Isolation and Identification of Antibiotic Resistant *Escherichia coli* from Clinical cases

THESIS

SUBMITTED TO THE

BIHAR AGRICULTURAL UNIVERSITY

(FACULTY OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY)

Sabour (Bhagalpur), BIHAR

In partial fulfilment of the requirements

FOR THE DEGREE OF

Master of Veterinary Science

IN

(VETERINARY MICROBIOLOGY)

BY

Dr. MD ARMANULLAH

Registration No – M/VMC/324/BVC/2015-16

(Veterinary Microbiology)

BIHAR VETERINARY COLLEGE

PATNA-800014

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DEPARTMENT OF VETERINARY MICROBIOLOGY
Bihar Veterinary College, Patna-800014.
(Bihar Agricultural University, Sabour, Bhagalpur, Bihar)

CERTIFICATE-I

This is to certify that the thesis entitled **"Isolation and Identification of Multidrug Resistant *Escherichia coli* from Clinical cases"** submitted in partial fulfilment of requirement for the degree of Master of Veterinary Science (Veterinary Microbiology) of the faculty of Post-Graduate Studies, Bihar Agricultural University, Sabour, Bhagalpur, Bihar is the record of bonafide research carried out by Dr. Md Armanullah, Registration No.- M/VMC/324/BVC/2015-16,under my supervision and guidance. No part of the thesis has been submitted for any other Degree or Diploma.

It is further certified that the assistance and help received during the course of this investigation and preparation of the thesis have been fully acknowledged.

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We, the undersigned members of the Advisory Committee of **Dr. Md Armanullah, Registration No. M/VMC/324/BVC/2015-16**, a candidate for the award of degree of Master of Veterinary Science with major in **Veterinary Microbiology**, have gone through the manuscript of the thesis and agree that the thesis entitled "**Isolation and Identification of Multidrug Resistant *Escherichia coli* from Clinical cases**" may be submitted by **Dr. Md Armanullah**, in partial fulfilment of the requirements for the degree.

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CERTIFICATE- III

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Md. Armanullah

Date: 21-0-2017 (Md. ARMANULLAH)

Place: B.V.C, Patna

DEDICATED

TO

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Abbreviations

%	:	Percentage
mg	:	Milli gram
µg	:	Micro gram
µl	:	Micro liter
µ	:	Micron
bp	:	Base pair
conc.	:	Concentration
DNA	:	Deoxy-ribonucleic acid
dNTPs	:	Deoxy-nucleotide triphosphates
DW	:	Distilled water
et al	:	et alibi
g	:	Gram
h	:	Hour
i.e.	:	That is
M	:	Molar
mA	:	Milli ampere
ml	:	Milliliter
mM	:	Millimole
Mw	:	Molecular weight
MHA	:	Mueller Hinton Agar
NA	:	Nutrient Agar
°C	:	Degree centigrade
p mol	:	Pico mole
PCR	:	Polymerase chain reaction
pH	:	- Log hydrogen ion concentration
rpm	:	Revolutions per minute
TAE	:	Tris Acetate EDTA
Taq	:	*Thermus aquaticus*
Tris	:	Tris-hydroxy methyl aminoethane
UV	:	Ultraviolet

V	:	Volts
Viz.	:	Namely
w/v	:	Weight by volume
MDR	:	Multi Drug Resitant
CLSI	:	Clinical and Laboratory Standard Institute
OF	:	Ofloxacin
AK	:	Amikacin
O	:	Oxytetracycline
AMC	:	Amoxyclav
CEC	:	Cefotaxime /Clavulanic Acid
CTR	:	Cefotaxime
CTX	:	Ceftriaxone
A/S	:	Ampicillin /Salbactum
NX	:	Norfloxacin
P	:	Penicillin
CIP	:	Ciprofloxacin
C	:	Chloramphenicol
GEN	:	Gentamicin
DO	:	Doxycycline
CO-T	:	Co-trimoxazole
U	:	Urine
M	:	Milk
U/D	:	Uterine Discharge

Introduction

1 Introduction

Escherichia coli is the head of gammaproteobacterium in the family *Enterobacteriaceae*, the enteric bacteria, which was first described by Theodor Escherich in 1885, as *Bacterium coli* commune, living in the intestinal tracts of healthy and diseased animals (Todar, 2005). The species *E. coli* comprises of gram-negative, cylindrical rods, usually motile, non-spore forming, facultative aerobic bacteria, which are about 1 μm in diameter with a cell volume of 0.6-0.7 μm^3 (Greenwood *et al.*, 2002).

E. coli are a very diverse species of bacteria found naturally in the intestinal tract of all humans and many other animal species. Commensal *E. coli* bacteria are often found among the gut flora of warm-blooded animals including humans (Eckburg *et al.*, 2005). Although most of the *E. coli* strains are non-pathogenic to animal and human beings, some are known to cause intestinal and extra-intestinal diseases (Kaper *et al.*, 2004 and Fairbrother *et al.*, 2006). A subset of *E. coli* is capable of causing enteric/diarrhoeal disease, and a different subset cause extra-intestinal disease, including urinary tract infection (UTI). Based on pathogenicity attributes, *E. coli* has been classified into different pathotypes viz., enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin producing *E. coli* (STEC) / enterohemorrhagic *E. coli* (EHEC) / verocytotoxin-producing *E. coli* (VTEC) (Paniagua *et al.*, 1997; Nataro and Kaper, 1998; Matar *et al.*, 2002; Kaper *et al.*, 2004 and Bischoff *et al.*, 2005). Other *E. coli* pathotypes have been proposed, such as cell detaching *E. coli* (CDEC); however, their significance remains uncertain (Clarke, 2001 and Abduch-Fabrega *et al.*, 2002).

The normal microbial flora of the bovine genital tract is made up of a dynamic mixture of aerobic, facultative anaerobic and strict anaerobic microorganisms. Under natural conditions this environment is stable, protecting the host from the setting in of pathogenic or potentially pathogenic saprophytic microorganisms. The normal microbial flora of this tract is composed by bacteria of the genera *Staphylococcus*,

Streptococcus and the coliform group (Hafez, 1993). Enterobacteriaceae, especially *Escherichia coli* have been isolated from the urogenital tract of cattle in low numbers (Torres *et al.*, 1994 and Otero *et al.*, 2000). *E. coli* is a major cause of bacterial diarrhea and urinary tract infection (UTI) in humans (Stamm, 2002). While *E. coli* is an important urinary tract infection agent in pigs (Brito *et al.*, 1999) and dogs (Johnson *et al.*, 2001), it does not have this importance in cattle in which the frequency of urinary tract infection (UTI) is low (Yeruham *et al.*, 2006). Adhesion of *E. coli* to the uroepithelium may protect bacteria from the effect of urinary lavage, increasing their ability to multiply and invade renal tissue (Korhonen *et al.*, 1988). The uropathogenic *E. coli* (UPEC) possess adherence factors called pilli or fimbriae, which allow them to successfully initiate infection. Specific adhesion is mediated by bacterial proteins termed adhesins which may or may not be associated with fimbriae. Pap (pyelonephritis-associated pilli), sfa (S fimbrial adhesion) and afa (afimbrial adhesion) operons are most commonly found to encode respectively, P, S and Afa adhesins (Le Bouguenec *et al.*, 1992). Besides bacterial adherence, several virulence factors may contribute to the pathogenicity of UPEC, including the production of α-hemolysin, colicin and aerobactin (Emody *et al.*, 2003)

Mastitis causes great economic losses to the dairy sector worldwide, mainly through reduction of milk yield (Seegers *et al.*, 2003). Mastitis has, in principal, been considered a problem in high producing cattle in developed countries, but through field investigations and improved recordings it is now recognized as a significant problem even in low yielding animals in developing countries, with a detrimental effect on animal production and public health (Harouna *et al.*, 2009., Mdegela *et al.*, 2009 and Tesfaye *et al.*, 2010). The prevalence of mastitis varies between countries and geographical regions, usually the highest prevalence being found in countries with a poor developed dairy sector and lack of udder health control programs. *E. coli* and *Klebsiella* spp. are coliforms that can cause mastitis (Schukken *et al.*, 2011). These bacteria are part of the normal bovine intestinal flora and contaminate the environment, often bedding material, via faeces. During the puerperal period, the cow is especially sensitive to coliform infections since the immune defence at this time is lower than normal. All gram-negative bacteria produce endotoxin and an intra mammary infection usually results in a severe inflammatory response. *E. coli* most

frequently induce acute form of clinical mastitis, often of serious character with a rapid progress and sometimes with a fatal outcome (Sandholm & Pyorala, 1995). *E. coli* can also cause subclinical mastitis, although less frequently, but these strains are different than those causing clinical mastitis (Dogan *et al.*, 2005). The frequency of *E. coli* isolated from mastitis cases may vary considerably depending on herd and country.

Inflammations of the uterus in cows, recently classified as puerperal metritis, clinical endometritis, subclinical endometritis, and pyometra represents one of the most important causes of (sub) infertility in dairy herds (Nakao *et al.*, 1992; Huszenicza *et al.*, 1999; LeBlanc *et al.*, 2002a; Kim and Kang, 2003; Maizon *et al.*, 2004; Gilbert *et al.*, 2005 and Sheldon *et al.*, 2006) because the occurrence of various types of intrauterine puerperal metritis and clinical endometritis in herds usually reaches 20–40% and the occurrence of subclinical endometritis is probably even higher (Sagartz and Hardenbrook, 1971; Markusfeld, 1987; Stevenson and Call, 1988; Peeler *et al.*, 1994; Gilbert *et al.*, 2005; Foldi *et al.*, 2006 and Sheldon *et al.*, 2006). Numerous pathogens have been involved in the postpartum uterine disease complex. *E. coli* are one of the most prevalent bacteria isolated from the uterine lumen of cows suffering from uterine infections. Also a range of other bacteria also have been isolated from clinically diseased cows. Since *E. coli* infections are mostly found during the first days or week after calving, this germ has been thought to pave the way for subsequent infections with other bacteria or viruses.

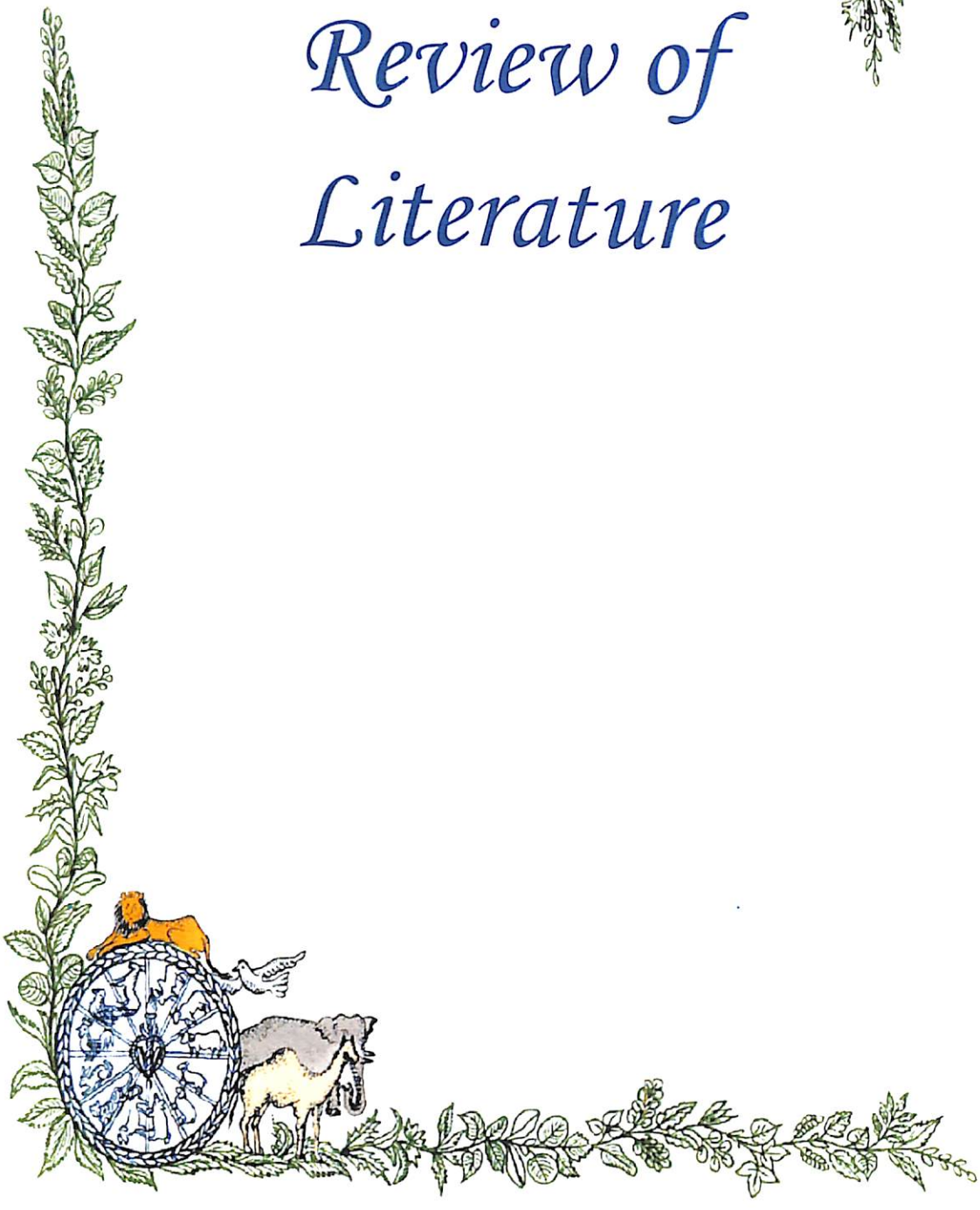
There are many kinds of antimicrobials used for preventing and controlling diseases, enhancing growth and increasing feed efficiency in food producing animals (CDC, 2005). The indiscriminate uses of antibiotics in treating animals results in resistance in micro-organisms (Philips *et al.*, 2004). Antibiotic usage is considered the most important factor promoting the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Levy, 1982; Witte, 1998).

In the recent years, Department of Microbiology has been receiving a large number of bovine samples from Teaching Veterinary Clinical complex, BVC, Patna for culture and sensitivity test and most of the isolates showing resistance to various antibiotics. Recently, due to the indiscriminate uses of antibiotics the incidence of

multiple drugs resistance in *E. coli* has been increased (Van den Bogaard, 1997; Witte, 1998; Khan *et al.*, 2005 and Sharada *et al.*, 2010). Thus, keeping in the view the reports of the grave situation due to emergence of multi drug resistant *E. coli,* the present work proposal was designed with the following objectives:

1. **To isolate and identify *Escherichia coli* from clinical samples of bovine**
2. **To generate antibiotic resistance profile of isolated *E. coli.***

Review of Literature



2 Review of Literature

E. coli, a member of the bacterial family of *Enterobacteriaceae*, is the most prevalent commensal inhabitant of the gastrointestinal tracts of humans and warm-blooded animals, as well as one of the most important pathogens (Kaper *et al.*, 2004). As a commensal it lives in a mutually beneficial association with hosts, and rarely causes disease. It is, however, also one of the most common human and animal pathogens as it is responsible for a broad spectrum of diseases.

Prior to the identification of specific virulence factors in pathogenic strains, *E. coli* was principally classified on the basis of the serologic identification of O (lipopolysaccharide, LPS) and H (flagellar) antigens (Kaper *et al.*, 2004). Based on the type of virulence factor present and host clinical symptoms, *E. coli* strains are classified into pathogenic types (pathotypes are defined as a group of strains of the same species causing a common disease): at least seven major pathotypes for enteric *E. coli*, whereas three *E. coli* pathotypes are extraintestinal strains (ExPEC) (Kaper *et al.*, 2004).

Intestinal pathogens spread through the faecal-oral route by ingestion of contaminated food or water. Enteropathogenic *E. coli* (EPEC) strains cause diarrhoea primarily in children, particularly under conditions of poor hygiene, as well as in animals (Kaper *et al.*, 2004). EHEC is a typically food-born pathogen causing haemorrhagic colitis or Hemolytic-uremic syndrome (HUS) (Kaper *et al.*, 2004). Typical enterohemorrhagic *E. coli* (EHEC) strains produce Shiga-like toxins (named Shiga toxin producing *E. coli*, STEC) similar to those produced by *Shigella*

dysenteriae making them the most virulent diarrhoeagenic *E. coli* known to date (Bilinski *et al.*, 2012). Enterotoxigenic *E. coli* (ETEC) are the most common pathogens causing travellers' diarrhoea with mild to severe watery diarrhoea in humans of all ages (Qadri *et al.*, 2005 and Al-Abr *et al.*, 2005). Enteroaggregative *E. coli* (EAEC) strains are associated with persistent diarrhoea in humans, and have been recognized as the cause of several outbreaks of diarrhoeal disease worldwide. EAEC, frequently found in the gut of asymptomatic humans, is the second foremost cause of travellers' diarrhoea worldwide. EAEC is frequently associated with diarrhoea in children in developing countries and in HIV-infected patients (Weintraub *et al.*, 2007 and Nataro *et al.*, 1998). Diffusely adherent *E. coli* (DAEC) causes diarrhoea particularly in children (Servin *et al.*, 2005). Enteroinvasive *E. coli* (EIEC) is frequent cause of watery diarrhoea and occasionally dysentery in both children and adults (Kaper *et al.*, 2004). EIEC strains are closely related to *Shigella* spp. Adherent-invasive *E. coli* (AIEC) is a recently emerged pathotype which has been associated with Crohn's disease lesions (Darfeuille *et al.*, 2002 and Nagroni *et al.*, 2012).

