# OCCURRENCE OF CARBAPENEMASE AND EXTENDED SPECTRUM BETA LACTAMASE PRODUCING MAJOR ENTERIC BACTERIA IN FARMED PIGS AND HOUSE FLIES FROM FARM PREMISES

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**(19-MVP-18)**

**THESIS**

**Submitted in partial fulfilment of the requirements for the degree of**

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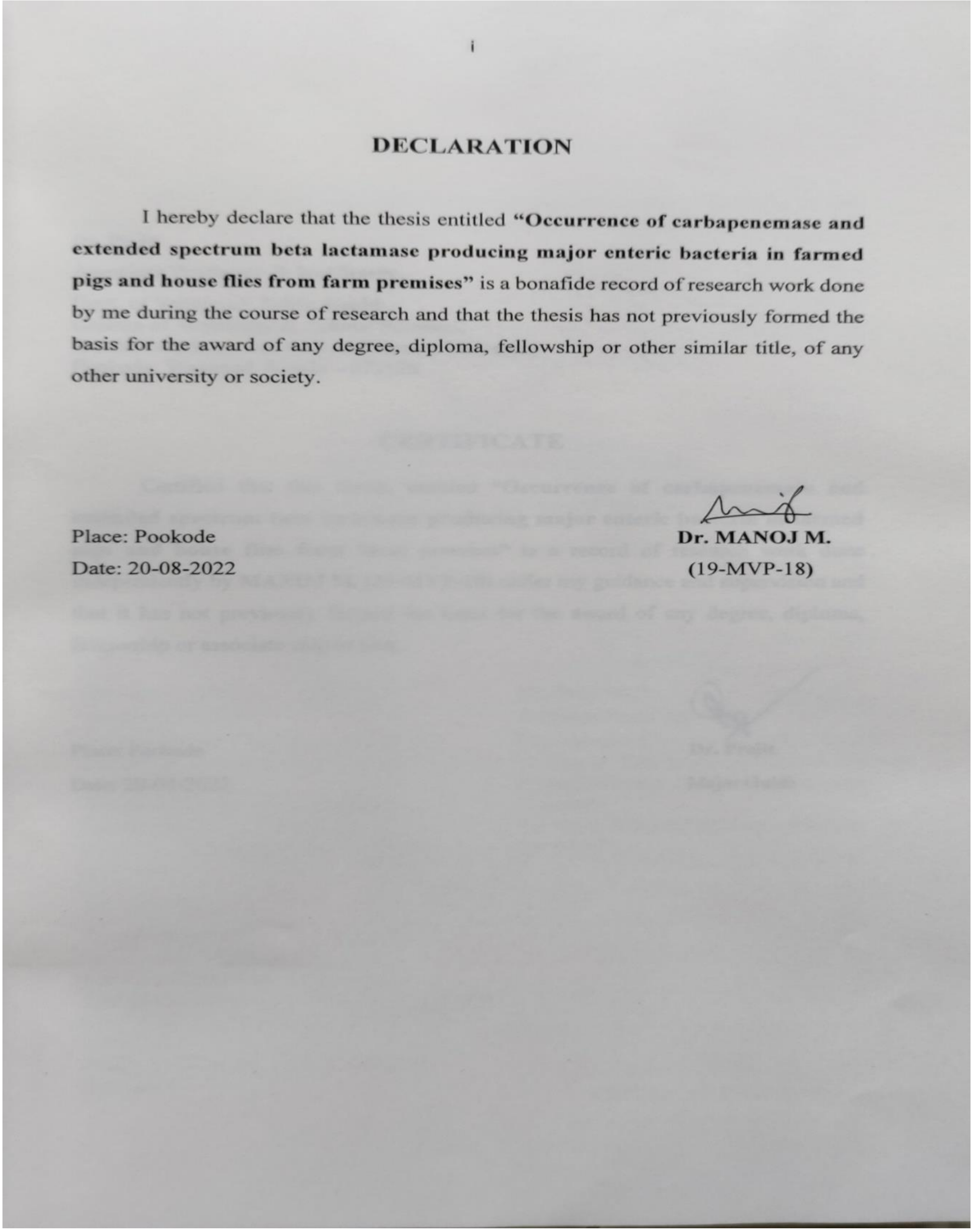

# DECLARATION

I hereby declare that the thesis entitled **"Occurrence of carbapenemase and extended spectrum beta lactamase producing major enteric bacteria in farmed pigs and house flies from farm premises"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other university or society.

