteriaceae. TEM-type and OXA-1enzymes are the major plasmid-borne L-lactamases implicated in amoxicillin clavulanic acid resistance in *Escherichia coli* isolates. A rapid and simple multiplex polymerase chain reaction (PCR) was developed by Colom *et al* (2003) which discriminates *bla*TEM, *blaSHV* and *blaOXA_1*genes by generating fragments of 516, 392 and 619 bp respectively. Multiplex PCR analysis of 51 amoxicillin clavulanate resistant *E. coli* isolates detected *bla*TEM and *blaSHV* genes in 45 and two strains, respectively, and only one strain harboured a *blaOXA_1*gene. Twenty-three of the 40 cefotaxime-resistant Enterobacteriaceae isolates produced amplicons with a size compatible with the presence of *bla*TEM (13 strains), *blaSHV* (six strains) genes or the association of both genes (four strains).

Bryan *et al* (2004) examined *E.coli* isolates isolated from 12 animal sources and humans for the presence and types of 14 tetracycline resistance determinants. They found 1,263 unique *E. coli* isolates from humans, pigs, chicken, turkeys, sheep, cows, goats, cats, dogs, horses, geese, ducks and deer. Of all the isolates, 31% were highly resistant to tetracycline. Tetracycline MICs for 61, 29, and 29% of *E. coli* isolates from pig, chicken and turkeys, respectively, were >233 g/ml. Muliplex PCR analyses indicated that 97% of these strains contained at least 1 of 14 tetracycline resistance genes [*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetKtetL*, *tetM*, *tetO*,

tetS, *tetA(P)*, *tetQ*, and *tetX*] examined. While the most common genes found in these isolates were *tetB*(63%) and *tetA*(35%), *tetC*, *tetD*, and *tetM*.

Sandvang *et al* (2000) investigated 78 gentamicin-resistant *Escherichia coli* strains from swine (27) and cattle (51) and they were characterized by phenotypic resistance, presence of selected aminoglycoside resistance genes, class 1 integrons and gene cassettes, and pulse-field gel electrophoresis. Gentamicin resistance was mainly encoded by the *ant(2'')-I* gene that was found in 76% of all the strains investigated, whereas the *aac(3)-IIa* gene was found in 14% of strains. The *ant(2'')-I* gene was predominant in strains from cattle, whereas the porcine strains contained both *ant(2'')-I*, *aac(3)-IIa*, and the *aac(3)-IVa* genes. Among the *E. coli* that were investigated, 20% contained class 1 integrons. Genes encoding resistance to trimethoprim (*dhfrI*, *dhfrIb* and *dhfrVII*), gentamicin, tobramycin, and kanamycin (*ant(2'')-Ia*, streptomycin and spectinomycin (*ant(3'')-Ia*) and streptothricin (*sat1*) were identified as gene cassettes. The most prevalent gene cassettes were *ant(3'')-Ia* (11 isolates) and the *dhfrI* (nine isolates).

Boerlin *et al* (2005) examined 318 *Escherichia coli* isolates from diarrheic and healthy pigs for their susceptibility to 19 antimicrobial agents. They were tested by PCR for the presence of resistance genes to tetracycline, streptomycin, sulfonamides, and apramycin and for 12 common virulence genes of porcine *E. coli*. Resistance profiles suggested that cephamycinases may be produced by >8% of enterotoxigenic *E. coli* (ETEC). Resistance to quinolones was detected only in enterotoxigenic *E. coli* (<3%). Associations were observed among *tetA*, *sul1*, *aadA*, and *aac(3)IV* and among *tetB*, *sul2*, and *strA*/*strB*, with a strong negative association between *tetA* and *tetB*.

Molina lopez *et al* (2011) examined molecular mechanisms associated with multidrug resistance (MDR) in a collection of *E.coli* isolates recovered from hospitalized animals in Ireland. PCR and DNA sequencing were used to identify genes associated with resistance. Class 1 integrons were prevalent (94.6%) and contained gene cassettes recognized previously and implicated mainly in resistance to aminoglycosides, β-lactams, and trimethoprim. The most frequently occurring phenotypes included resistance to ampicillin (97.3%), chloramphenicol (75.4%), florfenicol (40.5%), gentamicin (54%), neomycin (43.2%), streptomycin (97.3%),

sulfonamide (98.6%) and tetracycline (100%). The mobility of the resistance genes was demonstrated using conjugation assays with a representative selection of isolates. High-molecular-weight plasmids were found to be responsible for resistance to multiple antimicrobial compound.

Nazik *et al* (2011) investigated the prevalence of CTX-M-type beta-lactamase in extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli*. Antibiotic susceptibility test was performed using disc diffusion method and ESBL production was determined using a double-disc synergy test. The presence of CTX-M-type beta-lactamase genes was investigated through amplification using specific primers. The isolates displayed high rates of resistance to tested antibiotics: 87% to ampicillin-sulbactam (SAM), 77% to amoxicillin-clavulanic acid (AMC), 76% to co-trimoxazole (SXT), 70% to norfloxacin (NOR), 68% to ciprofloxacin (CIP), and 51% to gentamicin (GN). All isolates were found susceptible to imipenem (IPM), meropenem (MEM) and fosfomycin (FOS).

Martins *et al* (2016) investigated the presence of enteropathogenic *E. coli* (EPEC) in fecal samples from 130 healthy sheep (92 lambs and 38 adults) raised. EPEC was detected in 19.2% of the sheep examined, but only lambs were found to be positive. A total of 25 isolates were characterized and designated atypical EPEC (aEPEC) as tested negative for *bfpA* gene and BFP (Bundle forming pilus) production. The presence of virulence markers linked to human disease as *ehxA, paa,* and *lpfAO113* was observed in 60%, 24%, and 88% of the isolates, respectively. Of the 11 serotypes identified, eight were described among human pathogenic strains, while three (O1:H8, O11:H21 and O125:H19) were not previously detected in EPEC.

Sunde *et al* (2006) investigated the distribution of associations between the transferability of antimicrobial resistance genes in resistant *Escherichia coli* strains isolated from Norwegian meat and meat products ,Out of 241 strains examined, strong positive associations were found between the *tet*(A) determinant and the genetic elements *sul1, dfrA1* and *aadA1*. Negative associations were found between resistance genes encoding resistance to the same antimicrobial agent: *tet*(A)/*tet*(B), *sul1*/*sul2* and *strA*-*strB*/*aadA1*.Strains harbouring several genes encoding resistance to the same antimicrobial agent were significantly ($P < 0.0001$) more frequently multiresistant than others.

Mishra (2015) examined a total of 240 fecal samples of 60 cattle, buffalo, sheep and deer from Mathura and Kanpur zoo for the presence of polymerase chain reaction (PCR) positive *E. coli* and VTEC genes. 212 *E. coli* strains were isolated out of 240 fecal samples. PCR tested all of the *E. coli* isolates to detect six1, six2, eaeA and hlyA virulence genes. Of those, 25 have been classified as VTEC isolates. The prevalence of VTEC was 13.4 percent (8/60), 13.4 percent (8/60), 6.67 percent (4/60) and 8.33 percent (5/60) respectively in cattle, buffalo, sheep and deer.

Zenger *et al*., (1992) examined faecal samples in dogs, *E.coli* was isolated form the feces of a six-year old dog with a chronic diarrhea associated with intestinal anomalies. *E. coli* shiga toxins (six1 and six2) were present in 15% and 23% respectively, of diarrhoeic fecal samples of greyhound dogs (Staats *et al*., 2003). Haemolytic *E. coli* has also been isolated from other dogs with diarrhea (Starcic *et al*,.2002). Histopathogenic and electron microscopic examination of intestines of two cats revealed enterpathogenic *E.coli* in ileum, caecum and colon.

Parul *et al* (2016) examined a total of 177 probable *E. coli* samples from 216 calf feceal samples showing an overall *E. coli* prevalence of 81.94%. A total of 32 isolates (14.8%) were classified by PCR as VTEC. The prevalence in the VTEC isolates of verotoxic genes vt1, vt2, and combination of vt1+vt2 was found to be 12 (37.5%, 14 (43.75%) and 6 (18.75%), respectively. Certain virulent genes era and hylA were detected in 6 and 11 VTEC strains with 18.75% and 34.37% prevalence values respectively.

An overview on Risk Factors viz Managemental Practices, Sex , Breed, Age

Sancak *et al* (2004) analysed *s*amples of faeces from 57 dogs with acute diarrhoea, 82 dogs with chronic diarrhoea, 34 clinically healthy household dogs and 88 kennelled control dogs by hybridisation, using DNA probes to detect enteropathogenic *Escherichia coli* (EPEc) and enterotoxigenic *E coli* (ETEC), verocytotoxin-producing *E coli* (VTEC), enterohaemorrhagic *E coli* (EHEC), enteroinvasive *E.coli* (EIEc) and enteroaggregative *E coli* (EAggEC). Samples of duodenal juice from 60 of the 82 dogs with chronic diarrhoea were also examined. Significantly more of the dogs with diarrhoea were excreting EPEC (acute 35.1 per

cent, chronic 31.7 per cent) and VTEC (acute 24.6 per cent, chronic 28 per cent) than the kennelled dogs (EPEC 17.1 per cent, VTEC 0 per cent) or the household control dogs (EPEC 6 per cent, VTEC 5.9 per cent). Enteropathic *E coli* was also detected in the duodenal juice of 23 of 60 (38.3 per cent) of the dogs with chronic diarrhoea.

Nweze (2009) found that of the 520 stool samples in the diarrhoea group,majority, 102 (44.74%) were *Escherichia coli.* Fifty (49.02%) were enteropathogenic (EPEC), 22 (21.57%) were enterotoxigenic (ETEC) while 7.84% was EAEC. Sex had no effect on the distribution of diarrhoegenic bacteria,except for EIEC. The isolated *E. coli* strains from the diarrhea and healthy asymptomatic age-matched controlgroups examined by PCR for 16 virulence genes indicates that the detection of EAEC, ETEC, EPEC and EIECwere significantly associated with diarrhea.

CHAPTER III

MATERIALS AND METHODS

3.1 Location and place of Work

The present work was carried out in the Department of Veterinary Microbiology, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

3.2 Ethical Approval

The current study was ethically approved by Institutional Animal Ethics Committee, Guru Angad Dev Veterinary and Animal Sciences University, (GADVASU), Ludhiana (497/GO/Re/SL/02/CPCSEA).

3.3 Collection of samples

A total of 100 faecal samples were collected from healthy as well as diarrhoeic dogs, from dogs owners from Punjab and from small animal clinics GADVASU Ludhiana, Punjab. These samples were aseptically collected in sample collection container and immediately kept in ice box and transferred to the laboratory. The samples were then subjected to bacterial isolation, followed by identification of isolates, culture sensitivity test and PCR.

3.4 Preparation of media.

Media was prepared in the laboratory as per the standard procedures. Different media used were: Brain Heart Infusion Agar (BHI) (Himedia) (52 grams in 1000 ml DW); Eosin Methylene Blue (EMB) agar (Himedia) (35.96 grams in1000 ml DW); MacConkey's Lactose agar (MLA) (Himedia) (49.53 grams in 1000 ml DW) and Mueller-Hinton agar (MHA) (Himedia) (38 grams in 1000 ml DW). Media were prepared as recommended by the manufacturer and sterilized by autoclaving at 121 for 15 minutes at 15 psi.

3.5 Isolation of bacteria

Out of 100 samples, 66 samples were collected from diarrhoeic Dogs whereas 34 samples were collected from healthy dogs.. The faecal samples brought to the laboratory were inoculated on BHI, MLA, and EMB agar. The inoculated plates were incubated at 37°C for 16-24 hours. The suspected colonies after incubation were

subjected to Gram's staining for identification and subjected to various biochemical tests for confirmation of various bacteria including *E.coli.*

3.6 Identification of isolates

All the isolates showing colonies as that of *E. coli* were subjected to Gram's staining to differentiate between Gram positive and Gram negative bacteria. Gram's staining was done using Gram's staining kit (Himedia), which contained Crystal violet, Gram's iodine, Decolorizer and Safranin.

3.6.1 Biochemical identification

Oxidase test

To conduct this test, a well isolated colony was picked with the sterilized tooth pick and rubbed onto the oxidase disc (Himedia). The oxidase positive organisms changed the color of the disc to dark purple within 60 to 90 seconds whereas oxidase negative took more than 2 minutes to change the colour

Catalase test

It was performed by adding one drop of 3% hydrogen peroxide on to the bacterial suspention placed on a clean grease free glass slide. The evolution of gas bubbles after addition of 3% hydrogen peroxide indicated positive reaction and absence of evolution of gas bubbles indicated negative reaction.