Neonatal Meningitis *E. coli* (NMEC) is a major cause of Gram-negative neonatal bacterial meningitis in developed countries with neurologic sequelae in many of the survivors (Gaschignard *et al.*, 2011). In the last years, a significant increase in multidrug resistant NMEC strains has been observed (Pouillot *et al.*, 2012).

Avian pathogenic *E. coli* (APEC), an additional animal pathotype found in the intestinal microflora of healthy birds, is responsible for extraintestinal diseases in several avian species (Johnson *et al.*, 2007 and Rodriguez *et al.*, 2005). Recent studies have revealed that APEC and ExPEC have similarities in their serogroups and

virulence factors suggesting a possible source of food-borne diseases (Johnson *et al.*, 2007 and Rodriguez *et al.*, 2005).

2.1 Role of *Escherichia coli* in urinary tract infection in animals

Extraintestinal pathogenic *Escherichia coli* (ExPEC) are incredibly diverse *E. coli* pathotype, and this genetic diversity is reflective of its occurrence in and colonization of diverse and highly specialized ecological niches. These strains, which can reside in the gastrointestinal (GI) tract, differ from normal commensal strains in that they possess virulence traits that allow them to colonize more inhospitable environments, such as the urogenital tract (Smith *et al.*, 2007). Besides from these bacteria-specific traits, host-specific factors are also required for disease. For this reason, ExPEC is considered a necessary but not sufficient cause for extraintestinal *E. coli* infection, meaning that additional factors are required for illness to occur (Rothman *et al.*, 2008). Consequently, ExPEC is considered an opportunistic pathogen for causing ExPEC-related illness.

In general, ExPEC can colonize the intestine, and when given the opportunity in individuals who might be vulnerable in some way (e.g., compromised immune system) or the presence of specific risk factors such as increased sexual activity and use of spermicides (Foxman, 2014), the bacterium can be transferred to the urogenital tract where it can cause a urinary tract infection (UTI). UTIs represent a serious burden of illness globally (Foxman, 2002; 2014), and 70–95% of these infections are caused by strains of *E. coli* (George and Manges, 2010; Hooton, 2012), often termed uropathogenic *E. coli* (UPEC), which is a subset of ExPEC.

The time between GI colonization and subsequent extraintestinal infection can be lengthy, with some studies using a 6-month risk window for development of the UTI (Manges *et al.*, 2007). This extended timeframe between exposure to an ExPEC strain and subsequent illness makes it very challenging to establish a "source" for the strain given the constant ebb and flow of *E. coli* strains within human and animal GI tracts. Similar observations of a high rate of *E. coli* turnover in the GI tract have also been made in animal populations (Hinton, 1986 and Hinton *et al.*, 1986).

ExPEC require specific virulence factor (VF) to cause disease in animals, it should be expected that there will be similarities in ExPEC across animal species. The niches that the ExPEC strains colonize extraintestinally require specific bacterial adaptations, and because some of these requirements are similar across animals, similarities in the subsets of VF necessary for causing extraintestinal disease in different animal hosts are expected. Among *E. coli* that cause extraintestinal disease in poultry, also known as avian pathogenic *E. coli* (APEC), similarities in VF profiles have been observed with the human ExPEC strains (Rodriguez-Siek *et al.*, 2005., Johnson *et al.*, 2008a; Belanger *et al.*, 2011 and Danzeisen *et al.*, 2013). Similarity in VF profiles of ExPEC from companion animals and humans has also been observed (Johnson *et al.*, 2009a).

Extraintestinal pathogenic *E. coli* from both animals and humans have been shown to have the ability to cross host species barriers. In mouse models, APEC and human ExPEC strains were shown to have UTI-causing ability (Jakobsen *et al.*, 2010a, 2012). Neonatal meningitis caused by *E. coli* (NMEC) is a serious and often fatal disease. In rat models, APEC strains were capable of causing meningitis (Tivendale *et al.*, 2010), although considerable work still needs to be performed to fully understand

virulence requirements in these isolates (Logue *et al.*, 2012). Human ExPEC strains have also been shown to be virulent for chicks (Moulin-Schouleur *et al.*, 2007).

Of considerable and justified concern is the global dissemination of highly virulent ExPEC clones over the past decade (Petty *et al.*, 2014 and Riley, 2014), including the clonal complex known as sequence type 131 (ST131) which now causes the majority of extraintestinal infections in humans, including UTI (Nicolas-Chanoine *et al.*, 2008; Novais *et al.*, 2012 and Price *et al.*, 2013). This clonal complex is thought to be highly virulent and is commonly multidrug resistant, including resistance to the critically important antibiotic classes of fluoroquinolones and third and fourth generation cephalosporins. The transmission pathways for these clones to disseminate globally remain unclear. The ST131 pandemic in humans appears to have little relationship to animal agriculture, at least in its origins, and likely emerged through evolutionary pressures in human hospitals and the community (Platell *et al.*, 2011b and Johnson *et al.*, 2012a). This strain is also found in companion animals, including pets that share residence with humans (Ewers *et al.*, 2010; Platell *et al.*, 2010, 2011 and Osugui *et al.*, 2014) and has been documented to spread among dogs and cats within the household (Johnson *et al.*, 2009d). While the possibility for horizontal transfer of virulence genes among *E. coli* exists (Johnson and Nolan, 2009), the global distribution of these highly virulent clones in multiple hosts and clear evidence of direct horizontal transmission among hosts would appear to make food an unlikely vehicle by which to explain this pattern of disease.

The establishment of extraintestinal disease by ExPEC is complex, usually involving strains carrying necessary VF first colonizing the GI tracts of individuals at-risk for disease (Moreno *et al.*, 2008). There are no concrete criteria, however, for

defining an *E. coli* strain as ExPEC. Some groups have made the assumption that possessing at least two of the following genetic determinants warrants the ExPEC label: *pap*A and/or *pap*C (P fimbriae structural subunit and assembly), *sfa/foc*DE (S and F1C fimbriae), *afa/dra*BC (Dr binding adhesins), *iut*A (aerobactin system), and *kps*M II (type II capsule; Johnson *et al.*, 2007a). This minimal predictive set of ExPEC virulence genes is based upon genotyping of many virulence-associated genes, but this subjective definition has not been thoroughly validated, as strains meeting this definition do not always cause disease. Using a list of known VF as a criterion misses the remaining genes in the pathogen's genome that may also be required for disease, but these might not be in all strains with the "correct" VF distribution.

The focus of most discussion concerning antibiotic resistant ExPEC in animals has been on antibiotics used for growth promotion / feed efficiency (Nordstrom *et al.*, 2013 and Riley, 2014). The antibiotics approved for this use, at least in poultry, have little activity against *E. coli* or are unrelated to the resistances that are relevant for treatment of human UTIs. Trimethoprim and sulfamethoxazole are antibiotics important for treating human ExPEC infections, but neither is used for growth promotion in the U.S. Fluoroquinolones such as ciprofloxacin are also important for treating human UTIs; these compounds have never been approved for growth promotion and were withdrawn from use in U.S. poultry in 2005. In fact, the National Antimicrobial Resistance Monitoring System (NARMS) in the U.S. has only identified two *E. coli* isolates from chicken meat with ciprofloxacin resistance since 2002 (U.S. Food and Drug Administration, Center for Veterinary Medicine, 2012b). The prevalence of ciprofloxacin resistance in UPEC has been steadily increasing, with the rate apparently accelerating even after the withdrawal of fluoroquinolones from U.S.

poultry (Sanchez *et al.*, 2012). Enrofloxacin is used in companion animals, and enrofloxacin resistance in *E. coli* UTI in dogs has been documented (Cooke *et al.*, 2002). Although the isolates from this study had VF patterns that differed from the most common human ExPEC isolates (Johnson *et al.*, 2009a), transmission between humans and companion animals is well-documented, as described previously.

Within the U.S., the U.S. FDA Center for Veterinary Medicine has initiated the process of phasing out of the growth promotion/feed efficiency label for critically important antibiotics over a 3-year period, to be completed by December 2016 (U.S. Food and Drug Administration, Center for Veterinary Medicine, 2012a, 2013). Consequently, while more research is needed to clarify the relationships between remaining on-farm antimicrobial usage and AR in human pathogens, a continued focus on the growth promoters will prevent us from accurately understanding and mitigating the pandemic crisis of multidrug resistant ExPEC.

Resistance to critically important antibiotics can be frequently found in ExPEC isolates, including UPEC and APEC. Similarities in the VF profiles of resistant and susceptible strains from animals have been interpreted as evidence that resistance develops in animals because of antibiotic use and is then transferred to people (Johnson *et al.*, 2007a, 2009b). The conclusions reached in the study by Johnson *et al.* (2007a), in particular, are problematic and not necessarily supported by the data and yet these conclusions have been widely cited, including in high profile documents such as the recent President's Council of Advisors on Science and Technology (PCAST) report from the Executive Office of the President of the United States (President's Council of Advisors on Science and Technology, 2014). For example, in that study both resistant and susceptible poultry isolates differed from the human isolates. The

principal co-ordinates analysis that was based on virulence genotypes and phylogroups showed this distinction. Because it is felt that the resistant human isolates were more similar overall to the poultry isolates than to the susceptible human isolates, it is concluded that the resistant strains in humans must have been derived from poultry. Furthermore, the finding of similar resistant strains in meat-eaters and vegetarians was interpreted as consistent with human-to-human transmission or errors in reporting of poultry consumption rather than human strains being derived from a source other than chicken. If these illnesses were truly being sourced from poultry, one would expect to see the susceptible human and poultry isolates overlapping as well, a finding not supported by this study. Finally, the observation that resistant and susceptible poultry ExPEC isolates were similar with respect to virulence should indicate that resistance is unlinked from virulence, likely through the gain and loss of resistance-encoding plasmids. This is not synonymous with the conclusion that resistance develops in poultry and subsequently spreads to humans. While it is possible for the virulence traits to be co-located with AR traits within the bacterium, in avian-source strains it is more common to observe virulence-associated traits on a plasmid that has no resistance traits (Johnson *et al.*, 2012b).

2.2 Role of *Escherichia coli* in bovine mastitis in animals

Bovine mastitis (BM) is responsible for major economic losses on dairy farms worldwide, caused by the decrease in milk production, increase in health care costs and increase in culling and death rates (Melchior, Vaarkamp and Fink-Gremmels, 2006). Moreover, mastitis poses a threat to human health since it may be responsible for zoonoses and for food toxin infections (Blum *et al.*, 2008 and Fernandes *et al.*, 2011). Staphylococci are the bacteria most commonly isolated from BM (Leitner *et al.*,

2011) and *Staphylococcus aureus* is a common cause of this disease (Oliveira *et al.*, 2007). However, coagulase-negative staphylococci (CNS) have become the most common BM isolate in many countries and are now predominant over *S. aureus* in most countries and could therefore be described as emerging mastitis pathogens (Tremblay *et al.*, 2013). Apart from Staphylococci, Coliforms, Enterococci and Streptococci are also frequently isolated from cows with mastitis (Smulski *et al.*, 2011). Depending on their primary reservoir and mode of transmission, mastitis may be classified as 'contagious' or 'environmental'. The main contagious microorganisms are *S. aureus* and Streptococcus species, being their main source the mammary gland of infected cows. On the other hand, the primary source of environmental mastitis pathogens is the habitat of the cow, and Streptococcus species and Gram-negative bacteria (*Escherichia coli* and *Klebsiella*) are examples of microorganisms included in this group (Bogni *et al.*, 2011).

E. coli is the common infectious agents of BM being considered one of the major agents worldwide (Blum *et al.*, 2015, Kempf, Loux and Germon, 2015). In England and Wales, *E. coli* was one of the major mastitis pathogens most commonly isolated from clinical cases (Bradley *et al.*, 2007). *E. coli* is a pathogen able to infect the mammary gland by entering the udder via the teat canal (Lipman *et al.*, 1995). Although the improvement in managing and housing of dairy cattle, mastitis caused by this bacterium continue to be a problem in several countries. *E. coli* presents several virulence factors / mechanisms such as toxins, adhesins, invasins, capsule production, the ability to resist serum complement and iron scavenging. To be able to promote disease, *E. coli* needs the successful combination of virulence factors, being these ones required to overcome the host's selection pressure and to colonize, multiply and

survive in the udder (Kaper, Nataro and Mobley, 2004). Clinical *E. coli* mastitis can range from mild with only local signs to severe disease with systemic clinical signs. In severe cases, *E. coli* can cause acute tissue damage and complete loss of milk production and even the death of the infected animal. According to Burvenich *et al.* (2003), the severity of mastitis promoted by this microorganism is related with factors other than strains characteristics, namely host factors. Generally, intramammary *E. coli* infections are spontaneously eradicated by host defences. However, this is not the case of recurrent infections that are more difficult to treat. Recurrent coliform mastitis infections can be caused by the persistence of the microorganism within the mammary gland (Bradley and Green, 2001), being possibly the biofilm production responsible for this persistence. Accordingly, Fernandes *et al.* (2011) demonstrated that all their *E. coli* mastitis isolates were able to form biofilm although at different levels. Any *E. coli* pathotype even non-pathogenic strains can cause mastitis (Shpigel *et al.*, 2008). More recently, Suojala *et al.* (2011) showed that mastitis-causing *E. coli* are typical commensals. Blum and Leitner, (2013) evidenced that the most common virulence factors exhibited by this bacterium causative agent were *lpf*A (long polar fimbriae), *iss* (increased serum resistance) and *ast*A (enteroaggregative *E. coli* heat-stable enterotoxin 1). However, none of them was specific to mastitis *E. coli* pathogens since none characterized the majority of the strains studied. *lpf*A was reported as having a potential role in the improvement of virulence in the mammary gland by promoting epithelial adhesion (Dogan *et al.* 2012). *Iss* was repeatedly found in *E. coli* BM isolates. Shpigel *et al.* (2008) proposed that it can exist as an association of unknown factors with pathogenicity of mastitis *E. coli* persistent bovine IMI strains. On the other hand, Blum and Leitner, (2013) and Suojala *et al.* (2011) concluded that mastitis *E. coli* strains do not have known specific markers of virulence and therefore until now

none virulence factor was associated with their pathogenicity, being this pathotype still uncharacterized. Additionally, Fernandes *et al.* (2011) also shown that the pathogenicity of BM *E. coli* isolates is not mediated by a single and specific virulence factor but classified this bacterium as 'opportunistic pathogen with different virulence factors'. They reported several combinations of different virulence factors in the *E. coli* isolates. The *fim*H gene, responsible for type 1 fimbriae expression and which has an important role on biofilm formation, was observed in all the isolates tested. This is in agreement with previous studies that proved the existence of these adhesins both in commensal and pathogenic *E. coli* isolates (Fernandes *et al.*, 2011). Taking this into account and despite the numerous attempts, it was impossible to group these isolates into pathotypes able to cause mastitis. As general conclusion these researchers abolish the idea of the existence of a specific mammary pathogenic *E. coli*. No association was also found between virulence factors, phylogeny groups and antimicrobial resistance traits and the severity of mastitis caused by this microorganism (Suojala *et al.*, 2011). Several authors reported that a strain selection during infection can take place in the mammary gland (Blum *et al.*, 2008, Blum and Leitner, 2013).

3.2 Role of *Escherichia coli* in uterine infection in animals

Dairy cattle are remarkable amongst domestic animals as bacterial contamination of the uterine lumen is ubiquitous after parturition (Elliott *et al.*, 1968; Griffin *et al.*, 1974; Sheldon *et al.*, 2002). As the cervix dilates to allow passage of the calf at parturition, the anatomical barrier to bacterial contamination is breached, allowing microorganisms from the cow's skin, faeces and surrounding environment to enter the uterus (Sheldon and Dobson, 1999). In one study, 93% of uteri obtained within 2 weeks of calving were contaminated with bacteria and others have shown the

presence of bacteria in more than 60% of animals 3 weeks after parturition (Elliott *et al.*, 1968; Griffin *et al.*, 1974 and Sheldon *et al.*, 2002). Many cows spontaneously eliminate this contamination; however, at least 20% of cows fail to clear the bacteria and in 10% to 15% of animals infection persists within the uterus, causing endometritis (Griffin *et al.*, 1974; Borsberry and Dobson, 1989 and Dhaliwal *et al.*, 2001). Uterine infection and the associated inflammatory and immune responses compromise animal welfare, causing subfertility and infertility (Borsberry and Dobson, 1989; Sheldon and Dobson, 2004). Endometritis is associated with lower conception rates, increased intervals from calving to first service or conception, and more culls for failure to conceive (Borsberry and Dobson, 1989). Furthermore, the subfertility associated with endometritis persists even after clinical resolution of the disease. The financial losses associated with uterine infection depend on the direct cost of treatment, reduced milk yields and subfertility. Despite great effort to control uterine disease in cattle, the number of animals treated annually for endometritis reported by the National Animal Disease Information Service (NADIS, the UK Veterinary Sentinel Practice Network, http://www.nadis.org.uk) has steadily increased since 2001; in 2006, almost 6% of animals required direct intervention by a veterinarian. An increased incidence of uterine disease and the associated costs will have major implications for the economic sustainability of both the individual animal and the national herd.