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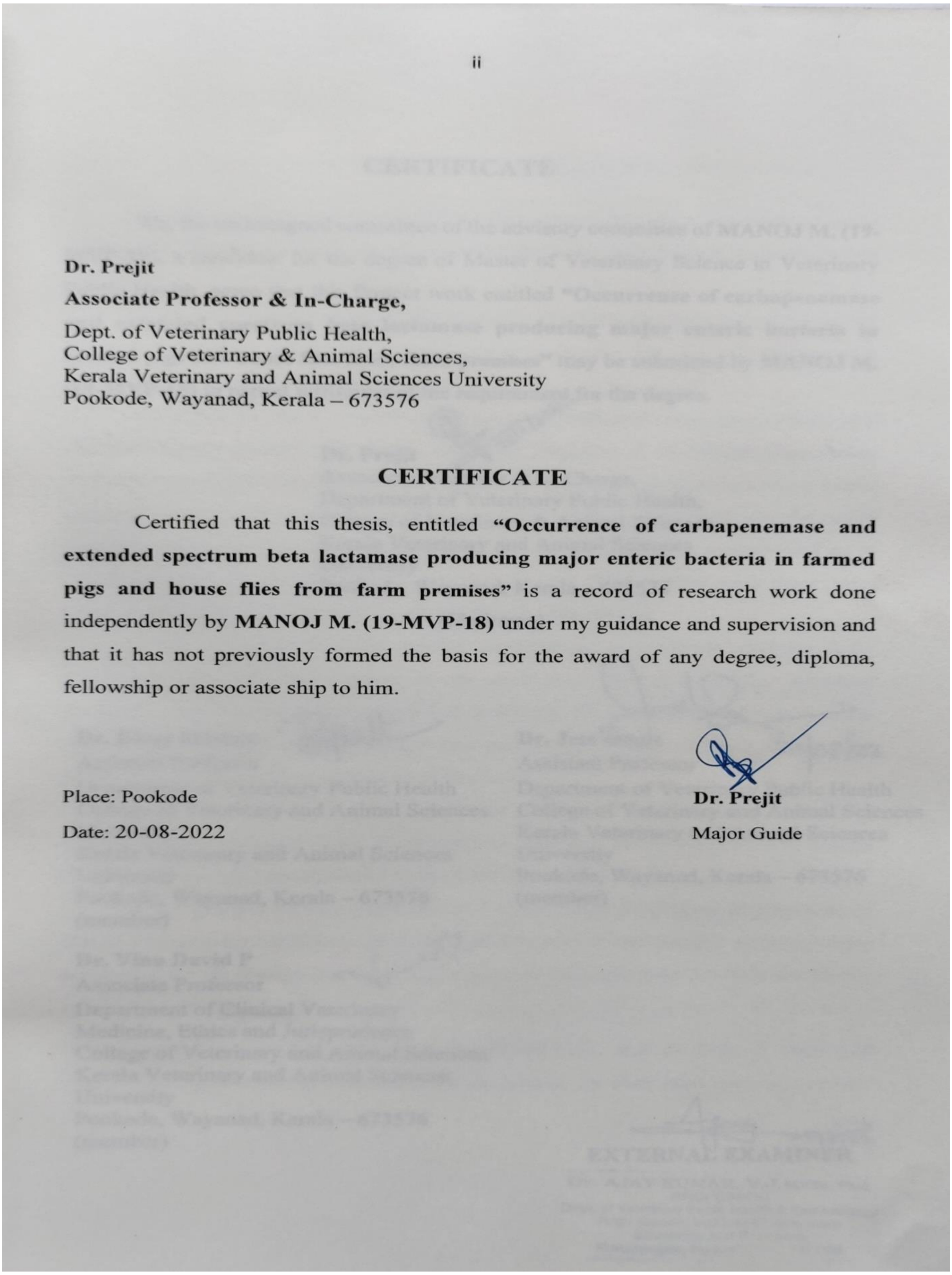