IMViC test

IMViC test was done by IMViC test kit (Himedia) *E.coli* is indole positive methyl red positive, Voges Proskauer and citrate negative.

Procedure

- Pick up the *E. coli* colonies by inoculation loop, then streak on to wells and incubate at 37º C for 18- 24 hrs.
- For Indole test, after inoculation and incubation, add 2-3 drops of Kovac's reagent in first well. If pink colour is obtained, then it is positive reaction.
- For MR test, add 1-2 drops of Methyl Red reagent in second well. If red colour obtained, the test is positive.
- For VP test, add 2-3 drops of Baritt reagent A and 1 drop of Baritt reagent B in third well. Pinkish red colour obtained in 5-10 minutes indicates a positive test.

- For citrate test, if colour changes to blue then it is positive for citrate utilization.

3.7 Maintenance of isolates

After the biochemical confirmation, isolates of *E. coli* were sub cultured on BHI agar slants and subcultured at regular intervals. Isolates of *Staphylococcus*, *Klebsiella* and *Bacillus* were also maintained in same manner.

3.8 Antibiogram study/ sensitivity testing

All the isolates obtained were tested for sensitivity to various antibiotics as per CLSI guidelines. Sixteen different antibiotics used were chloramphenicol (30mcg), Ampicillin/sulbactum (10/10 mcg), Nalidixic acid (5mcg), Tetracycline (30mcg), Doxycycline (30mcg), co-trimoxazole (25mcg), Gentamicin (10mcg), Erythromycin (15mcg), Methicillin (5mcg), Trimethoprim (5mcg),Streptomycin (10mcg), Ciprofloxacin (5mcg),Colistin (10mcg), Vancomycin(10mcg), Furazolidone (10mcg) and amikacin (30mcg). The antibiotic sensitivity test was performed on Mueller-Hinton Agar plates. Overnight grown culture of individual bacteria in BHI broth was uniformly spread onto a Mueller Hinton Agar plate with the help of a sterilized cotton swab. The discs were placed at appropriate distance and incubated for 16-24 hours. After incubation, the zone of inhibition was measured using a ruler and recorded in millimeters (mm). The diameter of zone of inhibition for various antibiotic discs was measured and isolates were designated as resistant or sensitive as per CSLI guidelines.

3.9 Molecular characterisation of isolates

3.9.1 Extraction of genomic DNA by hot-cold lysis method

About four to five colonies of the *E. coli* isolates were taken into 1ml of sterile distilled water/ normal saline in 1.5 ml microcentrifugation tube (MCT) to make a homogeneous suspension. The MCT containing the suspension was then placed into a water-bath preheated to 100°C. The suspension was boiled for 10 minutes at this Temperature. MCT containing the suspension was then immediately transferred to ice kept at -20°C. The suspension was centrifuged and supernatant was used as source of DNA. Extracted DNA was then stored at -20°C for further use.

3.9.3 Polymerase chain reaction (PCR) for detection of *E.coli*

Confirmation of *E. coli* isolates was done by PCR primers as per Riffen *et al* (2001) (Table 1).

Table 1: Sequence of Primers for detection of *E. coli*

	5' to 3' oligonucleotide Sequence	Amplicon Size (bp)	Reference
E. coli	F:ATCAACCGAGATTCCCCCA R:TCACTATCGGTCAGTCAGCAGGAG	232	Riffen *et al* (2001)

3.9.3.1 PCR protocol

Amplification reaction mixture (for one reaction) was prepared in a volume of 25 µl consisting of 12.5 µl mastermix (Promega), forward primer 20 pmol (1 µl), reverse primer 20 pmol (1 µl), 3 µl of Template DNA and 7.5 µl of nuclease free water (Table 2)

Table 2: Reaction Mixture for *E.coli*

2x Go Taq Green master mix (Promega)	12.5 µl
Forward primer 20 pmol/µl	1 µl
Reverce primer 20 pmol/µl	1 µl
Template DNA	3 µl
Nuclease free water	7.5 µl
Total	25 lµl

PCR cycling conditions are depicted in (Table 3) and consisted of an initial denaturation at 94°C for 5 minutes followed by 30 cycles each of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 10 minutes.

Table 3: *E. coli* PCR program using genus specific primer pair.

Step name	Temperature	Time	No. of cycles
Initial denaturation	94^{o}C	5 minutes	1
Denaturation	94^{o}C	45 seconds	30
Annealing	55^{o}C	45 seconds	
Extension	72^{o}C	60 seconds	
Final extension	72^{o}C	10 minutes	1

3.10 Detection of antibiotic resistance genes of *E. coli*

All the *E. coli* isolates were tested for the presence of *bla* TEM, *tetA* and *tetB* antibiotic resistance genes. PCR was done for detection of these genes by using primers, as shown in Table 4.

Table 4: Sequence of Primers used for the detection of antibiotic resistance genes *(bla* TEM*, tetA, tetB* and *aadA)* in *E. coli*

Sr. No.	Name of Primer Gene	Oligonucleotide sequence (5' to 3')	Amplicon Size (bp)	Reference
1	*bla* TEM	TEM-C: ATCAGCAATAAACCAGC TEM-H: CCCCAAGAACGTTTTC	516	Colom *et al* (2003)
2	*tetA*	*tetA*-L: GGCGGTCTTCTTCATCATGC *tetA*-R: CGGCAGGCAGAGCAAGTAGA	502	Patrick *et al* (2005)
3	*tetB*	F: CCTCAGCTTCTCAACGCGTG R: GCACCTTGCTGATGACTCTT	634	Momtaz *et al* (2012)
4	*aad*	F:TGATTTGCTGGTTACGGTGAC R:CGCTATGTTCTCTTGCTITTG	284	Van *et al (2008)*

3.10.1 PCR protocol for detection of *tet* A

For PCR, 25 µl reaction mixture was made consisting of 12.5 µl mastermix (Promega), forward primer (20pmol/ µl) 1 µl, reverse primer (20pmol/ µl) 1 µl, 3 µl of template DNA and 6.5 µl nuclease free water (Table 5). PCR was performed in a thermocycler (Veriti, ABI, USA) with the following conditions; an initial denaturation at 95°C for 4 minutes followed by 35 cycles each of denaturation at 95°C for 60 seconds, annealing at 64°C for 60 seconds and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 7 minutes (Table 6).

Table 5: PCR reaction mixture for antibiotic resistance gene *tetA*.

2x Go Taq Green master mix (Promega)	12.5 µl
Forward primer 20 pmol/µl	1 µl
Reverse primer 20 pmol/µl	1 µl
Template DNA	3 µl
Nuclease free water	7.5 µl
Total	25 µl

Table 6: *E. coli* PCR program for antibiotic resistance gene *tetA*.

Step name	Temperature	Time	No. of cycles
Initial denaturation	95°C	4 minutes	1
Denaturation	95°C	60 seconds	35
Annealing	64°C	60 seconds	
Extension	72°C	60 seconds	
Final extension	72°C	7 minutes	1

3.10.2 PCR protocol for detection of *tet* B

For PCR, 25 µl reaction mixture was made consisting of 12.5 µl mastermix (Promega), forward primer 20 pmol/ µl (1 µl), reverse primer 20 pmol/ µl (1 µl), 3 µl of Template DNA and 6.5 µl of nuclease free water (Table 7).

PCR was performed in a thermocycler (Veriti, ABI, USA) with the following conditions; an initial denaturation at 94°C for 4 minutes followed by 35 cycles each of denaturation at 94°C for 60 seconds, annealing at 64°C for 60 seconds and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 7 minutes (Table 8).

Table 7: PCR reaction mixture for antibiotic resistance gene *tetB*.

2x Go Taq Green master mix (Promega)	12.5 µl
Forward primer 20 pmol/µl	1 µl
Reverce primer 20 pmol/µl	1 µl
Template DNA	3 µl
Nuclease free water	7.5 µl
Total	25 µl

Table 8: *E. coli* PCR program for antibiotic resistance gene *tetB*.

Step name	TEMperature	Time	No. of cycles
Initial denaturation	95°C	4 minutes	1
Denaturation	95°	60 seconds	35
Annealing	64°C	60 seconds	
Extension	72°C	60 seconds	
Final extension	72°C	7 minutes	1

3.10.3 PCR protocol for detection of *bla* TEM

For PCR, 25 µl reaction mixture was made consisting of 12.5 µl mastermix (Promega), forward primer (20 pmol/µl) 1 µl, reverse primer (20 pmol/µl) 1 µl), 3 µl of Template DNA and 6.5 µl of nuclease free water (Table 9).

PCR was performed in a thermocycler (Veriti, ABI, USA) with the following conditions; an initial denaturation at 94°C for 4 minutes followed by 35 cycles each of denaturation at 94°C for 60 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 7 minutes. The PCR product was stored at -20°C after removing from thermocycler (Table 10).

Table 9: PCR reaction mixture for antibiotic resistance *bla* TEM

2x Go Taq Green master mix (Promega)	12.5 µl
Forward primer 20 pmol/µl	1 µl
Reverce primer 20 pmol/µl	1 µl
Template DNA	3 µl
Nuclease free water	7.5 µl
Total	25µl

Table 10: *E. coli* PCR program for antibiotic resistance gene *bla* TEM

Step name	Temperature	Time	No. of cycles
Initial denaturation	94^{o}C	5 minutes	1
Denaturation	94^{o}C	30 seconds	32
Annealing	54^{o}C	30 seconds	
Extension	72^{o}C	60 seconds	
Final extension	72^{o}C	10 minutes	1

3.10.4 PCR protocol for detection of *aadA*

For PCR, 25 µl reaction mixture was made consisting of 12.5 µl mastermix (Promega), forward primer 20 pmol/µl (1 µl), reverse primer 20 pmol/µl (1 µl), 3 µl of Template DNA and 6.5 µl of nuclease free water (Table 11).

PCR was performed in thermocycler (Veriti, ABI, USA) with the following conditions; an initial denaturation at 95^{0}C for 15 minutes followed by 30 cycles each of denaturation at 94^{0} C for 30 seconds, annealing at 58^{0} C for 30 seconds and extension at 72^{0}C For 1 minute. This was followed by a final extension at 72^{0}C for 10

minutes. The PCR product was stored at-20^{0}C after removing from thermoycler (Table 12).

Table 11: PCR reaction mixture for antibiotic resistance *aadA*

2x Go Taq Green master mix (Promega)	12.5 µl
Forward primer 20 pmol/µl	1 µl
Reverse primer 20 pmol/µl	1 µl
Template DNA	3 µl
Nuclease free water	7.5 µl
Total	25 µl

Table 12: *E. coli* PCR program for antibiotic resistance gene *aadA*

Step name	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95^{o}C	15 minutes	1
Denaturation	94^{o}C	30 seconds	32
Annealing	58^{o}C	30 seconds	
Extension	72^{o}C	60 seconds	
Final extension	72^{o}C	10 minutes	1

CHAPTER IV

RESULT AND DISCUSSION

4.1 Isolation and Identification of *E. coli* sp.

To serve the objectives of present study, a total of 100 samples were collected and processed for isolation of *E. coli.* From 100 samples, 67 isolates of *E. coli* were obtained. The details of isolation are shown in (Table 13). Pink colour coccobacilli were seen in Gram's staining (Fig 7). The *E. coli* cultures were confirmed biochemically *viz.* Catalase test (+ve) Oxidase (-ve) and IMViC test, [Indole (+ve), methyl red (+ve), Voges Proskauer (-ve) and citrate (-ve)](Fig 6). On BHI, colonies obtained were spherical, creamy and mucoid in nature(Fig 1)**.** On MLA, lactose fermenting pink colour colonies were obtained(Fig2) and on EMB, colonies showed green metallic sheen(Fig3). All the *E. coli* isolates were found to be positive in catalase, methyl-red positive and indole positive but negative to VP test which supports the findings of Beutin *et al* (1993). Results of MR, Indole test of the *E. coli* isolates were positive as reported by Buxton and Fraser 1977. The colony characteristics of the isolated *E. coli* in different media are similar with the colony characteristics of *E. coli* as stated by Ali *et al* (1998). They reported that the faecal isolates showed various colony characteristics and biochemical reactions in different bacteriological agar media.