The flora cultured *in vitro* from the early post partum uterus represents a wide spectrum of environmental contaminants and some anaerobic species, and bacteria isolated from the uterine lumen have been categorised according to their pathogenicity within the uterus (Ruder *et al.*, 1981; Olson *et al.*, 1984; Farin *et al.*, 1989; Noakes *et al.*, 1989; Bonnett *et al.*, 1991 and Laven *et al.*, 2000). Bacteria are classed as

'recognised uterine pathogens' associated with uterine endometrial lesions; 'potential pathogens' frequently isolated from the bovine uterine lumen and cases of endometritis, but not commonly associated with uterine lesions; and, 'opportunist contaminants' transiently isolated from the uterine lumen but not associated with endometritis (Sheldon *et al.*, 2002 and Williams *et al.*, 2005). The recognised uterine pathogens are *E. coli*, *Arcanobacterium pyogenes*, *Fusobacterium necrophorum*, *Prevotella melaninogenica* and *Proteus* species, and these bacteria are associated with greater endometrial inflammation and more severe signs of clinical uterine disease (Farin *et al.*, 1989; Bonnett *et al.*, 1991; Sheldon *et al.*, 2002 and W illiams *et al.*, 2005).

The bacterium *E. coli* is the most commonly isolated pathogen from the post partum uterus and, in the first few days after calving, dominates the uterine flora (Hussain *et al.*, 1990 and Huszenicza *et al.*, 1999). Of greater importance is the infection with *E. coli*, which appears to increase the susceptibility of the endometrium to subsequent infection with A. *pyogenes* (Williams *et al.*, 2007). These observations highlight the substantial impact *E. coli* has on post partum uterine health.

2.4 Prevalence of *E. coli* in clinical samples

Soomro *et al.* (2002) analysed hundred milk samples for the isolation of *E. coli* by inoculation on different bacteriological media and confirmation by biochemical tests, and reported a prevalence of 57%.

On the basis of microbial culture and biochemical tests urine samples collected from 7 days to 90 days old calves yielded prevalence of *E. coli* at 35%, α- haemolytic streptococci at 10 % , *Corynebacterium renale* at 14% and *Arcanobacterium pyogenes*

at 5 %. The prevalence for *Pseudomonas aeruginosa,* plasma coagulase negative *Staphylococcus* species and Proteus species was recorded to be 12 % each (Yeruham *et al.*, (2004). In a similar study conducted later, Yeruham *et al.* (2006) found *E. coli* as the most frequent cause of UTI in calves and cows (29% and 31 %, respectively).

Zinnah *et al.* (2007) analysed 100 samples, including human feces and urine, rectal swab of cattle, sheep and goat, cloacal swab of chicken, duck and pigeon, drain sewage and soil and reported a isolation of 10% *E. coli* from 10 different biological and environmental sources.

Ekera and Yardimcib, (2008) examined three hundred rectal faecal samples and 213 milk samples for isolation of *Escherichia coli* O157:H7 using standard cultural methods and reported a isolation of 3.7 % and 1.4 % *E. coli* from faecal samples and milk samples, respectively.

Braun *et al.*, (2008), conducted a study on 17 cattle with cases of pyelonephritis. Bacteriological examination of urine yielded *Corynebacterium renale* in 11 animals (64%), *Arcanobacter pyogenes* in two (11%) and *E. coli* in one (approx. 6%).

Kumar and Prasad, (2010) perform a study to isolate *E. coli* from milk and reported that out of 135 samples, 11 samples were positive for *E. coli.*

Ali and Abdelgadir, (2011) estimated the incidence of opportunistic pathogen, *E. coli*, in cow's milk in Khartoum State (Khartoum, Khartoum North and Omdurman). They analysed hundred milk samples and reported that 63% of the samples were *E. coli* positive.

Zahera *et al.* (2011) processed a total of 50 human urine samples and reported a prevalence of *E. coli* as 30%. They also reported that isolates showed a high sensitivity pattern to common drugs like tetracycline (22%), norfloxacin (18%) while, moderate sensitivity pattern to ampicillin (14%), cephalosporin (10%) but resistant pattern of common drugs like penicillin (22%) and cephalosporin (18%).

Tanzin *et al.* (2016) collected 34 milk samples from 17 different healthy cattle (n=14) and buffaloes (n=3) and analyzed in laboratory by staining, cultural and biochemical characteristics followed by polymerase chain reaction targeting 16 S rRNA of *E. coli* and antibiotic sensitivity pattern of the isolates. They reported the presence of *E. coli* in three samples (2 from cattle, 1 from buffalo). They also reported that most of the isolates were resistant to gatifloxacin and one isolate showed intermediate resistance to ofloxacin while sensitive to ciprofloxacin and levofloxacin.

Zdolec *et al.* (2016) collected milk samples from healthy cows without any signs of mastitis (n=17) and drug-treated cows with cured mastitis (n=19) and reported the occurrence of higher multi drug resistant *E. coli.*

Hariharan *et al.* (2016) analysed 52 culture positive urine samples from dogs in Grenada and reported that 65.5% of isolates were Gram-negative bacteria, with *E. coli* as the predominant species, followed by *Proteus mirabilis*, and *Pseudomonas aeruginosa*. Other Gram-negative isolates included *Klebsiella pneumoniae*, *Acinetobacter anitratus*, and *Serratia plymuthica*. They also reported that 19% isolates were resistant to enrofloxacin and more than two-thirds of isolates were resistant to tetracycline.

Sharma *et al.* (2016) reported that *E. coli* was the most frequent isolate throughout the three years (67.66 % of the total isolates). Isolates showed the lowest percentage of resistance against imipenem between 11.86 % (2012) and 11.36 % (2014). Resistance for nitrofurantoin decreased over the three consecutive years from 36.1 % (2012) to 18.15 % (2014). Over the successive years, resistance to ceftriaxone tends to increase from 53.39 % (2012) to 73.33 % (2014). *E. coli* showed absolute resistance (100 %) to cotrimoxazole and tetracycline. On an average over the three years *E. coli* showed high amount of resistance to fluoroquinolones (75 %) and aminoglycosides (67 %).

Barkalita *et al.* (2016) recovered 45 *E. coli* isolates from faecal samples of 77 diarrhoeic and 85 healthy animals and birds, 51 milk samples and 48 diarrhoeic human stool samples. They also reported that multiplex PCR based study reveal presence of 24 Shiga toxin-producing *E. coli* (STEC) and 21 as enteropathogenic *E. coli* (EPEC).

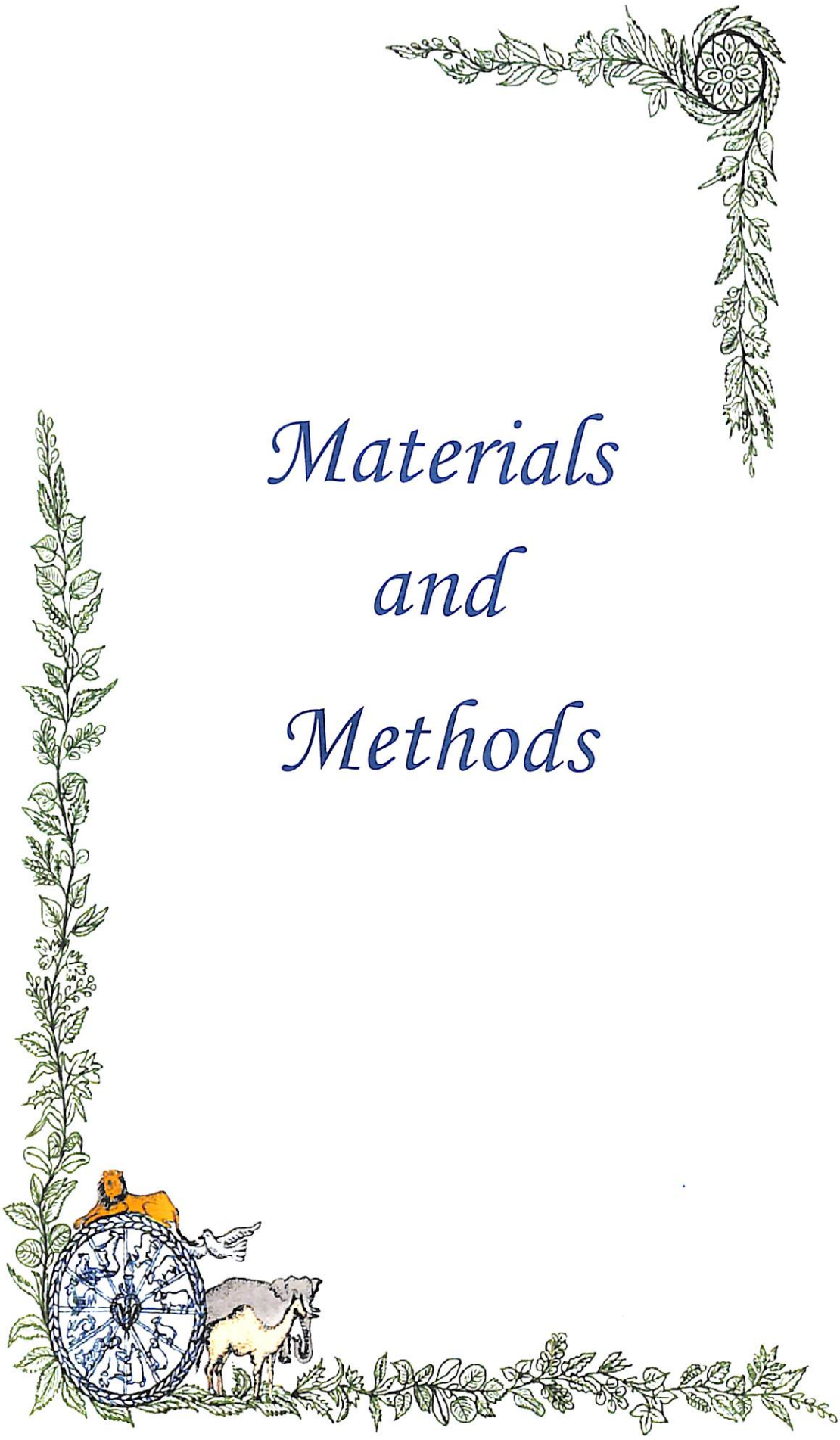
2.4 Antimicrobial resistance in *Escherichia coli:*

The discovery of "Penicillin" by Alexender Fleming in 1929 is considered as one of the most important breakthroughs in the history of human health care management. The use of antimicrobials has almost revolutionised the management of infectious diseases. They are in use for the last 70 years in both human and veterinary medicine. Unfortunately, the problem of antibiotic resistance emerged only a few years later the use of penicillin started for treating patients in 1944. As early as in 1948, Penicillin resistant *Staphylococcus aureus* strains were isolated from patients in Britain (Barber and Rozwadowksa, 1948) and *Mycobaterium* isolates resistant to *Streptomycin* were also observed (Crofton and Mitchison, 1948). The injudicious use of antimicrobial in human and veterinary health care along with its use in various

agricultural practices to prevent and control infectious diseases and as growth promoting agent in livestock has contributed in emergence of pathogens with ability to resist most of the antibiotics with are in use. World health organisation has defined antimicrobial resistance as "Antimicrobial resistance is the resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it" (Economou and Gousia, 2015). Besides other reasons, the use of antimicrobial agents as growth promoting substance has important bearing on the development of antimicrobial resistance in microbes. The use of antimicrobial growth promotants started in mid -1950s when Stokestad and Jukes reported the positive correlation between use of antibiotics and production performances in animals (Stokestad and Jukes, 1950). *E. coli* are considered to be normal commensal which constitute the part of intestinal flora. Obviously, these *E. coli* strains are non pathogenic. However, large numbers of *E. coli* pathotypes are known that have pathogenic potential and causes various diseases in animals and human. On the basis of pathologies produced, various pthotypes of *E. coli* were characterised such as, viz., enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin producing *E. coli* (STEC) / enterohemorrhagic *E. coli* (EHEC) / verocytotoxin-producing *E. coli* (VTEC) (Paniagua *et al.*. 1997; Nataro and Kaper, 1998). Because of its commensal existence in alimentary tract and as a pathogenic agent *E. coli* are exposed to good number of antimicrobial agents that leads to selection of strains showing resistance to these antimicrobials. Various studies conducted with an aim to evaluate the antibiotic resistance pattern shown by the *E. coli* have found varying percentage of multi drug resistant *E. coli* strains isolated from samples collected from animals. A large number of *E. coli* isolated from meat

producing animals were tested for antibiotic resistance pattern. In an investigation for antimicrobial resistance profiles of E. coli isolates from broiler chickens caecal content, of 600 *E. coli* isolates tested, 475 (79.2%) were resistant to one or more antimicrobials, 326 (54.3%) were resistant to three or more antimicrobials, 65 (10.8%) were resistant to five or more antimicrobials, and 15 (2.5%) were resistant to seven or more antimicrobials. The most common resistance was to tetracycline (69.2%), followed by streptomycin (48.2%), kanamycin (40.3%), and sulfisoxazole (38.0%) (Mainali *et al.*, 2013). A total of 374 E. coli isolates from mastitic milk samples were analyzed for antibiotic sensitivity pattern. The most frequently observed resistance was tetracycline (23.3%), followed by streptomycin (17.1%), ampicillin (16.6%), neomycin (11.8%), and trimethoprim / sulfamethoxazole (11.2%). Multidrug resistance was observed in 15.5% of isolates (Tark *et al.*, 2017). *E. coli* isolated from diarrheic neonatal calves were tested for antimicrobial susceptibility of multidrug-resistant (MDR) *E. coli* using PCR. Out of 23 virulent isolates, 20 (87.0%) were MDR; the highest prevalence of resistance was recorded for the macrolide-lincosides, followed by the tetracyclines and penicillins. Also, 17 of 23 (74.0%) virulent isolates were resistant to sulfadimethoxine, and 10 of 23 (43.5%) were resistant to trimethoprim-sulfamethoxazole. Additionally, 60 of 74 (81.0%) avirulent isolates were MDR (Barigaye *et al.*, 2012). From the 245 isolates from faecal samples of healthy goats, the E. coli harbouring any gene for Shiga toxins (stx 1/stx 2) was detected in 36 (14.7%). Upon subjected to antibiotic sensitivity test, the STEC isolates resistantce most frequently to erythromycin (80.5%), amikacin (52.7%), cephalothin (50%), kanamycin (41.6%), neomycin (36.1%) and gentamycin (36.1%) and less frequently to norfloxacin (2.7%), enrofloxacin (2.7%), and ciprofloxacin (2.7%). Multidrug resistance was observed in eleven STEC isolates (Mahanti *et al., 2015*).

Materials and Methods



3.1 MATERIALS

3.1.1 Design of study

The study was designed for isolation, biochemical and molecular confirmati and antibiotic sensitivity profiling of *Escherichia coli* isolated from bovine clinic samples. Urine, milk and uterine discharge were collected from clinical cases t processing in laboratory.

3.1.2 Sample collection area and period of study

All the samples were collected from bovine clinical cases presented at TVC Bihar Veterinary College, Patna during period of August 2016 to May 2017.

3.1.3 Experimental Sample

A total of two hundred (200) samples were collected and the details of sampl used in this present study were given in Table 1.

Table 1: Sample from different bovine clinical cases

SL. No.	Type of samples	No. of Sample
1.	Urine	127
2.	Milk	63
3.	Uterine discharge	10
	Total	**200**

3.1.4 Collection & transportation of samples

Approx. one ml of different samples was collected aseptically in a steril sample container, properly labeled as sample ID or case no. and caped to avoi

contamination. The collected samples were placed in container with ice cubes and transported to the laboratory. Processing of sample was initiated within 1 hr of collection.

3.1.5 Media, buffers and reagents

The media and chemicals used in this study were procured from reputed firms. Some of them included MacConkey broth, Eosin metylene blue agar, nutrient agar, nutrient broth, agar powder purified and Muller Hinton agar (Himedia, India). The detail of the preparation of media, buffers and reagents used in this study were listed in Appendix.

3.1.6 Chemicals used for molecular studies

All the chemicals used in the study were of molecular biology grade except the requirements for cultural isolation. The chemicals were procured from Thermo Scientific (USA), Fermentas (India), SRL (India), Xcelris (India) and other reputed firms. The chemicals used in PCR study included Dream *Taq* DNA polymerase (Himedia, India), 10 x PCR buffer (Fermentas), dNTP mixture (Fermentas), 6X gel loading dye (Thermo Scientific), Gene ruler 100 bp plus DNA ladder (Thermo Scientific), nuclease free water (Thermo Scientific, USA), and ethidium bromide (Sigma, USA).

3.1.7 Plasticwares and glasswares

Plasticwares used in this study were obtained from Himedia (India), Axiva (India), Greiner (Germany) and Axygen (USA) whereas Glasswares were obtained from Tarsons (India), Borosil (India) and Schott Duran (Germany). Glasswares were thoroughly washed and sterilized wherever necessary following the recommended procedures.