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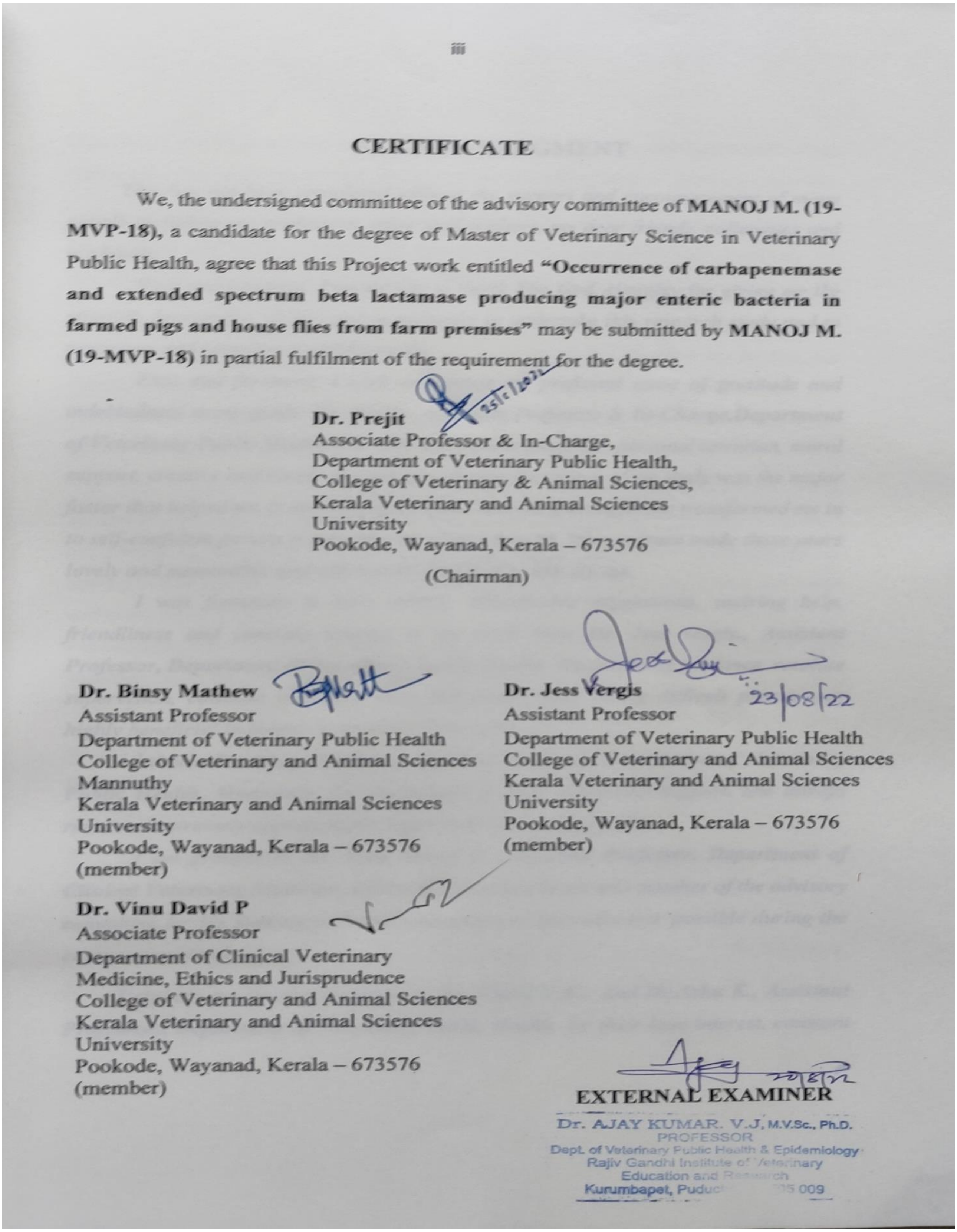

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We, the undersigned committee of the advisory committee of **MANOJ M. (19-MVP-18)**, a candidate for the degree of Master of Veterinary Science in Veterinary Public Health, agree that this Project work entitled **"Occurrence of carbapenemase and extended spectrum beta lactamase producing major enteric bacteria in farmed pigs and house flies from farm premises"** may be submitted by **MANOJ M. (19-MVP-18)** in partial fulfilment of the requirement for the degree.