4.2 Isolation and Identification of other than *E.coli* Bacteria

To serve the objective of present study, a total of 100 samples were collected and processed for isolation of various bacteria. Out of 100 samples, other bacteriae isolated were , *Staphylococcus* (14),*Klebsiella* (13) & *Bacillus* (6). All the isolates were identified by Gram's Staining(Fig 8). On MLA, the colonies of *Klebsiella* were found to be large, shining mucoid lastose in appearance(Fig 5) . The colonies of *Staphylococcus* on MSA were small, creamish yellow , slightly raised from the surface of agar(Fig4). Colonies of *Bacillus* were flat with irregular edges and creamish white in colour.

Table 13: Details of samples collected for isolation of various bacteria including *E. coli*

Samples	Breed	Age	Sex	Antibiotics	Isolation
E1	Labrador	3	M	Yes	*Klebsiella* sp
E2	Labrador	4	F	Yes	*E. coli*
E3	Shihtzu	1.5	M	Yes	*E. coli*
E4	German Shephard	3	M	Yes	*E. coli*
E5	German Shephard	8	M	No	*E. coli*, *Staphylococcus*
E6	Rottweiller	2.7	M	Yes	No Growth
E7	Labrador	91	M	No	*Klebsiella* sp
E8	Beagle	1	M	Yes	No Growth
E9	Beagle	2.5	F	No	*Staphylococcus* sp
E10	Labrador	5	M	Yes	No Growth
E11	Beagle	10	F	Yes	*E. coli*,*Klebsiella*
E12	Labrador	1	M	No	*E. coli*
E13	Pom	2	F	No	*E. coli*,*Staphylococcus*
E14	Retriever	1.5	F	Yes	*E. coli*
E15	German Shephard	6.5	M	No	*Bacillus sp*
E16	ND	4.5	F	Yes	No Growth
E17	ND	4	F	Yes	No Growth
E18	Dachshund	3	M	Yes	*Klebsiella sp*
E19	Beagle	3	M	Yes	*E. coli*
E20	Rottweiller	5	M	No	*E. coli*,*Klebsiella*
E21	Labrador	4.5	F	No	*E. coli*
E22	Saint bernard	2.5	M	Yes	*Klebsiella sp*
E23	Labrador	4	F	Yes	*Staphylococcus sp*
E24	Rottweiller	5.5	M	No	No Growth
E25	Pom	10	M	Yes	*Klebsiella* sp
E26	Labrador	7	F	No	*E. coli*
E27	Shihtzu	6	F	No	*E. coli*,*Bacillus*
E28	Labrador	7.5	F	Yes	No Growth
E29	Rottweiller	11	M	No	*E. coli*
E30	Pug	1	M	No	*E. coli*
E31	Labrador	2	M	Yes	*E. coli*
E32	German Shephard	3.5	M	Yes	*E. coli*,*Staphylococcus*
E33	Rottweiller	1.4	F	Yes	*E. coli*
E34	ND	1	M	Yes	*E. coli*,*Klebsiella*

Samples	Breed	Age	Sex	Antibiotics	Isolation
E35	German Shephard	1.2	M	Yes	*Staphylococcus* sp
E36	Dobermann	1	M	Yes	No Growth
E37	Labrador	1	M	Yes	*E. coli*
E38	Labrador	2	F	No	*Bacillus* sp
E39	Beagle	3	M	Yes	*Staphylococcus* sp
E40	Beagle	6	M	No	No Growth
E41	Retriver	1.5	M	Yes	*E. coli*
E42	Pug	2.4	M	Yes	*E. coli*
E43	Beagle	3.5	M	No	*E. coli*
E44	Saint Bernard	4.5	F	Yes	*E. coli*
E45	Pug	5	M	Yes	*E. coli*
E46	Labrador	2	F	Yes	*E. coli*
E47	ND	7	F	No	*E. coli*
E48	Husky	5	M	Yes	*Klebsiella* sp
E49	ND	9	M	Yes	*E. coli*
E50	Labrador	10	M	Yes	*E. coli*,*Klebsiella*
E51	Pug	4	M	No	*E. coli*
E52	Saint Bernard	7	M	Yes	*E. coli*
E53	Pom	8	F	No	*E. coli*
E54	ND	4.5	F	Yes	*E. coli*
E55	Dachshund	2	M	No	*E. coli*
E56	Labrador	1.5	F	No	*E. coli*, *Staphylococcus*
E57	German Shephard	2.5	F	Yes	No Growth
E58	Labrador	2	M	Yes	No Growth
E59	Pug	1	F	Yes	*E. coli*,*Bacillus*
E60	German Shephard	1.5	M	No	No Growth
E61	Labrador	1	M	Yes	*Bacillus*
E62	Pug	4	F	Yes	*E. coli*,*Staphylococcus*
E63	Rottweiller	5	M	Yes	*E. coli*
E64	Dachshund	4	F	Yes	No Growth
E65	Shihtzu	2.5	F	No	*E. coli*
E66	Husky	4.5	F	Yes	*Staphylococcus sp*
E67	Beagle	11	M	No	No Growth
E68	Pug	2	M	Yes	*E. coli*
E69	Pitbull	10	M	Yes	No Growth
E70	ND	2	M	Yes	*E. coli*

Samples	Breed	Age	Sex	Antibiotics	Isolation
E71	Labrador	1	F	Yes	*E. coli*
E72	Labrador	1	F	Yes	*E. coli*
E73	Labrador	8	F	No	*E. coli*
E74	Dachshund	2.5	M	No	No Growth
E75	Labrador	5	M	Yes	*E. coli*
E76	Pom	1.5	M	No	*Bacillus* sp
E77	ND	3.5	M	Yes	No Growth
E78	ND	4	F	Yes	*E. coli,Klebsiella*
E79	Labrador	3.5	M	No	No Growth
E80	Beagle	4	M	Yes	*Klebsiella* sp
E81	ND	3	M	Yes	*E. coli*
E82	Labrador	2	M	Yes	*E. coli*
E83	Pointer	2	M	No	*Staphylococcus* sp
E84	Rottweillwer	1.5	F	Yes	No Growth
E85	Pug	1	F	Yes	*E. coli*
E86	Beagle	1	M	Yes	*E. coli*, *Staphylococcus*
E87	Retriever	2	M	No	*E. coli*
E88	Labrador	1	M	Yes	No Growth
E89	German Shephard	2	F	Yes	*E. coli*, *Klebsiella*
E90	Dobermann	2.5	F	No	*E. coli,Staphylococcus*
E91	Husky	8	M	Yes	*E. coli*
E92	Labrador	7	M	No	*E. coli*
E93	Beagle	1.5	M	Yes	*E. coli*
E94	ND	6	M	Yes	*E. coli*
E95	Dachshund	7	F	Yes	No Growth
E96	Beagle	5	M	No	*E. coli*
E97	Labrador	4	F	No	*E. coli*
E98	Labrador	5	M	Yes	No Growth
E99	Labrador	2	M	Yes	*E. coli*
E100	Rottweiller	1.5	M	Yes	*E. coli*

Table 14: No of samples Obtained from diseased and healthy dogs.

Animal	No. of samples obtained from diseased dogs	No. of samples obtained from healthy dogs
Dogs	66	34
Total	-	100

4.2 Culture sensitivity test of *E. coli* isolates

A total of 67 *E. coli* isolates isolated from faeces of diarrhoeic dogs were screened for antibiogram against 12 antibiotic agents Nalidixic acid(5mcg) Tetracycline(30mcg), Ampicillin /Sulbactum (10/10mcg), Trimethoprim(5mcg), Streptomycin(10mcg), Colistin(10mcg) , Ciprofloxacin(5mcg), Amikacin(30mcg) , Co-trimoxazole (25mcg) Furazolidone(10mcg), Gentamicin (10mcg) Doxycycline(30mcg). The results were interpreted as per CLSI guidelines (Table 15). Majority of isolates were resistant to Ampicillin & Sulbactam (100%) ,Nalidixic acid (91.7%). In dogs, resistance to other antimicrobial agents in decreasing order was Amikacin (81%), Trimethoprim(58.6%), Tetracycline (58.6%), Furazolidone (55.1%), Gentamycin (51.7%), Doxycycline(48.2%) Colistin (46.5%),Cotrimaxazole (43.1%), Ciprofloxacin (37.9%), Streptomycin (29.3%)(Table 15 and Table 19). The results that we arrived at in our study were same as were reported by Medina *et al* (2011) They reported that overall percentage of resistant *E. coli* strains to tetracycline, Trimethoprim, and Furazolidone was very high (60%). Moreover, a high level of resistance (approximately 40%) was also detected to Streptomycin & Ciprofloxacin. The AEEC strains were very susceptible (90%) to Ampicillin and sulbactum, Nalidixic acid, Amikacin. In another study conducted by Koladka *et al* (2016) ampicillin (50 resistant strains) were most frequently found. In case of amoxicillin with clavulanic acid, high percentage of resistant strains was also found. No resistance to amikacin was observed. Highly susceptible strains to cefotaxime, trimethoprim-sulfamethoxazole, were detected. They analysed that *E. coli* strains (74 %) were resistant to one or more antibiotics. Lee (2009) examined 22 antibiotics in his study and found that, ampicillin was the most common antibiotic that the *E. coli* isolates were resistant to followed by tetracycline and streptomycin. None of the isolates were resistant to fluoroquinolones, such as ciprofloxacin, ofloxacin and norfloxacin, and to ceftriaxone, amikacin and imipenem. Susceptibility and resistance of different antibiotics was measured in vitro as per CLSI Guidelines.

4.3 Culture Senstivity test result of *Staphylococcus* sp

A total of 14 *Staphylococcus* isolates isolated from faeces of diarrhoeic dogs were screened for antibiogram against 10 antibiotic agents Tetracycline(30mcg), Erythromycin(15mcg), Chloramphenicol(30mcg), Ampicillin /Sulbactum (10/10mcg), Trimethoprim(5mcg), Vancomycin(10mcg), Amikacin(30mcg) , Methicillin(5mcg), *C*o-trimoxazole (25mcg) , Doxycycline(30mcg). The results were interpreted as per CLSI guidelines.Majority of isolates were resistant to amikacin (85.7%) Ampicillin and sulbactam (71.4%), trimethoprim and doxycycline (57.1%) erythromycin (50%), vancomycin (42.8%) and tetracycline (35.7%) (Table 17&21).

In a study by Sarangi *et al* (2009) they stated that newer drugs like levofloxacin, enrofloxacin. chloramphenicol and gentamicin were effective in treatment of *Staphylococcus* mastitis which is relevant with the findings of current study too. Kour (2017) reported sensitivity to chloramphenicol (93.02%), gentamicin (76.74%) and ofloxacin (76.74%) whereas resistance to penicillin (97.61%), amoxicillin (86.04%) and streptomycin. Kulaste (2019) showed that there was high sensitivity to sparfloxacin (96.88%) followed by tetracycline, gentamicin, vancomycin. doxycycline (90.63%), co-trimoxazole, erythromycin, cephalexin, gatifloxacin, teicoplanin, azithromycin (87.5%), ciprofloxacin, chloramphenicol and ofloxacin (84.38%) and resistance to amoxicillin (68.75%) for *E.coli* isolates which is similar to finding of our study.

4.3 Culture Senstivity test result of *Klebsiella* sp

A total of 13 *Klebsiella* isolates isolated from faeces of diarrhoeic dogs were screened for antibiogram against 11 antibiotic agents Nalidixic acid(5mcg) Tetracycline(30mcg), Chloramphenicol(30mcg), Ampicillin /Sulbactum (10/10mcg), Trimethoprim(5mcg), Streptomycin(10mcg), Colistin(10mcg) , Ciprofloxacin(5mcg), co-trimoxazole (25mcg) , Gentamicin (10mcg) ,Doxycycline(30mcg). The results were interpreted as per CLSI guidelines (Table 16 & 20) Majority of isolates were resistant to ampicillin and sulbactam (76%), followed by nalidixic acid (64.2%), doxycycline(61.5),trimethoprim (53.8%) each of colistin (46.1%), and chloramphenicol(42.1%). Sikarwar and Batra (2011) observed that *Klebsiella pneumoniae* were susceptible to ofloxacin, gentamicin, amikacin, pefloxacin nd ciprofloxacin whereas resistance to carbenicillin, piperacillin, ampicillin, co-

trimoxazole, cefotaxime, chloramphenicol and tetracycline which are similar to the findings of the present study. Kour et al (2017) reported that isolated *Klebsiella* spp were sensitive to chloramphenicol, co-trimoxazole, gentamicin, ciprofloxacin, doxycycline and resistant to methicillin, penicillin, teicoplanin, azithromycin, vancomycin, amoxicillin which is absolutely similar to the findings of the present study, Singh *et al* (2018) reported an antibiotic sensitivity testing of *Klebsiella* isolates that they were sensitive to azithromycin, gentamicin, cephalexin, ciprofloxacin, cotrimoxazole and doxycycline and resistant to amoxicillin, teicoplanin, erythromycin. sparfloxacin and gatifloxacin similar to the findings of the present study. Kulaste (2019) observed high sensitivity of *Klebsiella* sp. to chloramphenicol, azithromycin and sparfloxacin (100%) each, followed by ciprofloxacin, gentamicin, ofloxacin and gatifloxacin (87.5%), cotrimoxazole, erythromycin, cephalexin. vancomycin, doxycycline (75%) each tetraycline (62.5%).