3.1.8 Equipments

Important equipment used in the study were electronic balance (Denver, USA), Centrifuge (REMI, India),deep freeze (-20°C) (Blue Star, India), Gel documentation system (Biorad, USA), Incubator (REMI, India), pH meter (LABMAN), variable pipette set (Tarson ,India), horizontal Gel electrophoresis apparatus (Thermoscientific,

China), vertical SDS-PAGE apparatus (BioRad, USA), refrigerated centrifuge (Remi, India), shaker incubator (Julabo-Shake Temp, SW22, Switzerland), water bath (YSI, India), vortexing (Tarson, India), autoclave ((Instrumentation India, India), and water distillation apparatus (Millipore India Pvt. Ltd., New Delhi, India), laminar air flow bench (Ikon instruments, India), microwave oven (LG, India) and micropipette (Eppendorf, Germany).

3.1.9 Oligonucleotide Primers

Primers used in the study were custom synthesized from Xcelris (India). The details of the primers are given in Table 2.

Table2. Oligonucleotide primers used in this study.

SL. NO.	*Primer Sequences (5 '- 3 ')*	Target gene/locus	Expected Product size (bp)	References
1.	ECA75F: GGAAGAAGCTTGCTTC TTTGCTGAC	16srRNA	544	Sabat *et al.*, 2000
	ECR619R: AGCCCGGGGATTTCAC ATCTGACTTA			

F: Forward primer, R: Reverse primer

3.2 METHODS

3.2.1 Enrichment:

Approximately 1 ml of samples were inoculated with 10 ml of pre-sterilized MacConkey broth in front of gas flame and incubated for 24 hr at 44°C (Feng *et al.*, 2016).

3.2.2 Isolation on selective media:

Eosin methylene blue agar was used as selective media for isolation of *E. coli* from the collected clinical samples. A loop full of MacConkey broth grown overnight and showed the turbidity was streaked on the EMB agar and incubated at 37°C for 24 hr. Characteristic colonies of the *E. coli* on EMB agar i.e., purple with black centre and green metallic sheen colonies were selected for further studies.

3.2.3 Biochemical characterization of presumptive *E. coli* isolates

The colonies of presumptive *E. coli* on EMB agar were confirmed by biochemical test using KB001 HiIMViC Biochemical test kit (Himedia, India) in order to confirm *E. coli* isolates involved in clinical cases of bovine. A single presumptive colony from each clinical samples were grown in 5 ml nutrient broth for 4-6 h at 37^{0}C and 0.5 µl of culture was inoculated on each well of test kit and incubated for 24 h at 37^{0}C. The results were interpreted after addition of reagents in wells as per manufacturer guidelines with the help of result interpretation chart (Himedia, India).

3.2.4 Preservation of the isolates:

All biochemically confirmed isolates were preserved in nutrient agar stab (0.9% agar) with overnight incubation at 37°C and stored at room temperature.

3.2.5 Re-use of the preserved isolates

The preserved isolates in nutrient agar stabs were revived in the nutrient broth with 24 hr incubation at 37°C. The DNA templates (lysate) were prepared from these broth cultures by snap and chill method for screening with PCR assay and in order to perform antibiotic sensitivity test of confirmed isolates.

3.2.6 Template DNA Preparation by snap chill method (Swetha *et al.*, 2015):

About 1.5 ml of overnight culture (sample) grown in nutrient broth were taken in eppendorf (2 ml) tube and centrifuged at 10,000 rpm for 10 min to pellet the bacteria and supernatant was discarded.

1. One ml normal saline was added to the bacterial pellet and vortexed.

2. Again centrifuged at 10,000 rpm for 10 min to obtain the pellet & supernatant was discarded.
3. Then 100 µl distilled water was added to the bacterial pellet and subjected to boiling for 8 min.
4. After boiling it was immediately transferred to -20°C till preparation of reaction mixture for PCR assay. This was considered as bacterial lysate.
5. Then the tube was taken out of freezer and kept at room temperature for thawing and centrifuged at 4,000 rpm for 3 min before use.
6. Supernatant was taken as genomic DNA template (bacterial lysate).

3.2.7 Detection of *E.coli* by 16S rRNA PCR-assay:

A PCR assay was standardized for the direct detection of *E. coli* by identifying species specific *16SrRNA* gene as per the method described by Sabat *et al.* (2000) with some modification. The PCR was performed using the bacterial lysate as template DNA prepared by snap chill method. Amplification reaction was performed in 25 µl reaction volume each containing 2.5 µl 10X PCR amplification buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM $MgCl_2$), 2.5 µl of dNTP (2.0 mM), 2.0 µl (10 pmol) of forward and reverse primers of 16SrRNA gene, 0.2 µl Taq DNA polymerase (5 unit/µl), 5.0 µl of bacterial lysate and nucleus free water upto 25 µl was used to amplify 16SrRNA gene at 544 bp.

The PCR programme included initial denaturation at 94°C for 3 min followed by 40 cycles of denaturation (94°C for 30 sec), annealing (72°C for 45 sec) and extension (72°C for 45 sec) with final extension at 72°C for 10 min.

3.2.8 Agarose gel electrophoresis

The gel casting tray was placed on a levelled surface and open sides were sealed with adhesive tape. The gel comb was the placed across the casting tray, so that the teeth of comb remain 1 mm above the base of tray.

Agarose gel (1.5%) was prepared by boiling molecular grade agarose in 1X Tris Acetate-EDTA buffer to dissolve completely. After boiling and cooling to 50°C, 1% ethidium bromide solution was added to give a final concentration of 0.5µg/ml

agarose gel solution. The molten agarose was poured onto gel casting tray and left undisturbed for about half an hour to solidify the gel.

After solidification, the comb was taken out and adhesive tape was removed. The gel with casting tray was then submerged in electrophoresis tank filled with sufficient quantity of 1X TAE electrophoresis buffer (about 1mm above the surface of the gel) with the wells at cathode end of the tank.

With the help of micropipette 10µl of PCR product was mixed with 2 µl of loading dye (6X) and loaded into wells. Electrophoresis was performed at 5 V/cm for 1 h and the progress of mobility was monitored by migration of bromophenol dye. To estimate the length of fragments, a 100-bp plus DNA ladder was run on each gel, and the amplicons were observed and documented under UV transilluminator/ gel documentation system.

3.2.9 Antibiotic susceptibility pattern

The antibiotic susceptibility pattern was performed by disc diffusion method (Wayne, 2002). For this first of all, the test isolate was inoculated overnight in nutrient broth at 37^0C. About 100 µl of the growth culture was spread on Mueller-Hilton agar plates with sterile L-shaped spreader and antibiotic disc of 5 mm were sticked to the plates with forcep, belonging to 15 antibiotics namely- ampicillin/ sulbactum (10/10 µg) ciprofloxacin (5µg), amoxiclav (30µg), ofloxacin (5µg), amikacin (30µg), cefotaxime/ clavulinic acid (30/10µg), cefotaxime (30µg), ceftriaxone (30µg), chloramphenicol (30µg), penicillin-G (10µg), gentamicin (10µg), norfloxacin(10µg), oxytetracyclin (30µg), co-trimoxazole (25µ), and doxycycline hydrochloride (30µg). All antibiotic disc containing plates were incubated for 18-24 h at 37^0C. The zone of inhibition of growth was measured by the scale and results were interpreted according to the guidelines of CLSI, (2014).

3.2.10 Prevalence of multidrug resistant *E. coli* isolates among bovine clinical samples

The result obtained from the antibiotic sensitivity testing of all *E. coli* isolates were analyzed for categorization of isolates as MDR *E. coli* or not MDR *E. coli* based on the recommendations of Magiorakos *et al.* (2012). They defined MDR as acquired

non-susceptibility to at least one agent in three or more antimicrobial categories. In this study, a total of six antimicrobial categories including nine antimicrobial agents were used to classify the isolates as MDR (Magiorakos *et al.*, 2012). The categories wise list of antimicrobial agents used in this study were listed in table 3:

Table 3. Antimicrobial categories used for MDR study

Antimicrobial categories	A		B		C	D		E	F
	Aminoglycosides		**Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins**		**Fluoroquinolones**	**Penicillins + β-lactamase inhibitors**		**Phenicols**	**Tetracyclines**
Antimicrobial agent	Gentamicin	Amikacin	Cefotaxime	Ceftriaxone	Ciprofloxacin	Amoxicillin-clavulanic acid	Ampicillin-sulbactam	Chloramphenicol	Doxycycline

Table 4. Incidence of *Escherichia coli* in bovine clinical samples

Sr. No.	Sample type	No. of sample screened	Growth at 44^{0}C	Metallic sheen on EMB	16SrRNA positive
1.	Urine	127	95	72 (56.69)	58 (45.67)
2.	Milk	63	34	17 (26.94)	12 (19.05)
3.	Uterine discharge	10	03	01 (10.00)	1 (10.00)
Total		**200**	**132**	**90 (45.00)**	**71 (35.50)**

* Value in the parenthesis includes percentage

Results

The results of study for the isolation by conventional method and identification by biochemical and molecular methods of *Escherichia coli* from bovine clinical samples submitted in the department of Veterinary Microbiology for microbiological investigations are presented in the section. The isolates were also subjected to antibiotic susceptibility profiles to determine the susceptibility and resistance pattern and the results thereof are also presented.

4.1. Isolation of *Escherichia coli* by conventional method

A total of 200 bovine clinical samples including urine, milk and uterine discharge were allowed for enrichment in MacConkey broth with overnight incubation at 44^0C for 24 h followed by streaking on the Eosin methylene blue agar (EMB agar) (Feng *et al.*, 2016). By conventional enrichment and plating on EMB agar typical colony of *E. coli* (purple with black centre and green metallic sheen colonies) was produced by 90 samples (45.00%) after 24 h incubation at 37^0C (Table 4, Fig.1-2).

By conventional method, the distribution of *E. coli* were found in 72 (56.69%) of cattle urine, 17 (19.05%) of cattle milk and 01 (10.00%) of uterine discharge. (Table 4).

4.2. Biochemical characterization of *Escherichia coli*

The colonies of presumptive *E. coli* on EMB agar were confirmed by biochemical test using KB001 HiIMViC Biochemical test kit (Himedia, India) in order to confirm *E. coli* isolates involved in clinical cases of bovine. The KB001 HiIMViC Biochemical test kit result showed that 71 presumptive *E. coli* isolates were positive for characteristics biochemical reactions that included Indol, methyl red, glucose, lactose, sorbitol (variable), mannitol, and rhamnose whereas negative for Voges Proskauer's, citrate utilization and sucrose fermentation (Table 4, Fig. 3).

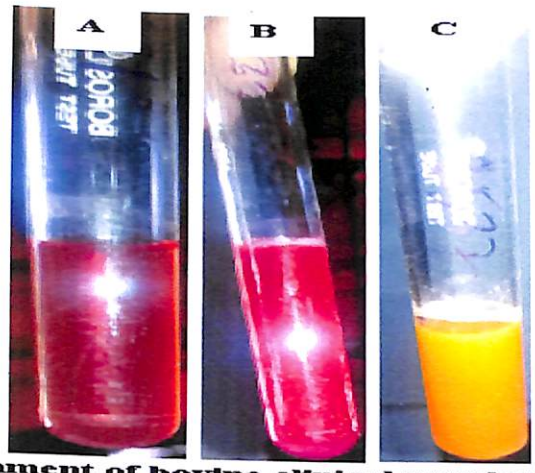


Fig. 1. Enrichment of bovine clinical samples for isolation of *Escherichia coli* in MacConkey broth

A. Bovine clinical sample with no growth

B & C. Bovine clinical sample with growth

Fig. 2. Green metallic sheen colonies of *E. coli* on EMB agar

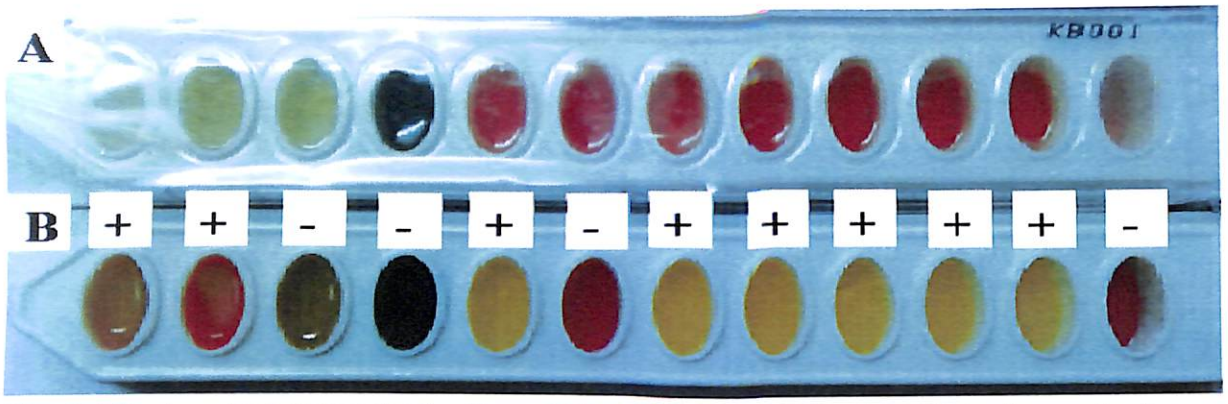


Fig. 3. Biochemical characterization of *E. coli* isolates of bovine clinical samples by HiMViC biochemical test kit

1-12: Indole, Methyl red, Voges Proskauer's, Citrate utilization, Glucose, Adonitol, Lactose, Sorbitol, Mannitol, Rhamnose and Sucrose.

A: Un-inoculated test kit

B: Test kit inoculated with sample

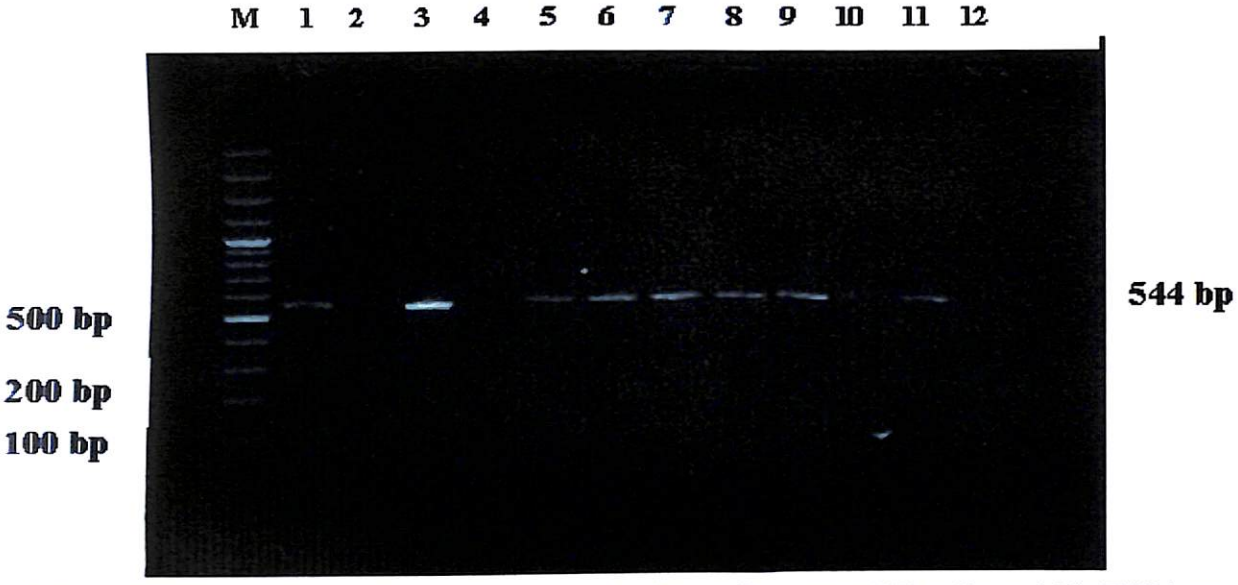


Fig. 4. PCR confirmation of *Escherichia coli* by amplification 16SrRNA gene

M : 100 bp plus DNA ladder

L1 : Positive control with amplicon of 544 bp

L3, L5-11: *E. coli* isolates from clinical samples with positive amplicons

L2-L4: No amplicon produced by the sample isolates

L12 : Negative control

4.3. PCR detection of 16SrRNA gene of *E. coli*

The metallic sheen producing colonies / isolates (n=90) from bovine clinical samples (n=200) were screened for molecular confirmation of *E. coli* by detection of the presence of *16S rRNA* gene by PCR (Fig. 4). This showed a distribution of *16S rRNA* gene in 71 (35.50%) of total bovine clinical samples (n=200) and 78.89% of metallic sheen on EMB producing isolates (n=90) (Table 4, Fig. 5).

The clinical urine samples (n=127) showed a distribution of *E. coli* in 58 (45.67%) samples (Table 4, Fig. 5).

In bovine clinical milk samples (n=63), the distribution of *E. coli* was found as 12 (19.05%) whereas, the distribution of *E. coli* in bovine uterine discharge (n=10) was found to be 01 (10.00%) (Table 4, Fig. 5).