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***MANOJ M.***

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| | |
|---|---|
| % | Per cent |
| µl | Microliter |
| µg | Microgram |
| ABST | Antibiotic Sensitivity Test |
| Bp | Base pair |
| BPW | Buffered peptone water |
| BHI | Brain heart infusion agar |
| $CaCl_2$ | Calcium chloride |
| CFU | Colony forming units |
| CLSI | Clinical and Laboratory Standards Institute |
| DEC | Diarrhoeagenic *Escherichia coli* |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxy nucleoside triphosphates |
| EDTA | Ethylene diamine tetra acetic acid |
| EHEC | Enterohaemorrhagic *Escherichia coli* |
| EAEC | Enteroaggregative *Escherichia coli* |

| | |
|---|---|
| *et al*., | and co-workers |
| Fig. | Figure |
| g/L | Gram per liter |
| Mg | Milli gram |
| MgCl2 | Magnesium chloride |
| Min | Minute |
| Ml | Milli liter |
| mM | Milli mole |
| mPCR | Multiplex Polymerase chain reaction |
| Ng | Nano gram |
| pH | Log hydrogen ion concentration |
| °C | Degree Celsius |
| Sec. | Second (S) |
| spp. | Species |
| Taq | Thermus aquaticus |
| TAE | Tris acetate EDTA |
| TE | Tris-ethylene diamine tetra acetic acid |
| Tris | Tris-hydroxy methyl aminoethane |
| viz. | Namely |
| v/v | Volume by volume |

# 1. INTRODUCTION

Antibiotics are biological and semi-synthetic agents that can kill (bactericidal effect) or stop bacteria's growth (bacteriostatic effect). Their life-saving properties have been lauded since the initial clinical use of penicillin in the 1940s. As a result, human and animal life expectancies were low throughout the globe. After the discovery of antibiotics in the 1940s, veterinary medicine was able to treat formerly life-threatening bacterial illnesses for the first time and rendered protection from bacterial infections through antibiotic prophylaxis and antisepsis. Antibiotic resistance occurs when bacteria can multiply in the presence of therapeutic antibiotic dosages. Bacteria develop antibiotic resistance in various ways, including their inherent capacity to divide into millions of cells in a matter of minutes, which could result in daughter cells with genomes that differ from the parent cells.

Antibiotic resistance among bacteria is increasingly rendering all antibiotic classes ineffective (Martinez, 2012). Consequently, hitherto treatable infections are proving difficult to manage, as there are minimal/no antibiotics available to treat extremely drug-resistant (XDR) bacteria. Increased health care costs for animals from resorting to more expensive and more toxic alternatives, prolonged hospitalization in treating even superficial infections, and higher morbidities and mortalities are some of the consequences of antibiotic resistance. There have been reports of resistance to all antibiotics currently in clinical use, and bacteria develop resistance faster than the design, development, and clinical trial of new ones (Van den Bogaard and Stobberingh 2000; Nordmann, 2014; Tiseo *et al.*, 2020). Antibiotic resistance has thus become one of the most critical global public health threats of this century. Without a concerted global effort, the world risks returning to the pre-antibiotic era, where simple infections will be fatal, and routine surgeries will not be safe, as there will be no prophylactic antibiotics to prevent surgical sites nosocomial infections.