4.4 Culture senstivity result of *Bacillus* sp

A total of six *Bacillus* isolates isolated from faeces of diarrhoeic dogs were screened for antibiogram against eight antibiotic agents Tetracycline(30mcg), Chloramphenicol(30mcg),Ampicillin/Sulbactum(10/10mcg),Gentamicin(10mcg), Doxycycline(30mcg), erythromycin (15mcg), vancomycin (10mcg), amikacin (30mcg). The results were interpreted as per CLSI guidelines (Table 18&22). Majority of isolates were resistant to Gentamycin (94%), Amikacin , Ampicillin & Salbactum, Doxycycline (66.6%) &Vancomycin (33.3%).

Bhalla *et al* (2003) screened140 stool samples obtained from 37 study patients for presence of Bacteria. Forty-nine (61%) of 80 stool samples obtained during therapy with an antianaerobic regimen were positive for antibiotic-resistant, Gram-negative Bacillus, whereas only 14 (23%) of 60 samples obtained 4 or more weeks after completion of such therapy were positive ($P < .001$). Twenty-four (65%) of the 37 patients had one or more stool cultures positive for a Gram-negative Bacillus resistant to ciprofloxacin, ceftazidime, or piperacillin/tazobactam.

Table 15: Culture sensitivity of *E. coli* isolated from diarrhoiec Dogs faecal samples

Antibiotics	Number of isolates from dogs(n=67)	% Resistant
Ampicillin & Sulbactam (10/10mcg)	58	100
nalidixic Acid (5mcg)	50	91.7
Amikacin (30mcg)	47	81
Trimethoprim (5mcg)	34	58.6
Furazolidone (10mcg)	32	55.1
Gentamicin (10mcg)	29	51.7
Doxycycline (30mcg)	28	48.2
Colistin (10mcg)	27	46.5
Cotrimaxazole (25mcg)	25	43.1
Tetracycline (30mcg)	24	41.37
Ciprofloxacin (5mcg)	22	37.9
Streptomycin (10mcg)	15	29.3

Table 16: Culture sensitivity test results of *Klebsiella* isolates

Antibiotics	Number of isolates from dogs(n=13)	% Resistant
Ampicillin & Sulbactam (10/10mcg)	10	76
nalidixic Acid (5mcg)	9	64.2
Trimethoprim (5mcg)	7	53.8
Doxycycline (30mcg)	8	61.5
Gentamicin (10mcg)	8	61.5
Colistin (10mcg)	6	46.1
Chloramphenicol (30mcg)	6	42.1
Streptomycin (10mcg)	6	37.9
Ciprofloxacin (5mcg)	5	38.4
Tetracycline (30mcg)	4	30.7
Cotrimaxazole (25mcg)	4	30.7

Table 17: Culture sensitivity test results of *Staphylococcus* isolates

Antibiotics	Number of isolates from dogs(n=14)	% Resistant
Amikacin (30mcg)	12	85.7
Ampicillin & Sulbactam (10/10mcg)	10	71.4
Trimethoprim (5mcg)	8	57.1
Doxycycline (30mcg)	8	57.1
Erythromycin (15mcg)	7	50
Chloramphenicol (30mcg)	7	50
Methicillin (5mcg)	7	50
Vancomycin (10mcg)	6	42.8
Tetracycline (30mcg)	5	35.7
Cotrimaxazole (25mcg)	5	35.7

Table 18: Culture sensitivity test results of *Bacillus* isolates

Antibiotics	Number of isolates from dogs(n=6)	% Resistant
Gentamicin (10mcg)	5	94
Amikacin (30mcg)	4	66.6
Ampicillin & Sulbactam (10/10mcg)	4	66.6
Doxycycline (30mcg)	4	66.6
Tetracycline (30mcg)	3	50
Erythromycin (15mcg)	3	50
Chloramphenicol (30mcg)	3	50
Vancomycin (10mcg)	2	33.3

Table 19: Culture sensitivity test results of *E. coli* isolates.

Culture sensitive test result of isolates isolated from dogs faecal Samples.												
ISOLATES	**NALIDIXIC ACID**	**TETRACYCLINE**	**AMPICILLIN&SULBACTAM**	**TRIMETHOPRIM**	**STREPTOMYCIN**	**COLISTIN**	**CIPROFLOXACIN**	**AMIKACIN**	**CO TRIMAXAZOLE**	**FURAZOLIDONE**	**GENTAMICIN**	**DOXYCYCLINE**
E2	R	S	R	S	S	S	S	R	S	S	S	I
E3	R	S	R	S	S	S	S	R	S	I	R	R
E4	R	S	R	S	S	S	S	R	S	I	S	S
E5	R	R	R	S	S	R	S	I	I	R	S	S
E11	R	R	R	R	S	R	R	R	R	R	R	R
E12	R	R	R	R	S	S	R	R	R	R	R	R
E13	R	R	R	R	R	S	R	R	R	R	R	R
E14	R	R	R	R	R	S	R	R	R	R	R	R
E19	R	R	R	R	R	S	R	R	R	R	R	R
E20	R	R	R	R	S	S	R	R	R	R	R	R
E21	R	S	R	R	R	S	R	I	R	R	S	R
E26	R	R	R	R	S	S	I	R	R	R	S	R
E27	S	R	R	R	R	S	R	R	R	S	S	R
E29	R	R	R	R	S	R	I	S	S	S	S	S
E30	R	S	R	S	I	R	S	R	S	S	I	S
E31	R	R	R	R	R	S	R	R	R	I	R	R
E32	R	S	R	R	S	S	R	R	R	R	R	R
E34	R	R	R	R	I	R	R	I	R	R	S	S
E37	R	S	R	S	I	S	S	R	S	R	S	R
E41	S	R	R	S	R	R	S	I	I	S	S	S
E42	R	R	R	R	R	S	R	R	S	S	R	R
E43	I	S	R	S	R	R	S	R	R	I	S	R
E44	R	R	R	R	I	R	R	R	R	R	R	R
E45	R	R	R	R	I	R	R	R	R	R	R	R
E46	R	R	R	R	I	S	R	R	R	S	R	R

E47	I	R	R	R	I	R	I	R	R	I	R	S
E49	R	R	R	R	S	S	I	R	R	R	R	R
E50	R	S	R	R	S	R	I	R	S	R	S	S
E51	R	S	R	R	R	S	R	R	S	R	S	S
E53	R	R	R	R	R	R	I	R	I	R	R	R
E54	R	S	R	R	S	R	I	R	S	R	R	I
E55	R	S	R	S	S	R	I	R	S	R	S	S
E56	R	S	R	S	S	R	I	R	S	R	S	S
E59	R	S	R	R	S	R	I	R	R	S	R	R
E62	I	I	R	R	S	R	S	R	S	S	R	I
E64	R	I	R	S	S	R	S	R	I	I	S	S
E65	R	S	R	S	S	S	I	I	R	S	S	S
E68	R	S	R	R	R	S	R	R	R	S	R	S
E70	R	S	R	S	S	R	I	S	S	S	S	S
E71	R	S	R	S	S	S	S	R	I	R	S	S
E73	R	S	R	S	I	R	S	R	S	R	S	S
E75	R	S	R	R	S	S	S	R	R	R	S	R
E78	R	S	R	R	S	S	S	R	S	I	S	R
E83	R	S	R	S	R	R	I	R	S	R	S	I
E82	R	S	R	S	I	R	I	I	S	S	R	R
E85	R	S	R	R	S	R	S	S	S	R	R	S
E86	R	S	R	R	S	S	S	R	S	R	R	S
E87	R	R	R	S	S	R	S	R	S	R	R	I
E89	R	S	R	S	I	S	S	R	S	S	S	S
E90	R	S	R	S	S	S	S	R	R	R	S	I
E91	R	R	R	R	S	S	S	R	R	S	R	R
E92	R	S	R	S	S	S	R	R	S	S	S	S
E93	R	S	R	S	R	S	S	R	S	R	S	S
E94	R	S	R	S	R	S	S	I	I	R	R	R
E96	I	S	R	R	S	R	R	R	S	S	R	S
E97	R	S	R	R	S	S	S	S	S	S	R	I
E99	R	R	R	R	I	R	R	R	R	S	R	R
E100	R	R	R	S	S	R	R	R	S	R	S	R

Table 20: Culture sensitivity test results of *Klebsiella* isolates.

Culture sensitive test result of isolates isolated from dogs faecal Samples.											
ISOLATES	**NALIDIXIC ACID**	**TETRACYCLINE**	**CHLORAMPHENICOL**	**AMPICILLIN&SULBACTAM**	**TRIMETHOPRIM**	**STREPTOMYCIN**	**COLISTIN**	**CIPROFLOXACIN**	**CO TRIMAXAZOLE**	**GENTAMICIN**	**DOXYCYCLINE**
E1	R	S	R	R	S	I	R	I	S	R	R
E7	R	S	R	R	R	S	R	S	S	R	S
E11	S	S	I	R	R	S	S	S	S	R	S
E18	R	R	S	R	S	R	R	S	S	S	R
E20	R	S	S	S	S	S	S	S	R	S	I
E22	S	R	R	R	R	S	S	R	R	R	R
E25	I	S	S	R	S	S	S	R	S	S	S
E34	R	S	S	R	S	R	S	S	S	S	S
E48	R	S	S	S	S	R	S	S	I	R	R
E50	S	S	S	R	R	S	R	R	S	R	S
E78	R	S	R	R	R	S	S	S	S	R	R
E81	R	R	R	S	R	I	R	R	R	R	R
E89	R	R	S	R	S	R	R	R	S	S	R

Table 21: Culture sensitivity test results of *Staphylococcus* isolates.

Culture sensitive test result of isolates isolated from dogs faecal Samples.										
ISOLATES	**TETRACYCLINE**	**ERYTHROMYCIN**	**CHLORAMPHENICOL**	**AMPICILLIN &**	**TRIMETHOPRIM SALBACTUM**	**VANCOMYCIN**	**AMIKACIN**	**METHICILLIN**	**CO TRIMAXAZOLE**	**DOXYCYCLINE**
E5	S	S	R	R	S	R	I	S	S	R
E9	S	R	R	S	R	S	S	R	S	S
E13	R	R	S	R	R	S	R	R	R	S
E23	R	S	S	R	S	R	R	S	S	R
E32	S	S	S	S	S	S	R	S	R	I
E35	R	R	R	R	R	R	R	S	R	R
E39	S	S	S	R	S	S	R	R	S	S
E52	R	S	R	S	R	R	I	R	S	S
E56	S	R	S	R	S	S	S	S	I	R
E63	S	R	S	S	R	R	R	R	S	S
E66	S	S	R	R	R	S	R	S	S	S
E78	S	R	R	I	R	S	R	S	R	R
E86	S	R	S	R	S	I	R	R	S	R
E90	R	S	I	R	R	S	R	R	R	S

Table 22: Culture sensitivity test results of Bacillus *i*solates.

Culture sensitive test result of isolates isolated from dogs faecal Samples.								
ISOLATES	**TETRACYCLINE**	**ERYTHROMYCIN**	**CHLORAMPHENICOL**	**AMPICILLIN&SULBACTAM**	**VANCOMYCIN**	**AMIKACIN**	**GENTAMICIN**	**DOXYCYCLINE**
E15	S	R	I	R	R	I	R	R
E27	S	S	S	R	R	R	R	S
E38	R	R	R	R	S	R	R	S
E59	R	S	S	S	I	R	R	R
E61	S	R	S	R	R	S	S	R
E76	R	R	R	I	R	R	R	R

4.3 Molecular detection of *E.coli*

Confirmation of *E. coli* isolates was done by using genus specific primers as per Riffen *et al* (2001). Out of 67 samples of *E.coli* identified by biochemical tests, 67 were confirmed by genus specific PCR. An amplification band of 232bp was obtained in all 67 isolates as well as in positive control (Fig.14).