4.4. Antibiotic susceptibility testing

4.4.1 Antibiotic susceptibility testing of bovine clinical isolates of *E. coli*

Antibiogram study of *16Sr RNA* PCR confirmed *E. coli* isolates from bovine clinical samples were performed using 15 antibiotic discs of ampicillin/ sulbactum (10/10 µg) ciprofloxacin (5µg), amoxiclav (30µg), ofloxacin (5µg), amikacin (30µg), cefotaxime/ clavulinic acid (30/10µg), cefotaxime (30µg), ceftriaxone (30µg), chloramphenicol (30µg), penicillin-G (10µg), gentamicin (10µg), norfloxacin(10µg), oxytetracyclin (30µg), co-trimoxazole (25µ), and doxycycline hydrochloride (30µg) (Fig. 6).

The antibiotic susceptibility study of *E. coli* isolates from bovine clinical samples revealed that 98.59% isolates were resistant to penicillin G, 83.3 % to cefotaxime / clavulinic acid, 78.87% to cefotaxime, 63.38 % ceftriaxone, 57.75% to oxytetracyclin, 43.66% to ciprofloxacin, 38.02% to norfloxacin, 35.21% to co-trimoxazole and ofloxacin, 30.98% to amoxiclav, 23.94% to doxycycline hydrochloride, 21.23% to gentamicin, 7.04% to amikacin and ampicillin / sulbactum and 5.63% to chloramphenicol (Fig. 7).

The *E. coli* isolates from bovine clinical samples showed a susceptibility of 53.52 % to ciprofloxacin, 25.35% to ceftriaxone, 46.48% to doxycycline

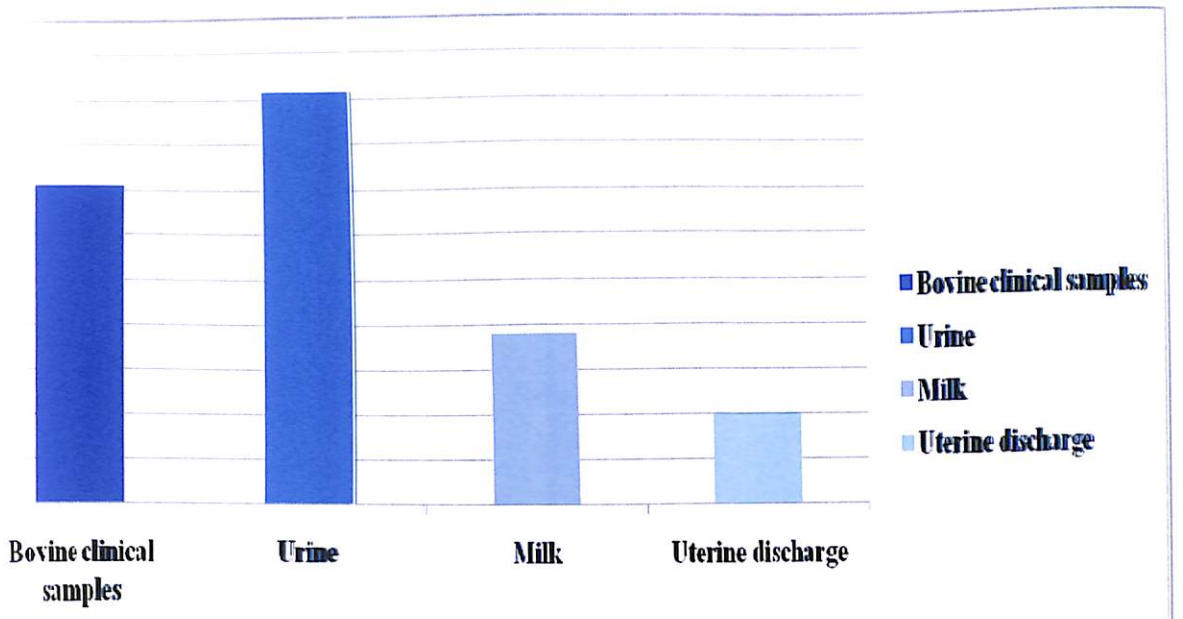
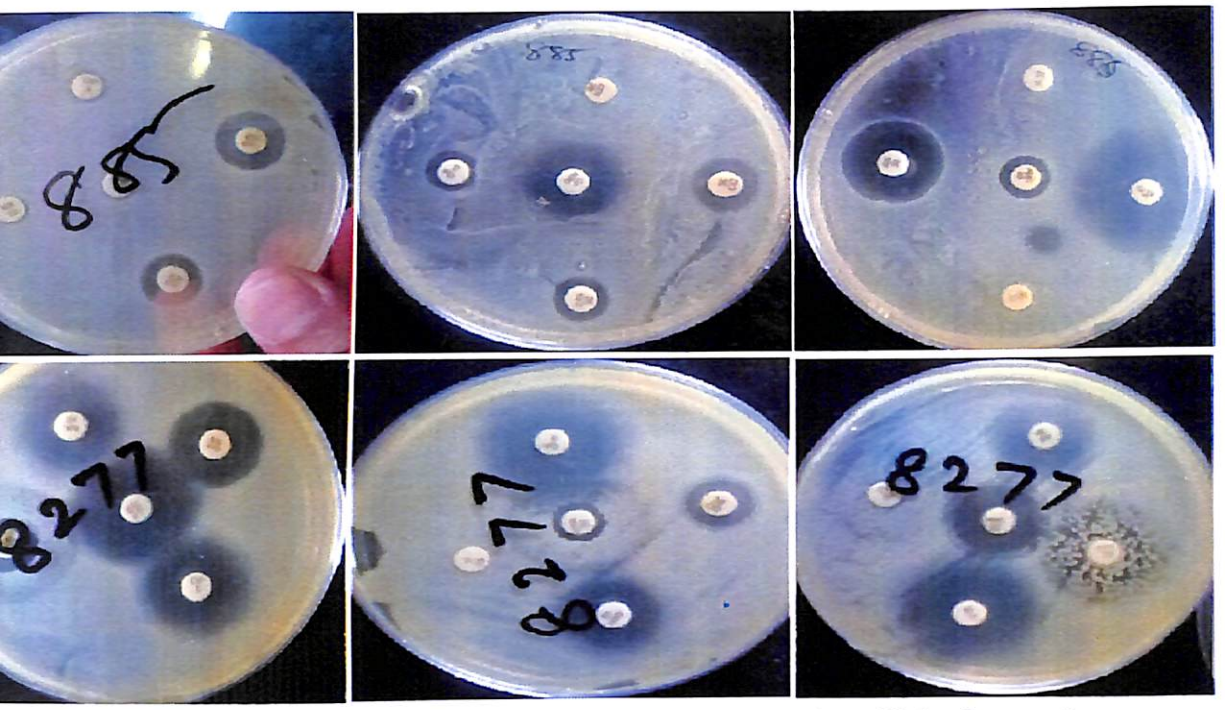


Fig. 5. Occurance of *Escherichia coli* in bovine clinical samples



S. Antibiotic sensitivity profile of *E. coli* isolates of bovine clinical samples on Mueller Hinton agar

A/S:Ampicillin/Sulbactum (10/10µg), CIP:Ciprofloxacin (5µg), AMC:Amoxiclav (30µg), OF:Ofloxacin (5µg), AK:Amikacin (30µg), CEC:Cefotaxime/Clavulinic acid (30/10µg), CTX:Cefotaxime (30µg), CTR:Ceftriaxone (30µg), C:Chloramphenicol (30µg), P:Penicillin-G (10µg), GEN:Gentamicin (10µg), NX:Norfloxacin (10µg), O:Oxytetracyclin (30µg), COT:Co-trimoxazole (25µ), and DO:Doxycycline hydrochloride (30µg).

hydrochloride, 57.75% to gentamicin, 59.15% to co-trimoxazole, 42.25% to oxytetracyclin, 1.40% to penicillin-G, 85.91% to chloramphenicol, 35.21% to amoxiclav, 88.73% to amikacin, 8.45% to cefotaxime, 56.34% to norfloxacin, 61.97% to ofloxacin, 50.70% to ampicillin / sulbactum and 16.90% to cefotaxime / clavulinic acid (Fig. 7).

The isolates also showed an intermediate susceptibility of 42.25% to Ampicillin / sulbactum, 33.80% to amoxiclav, 29.58% to doxycycline hydrochloride, 21.23% to gentamicin, 12.68% to cefotaxime, 11.27% to ceftriaxone, 8.45% to chloramphenicol, 5.63 % to co-trimoxazole and norfloxacin, 4.22% to amikacin and 2.81% to ciprofloxacin and ofloxacin (Fig. 7).

4.4.2 Antibiotic susceptibility testing of bovine clinical Urine isolates of *E. coli*:

The antibiotic susceptibility study of *E. coli* isolates from bovine clinical urine samples revealed that 98.27% isolates were resistant to penicillin G, 82.76% to cefotaxime / clavulinic acid, 81.03% to cefotaxime, 67.24% to ceftriaxone, 60.34% to oxytetracyclin, 48.27% to ciprofloxacin, 43.10% to norfloxacin, 41.38% to ofloxacin, 39.65% to co-trimoxazole, 34.48% to amoxiclav, 24.13% to doxycycline hydrochloride, 20.69% to gentamicin, 6.89% to amikacin and ampicillin/ sulbactum and 5.17% to chloramphenicol (Table 5, Fig. 8).

The isolates showed a sensitivity of 87.93% to amikacin, 84.48% to chloramphenicol, 58.62% to gentamicin, 55.17% to co-trimoxazole and ofloxacin, 51.72% to norfloxacin, 50.00% to ampicillin/ sulbactum, 48.27% to ciprofloxacin, 43.10% to doxycycline hydrochloride, 39.65% to oxytetracyclin, 29.31% to amoxiclav, 22.41% to ceftriaxone, 17.24% to cefotaxime/ clavulinic acid, 6.89% to cefotaxime and 1.72% to penicillin-G (Table 5, Fig. 8).

The isolates also showed an intermediate susceptibility of 43.10% to ampicillin / sulbactum, 36.21% to amoxiclav, 32.76% to doxycycline hydrochloride, 20.69% to gentamicin, 12.07% to cefotaxime, 10.34% to ceftriaxone and chloramphenicol, 5.17% to co-trimoxazole, amikacin, norfloxacin and 3.44% to ofloxacin and ciprofloxacin (Table 5, Fig. 8).

Sl. No	Sample ID	Antibiotics														
		CIP	CTR	DO	GEN	COT	O	P	C	AMC	AK	CTX	NOR	OF	A/S	CEC
1.	159	S	I	S	I	S	S	R	S	I	S	R	S	S	S	R
2.	160	S	S	R	R	S	R	R	S	I	S	R	S	S	S	S
3.	162	S	S	S	R	S	S	R	S	S	I	R	S	S	I	R
4.	169	R	R	R	R	R	R	R	S	R	S	R	R	R	I	R
5.	172	R	R	R	R	R	R	R	S	R	S	R	R	R	I	R
6.	219	R	R	S	S	R	S	R	S	I	S	R	R	S	S	S
7.	248	S	R	S	S	S	R	R	I	S	S	R	S	S	S	S
8.	264	R	R	S	S	R	R	R	S	R	S	R	R	R	I	R
9.	396	S	R	R	S	S	S	R	I	I	S	R	R	R	I	R
10.	512	R	R	S	S	S	S	R	S	S	S	R	R	R	I	R
11.	534	S	R	R	S	S	S	R	I	I	S	R	R	R	I	R
12.	555	R	R	R	S	S	R	R	S	R	S	R	R	R	S	R
13.	608	R	R	I	R	R	R	R	S	R	I	R	R	R	R	R
14.	618	S	R	S	I	S	R	R	S	S	R	R	S	S	S	S
15.	619	S	S	S	S	S	S	R	S	S	S	I	S	S	S	S
16.	630	S	R	S	S	S	R	R	S	I	S	R	S	S	S	R
17.	797	S	I	R	S	S	S	R	S	R	S	R	S	S	R	R
18.	885	R	R	I	S	R	R	R	S	R	S	R	R	R	I	R
19.	907	S	S	S	S	S	S	R	S	S	S	I	S	S	S	R
20.	1001	R	R	I	S	R	R	R	S	I	S	R	I	I	S	R
21.	2253	S	R	S	S	S	S	R	S	I	S	R	R	S	S	R
22.	2344	R	R	R	S	R	R	R	S	R	S	R	R	R	R	R
23.	2537	S	S	R	S	S	S	R	S	S	S	S	S	S	S	S
24.	2792	S	R	R	S	I	S	R	S	S	S	I	S	S	I	R
25.	2803	I	S	R	S	S	R	R	I	I	R	I	S	S	I	S
26.	2837	S	R	I	S	S	R	S	S	I	S	R	R	R	I	R
27.	2847	R	R	I	R	R	R	R	S	R	S	R	R	R	I	R
28.	2948	I	R	S	I	S	R	R	S	I	S	R	R	I	S	R
29.	2949	R	R	R	I	R	R	R	R	R	S	R	R	R	I	R
30.	2996	R	R	I	R	R	S	R	I	R	S	R	R	R	I	R

Sl. No	Sample ID	Antibiotics														
		CIP	CTR	DO	GEN	COT	O	P	C	AMC	AK	CTX	NOR	OF	A/S	CEC
31.	3029	R	R	S	I	S	S	R	S	S	R	R	R	R	S	R
32.	3056	R	R	I	S	R	R	R	R	R	S	R	S	S	S	R
33.	3144	S	S	S	S	S	S	R	S	S	S	R	S	S	S	R
34.	3221	S	R	S	S	R	S	R	S	I	S	R	S	S	I	R
35.	3302	S	S	S	I	S	S	R	S	S	S	R	S	S	S	R
36.	3330	R	R	R	R	S	R	R	S	R	S	R	S	R	I	R
37.	3644	R	R	I	S	S	R	R	S	I	S	R	S	S	S	R
38.	3649	S	R	S	S	S	R	R	S	S	S	R	S	S	S	R
39.	3690	S	S	S	S	S	S	R	S	I	S	S	S	S	S	S
40.	4165	R	R	I	R	I	R	R	I	I	S	R	S	R	I	R
41.	4184	S	I	S	I	S	S	R	S	S	S	I	S	S	S	R
42.	5476	R	R	I	R	R	R	R	S	I	S	R	S	S	S	R
43.	5618	S	S	S	S	S	S	R	S	S	S	R	S	S	S	R
44.	5684	S	S	S	S	S	S	R	S	I	S	S	S	S	S	S
45.	6113	R	R	S	S	R	R	R	S	I	S	R	S	S	I	R
46.	6356	S	R	S	S	S	R	R	S	S	S	R	S	S	S	R
47.	6529	S	S	S	I	S	S	R	S	S	S	I	S	S	S	S
48.	6530	R	S	I	S	S	S	R	S	R	S	S	R	R	S	S
49.	6658	S	R	I	I	R	R	R	S	I	S	R	I	S	S	R
50.	6986	R	R	I	I	R	R	R	S	R	S	R	R	R	I	R
51.	6989	S	R	S	S	S	R	R	S	S	S	R	S	S	R	R
52.	7091	R	R	I	R	R	R	R	S	R	S	R	R	R	I	R
53.	7222	R	R	I	I	R	R	R	R	R	S	R	S	S	I	R
54.	7224	S	I	I	S	R	R	R	S	R	R	I	S	S	I	R
55.	7250	R	I	I	S	R	R	R	S	R	S	R	R	R	I	R
56.	7557	R	I	R	S	R	R	R	S	R	S	R	R	R	I	R
57.	7601	R	R	I	R	R	R	R	S	I	I	R	R	R	I	R
58.	8083	R	R	I	I	I	R	R	S	I	S	R	I	R	S	R

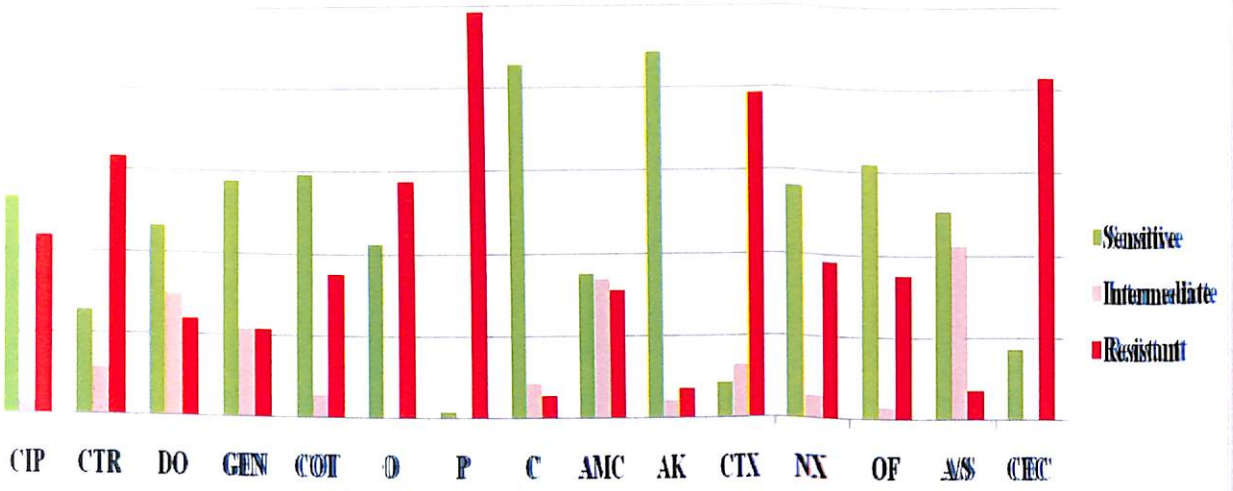


Fig. 7. Antibiotic sensitivity profile of *E. coli* isolates of bovine clinical samples