Antibiotics are widely employed in agriculture, notably in the production of food animals, where they are utilized not only to treat infections but also as prophylactics to prevent and limit disease spread and sometimes as growth promoters, where they are given at low doses over an extended period. The microorganisms that acquired resistance determinants develop enhanced fitness and competitive advantage over other microbes benefit from the ongoing selective pressure within the food animal production ecosystem. Several investigations have confirmed resistant infections from these modern animals farming facilities, including *Salmonella* spp. and *E. coli*. Many of these pathogens have also been isolated from people close to these facilities (Dohmen *et al.*, 2015; Pawade *et al.*, 2019).

Antimicrobial usage in farm animals raises the question of whether and to what extent it poses a risk to human health. Surprisingly, little research has been published that specifically addresses this issue. We know from several reports of widespread resistance in farm animals and, more lately, the first reports of carbapenem that the direction of resistance movement (if any) between human and livestock populations is unknown. Carbapenems, for example, are not administered to animals; therefore, resistance came from somewhere else, most likely humans. ESBL-producing *E. coli* and *Salmonella* spp. isolates from humans and animals have been the subject of a lot of investigation (Murray *et al.*, 2022). However, in terms of animal studies in India, there are only a few findings available.

According to the 20th livestock census, (2019) India had a pig population of 9.06 million, of which Kerala contributed 1.5 per cent. Compared to the previous year, India showed a decline of 12.03 per cent in pig production. Food-producing animals, notably pigs, are known to be critical reservoirs of antibiotic-resistant *Enterobacteriaceae* and potential transmission sources to humans (Fournier *et al.*, 2021). In pig farming, antibiotics are used as growth promoters, prophylactic, metaphylactic, and therapeutic purposes. Such antimicrobial agents elicit ‘selection pressure’ on pathogenic bacteria contributing to the development of anti-microbial resistance that can be critically important in human medicine. Various literature showcases the prevalence or occurrence of ESBL and carbapenem resistant *Enterobacteriaceae* in swine industry (Mandakini *et al.*, 2015; Pruthvishree *et al.*, 2017). Considering the poor hygiene status of some of the pig farms in developing countries, the presence of house flies is very high. Moreover, there are many documented reports of antimicrobial genes in house flies (Cervelin *et al.*, 2018; Akter *et al.*, 2020).

Insects are one of the most varied taxa in the animal kingdom, with over a million species documented, accounting for more than half of all known living organisms. Given its vast diversity of species and its close interaction with humans, it is surprising that only relatively limited information is available regarding the impact of insects and the implications of insect-associated bacteria on human health (Asril *et al.*, 2022). Around 90 per cent of insects in the human habitat are houseflies. As per the CDC, house flies are considered cleanliness indicators in public places (CDC, 2014). Flies from garbage and sewage from farms pick up pathogenic organisms (includes MDR organisms) and transfer them to human and animal food via their mouthparts, vomitus, faeces, and contaminated body parts. The movement of flies from animal or human faeces to food that will be eaten uncooked by humans is of particular concern (Olagunju, 2022). In addition, some diseases can be harboured in the mouthparts or alimentary canal of flies for several days after being fed and then spread when the flies defecate or regurgitate (Park *et al.*, 2019). The role of houseflies in disseminating various diseases like malaria and cholera is already documented (Olagunju, 2022). Due to their presence in all places, house flies can be considered "one health" indicator of AMR transmission in humans, animals, and their environments. Several studies have demonstrated antibiotic resistance among microbes in houseflies and their function in food chain transmission (Gwenzi *et al.*, 2021).

Thus, the present study was envisaged to understand the occurrence of zoonotic enteric bacterial pathogens and the plausibility of these pathogens harbouring antibiotic resistance genes among in-house reared pigs and houseflies trapped in the farm and non-farm settings.