4.4 Detection of antibiotic resistance genes

All the 67 isolates of *E. coli* which were confirmed by genus specific PCR were tested for the presence of antibiotic resistance genes by PCR. Out of 67 isolates, 43 isolates were positive for the presence of *bla* TEM gene, 36 for *tetA* , 23 for *tetB* **a**nd 11 for *aadA*. The details of various antibiotic resistance genes detected are depicted in Table 22.

4.4.1 Molecular detection of *bla* TEM gene for penicillin resistance

DNA was extracted from *E.coli* isolates & subjected to PCR for detection of *bla* TEM by using primer pair as per Colom *et al* (2003). An amplicon size of 516 bp was obtained in positive control as well as in the positive isolates obtained from dogs (Fig 15). A total of 67 isolates were tested for the presence of *bla* TEM amr

genes. Out of 67 isolates, 43 isolates were positive for the presence of *bla* TEM genes. Aslam *et al* (2009) also studied same gene in *E. coli* isolates, they found that the majority of streptomycin-resistant *E. coli* isolates (76%) were positive for the *strA* and *strB* genes together. The *bla(CMY), bla(*TEM*),* and *bla(SHV)* genes were found in 12%, 56%, and 4%, of ampicillin-resistant *E. coli* isolates respectively**.** In a study carried by Colom *et al* (2003), *bla* TEM was detected in 45 out of 51 amoxycillin clavulinic acid resistant isolates. In another study, *bla* TEM was found in 97 % of ampicillin resistant strain of *E. coli* (Medina *et al* 2011). Broad-spectrum β-lactamase (TEM-1) and ESBL (CTX-M) -producing *E. coli* (*n* = 59) were observed by (Conrad *et al* 2018) in 23.9% fecal samples. However, resistance determinants were only identified in 10.2% of these samples (21 isolates) in which *bla* TEM-*1* was most common (*n* = 10), followed by CTX-M-15 (*n* = 8), and CTX-M-1 (*n*= 3). Delmani *et al* (2017) studied the molecular characteristics of *blaTEM* gene, and the associated ampicillin resistance mechanisms present in *E. coli*. In this study, 150 unrelated *E. coli* were isolated from different clinical sources like urine, blood, pus and abscess. PCR results showed the presence of *blaTEM* gene which has been found responsible for Ampicillin resistance in 5 of the 14 isolated *E. coli* strains; the gene was located on a plasmid having a size of 1190 bp. The *blaTEM* gene found in this plasmid showed strong correlation between genotype conferred resistance determined by PCR and antibiotic susceptibility patterns. Shahi *et al* (2013) detected bla_{TEM}, bla_{SHV} and bla_{OXA} in *E. coli* isolates. Altogether 16 *E. coli* strains were successfully isolated from biopsy and/or swab samples of 15 (35.71%) patients. Amplification of β-lactamase genes by multiplex PCR showed the presence of $bla_{\text{CTX-M}}$ like genes in 10 strains, bla_{TEM} and bla_{OXA} in nine strains each, and bla_{SHV} in eight of the total 16 strains of *E. coli.* Bailey *et al* (2011) examined the distribution of the closely related *blaTEM* transposons *Tn1, Tn2 and Tn3,* or *blaTEM*- containing fragments of them, in ampicillin-resistant human commensal *Escherichia coli* isolates. They recovered 25 ampicillin-resistant *E. coli* strains from the faecal flora of healthy humans that carried the *blaTEM* gene.

4.4.2 Molecular detection of gene for teteracycline resistance (*tet A)*

DNA was extracted from *E. coli* isolates. The extracted DNA was subjected to polymerase chain reaction (PCR) by using primer pair as per Patrick *et al* (2005). An amplicon size of 502 bp was obtained in positive control as well as in the positive

isolates. (Fig 16). A total of 67 isolates were tested for the presence of *tetA* gene. Out of 67 isolates, 36 isolates (62 %) were positive for the presence of *tet A* genes. These results are in agreement with the studies conducted by Medina *et al* (2011). According to their study, 76.7 % isolates were found positive for *tet A* gene. Karami *et al* (2006) assessed the carriage of tetracycline resistance genes, persistence in the microbiota, fecal population counts and virulence factor genes in 309 commensal, intestinal *Escherichia coli* strains Tetracycline resistance was identified in 12% of strains, all of which carried either *tet(A)* (49%) or *tet(B)* (51%) genes. Resistance to other antibiotics occurred in 50% of *tet*(A)- positive strains, 42% of *tet(B)*-positive strains and 13% of tetracycline-sensitive strains. However, colonization with tetracycline-resistant strains was founded to be unrelated to treatment with antibiotics. Strains that were *tet*(B) or *tet(A)* positive carried the genes for P fimbriae. Jurado *et al* (2014) determined the occurrence of *tet*(A), *tet*(B), and *tet*(M) genes in doxycycline-resistant *E. coli* isolates from pigs, as well as the detection of mobile genetic elements linked to *tet*(M) in *E. coli* and its possible transfer from Enterococci. The *tet*(A) was most frequently detected gene (87.9%) in doxycycline-resistant isolates, *tet*(M) was found in 13.1% *E. coli* isolates. The *tet*(M) gene was detected in relation with conjugative transposons in 10 out of 36 Enterococci isolates analyzed but not in any of *E. coli* isolates positive for *tet*(M). Boerlin *et al* (2005) carried out studies on antimicrobial resistant and virulence genes of *E. coli* isolates from swine in Ontorio, and in that study they found 98 % of *E. coli* isolates were having *tet A* gene. Aslam *et al* (2009) also studied same genes in *E. coli* isolates, and they found that about 50% of tetracycline-resistant *E. coli* isolates were positive for *tet(A)* (14%), *tet(B)* (15%), or *tet(C)* (21%) genes or both *tet(B)* and *tet(C)* genes together (3%).

4.4.3 Molecular detection of gene for teteracycline resistance (*tet* B*)*

DNA were extracted from *E.coli* strains and from suspected *E. coli* isolates. The extracted DNA was subjected to polymerase chain reaction (PCR) for detection of *tet B* gene by using primer pair as per Momtaz et al (2012). An amplicon size of 634 bp was obtained in positive control as well as in the positive isolates obtained from sheep and goats (Fig 17). A total of 67 isolates were tested for the presence of *Tet B* genes**.** Out of 67 isolates, 23 isolates (39.65 %) were positive for the presence of *Tet B* genes. These results are in accordance with those of Guerra *et al* (2003). In their study for the phenotypic and genotypic characterization of antimicrobial resistance in *Escherichia coli,* they found 42 % of isolates were having *tet B* gene. Gow *et al* (2008) examined associations among the genetic determinants of antimicrobial resistance (AMR) in 207 faecal generic *Escherichia coli* isolatesand , they found that most common resistance genes in the study sample (207 isolates) were *sul2* (48.3%), *tet(B)* (45.4%), and ant(3)-Ia (*aadA1*) (19.3%). Aslam *et al* (2009) also studied same genes in *E. coli* isolates and, they found that about 50% of tetracycline-resistant *E. coli* isolates were positive for *tet(A)* (14%), *tet(B)* (15%), or *tet(C)* (21%) genes or both *tet(B)* and *tet(C)* genes together (3%). Boerlin *et al* (2005) carried out studies on antimicrobial resistant and virulence genes of *E. coli* isolates from swine in Ontorio, and in that study they found 98% of tetracycline isolates having *tet B* gene. Olowe *et al* (2013) determined the tetracycline resistance in 203 *Escherichia coli* isolates. Antibiotic susceptibility to a panel of eight antibiotics was also performed, and resistance genes were detected with the polymerase chain reaction (PCR) technique. Result of the disk diffusion antibiotic susceptibility test of *E. coli* isolates showed 96.1% isolates to be resistant to ampicillin, 77.8% to tetracycline, 37.9% to cotrimoxazole, 38.4% to nalidixic acid, 20.7% to ofloxacin, 17.7% to ceftriaxone, 11.8% to gentamycin and 2% to nitrofurantoin. A total of 162 isolates had minimum inhibitory concentration (MIC) of tetracycline ≥ 128 μg/ml. The polymerase chain reaction (PCR) detected *tet*A gene in 89 (43.8%) isolates, *tetB* gene in 65 (32.0%), and both *tetA* and *tetB* genes in 9 (4.4%) isolates.

4.4.4 Molecular detection of gene for resistance (*aadA)*

E. coli isolates were taken and DNA were extracted from them. These DNA were subjected to polymerase chain reaction (PCR) for detection of *aadA* gene by using primer pair as per (Van *et al* (2008). An amplicon size of 284 bp was obtained

in positive control as well as in the positive isolates obtained from dogs (Fig 18). A total of 67 isolates were tested for the presence of *aadA* genes. Out of 67 isolates, 11 isolates were positive for the presence of *aadA* genes. Resistance was not frequently observed to Tetracycline (77.8%). Streptomycin (39.4%) Nalidixic acid (34.3%) and Gentamicin (24.2%). In addition, the isolates also displayed resistance to Fluoroquinolones (Ciprofloxaxin 16.2%, Norfloxacin 17.2%, and Enrolfloxacin 21.2%). Thirty-eight multi-resistant isolates showing the highest rates of resistance of antibiotic resistance of these antibiotics (52.6-63.2%). Thirty-eight multi resistant isolates were selected further for the examination of antibiotic resistance genes and were also evaluated for virulence gene profiles by multiplex and uniplex polymerase chain reaction. The beta-lactam TEM gene and tetracycline resistance *tet A*, *tetB* genes were frequently detected in the tested isolates (84.2% and 89.5% respectively). Genes which are responsible for resistance to Streptomycin(*addA*) (68.4%), Chloramphenicol (*cmlA*) (42.1%), Sulfonamides *(sull)*(39.5%), Trimethoprim (*dhfrV)* (26.3%) and Kanamycin (*aphA-1)* (23.7%) were also widely distributed. Plasmid-mediated *ampC* genes were detected in *E. coli* isolates from chicken and pork.

4.4.4 Comparison of phenotypic and genotypic results for antibiotic resistance

Some of the isolates which showed phenotypic resistance to tetracycline were not found to carry *tetA* and *tetB* . Also, some of the isolates which were phenotypically resistant to β- lactams were not found to carry *bla* TEM gene. Also, some isolates which did not show phenotypic resistance were found to carry the antibiotic resistance genes.

Table 23: Antibiotic resistance genes profile of *E. coli* isolated from dogs.

ISOLATES	*tet A*	*tet B*	*bla TEM*	*aadA*
E2	+	+	-	-
E3	+	-	+	-
E4	+	-	+	-
E5	-	+	+	-
E11	+	+	+	-
E12	+	-	+	-
E13	-	-	-	-
E14	+	-	+	-
E19	+	-	+	-
E20	-	-	+	-
E21	+	-	+	-
E26	-	+	+	-
E27	+	-	-	+
E29	+	-	-	-
E30	+	-	+	-
E31	+	+	+	+
E32	-	-	+	-
E34	-	-	-	-
E37	+	-	+	-
E41	-	+	+	+
E42	+	+	+	+
E43	-	+	-	-
E44	-	-	+	-
E45	-	+	+	+
E46	+	+	+	-
E47	-	-	+	-
E49	+	+	-	-
E50	-	+	-	-
E51	+	-	+	+
E53	+	-	+	-
E54	+	-	+	-
E55	+	+	+	-
E56	-	+	-	-
E59	-	-	-	-
E62	+	+	-	-
E64	+	-	+	-

ISOLATES	*tet A*	*tet B*	*bla TEM*	*aadA*
E65	-	-	+	-
E68	+	-	+	-
E70	+	-	+	-
E71	+	+	+	-
E73	+	-	+	-
E75	-	+	+	-
E78	-	-	-	+
E83	+	-	-	-
E82	-	+	+	-
E85	+	-	+	-
E86	+	+	+	+
E87	-	+	+	-
E89	-	-	+	-
E90	+	-	+	-
E91	+	-	+	+
E92	-	-	+	+
E93	+	-	+	-
E94	+	+	-	-
E96	+	-	+	-
E97	+	-	+	-
E99	-	+	+	-
E100	+	+	-	+

Statistical Analysis

Association of diarrhoiec status with managemental practices , sex, breed of animal was found to be highly significant using χ^2 test at 1% level of significance.The probability of developing diarrhoea is less in dogs raised in good and hygenic conditions than animals raised under poor and unhygenic conditions.T test was used for study of association between diarrhoea and age(Table 24).The Dogs in the given age group were found more diarrhoiec than healthy.Sex had no effect on the distribution of diarrhoegenic bacteria . Also , using X^2test the association between phenotypic and genotypic resistance of *Escherchia coli* to Tetracycline ,Methiciliin, Ampicillin&sulbactam were found to be significant at 1% and 5% level of significance and no significant association was found between genotypic and phenotypic resistance to Trimethoprim.(Table25)