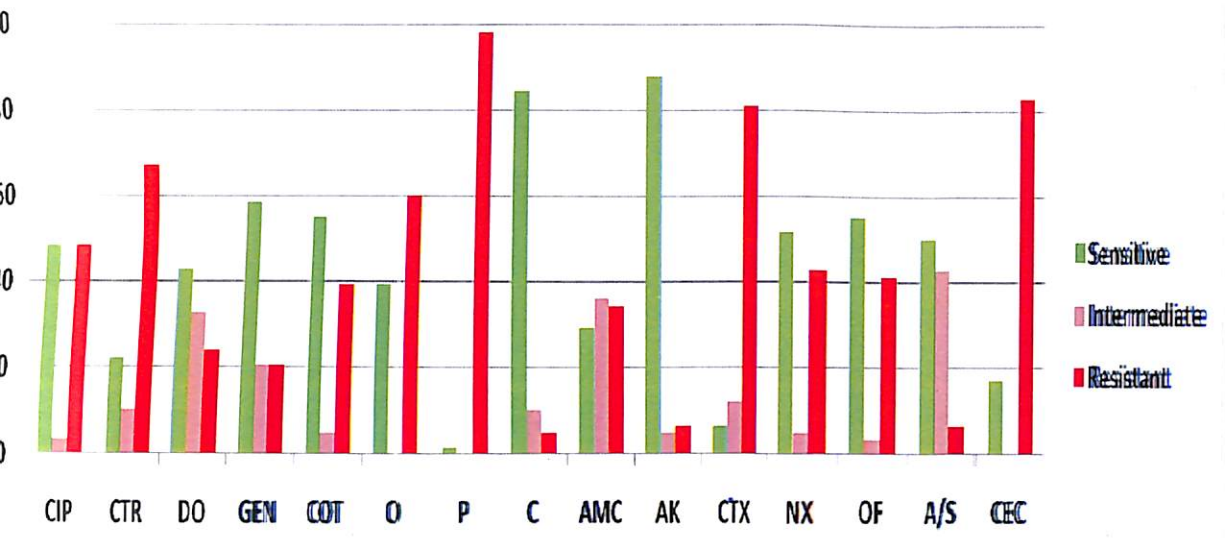


Fig.8. Antibiotic sensitivity profile of bovine urine isolates of *E. coli*

4.4.3 Antibiotic susceptibility testing of bovine clinical Milk isolates of *E. coli*:

The antibiotic susceptibility study of bovine clinical urine isolates of *E. coli* (58) revealed that all isolates were resistant to penicillin-G while, 83.33% showed resistant to cefotaxime / clavulinic acid, 66.67% to cefotaxime, 41.67% to ceftriaxone, doxycycline hydrochloride, gentamicin, co-trimoxazole, oxytetracyclin and amoxiclav, 16.67% to ciprofloxacin and 8.33% to amikacin, norfloxacin, ofloxacin and chloramphenicol (Table 6, Fig. 9).

In sensitive group, a sensitivity of 83.33% to ciprofloxacin, 41.67% to ceftriaxone, 66.67% to doxycycline hydrochloride, 58.33% to gentamicin, 75.00% to co-trimoxazole, 41.67% to oxytetracyclin, 0% to penicillin-G, 91.66% to chloramphenicol 66.67% to amoxiclav, 91.66% to amikacin, 41.67% to cefotaxime, 83.33% to norfloxacin, 91.66% to ofloxacin, 58.33% to ampicillin / sulbactum, 41.67% to cefotaxime / clavulinic acid were found (Table 6, Fig. 9).

Among clinical milk isolates of *E. coli* an intermediate susceptibility of 16.67% to ceftriaxone, 41.67% to doxycycline hydrochloride, 25.00% to gentamicin, 8.33% to co-trimoxazole, 41.67% to amoxiclav, 41.67% to cefotaxime, 8.33% to norfloxacin and 41.67% to ampicillin/ sulbactum were found (Table 6, Fig. 9).

4.4.4 Antibiotic susceptibility testing of bovine clinical uterine discharge isolates of *E. coli*:

The antibiotic susceptibility study of *E. coli* isolates of uterine discharge revealed that the isolate was resistant to ciprofloxacin, ceftriaxone, Doxycycline hydrochloride, gentamicin, oxytetracyclin, penicillin-G, cefotaxime, norfloxacin, ampicillin/ sulbactum and cefotaxime/ clavulinic acid, while sensitive to co-trimoxazole, chloramphenicol, amikacin and ofloxacin. The amoxiclav had intermediate effect on this isolate (Table 7, Fig. 10).

4.4.5 Prevalence of multidrug resistant *E. coli* isolates of bovine clinical samples

The result obtained from the antibiotic sensitivity testing of all *E. coli* isolates were analyzed for categorization of isolates as multidrug resistant (MDR) *E. coli* based on the recommendations of Magiorakos *et al.* (2012) which define MDR as acquired non-susceptibility to at least one agent in three or more antimicrobial categories.

Table 6. Antibiotic sensitivity profile of *Escherichia coli* isolates from bovine clinical milk samples

Sl. No	Sample ID	Antibiotics														
		CIP	CTR	DO	GEN	COT	O	P	C	AMC	AK	CTX	NOR	OF	A/S	CEC
1.	463	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S
2.	464	S	I	S	I	S	R	R	S	I	R	R	S	S	I	R
3.	691	R	R	I	R	S	S	R	S	I	S	R	R	R	I	R
4.	698	S	R	S	S	R	R	R	S	S	S	R	S	S	S	R
5.	791	S	S	S	I	S	S	R	S	R	S	R	S	S	S	R
6.	1821	S	R	R	S	R	R	R	S	R	S	R	S	S	I	R
7.	3002	S	S	R	S	I	S	R	S	S	S	R	S	S	S	R
8.	3145	S	I	S	S	S	R	R	S	S	S	R	S	S	S	R
9.	3242	S	S	S	S	S	R	R	R	S	S	I	S	S	S	R
10.	3738	S	S	S	S	S	S	R	S	S	S	I	S	S	S	S
11.	4153	S	R	S	R	S	R	R	S	S	S	R	I	S	I	R
12.	8277	S	R	I	I	S	R	R	S	S	S	S	S	S	I	R

Table 7. Antibiotic sensitivity profile of *Escherichia coli* isolates from bovine clinical uterine discharge samples

Sl. No	Sample ID	Antibiotics														
		CIP	CTR	DO	GEN	COT	O	P	C	AMC	AK	CTX	NOR	OF	A/S	CEC
1.	708	R	R	R	R	S	R	R	S	I	S	R	R	S	R	R

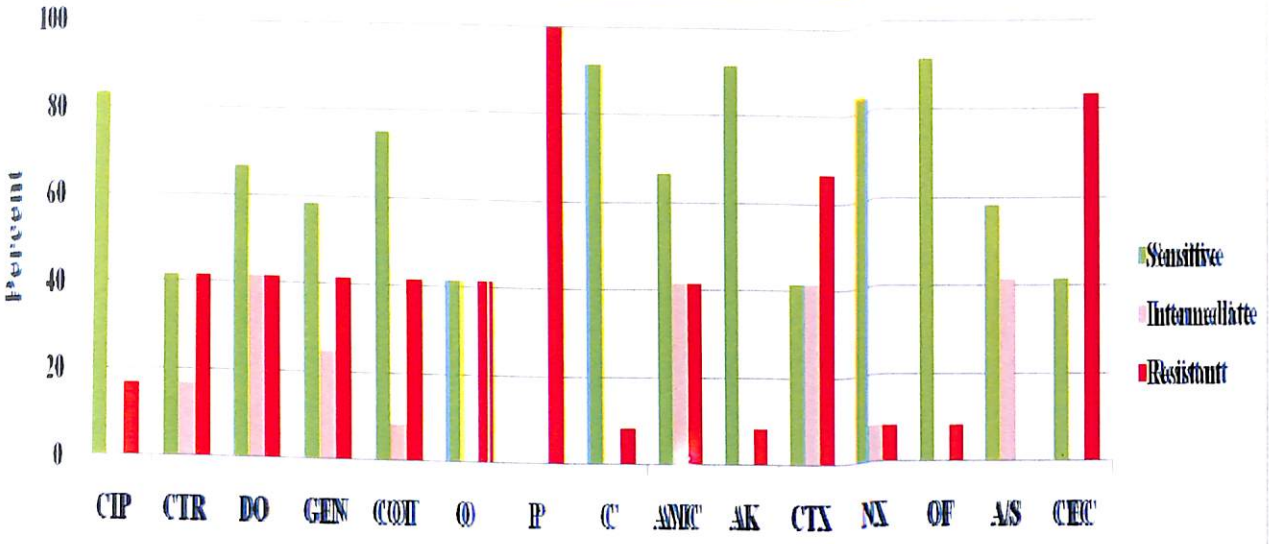


Fig. 9. Antibiotic sensitivity profile of bovine milk isolates of *E. coli*

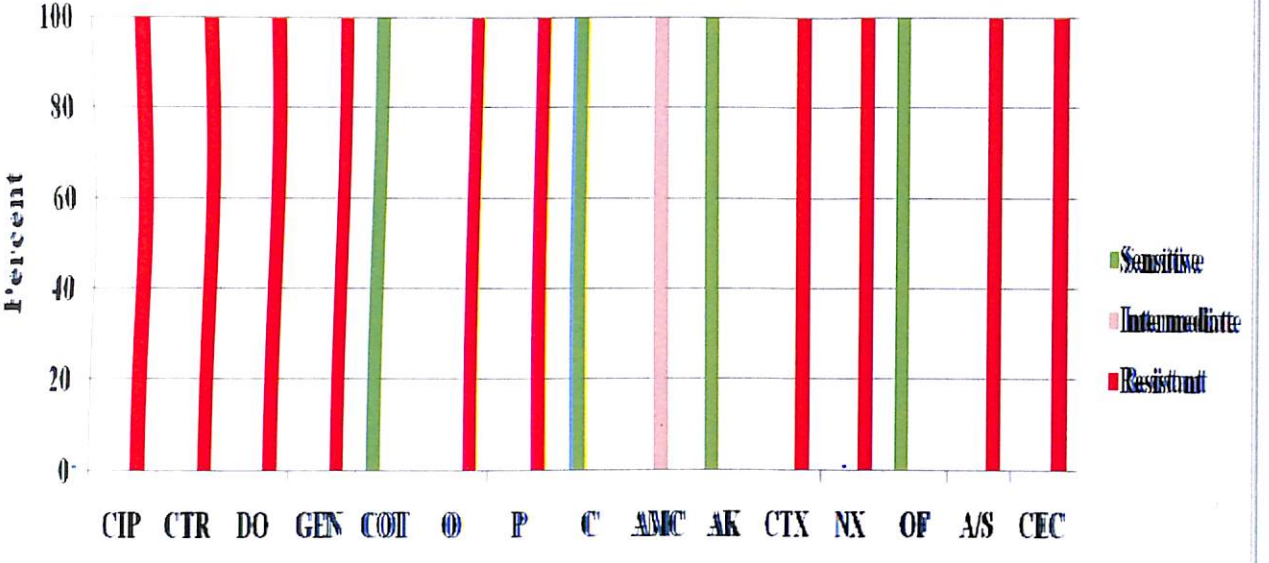


Fig. 10. Antibiotic sensitivity profile of bovine uterine discharge isolates of *E. coli*

In this study, a total of six antimicrobial categories with nine antimicrobial agents including aminoglycosides, extended-spectrum cephalosporins (gentamicin, amikacin), 3rd and 4th generation cephalosporins (cefotaxime, ceftriaxone), fluoroquinolones (ciprofloxacin), penicillins + β-lactamase inhibitors (amoxicillin-clavulanic acid, ampicillin-sulbactam), phenicols (chloramphenicol) and tetracyclines (doxycycline) were used (Table 3).

Based on the finding of present study, a total of 66.20% (47/71) isolates of bovine clinical samples were categorized as MDR. The distribution of MDR *E. coli* from 71 16S r RNA confirmed bovine clinical samples showed a total of 70.69% (40/58), 50.00% (06/12) and 100% (1/1) *E. coli* isolates from urine, milk and uterine discharge were MDR (Table 8, 9,10).

Among 58 *E. coli* isolates from clinical urine samples 22.41(13/58), 18.96 (11/58), 18.96 (11/58) and 8.6% (5/58) were resistant to 3, 4, 5 and 6 anti microbial categories, respectively (Table 8, Fig. 10). While, out of 12 *E. coli* isolates from clinical milk samples 33.33% (4/12), 8.33% (1/12) and 8.33% (1/12) were resistant to 3, 4 and 5 antimicrobial categories, respectively (Table 9, Fig. 11). A single isolate from uterine discharge showed a resistant with 5 antimicrobial categories (Table 10).

Antimicrobial categories		Aminoglycosides		Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins		Fluoroquinolones	Penicillins + β-lactamase inhibitors		Phenicols	Tetracyclines	Isolates susceptible with no. of Antimicrobial categories
		Antimicrobial agent									
Sl. No.	Isolates ID	Gentamicin	Amikacin	Cefotaxime	Ceftriaxone	Ciprofloxacin	Amoxicillin-clavulanic acid	Ampicillin-sulbactam	Chloramphenicol	Doxycycline	
1.	159	R	S	R	R	S	R	S	S	S	3
2.	160	R	S	R	S	S	R	S	S	R	4
3.	162	R	R	R	S	S	S	R	S	S	3
4.	169	R	S	R	R	R	R	R	S	R	5
5.	172	R	S	R	R	R	R	R	S	R	5
6.	219	S	S	R	R	R	R	S	S	S	3
7.	248	S	S	R	R	S	S	S	R	S	2
8.	264	S	S	R	R	R	R	R	S	S	3
9.	396	S	S	R	R	S	R	R	R	R	4
10.	512	S	S	R	R	R	S	R	S	S	3
11.	534	S	S	R	R	S	R	R	R	R	4
12.	555	S	S	R	R	R	R	S	S	R	4
13.	608	R	R	R	R	R	R	R	S	R	5
14.	618	R	R	R	R	S	S	S	S	S	2
15.	619	S	S	R	S	S	S	S	S	S	1
16.	630	S	S	R	R	S	R	S	S	S	2
17.	797	S	S	R	R	S	R	R	S	R	3
18.	885	S	S	R	R	R	R	R	S	R	4
19.	907	S	S	R	S	S	S	S	S	S	1
20.	1001	S	S	R	R	R	R	S	S	R	4
21.	2253	S	S	R	R	S	R	S	S	S	2
22.	2344	S	S	R	R	R	R	R	S	R	4
23.	2537	S	S	S	S	S	S	S	S	R	1
24.	2792	S	S	R	R	S	S	R	S	R	3
25.	2803	S	R	R	S	R	R	R	R	R	6
26.	2837	S	S	R	R	S	R	R	S	R	3
27.	2847	R	S	R	R	R	R	R	S	R	5
28.	2948	R	S	R	R	R	R	S	S	S	3
29.	2949	R	S	R	R	R	R	R	R	R	6
30.	2996	R	S	R	R	R	R	R	R	R	6
31.	3029	R	R	R	R	R	S	S	S	S	3
32.	3056	S	S	R	R	R	R	S	R	R	5
33.	3144	S	S	R	S	S	S	S	S	S	1
34.	3221	S	S	R	R	S	R	R	S	S	3
35.	3302	R	S	R	S	S	S	S	S	S	2

Antimicrobial categories		Aminoglycosides		Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins		Fluoroquinolones	Penicillins + β-lactamase inhibitors		Phenicols	Tetracyclines	Isolates susceptible with no. of antimicrobial categories
		Antimicrobial agent									
Sl. No.	Isolates ID	Gentamicin	Amikacin	Cefotaxime	Ceftriaxone	Ciprofloxacin	Amoxicillin-clavulanic acid	Ampicillin-sulbactam	Chloramphenicol	Doxycycline	
36.	3330	R	S	R	R	R	R	R	S	R	5
37.	3644	S	S	R	R	R	R	S	S	R	4
38.	3649	S	S	R	R	S	S	S	S	S	1
39.	3690	S	S	S	S	S	R	S	S	S	1
40.	4165	R	S	R	R	R	R	R	R	R	6
41.	4184	R	S	R	R	S	S	S	S	S	2
42.	5476	R	S	R	R	R	R	S	S	R	5
43.	5618	S	S	R	S	S	S	S	S	S	1
44.	5684	S	S	S	S	S	R	S	S	S	1
45.	6113	S	S	R	R	R	R	R	S	S	3
46.	6356	S	S	R	R	S	S	S	S	S	1
47.	6529	R	S	R	S	S	S	S	S	S	2
48.	6530	S	S	S	S	R	R	S	S	R	3
49.	6658	R	S	R	R	S	R	S	S	R	4
50.	6986	R	S	R	R	R	R	R	S	R	5
51.	6989	S	S	R	R	S	S	R	S	S	2
52.	7091	R	S	R	R	R	R	R	S	R	5
53.	7222	R	S	R	R	R	R	R	R	R	6
54.	7224	S	R	R	R	S	R	R	S	R	4
55.	7250	S	S	R	R	R	R	R	S	R	4
56.	7557	S	S	R	R	R	R	R	S	R	4
57.	7601	R	R	R	R	R	R	R	S	R	5
58	8083	R	S	R	R	R	R	S	S	R	5