Thus, the main objectives considered in this study include

1. Isolation and identification of *Escherichia coli* and *Salmonella* spp. from farmed pigs and house flies (*Musca domestica*) from farm premises
2. Molecular confirmation of the isolates by Polymerase Chain Reaction (PCR)
3. Detection of extended-spectrum β-lactam and carbapenem resistance by phenotypic and genotypic methods

# 2. REVIEW OF LITERATURE

## 2.1. *Escherichia coli* – isolation and importance

Theodore Escherich, a German pediatrician, first discovered *Escherichia coli* in 1885. Within the *Enterobacteriaceae* family, the type species of the genus Escherichia contains predominantly motile gram-negative bacilli. For a long time, the pathogen was used to indicate the presence of fecal contamination and unsanitary processing. The most common agars used to recover *E. coli* from feces were MacConkey and Eosin Methylene Blue (EMB). Diarrhoeagenic *E. coli* can also be isolated using blood agar plates (Edwards and Ewing, 1972). EMB agar was considered a suitable medium for isolating *E. coli* from faeces because it tends to form distinctive colonies with a greenish-metallic tinge that were not produced by other bacteria of the family Enterobacteriaceae (Merchant and Packer, 1967).

Using a variety of virulence factors, pathogenic *E. coli* was classified into different pathotypes that cause a common disease *Viz*; Enterotoxigenic *E. coli* (ETEC), which causes diarrhea without fever; enteropathogenic *E. coli* (EPEC) causing travelers' diarrhea; enterohemorrhagic *E. coli* (EHEC), which cause bloody diarrhea without fever; enteroinvasive *E. coli* (EIEC), which causes dysentery like diarrhea without fever and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998).

*E. coli* was chosen as the indicator bacterium because changes in antibiotic resistance in this species can be considered as an early warning system for developing resistance in associated pathogenic bacteria (Van den Bogaard *et al.*, 2000).

## 2.2. *Salmonella* spp. – Isolation and Importance

*Salmonella* spp. was named in recognition to Dr. Daniel Salmon, a veterinary bacteriologist at the United States Department of Agriculture. *Salmonella enterica* is a small gram-negative bacillus that belongs to the *Enterobacteriaceae* family of bacteria. *Salmonella enterica* subspecies enterica, *Salmonella enterica* subspecies *salamae*, *Salmonella enterica* subspecies *arizonae*, *Salmonella enterica* subspecies *diarizonae*, *Salmonella enterica* subspecies *houtenae*, and *Salmonella enterica* subspecies *indica* are the six subspecies of *Salmonella enterica*. Based on the antigen component of the lipopolysaccharide, these subspecies can be divided into about 50 serogroups (Nunes *et al*., 2003).

*Salmonella* spp. is one of the most critical foodborne zoonotic agents globally, and antibiotic resistance in *Salmonella* spp. is becoming a growing threat to human health. Antimicrobial resistance surveillance programs for certain zoonotic and animal infections, including *Salmonella* spp., had been mandated by new legislation in all EU Member States (Carlson and Wu, 2003). To detect *Salmonella* spp., a PCR targeting the *invA* gene was efficient, sensitive, and specific. The *invA* gene was used for molecular detection of *Salmonella* species; this gene encodes for a bacterial membrane protein required for epithelial cell invasion (Lampel *et al.*, 2000).

Pre-enrichment in BPW and selective enrichment with Tetrathionate Broth, Selenite Cysteine Broth, or Rappaport Vassiliadis (RV) Broth, followed by selective plating on Hektoen Enteric Agar and Xylose Lysine Deoxycholate Agar and finally molecular confirmation with PCR was a standard protocol used for *Salmonella* spp. isolation (Nambiar *et al*., 2009).

## 2.3. Prevalence of *E. coli* and *Salmonella* species in domestic pigs

PCR-based pathogen detection was used to rapidly identify Salmonella from clinical specimens, food samples, and faecal samples (Rahn *et al*., 1992).

Feder *et al.* (2003) detected *E. coli* O157:H7 and established that potentially pathogenic *E. coli* O 157:H7 could be found in pigs in the United States.

Paixão *et al*. (2013) found seven positive samples out of 210 faecal samples examined from pigs for *Salmonella Enteritidis*.

Valentin *et al.* (2014) reported Extended-Spectrum-Lactamase producing *E. coli* from pig slurry in Estonia. From 2011 to 2014, a total of 347 *E. coli* strains were identified, of which 144 were from humans, 88 from animals, and 115 from the environment. The *E. coli* strains were explicitly chosen for their ability to produce ESBL.