Table 24: χ^2 Test for the study of association between diarrhoiec status and various parameters

Parameters	Levels	Number	χ^2 value
Management	Good	18	18.36
	Poor	82	
Sex	Male	58	3.29
	Female	42	
Breed	Random	100	12.25

Table 25: T Test for the study of association between diarrhoiec status and Age

Diarrhoeic	**Number**	**Mean**
0	18	3.25
1	82	4.08

Table 26: Statistical analysis of association between genotypic and phenotypic resistance to various antibiotics among *Escherchia coli*

	χ^2 value	**P value**	***Remarks***
Association between presence of *TET A* and phenotypic Methiciliin	4.66	0.037	Significant at 5% level of significance
Association between presence of *TET B* and phenotypic Ampicillin&salbactum	3.56	0.045	Significant at 5% level of significance
Association between presence of *BLATEM* and phenotypic Tetracycline,	14.47	0.0009	Highly significant at 1% level of significance
Association between presence of *AAD* and phenotypic Trimethoprim,	0.95	0.33	Non-significant

Sancak *et al* (2004) analysed samples of faeces from 57 dogs with acute diarrhoea, 82 dogs with chronic diarrhoea, 34 clinically healthy household dogs and 88 kennelled control dogs by hybridisation, using DNA probes to detect enteropathogenic *Escherichia coli* (EPEc) and enterotoxigenic *E coli* (ETEC),

verocytotoxin-producing *E coli* (VTEC), enterohaemorrhagic *E coli* (EHEC), enteroinvasive *E.coli* (EIEc) and enteroaggregative *E coli* (EAggEC). Samples of duodenal juice from 60 of the 82 dogs with chronic diarrhoea were also examined. Significantly more of the dogs with diarrhoea were excreting EPEC (acute 35.1 per cent, chronic 31.7 per cent) and VTEC (acute 24.6 per cent, chronic 28 per cent) than the kennelled dogs (EPEC 17.1 per cent, VTEC 0 per cent) or the household control dogs (EPEC 6 per cent, VTEC 5.9 per cent). Enteropathic *E coli* was also detected in the duodenal juice of 23 of 60 (38.3 per cent) of the dogs with chronic diarrhoea..

Nweze (2009) found that of the 520 stool samples in the diarrhoea group,majority, 102 (44.74%) were *Esherichia coli.* Fifty (49.02%) were enteropathogenic (EPEC), 22 (21.57%) wereenterotoxigenic (ETEC) while 7.84% was EAEC. Sex had no effect on the distribution of diarrhoegenic bacteria,except for EIEC. The isolated *E. coli* strains from the diarrhea and healthy asymptomatic age-matched controlgroups examined by PCR for 16 virulence genes indicates that the detection of EAEC, ETEC, EPEC and EIECwere significantly associated with diarrhea.

Conclusion

Out of 100 samples processed, 67 suspected *E.coli* isolates were identified on the basis of cultural characteristics and biochemical tests and all the isolates were identified through genus specific PCR. Other bacteria identified out of 100 samples were *viz. Staphylococcus* (14/100) , *Klebsilla* (13/100) and *Bacillus* (6/100).Culture Sensitivity Test of *E.coli* isolated from dogs, revealed highest resistance to ampicillin/ Sulbactam (100%), Nalidixic acid (91.7%).The molecular detection of AMR genes via PCR showed that maximum number of isolates harboured *bla TEM* gene followed by *tet A* gene. Using χ^2 test association between phenotypic and genotypic resistance of *Escherchia coli* to tetracycline, Methicillin, Ampicillin/ Sulbactam,was found significant at 1% and 5% level of significance and no significance was observed to Trimethoprim.

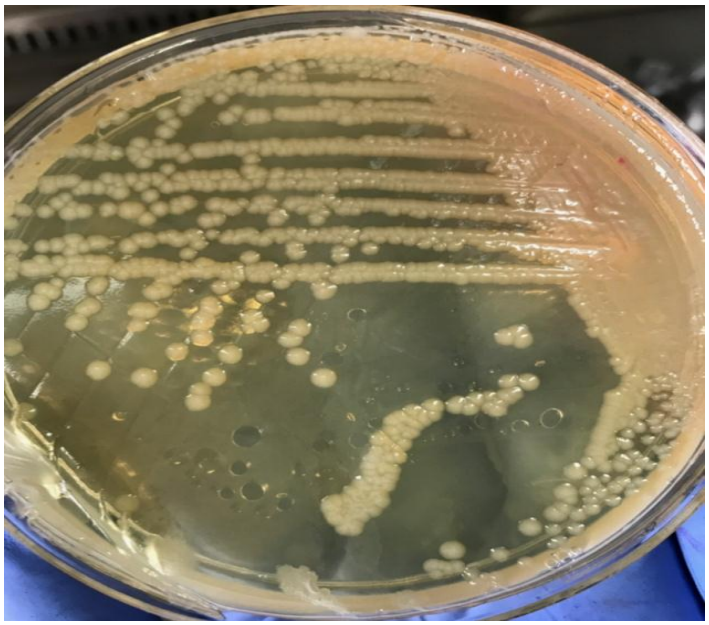

Fig 1 *E. coli* on BHI showing cream colour, mucoid type colonies

Fig 2 *E. coli* on MLA showing pink coloured colonies

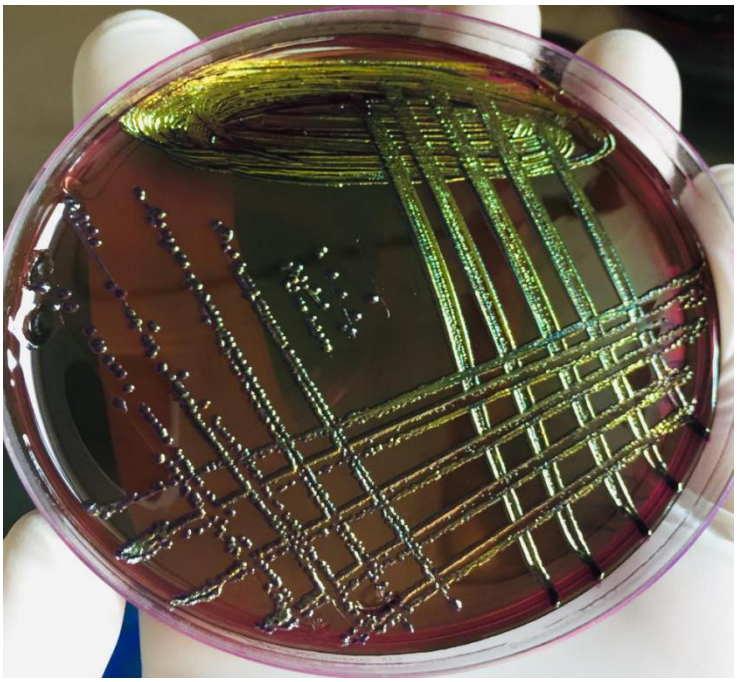

Fig 3 *E. coli* on EMB showing green metallic sheen

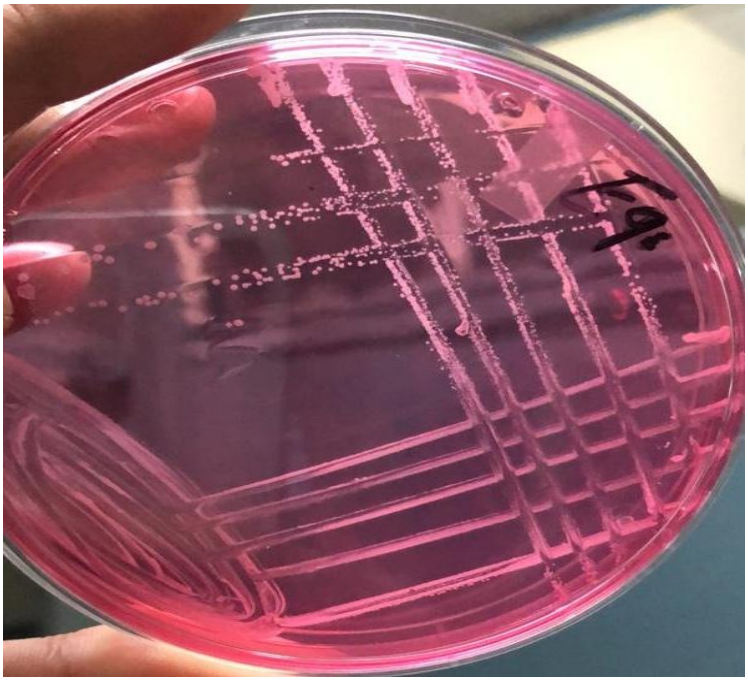

Fig 4 *Staphylococcus* on MSA Agar showing small, creamish yellow ,slightly raised colonies