Table 9. Multidrug-resistant (MDR) *Escherichia coli* isolates from bovine clinical milk samples

Antimicrobial categories		Aminoglycosides		Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins		Fluoroquinolones	Penicillins + β-lactamase inhibitors		Phenicols	Tetracyclines	Isolates susceptible with no. of Antimicrobial categories
		Antimicrobial agent									
Sl. No.	Isolates ID	Gentamicin	Amikacin	Cefotaxime	Ceftriaxone	Ciprofloxacin	Amoxicillin-clavulanic acid	Ampicillin-sulbactam	Chloramphenicol	Doxycycline	
1.	463	S	S	S	S	R	S	S	S	S	1
2.	464	R	R	R	R	S	R	R	S	S	3
3.	691	R	S	R	R	R	R	R	S	R	5
4.	698	S	S	R	R	S	S	S	S	S	1
5.	791	R	S	R	S	S	R	S	S	S	3
6.	1821	S	S	R	R	S	R	R	S	R	3
7.	3002	S	S	R	S	S	S	S	S	R	2
8.	3145	S	S	R	R	S	S	S	S	S	1
9.	3242	S	S	R	S	S	S	S	R	S	2
10.	3738	S	S	R	S	S	S	S	S	S	1
11.	4153	R	S	R	R	S	S	R	S	S	3
12.	8277	R	S	S	R	S	S	R	S	R	4

Table 10. Multidrug-resistant (MDR) *Escherichia coli* isolate from bovine clinical uterine discharge samples

Antimicrobial categories		Aminoglycosides		Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins		Fluoroquinolones	Penicillins + β-lactamase inhibitors		Phenicols	Tetracyclines	Isolates susceptible with no. of Antimicrobial categories
		Antimicrobial agent									
Sl. No.	Isolates ID	Gentamicin	Amikacin	Cefotaxime	Ceftriaxone	Ciprofloxacin	Amoxicillin-clavulanic acid	Ampicillin-sulbactam	Chloramphenicol	Doxycycline	
1.	708	R	S	R	R	R	R	R	S	R	5

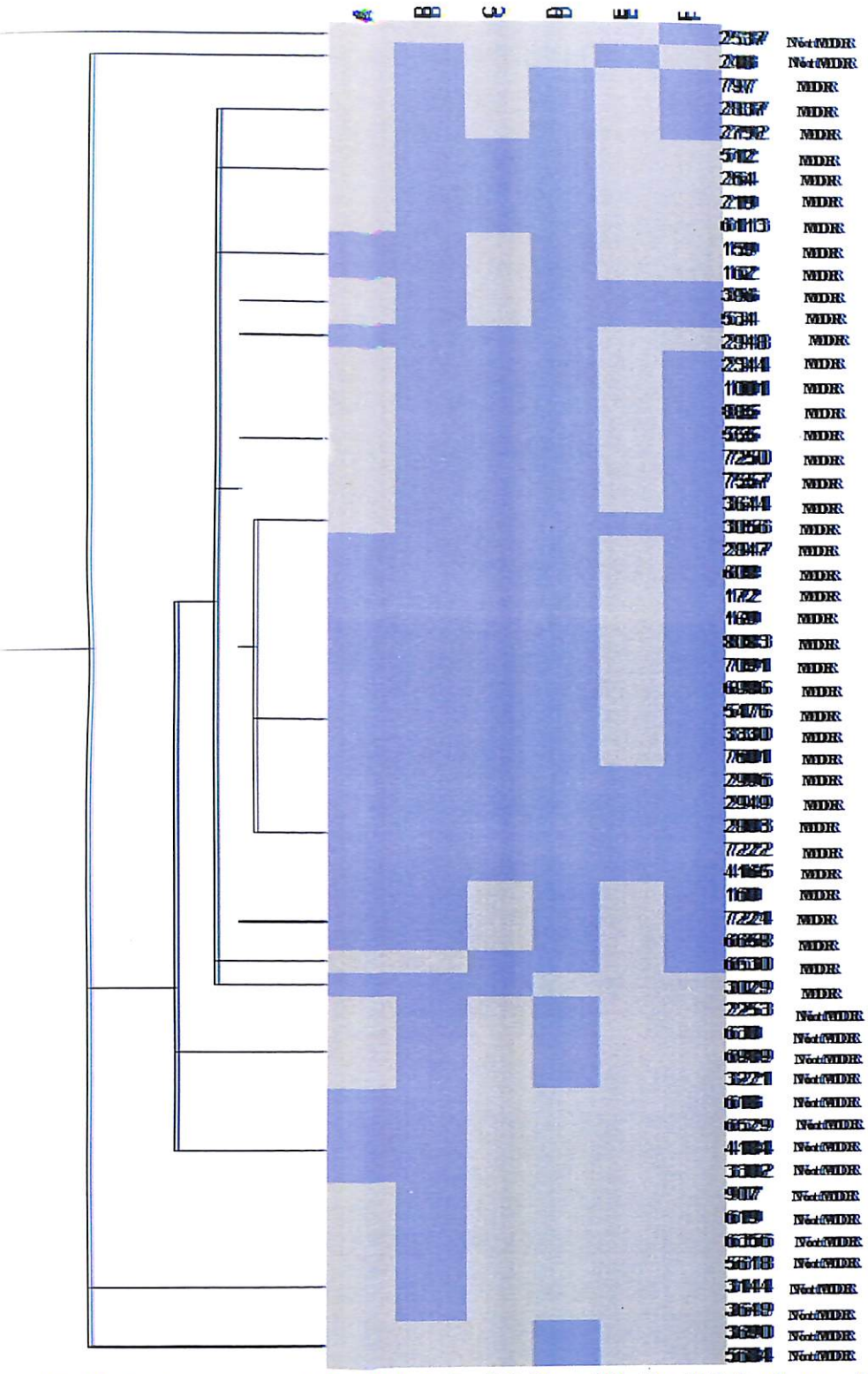


Fig. 11. Heat map representation MDR *E. coli* isolates of bovine clinical urine samples tested against six group of antimicrobial categories.

The right vertical axis lists the isolates ID and the horizontal axis is labelled with antimicrobial categories ((A: Aminoglycosides; B: Extended-spectrum cephalosporins, 3rd and 4th generation cephalosporins; C: Fluoroquinolones, D: Penicillins + β-lactamase inhibitors; E: Phenicols; F: Tetracyclines)).

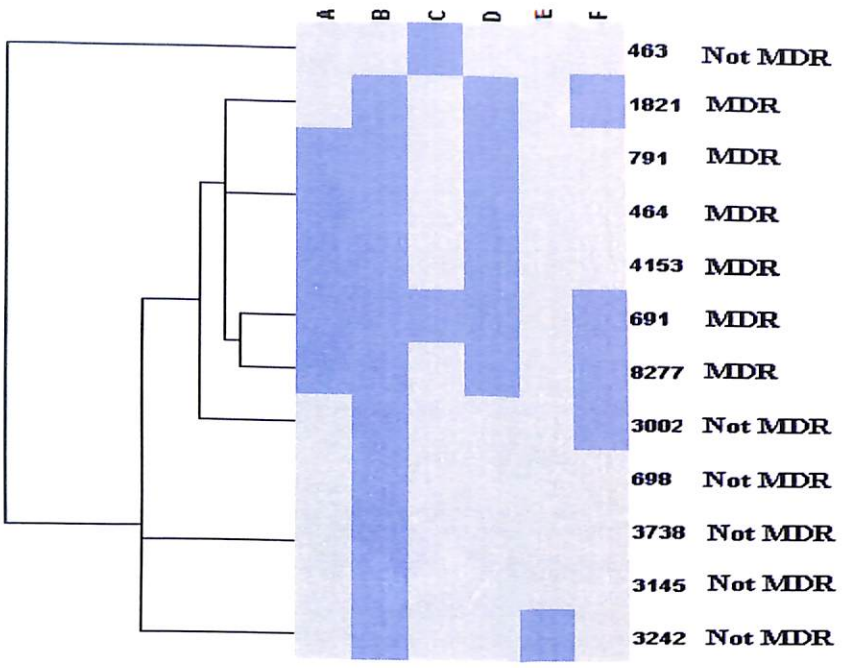


Fig. 12. Heat map representation MDR *E. coli* isolates of bovine clinical milk samples tested against six group of antimicrobial categories.
The right vertical axis lists the isolates ID and the horizontal axis is labelled with antimicrobial categories (A: Aminoglycosides; B: Extended-spectrum cephalosporins, 3rd and 4th generation cephalosporins; C: Fluoroquinolones, D: Penicillins + β-lactamase inhibitors; E: Phenicols; F: Tetracyclines).

Discussions

5 Discussion

Escherichia coli are gram-negative, cylindrical rods, found naturally in the intestinal tract of all humans and many animal species. Although most of the *E. coli* strains are non-pathogenic to animal and human beings, some are known to cause intestinal and extra-intestinal diseases (Kaper *et al.,* 2004; Fairbrother *et al.,* 2006). A subset of *E. coli* is capable of causing enteric/diarrhoeal disease, and a different subset cause extra-intestinal disease, including urinary tract infection (UTI), mastitis and uterine infections. The different pathotypes of *E. coli* viz., enteropathogenic, enterotoxigenic, enteroinvasive, enteroaggregative, diffusely adherent, and enterohemorrhagic involved in diseases different spectrum in animals and man (Bischoff *et al.*, 2005).

The focus of attention in the current study was to evaluate the role of *E. coli* in bovine diseases and to generate the antibiotic sensitivity profile of *E. coli* isolates. Primarily this organism was prioritized because *E. coli* is a major cause of bacterial diarrhea and urinary tract infection (UTI) in humans (Stamm, 2002). While *E. coli* is an important urinary tract infection agent in pigs (Brito *et al.*, 1999) and dogs (Johnson *et al.*, 2001), it does not have this importance in cattle in which the frequency of urinary tract infection (UTI) is low (Yeruham *et al.*, 2006). The uropathogenic *E. coli* (UPEC) possess adherence factors called pilli or fimbriae, which allow them to successfully initiate infection. *E. coli* can also cause subclinical mastitis, although less frequently, but these strains are different than those causing clinical mastitis (Dogan *et al.*, 2005). The frequency of *E. coli* isolated from mastitis cases may vary considerably depending on herd and country. *E. coli* are one of the most prevalent bacteria isolated from the uterine lumen of cows suffering from uterine infections. Since *E. coli* infections are mostly found during the first days or week after calving, this germ has been thought to pave the way for subsequent infections with other bacteria or viruses.

To address the research question, *E. coli* was isolated from bovine clinical samples (urine, milk and uterine discharge) of animals presented at TVCC, BVC, Patna and was confirmed by biochemical and molecular methods. The isolates were

screened biochemically by Hi IMViC test kit (Himedia) and confirmed for species by species specific 16SrRNA-PCR (Sabat *et al.*, 2000). Further, all confirmed isolates from bovine clinical samples were analyzed for antibiotic susceptibility using 15 antibiotics including ampicillin/ sulbactum (10/10 μg) ciprofloxacin (5μg), amoxiclav (30μg), ofloxacin (5μg), amikacin (30μg), cefotaxime/ clavulinic acid (30/10μg), cefotaxime (30μg), ceftriaxone (30μg), chloramphenicol (30μg), penicillin-G (10μg), gentamicin (10μg), norfloxacin(10μg), oxytetracyclin (30μg), co-trimoxazole (25μ), and doxycycline hydrochloride (30μg).

In this study a total of 200 bovine clinical samples including urine, milk and uterine discharge were allowed for isolation of *E. coli* by enrichment and selective plating. It showed a distribution of presumptive *E. coli* in 56.69% of urine, 26.94% of milk and 10.00% of uterine discharge samples. All 90 presumptive *E. coli* isolates from bovine clinical samples were screened by biochemical and molecular tests; it showed a prevalence of 35.50% *E. coli* in bovine clinical samples including 45.67%, 19.05% and 10.0% in urine, milk and uterine discharge samples, respectively.

The prevalence of *E. coli* in clinical bovine urine samples was 45.67% which was in concordance with the earlier reports of Sharma *et al.* (2006) who isolated *E. coli* in 57.10% cases of uroperitoneum. However, in contrast to the finding of present study Kushwaha *et al.* (2012) analysed 31 urine samples from buffalo calves suffering from obstructive urolithiasis, and reported a prevalence of 16.12% *E. coli.*

In the present study, *E. coli* was isolated from 19.05% of bovine clinical milk samples. The prevalence of *E. coli* in clinical milk samples (19.05%) was in concordance with the earlier reports of Sumathi *et al.* (2008) and Lamey *et al.* (2013) as they isolated *E. coli*, from 20% and 18.47%, respectively from all tested samples. *E. coli* has been successfully isolated from bovine mastitis cases by number of investigators in India (Ranjan *et al.*, 2011; Kurjogi and Kaliwal, 2011; Hegade *et al.*, 2012 and Palaha *et al.*, 2012) and outside India by many researchers (Dopfer *et al.*, 1999; Bradley and Green 2001; Lira *et al.*, 2004; Momtaz *et al.*, 2012; Abera *et al.*, 2013; Alekish *et al.*, 2013; Tesfaye *et al.*, 2013., Mahamoud *et al.*, 2015 and Iraguha *et. al.*, 2015). All these investigators have recorded prevalence of *E. coli* from bovine

mastitis in the range of 6 to 35 per cent. So, present findings are consistent to these findings in which overall prevalence 19.05 per cent was recorded.

In the present study, *E. coli* was isolated from 10.00% of bovine clinical uterine discharge samples. In the present study, the isolation rate of *E. coli* from bovine clinical uterine discharge samples was somewhat in accordance with Azawi, (2008), who reported 18.4% isolation rate of *E. coli* from buffalo uterus. In contrast to the finding of present study 24.27% prevalence of *E. coli* in uterine washings/fluid from Mumbai, India was reported by Ingale *et al.*, (2016) and a higher prevalence of 36.66% *E. coli* in clinical cases of endometritis from Namakkal district of Tamil Nadu were reported by Udhayavel *et al.* (2013). The increased incidence of *E. coli* in uterus might be due to unhygienic practices during artificial insemination and during parturition results in contamination of uterus with dung, which is the main source for *E. coli.*

In veterinary hospitals, the ongoing usage of antimicrobial compounds for treatment of animals increases the selective pressure for emergence of MDR organisms and dissemination of resistance (Dunowska *et al.*, 2006 and Nam *et al.*, 2010). Antimicrobial agents frequently used in referral veterinary hospitals include broad-spectrum-activity drugs, such as β-lactams and new cephalosporins, as well as fluoroquinolones. Thus, hospitalized animals may constitute an important reservoir of antimicrobial resistance (Guardabassi *et al.*, 2004).

The high prevalence of penicillin resistant (98.27% of urine, 100% of milk and uterine discharge), cefotaxime/ clavulinic acid resistant (82.76% of urine, 83.33% of milk and 100% of uterine discharge) and cefotaxime (81.03% of urine, 66.67% of milk and 100% of uterine discharge) observed in this study suggests that bovine can be a significant reservoir of penicillin and cefotaxime or cefotaxime /clavulinic acid resistant *E. coli.*

Although the prevalence of ceftriaxone resistant (67.24% of urine, 41.67% of milk and 100% of uterine discharge), oxytetracyclin resistant (60.34% of urine, 41.67% % of milk and 100% of uterine discharge), ciprofloxacin resistant (48.27% of urine, 16.67% of milk and 100% of uterine discharge) norfloxacin resistant (43.10% of urine, 8.33% of milk and 100% of uterine discharge), co-trimoxazole resistant (39.65% of urine, 41.67% of milk and 0% of uterine discharge), ofloxacin resistant (41.38% of

urine, 8.33% of milk and 0% of uterine discharge), amoxiclav resistant (34.48% of urine, 41.67% of milk and 0% of uterine discharge), doxycycline hydrochloride resistant (24.13% of urine, 41.67% of milk and 100% of uterine discharge), gentamicin resistant (20.69% of urine, 41.67% of milk and 100% of uterine discharge), amikacin resistant (6.89% of urine, 8.33% of milk and 0% of uterine discharge), ampicillin/ sulbactum resistant (6.89% of urine, 0% of milk and 100% of uterine discharge) and chloramphenicol resistant (5.17% of urine, 8.33% of milk and 0% of uterine discharge) were lower than those of penicillin and cefotaxime or cefotaxime / clavulinic acid resistant *E. coli* but their role as reservoirs of antimicrobial resistance determinants should not be overlooked.