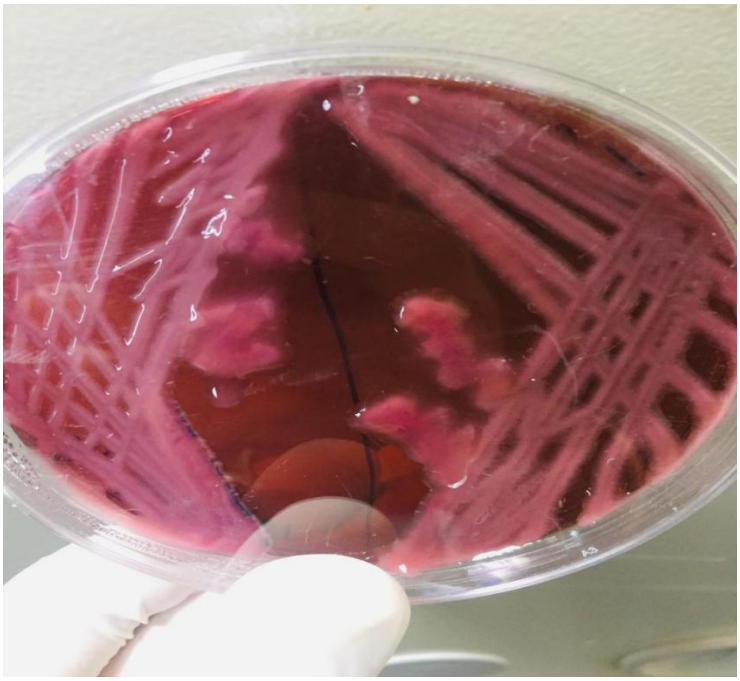

Fig 5 *Klebsiella* on MLA agr showing large, shining mucoid in apperance

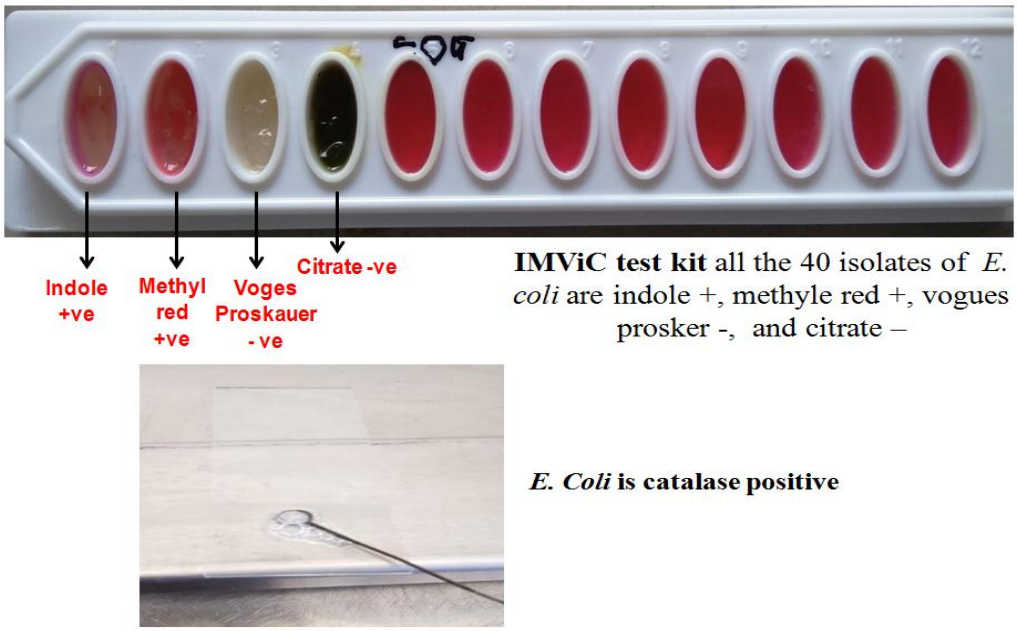

Fig 6 Biochemical test for identification of *E. coli*

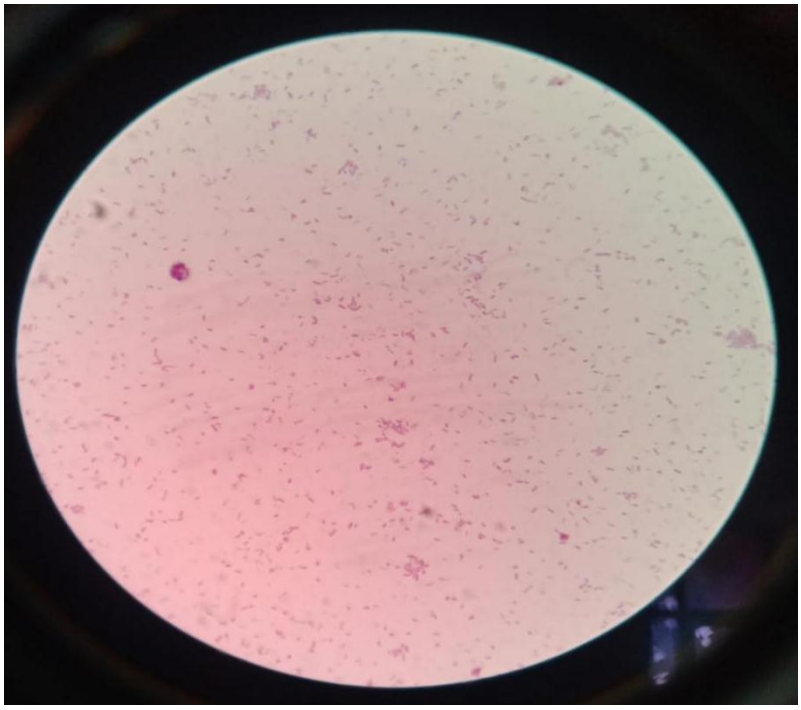

Fig 7 *E. Coli* Gram negative bacteria shows pink colour Coccobacilus

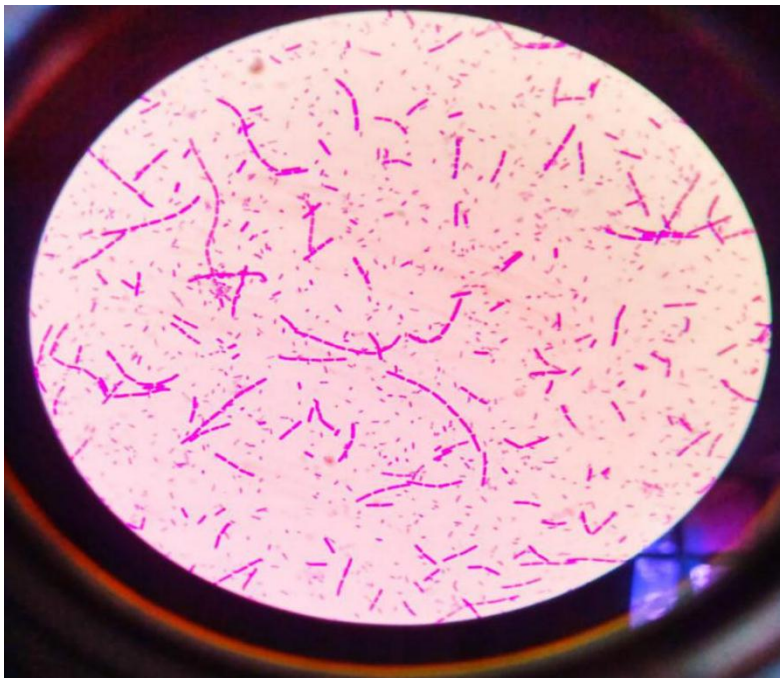

Fig 8 *Bacillus* rod shaped Bacteria

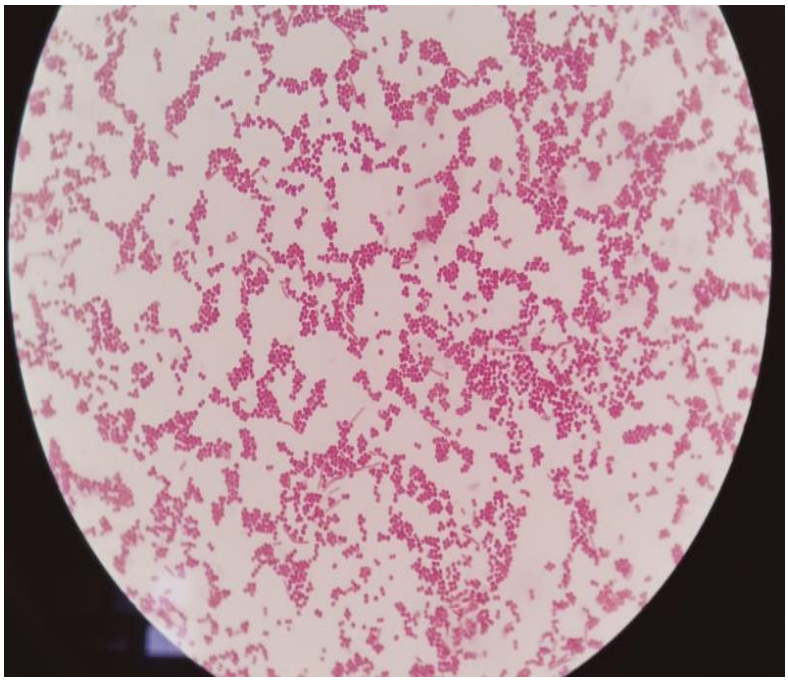

Fig 9 *Staphylococcus* cocci in cluster

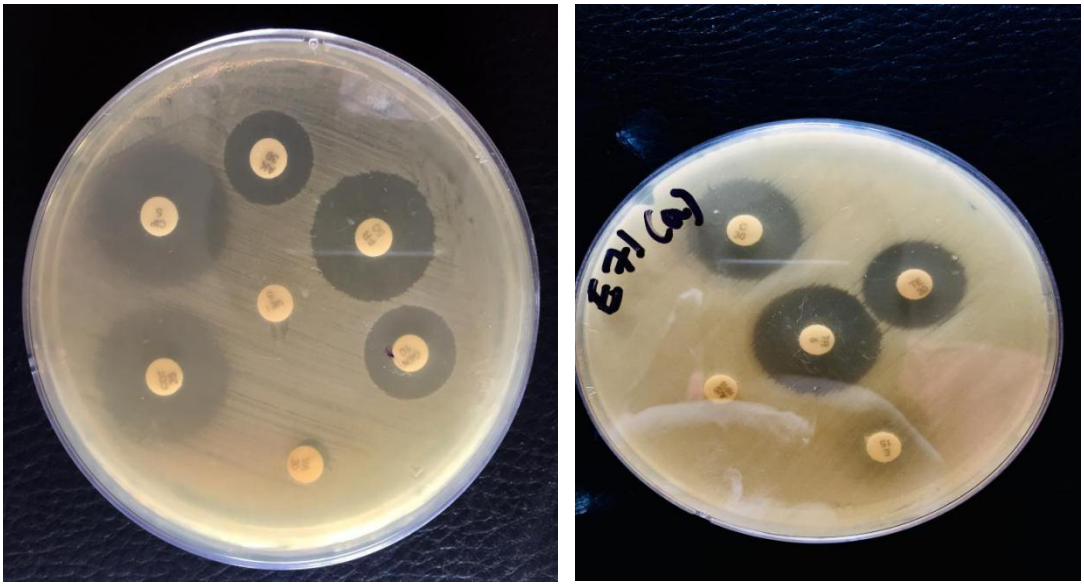

Fig 10 Disc diffusion test results of *E. coli* isolates on MHA agar

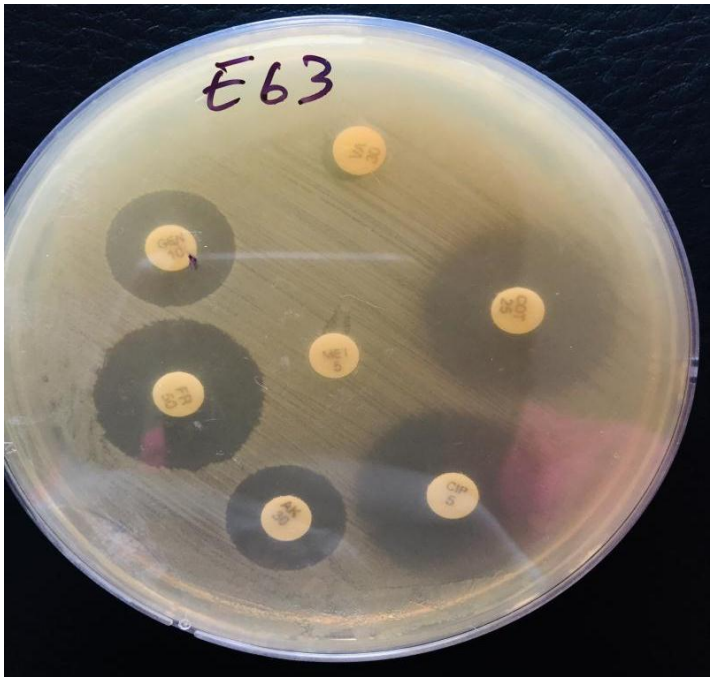

Fig 11 Disk Diffusion Test of *Staphylococcus* isolates on MHA agar

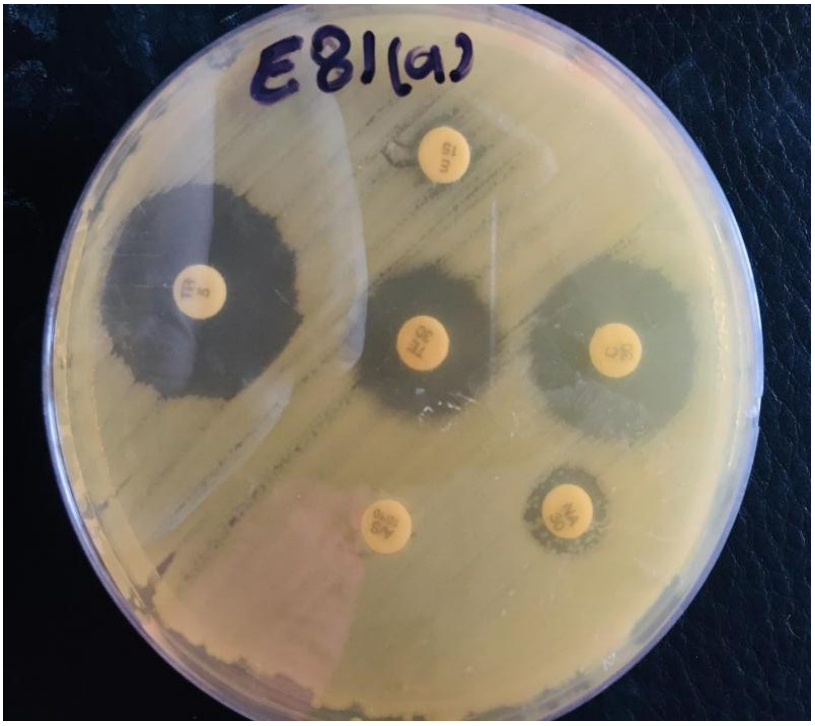

Fig 12 Disk Diffusion Test of *Klebsiella* isolates on MHA agar

Fig 13 Disk Diffusion Test of *Bacillus* isolates on MHA agar

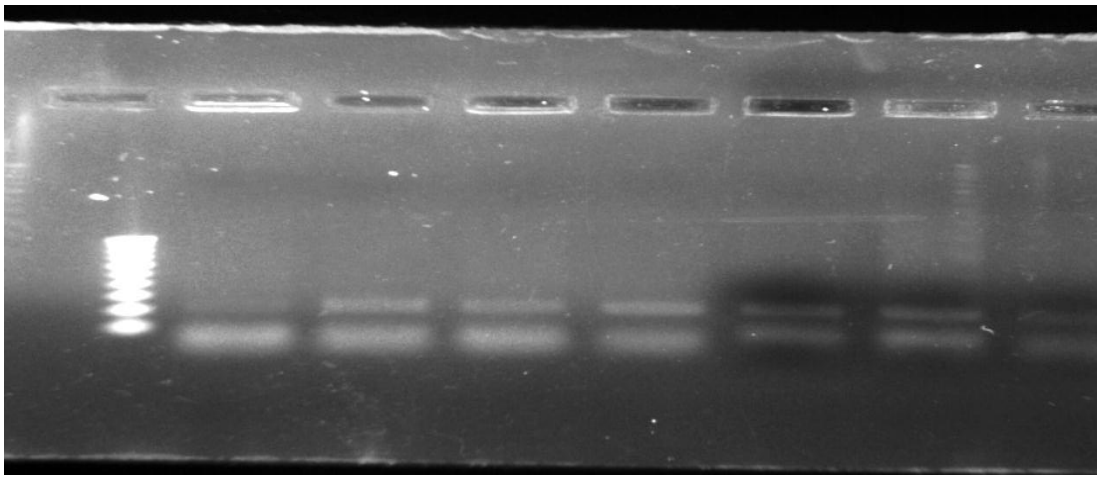

Fig 14 Gel electrophoresis of PCR amplified fragment from *E.coli* isolates by using genus specific primer pair (L1=100 bp DNA ladder, L2,3,5,11,12,14 =positive samples L=1 negative control)

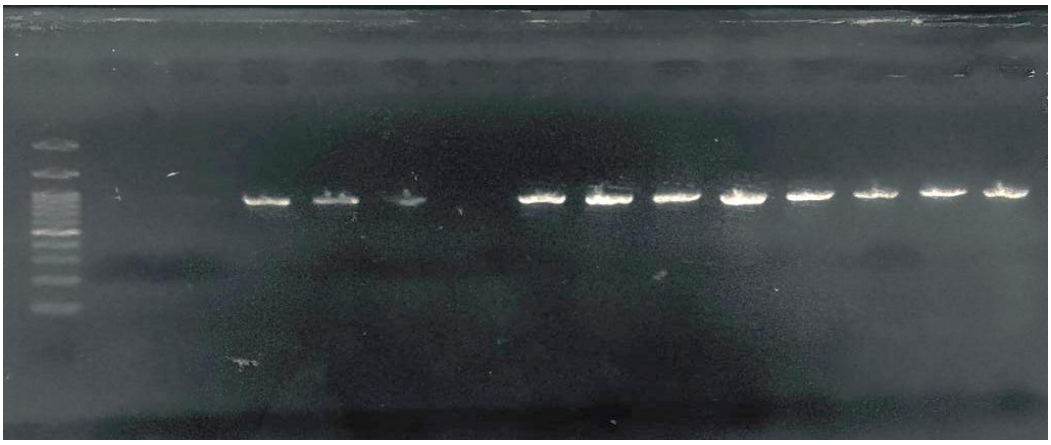

Fig 15 PCR amplification of bla TEM resistance gene in E. coli (L1=100 bp DNA ladder L =3 negative control L=3,4,5,11,12,14,19,20,21,26 positive samples)

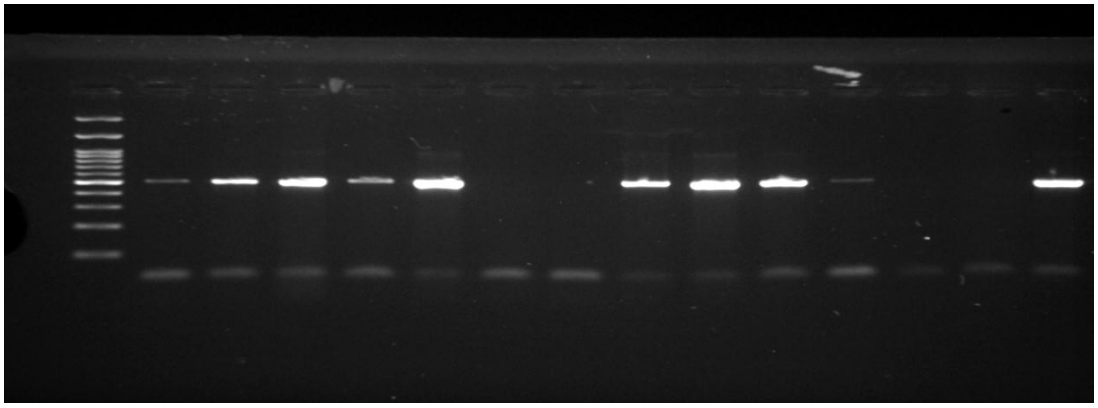

Fig 16 PCR amplification of tet A resistent gene in E. coli (L1=100 bp DNA ladder L =8 negative control L=2,3,4,5,8,9,10,14 positive samples L= 6,7,11,12,13 negative sample)

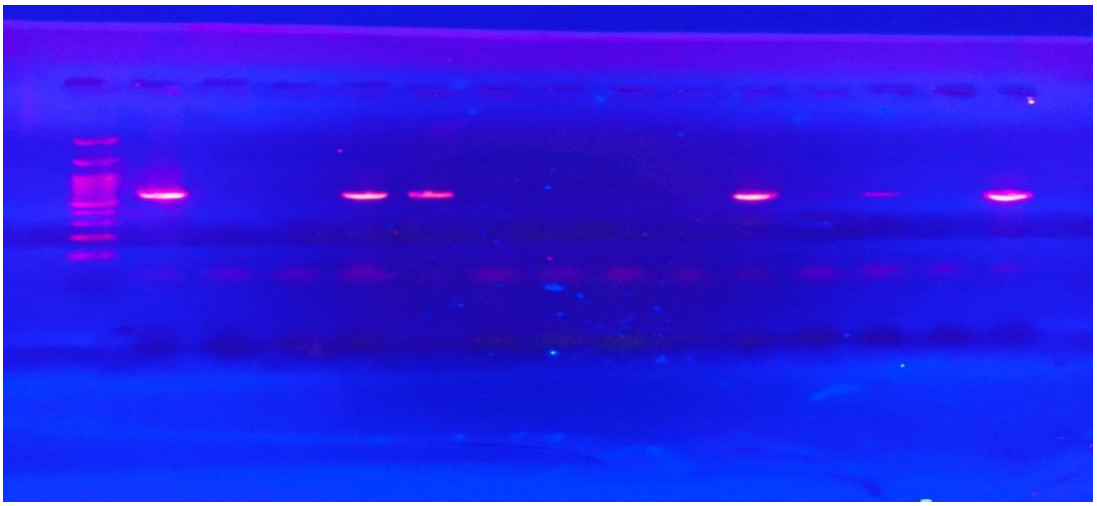

Fig 17 PCR amplification of tet B resistent gene in E. coli (L1=100 bp DNA ladder L =3 negative control L= 5,11,26,31,41 positive samples L= 3,4,12,13,19,20,27,29,32 negative sample)

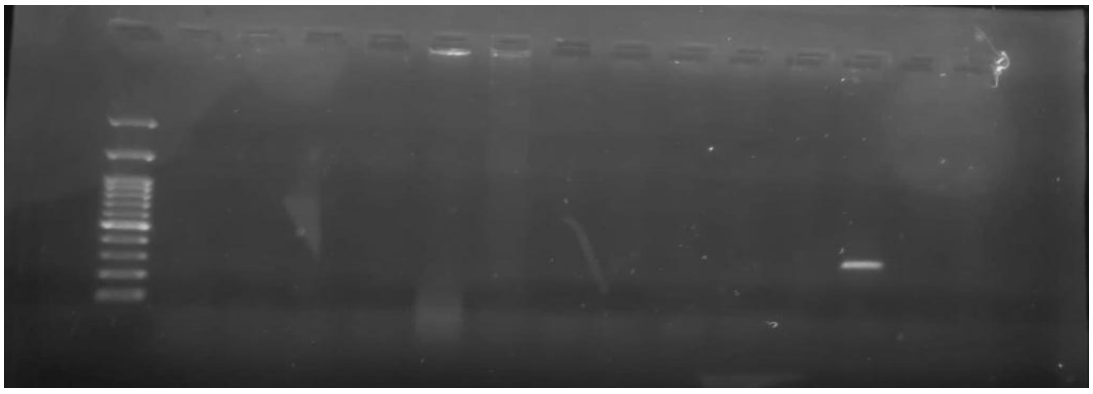

Fig 18 PCR amplification of aad resistent gene in E. coli (L1=100 bp DNA ladder L =3 negative control L= 27 positive samples L= 2,3,11,12,13,19,20,26 negative sample)

CHAPTER V

SUMMARY AND CONCLUSIONS

Antibiotic resistance is the ability of microorganisms to survive and reproduce in the presence of antibiotic with emergence of resistance, new antibiotics are released & widely used. Some of bacterial pathogens have developed MDR i.e. multidrug resistance which means resistance against three or more antibiotics. Antibiotic resistance is serious problem in all parts of the world. MDR in *E.coli* has become a major issue which has grown not only in humans but also in animals.

The present investigation were carried out in the department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana in which a total of 100 faecal samples were collected from healthy as well as diarrhoeic dogs from dog owners of the Punjab state and from small animal clinics of Guru Angad Dev Veterinary and Animal Science University, Ludhiana. The samples were projected to bacterial isolation followed by identification of isolates, culture sensitivity test and polymerase chain reaction (PCR). Out of 100 isolates, 66 were collected form diarrhoeic dogs and 34 were from healthy dogs. All the isolates were tested for sensitivity to various antibiotics as per CLSI guidelines. 16 different antibiotics were used. These antibiotics are Nalidixic acid Tetracycline (30mcg) Erythromycin(15mcg), Chloramphenicol (30mcg) Ampicillin/ sulbactum (10/10mcg) Trimethoprim(5mcg), Streptomycin(10mcg) Colistin(10mcg), Vancomycin(10mcg) Ciprofloxacin(5mcg), Amikacin(30mcg), Methicillin(5mcg) Co-trimoxazole (25mcg) Furazolidone (10mcg), Gentamicin (10mcg) Doxycycline (30mcg).

5.1 Isolation and identification results for *E.coli* &other bacteria

All the isolates were identified by Gram's Staining. The *E.coli* on MLA, lactose fermenting pink colour colonies were obtained and on EMB, colonies showed green metallic sheen. The colonies of *Klebsiella o*n MLA, were found to be large, shining mucoid lastose in appearance . The colonies of *Staphylococcus* on MSA were small, creamish yellow , slightly raised from the surface of agar. Colonies of *Bacillus* were flat with irregular edges and creamish white in colour.

E.coli isolates were taken and DNA were extracted them subjected to Polymerase Chain Reaction (PCR) for detection of *bla TEM* genes, *tetA* genes, *tetB* genes and *aadA* genes. Out of 67 isolates, 43 were positive for presence of *bla TEM* genes. Extracted DNA were subjected to PCR by using primer pair for *tetA* genes investigation. An amplification size of 502bp was obtained in positive control as well as in positive isolates. Out of 67 isolates, 36 were positive for presence of *tetA* genes. Extracted DNA were also subjected to PCR by using primer pair for *tetB* genes investigation. An amplification size of 634bp was obtained in positive control as well as in positive isolates. Out of 67 isolates, 23 were found positive for presence of *tetB* genes. Extracted DNA were subjected to PCR by using primer pair for *aadA* genes investigation. An amplification size of 284bp was obtained in positive control as well as in positive isolates. Out of 67 isolates, 11 were positive for presence of *aadA* genes

Conclusions

- Out of 100 samples processed, 67 suspected *E.coli* isolates were identified on the basis of cultural characteristics and biochemical tests and out of 67 isolates, 67 were identified through genus specific PCR.
- Some other bacteria identified out of 100 samples were *viz. Staphylococcus* (14/100), *Klebsilla* (13/100) and *Bacillus* (6/100).
- Culture Sensitivity Test of *E.coli* isolated from dogs, revealed highest resistance to ampicillin/ Sulbactam (100%), Nalidixic acid (91.7%).
- Culture Sensitivity Test of *Staphylococcus* isolated from dogs, revealed highest resistance to Amikacin (85.7%), Ampicillin and Sulbactam (71.4%).
- Culture Sensitivity Test of *Klebsiella* isolated from dogs, revealed highest resistance to Ampicillin and Salbactum (76%), Nalidixic acid (64.2%).
- Culture Sensitivity Test of *Bacillus* isolated from dogs, revealed highest resistance to Gentamicin (94%), Amikacin (66.6%).
- The molecular detection of AMR genes via PCR showed that maximum number of isolates harboured *bla TEM* gene followed by *tet A* gene.

- Using χ^2 test association between phenotypic and genotypic resistance of *Escherchia coli* to tetracycline, Methicillin, Ampicillin/ Sulbactam, found significant at 1% and 5% level of significance and no significance to Trimethoprim.

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