Intermediate resistance refers to those *E. coli* species that were not clearly resistant or susceptible (Adzitey, 2015). Alongwith the prevalence of resistant *E. coli*, a prevalence of ampicillin / sulbactum intermediate resistant (43.10% of urine and 41.67% of milk), amoxiclav intermediate resistant (36.21% of urine, 41.67% of milk and 100% of uterine discharge), doxycycline hydrochloride intermediate resistant (32.76% of urine and 41.67% of milk), gentamicin intermediate resistant (20.69% of urine and 25.00% of milk), cefotaxime intermediate resistant (12.07% of urine and 16.67% of milk), ceftriaxone intermediate resistant (10.34% of urine and 41.67% of milk), chloramphenicol intermediate resistant (10.34% of urine), co-trimoxazole intermediate resistant (5.17% of urine and 8.33% of milk), amikacin intermediate resistant (5.17% of urine), norfloxacin intermediate resistant (5.17% of urine and 8.33% of milk), ofloxacin intermediate resistant (3.44% of urine) and ciprofloxacin intermediate resistant (3.44% of urine) were also found under this study. It has been suggested in clinical diagnoses that patients with intermediate results can be given a higher dosage of antibiotics (Lorian, 2005). The organisms that exhibit intermediate resistance also have the tendency to easily become resistant (Adzitey *et al.*, 2012b). The use of antibiotics in the treatment of diseases and as growth promoters in farm animals and other factors have been linked to the development of resistant microorganisms (Krumperman, 1983; Schroeder *et al.*, 2002; Aarestrup *et al.*, 2008 and Adzitey *et al.*, 2012b).

The antimicrobial resistance study of *E. coli* isolates of uterine discharge revealed a high resistant or sensitivity percentage due to the number of *E. coli* isolated

and used under the study was only one. The result of the finding of present study was somewhat supported by the findings of others with respect to antibiotic sensitivity profile of *E. coli* isolates. Rangel and Marin, (2009) tested 231 *E. coli* isolates from bovine mastitic milk and reported a high antimicrobial drug resistant especially for amoxicillin+clavulanic acid (85.7%), ceftriaxone (82.2%) and cotrimoxazole (68.8%) that was somewhat similar to the finding of present study. Chandrasekaran *et al.* (2014) also studied the prevalence of drug resistant *E. coli* isolates of mastitis in Tamil Nadu and reported a resistance to amoxicillin (53%), oxytetracycline (58%), penicillin G (60.5%), oxacillin (56.3%), gentamicin (43.7%), enrofloxcain (43.7%), amoxicillin + sulbactam (49.6%) and ceftriaxone (13.4%). Sabir, *et al.* (2014) studied antibiotic sensitivity of *E. coli* isolates of human urine samples and reported that the organisms were highly resistant to penicillin (100%), amoxicillin (100%) and cefotaxime (89.7%), followed by intermediate level of resistance to ceftazidime (73.8%), cephradine (73.8%), tetracycline (69.4%), doxycycline (66.6%), augmentin (62.6%), gentamycin (59.8%), cefuroxime (58.2%), ciprofloxacin (54.2%), cefaclor (50%), aztreonam (44.8%), ceftriaxone (43.3%), imipenem (43.3%), and low level of resistance to streptomycin (30%), kanamycin (19.9%), tazocin (14%), amikacin (12.7%) and lowest to norfloxacin (11.2%). In other study, Ingale *et al.* (2016) performed antibiotic sensitivity study on *E. coli* strains isolated from uterus of buffaloes and determined its in-vitro sensitivity to commonly used antibiotics and reported a good antibiotic sensitivity to tetracycline (100%), cotrimoxazole (100%), gentamicin (90%) and chloramphenicol (88%) with quite resistant to nitrofurantoin (48%) and amoxicillin (41%).

Magiorakos *et al.* (2012) defined MDR as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. Based on the finding of present study, a total of 66.20% isolates of bovine clinical samples were categorized as MDR (resistant to ≥3 to 6 antimicrobial categories). The prevalence of MDR *E. coli* in bovine clinical samples was 70.69%, 50.00% and 100% in urine, milk and uterine discharge samples, respectively. In this study among 58 *E. coli* isolates from clinical urine samples 22.41%, 18.96%, 18.96% and 8.6% were resistant to 3, 4, 5 and 6 anti microbial categories, respectively. While, out of 12 *E. coli* isolates from clinical milk samples 33.33%, 8.33% and 8.33% were resistant to 3, 4 and 5 antimicrobial

categories, respectively. A single isolate from uterine discharge showed a resistant with 5 antimicrobial categories. The prevalence of MDR *E. coli* in clinical milk samples (50.00%) was nearer with the earlier reports of Todorovic *et al.* (2017) who characterized multidrug-resistant (MDR) *E. coli* isolates collected from Serbia from bovine clinical mastitis cases and diseased pigs, during the years 2013–2014, and reported a prevalence of 45.83% isolates as MDR. A slight lower prevalence of 40% MDR *E. coli* isolates of healthy lactating cattle was reported by Sawant *et al.*, (2007). A higher prevalence of 81% MDR *E. coli* from human urine samples was reported by Sabir, *et al.* (2014). In regard with the finding of the present study Brennan *et al.* (2016) isolated 150 *E. coli* from faecal samples collected from cattle with suspected enteric infection or milk-aliquots collected from cattle with suspected mastitis in France and Germany and expressed MDR as resistance to ≥3 drug classes and reported 30% isolates.

The higher prevalence rate of *E. coli* observed in the present study underscores the role of this pathogen in bovine urinary tract and udder infections. Although, the role of *E. coli* as a cause of environmental mastitis is well documented, the reports on its involvement in urinary tract infection in bovine are meagre. The most common cause of cystitis, urethritis and pyelonephritis in cattle is considered to be *Corynebacterium* species viz., *Corynebacterium renale, Corynebacterium cystidis and Corynebacerium pilosum* (Wallace *et al.,* 1990). In this study, the isolation of *E. coli* from 45.67% of urine sample indicates that the role of *E. coli* in setting urinary tract infections in cattle is highly under reported. Rebhun *et al.,* 1989, in a bacteriological study to investigate the cases of pyelonephritis in cattle, isolated *E. coli* in 9 (9/15) and *Corynebacterium renale* in 6 (6/15) urine samples. Similarly, Yeruham *et al.,* 2006, reported *E. coli* as most frequent cause of UTI in calves (29%) and in cows (31 %). The results of present finding are in agreement with these reports. Although, there is no dearth of reports that claims uropathogenic *E. coli* as primary pathogen involved in UTI cases in human (Hadifar *et al.,* 2017), we were surprised to find only three reports that indicated involvement of *E. coli* as a cause of UTI infections in cattle. This is even more intriguing considering the presence of *E. coli* in animal's intestine as a part of normal flora, its high build in the animal's surroundings, known ability of *E. coli* to colonise urinary tract and possession of virulence factors capable of inducing

pathology in urinary tract. Thus, investigations in this direction to ascertain role of *E. coli* in establishing UTI infections in cattle is highly warranted to appreciate the actual scenario.

The ability of microbes to resist antibiotics was realized as early as 1948, barely four years after the use of penicillin started for treating infections. In following decades a large number of antimicrobial agents were discovered with differing mechanism of actions offering good number of alternatives for clinicians to treat infections. This has largely shadowed the underlying problem with replacement of antibiotic sensitive strains of pathogens with antibiotic resistant ones under the selective pressure created by liberal and imprudent use of antibiotics. Microbes are very versatile life forms that have shown enormous power of adaptability in conquering various adverse conditions. Compounded with generation time as low as in minutes, ability to mutate fast and various possible modes of transfer of genetic material, they are quite malleable to resist adversities. The interplay of multiple resistance mechanism either via the acquisition of extraneous resistance determinants or spontaneous mutation may leads into the development multiple drug resistance (Cag *et al.*, 2016). Apart from therapeutic use, antibiotics are also used in prevention and control of diseases (as in viral infections, surgeries), to control spread of disease (administering antibiotics to whole flock while only few members are infected), as growth promoting substance in livestock (use of sub therapeutic level of antibiotics to improve weight gain). A bacterial species like *E. coli* which forms the part of normal flora of intestinal tract in human and animals are invariably subjected to antibiotic exposure. This is reflected in reports from world over showing rapid rise in emergence of antibiotic resistant *E. coli* strain from various sources viz., human UTI infections, sepsis and septic shock (Iredell *et al.*, 2016), various samples from meat producing animals (Marshall and Levy, 2011 and Onen *et al.*, 2015) and interestingly from environmental samples (Stephanie, 2015). The *E. coli* isolates showing very high resistance to some of the antibiotics and high prevalence of MDR strains, as found in the present study, is pointing towards an alarming situation that warrants further investigations and instituting the frameworks to address the issue.

Summary and Conclusions

6 Summary and Conclusions

The current study was performed to evaluate the role of *E. coli* in bovine diseases and to generate the antibiotic sensitivity profile of *E. coli* isolates. A total of 200 bovine clinical samples including urine, milk and uterine discharge were subjected to isolation of *E. coli* by enrichment and selective plating. The biochemical and molecular method showed a prevalence of 35.50% *E. coli* in bovine clinical samples including 45.67%, 19.05% and 10.0% in urine, milk and uterine discharge samples, respectively. In veterinary hospitals, the ongoing usage of antimicrobial compounds for treatment of animals increases the selective pressure for emergence of MDR organisms and dissemination of resistance. The high prevalence of penicillin resistant (98.27% of urine, 100% of milk and uterine discharge), cefotaxime/ clavulinic acid resistant (82.76% of urine, 83.33% of milk and 100% of uterine discharge) and cefotaxime (81.03% of urine, 66.67% of milk and 100% of uterine discharge) observed in this study suggests that bovine can be a significant reservoir of penicillin and cefotaxime or cefotaxime /clavulinic acid resistant *E. coli.*

Although the lower prevalence of ceftriaxone, oxytetracyclin, ciprofloxacin, norfloxacin, co-trimoxazole, ofloxacin, amoxiclav, doxycycline hydrochloride, gentamicin, amikacin, ampicillin/ sulbactum and chloramphenicol resistant were observed than those of penicillin and cefotaxime or cefotaxime / clavulinic acid resistant *E. coli* but looking to the constant selection pressure, emergence of stains showing higher resistance to these agents doesn't look very far.

Intermediate resistance refers to those *E. coli* species that were not clearly resistant or susceptible. Along with the prevalence of resistant *E. coli*, a prevalence of ampicillin / sulbactum, amoxiclav, doxycycline hydrochloride, gentamicin, cefotaxime, ceftriaxone, chloramphenicol, co-trimoxazole, amikacin, norfloxacin, ofloxacin and ciprofloxacin intermediate resistant were also found under this study. The organisms that exhibit intermediate resistance also have the tendency to easily become resistant.

Based on the finding of present study, a total of 66.20% isolates of bovine clinical samples were categorized as MDR (resistant to ≥3 to 6 antimicrobial categories). The prevalence of MDR *E. coli* in bovine clinical samples was 70.69%, 50.00% and 100% in urine, milk and uterine discharge samples, respectively. In this study among 58 *E. coli* isolates from clinical urine samples 22.41%, 18.96%, 18.96% and 8.6% were resistant to 3, 4, 5 and 6 antimicrobial categories, respectively. While, out of 12 *E. coli* isolates from clinical milk samples 33.33%, 8.33% and 8.33% were resistant to 3, 4 and 5 antimicrobial categories, respectively. A single isolate from uterine discharge showed a resistant with 5 antimicrobial categories.

Discovery of antibiotics is considered as corner stone of innovation that had most profound impact on medical and veterinary health care practices. Unfortunately, within a span of almost 70 years the "The Golden era" in the management of microbial infection is losing sight. The magnitude of the problem can be realized with the fact that WHO has recognized emergence of antimicrobial resistance as "global emergency" and marked it as one of the top health challenges facing 21 century.

The findings of the present study provides a novel insights into the epidemiological characteristics of bovine clinical *E. coli* isolates in Patna, Bihar and suggest the need for the prudent use of antimicrobial agents in husbandry and livestock clinics along with the urgent need to establish a national antibiotic resistance monitoring program.

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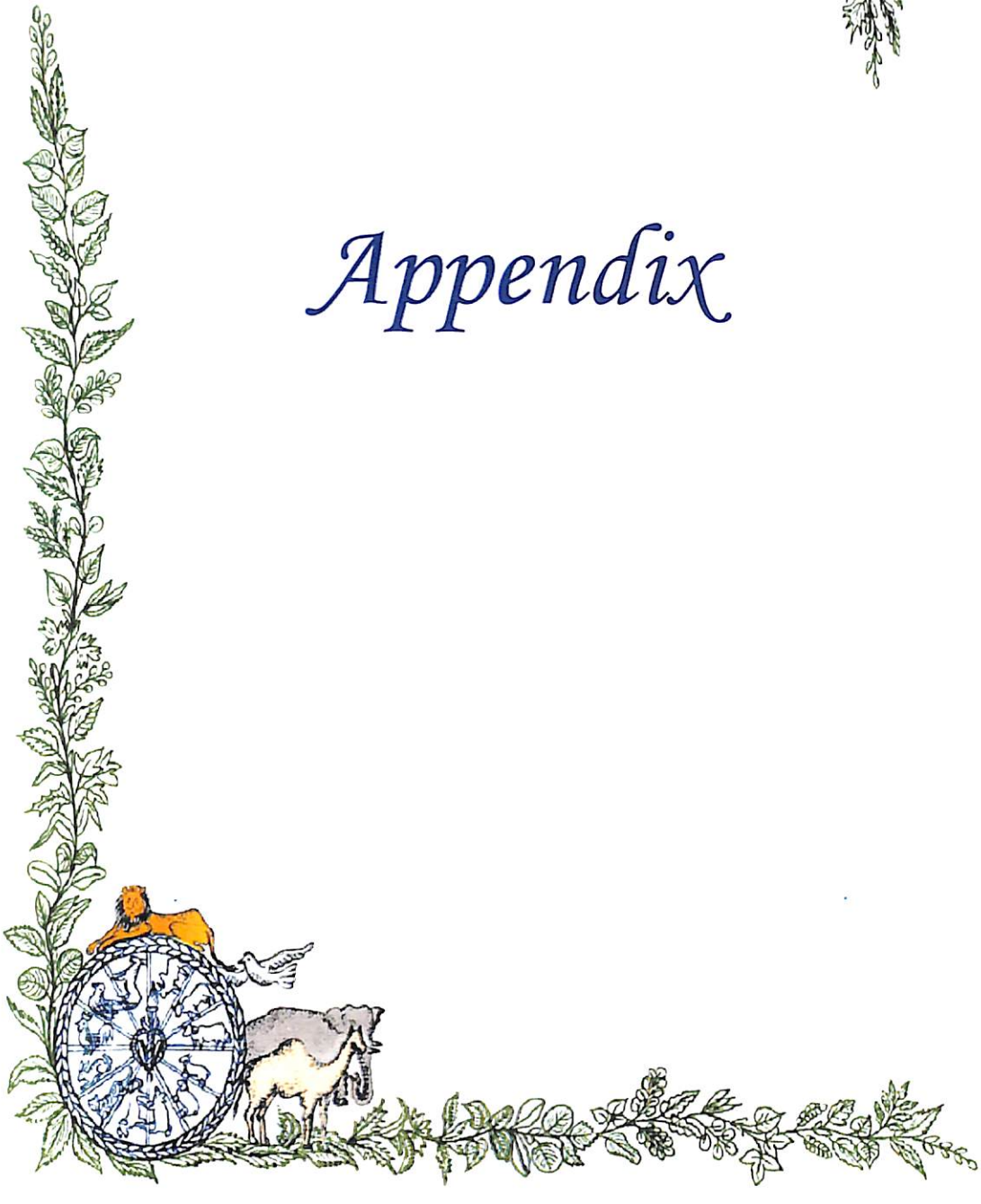
Appendix



MacConkey Broth

Gelatin peptone	20.0 g
Lactose monohydrate	10.0 g
Dehydrated bile	5.0 g
Bromo cresol purple	0.01 g
Distilled water	1000 ml

Ingredient were mixed properly by heating, evenly distributed 10 ml in 15 ml capacity test tubes, sterilized by autoclaving at 15 psi for 15 minutes and was stored at room temperature.

Eosin Methylene Blue (EMB) Agar

Peptic digest of animal tissue	10.0 g
Dipotassium phosphate	2.0 g
Lactose	5.0 g
Sucrose	5.0 g
Eosin - Y	0.4 g
Methylene blue	0.065 g
Agar	13.5 g
Distilled water	1000 ml

Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates.

Mueller Hinton Agar

Meat, infusion solids from 300g	2.0 g
Casein acid hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1000 ml

Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates.

Nutrient stab

Nutrient broth	1.3g
Bacteriological agar	0.9g
Distilled water	100ml

Ingredient were mixed properly by heating, evenly distributed 10 ml in 15 ml capacity test tubes, sterilized by autoclaving at 15 psi for 15 minutes and was stored at room temperature.

Nutrient broth

Peptone	10.0 g
Beef extract	10.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml

Ingredient were mixed properly by heating, evenly distributed 10 ml tubes in tubes (15 ml capacity), sterilized by autoclaving at 15 psi for 15 minutes and was stored at room temperature.

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS:

TAE buffer (50 X)

Tris base	60.50 g
Glacial acetic acid	14.25 ml
0.5 M EDTA solution (pH 8.0)	25.0 ml

The final volume was made up to 250 ml with double distilled water.

Ethidium bromide stock (10 mg / ml)

Ethidium bromide	10 mg
Double distilled water	1 ml

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