Ainslie-Garcia *et al*. (2018) investigated the association between faecal shedding of *Salmonella* spp. in pigs at different production stages and the presence of *Salmonella* spp. in tissues at slaughter on a farm. From birth to slaughter, faeces and tissues were collected from 14 groups of pigs from eight commercial farrowing farms (a total of 809 animals) and cultured for *Salmonella* spp. *Salmonella* spp. was detected in 13 per cent (421/3339) of the faecal samples. Overall, 35 per cent (284) of the pigs shed *Salmonella* spp. at least once and 12 per cent (99) shed several times. *Salmonella* spp. excretion increased with pig age ($P \geq 0.01$) and summer ($P \geq 0.01$).

De Koster *et al.* (2021) screened 817 pig faecal samples collected from 31 multiplier pig farms in Belgium and Netherlands for the presence of antibiotic-resistant *E. coli*. They could not find carbapenems resistance in *E. coli*. However, ESBL- *E. coli* were found in significantly higher numbers in Belgian farms (37 per cent) than in Dutch farms (33 per cent).

Gwenzi *et al*. (2021) studied the prevalence of *Salmonella* spp. serotypes in non-diarrheal pigs in India by analyzing fecal samples from 194 randomly selected pigs. *Salmonella* spp. was detected in 11.3 per cent of the apparently healthy pigs with no diarrhoea. Isolated serotypes were *S. Weltevreden* (81.8 per cent) and *S. Enteritidis* (18.2 per cent). A total of 18.2 per cent of the isolates were multidrug resistant (three antibiotic classes) with a variety of antibiotic resistance genes on their genomes, including ESBL ($bla_{TEM}$ *and* $bla_{OXA}$).

Antimicrobial resistance and the occurrence of *Salmonella* spp. from pig farms were reported by Ye *et al*. (2021). *Salmonella* spp. was found in 64 (15 per cent) faecal samples that were collected.

Igbinosa *et al*. (2021) conducted a study to characterize *Salmonella* spp. serovars from commercial pig farms in Benin City, Nigeria. On screening 81 samples from farms, 15 *Salmonella* ser. Enteritidis, 11 *Salmonella ser.* Typhimurium and 58 other *Salmonella* spp. serovars were isolated.

Chai *et al*. (2022) examined 400 faecal swab samples collected from rabbits, pigs, and poultry farms in Malaysia for the antibiogram profiles of ESBL-producing *E. coli* and CREC. Suspected *E. coli* isolates were confirmed by PCR test and isolation. The Kirby-Bauer disk diffusion technique was used to assess the antibiogram of the *E. coli* isolates, and twelve ESBL-producing *E. coli* (3 per cent; 12/400) were identified.

Non-typhoid *Salmonella* spp. (NTS) serovars were found in food producing animals and pose a threat to food safety and public health (Karabasanavar *et al*., 2022).

## 2.4. Prevalence of *E. coli* and *Salmonella* species in houseflies

Iwasa *et al*. (1999) conducted a study to detect *E. coli* O157:H7 from *Musca domestica* (Diptera: Muscidae). A total of 310 fly samples were obtained from four different farms including pig farm. *E. coli* serotype O157:H7 was isolated from 5 (1.81 per cent) of 275 houseflies (*M.domestica*) collected from a cattle farm, whereas no isolates were obtained from pig or poultry farms.

Olsen and Hammack (2000) isolated *Salmonella* spp. from the housefly (*Musca domestica*) sampled from caged layer houses. Twenty-two species of flies were collected from 18 traps for microbiological examination. There were 15 pools of houseflies, *M. domestica*, ranging from 1 to 19 adult flies per pool. *Salmonella* spp. was found in four of the 22 fly pools (18.81 per cent).

*Salmonella* spp. was isolated from muscoid flies in commercial animal operations in San Bernardino County, California, by Mian *et al.* (2002). *Salmonella* Enteritidis was detected in adult flies (2,686 muscoid flies) obtained from three dairies and eight commercial poultry farms. *Musca domestica* accounted for 93.3 per cent of the fly population. In eleven (12.94 per cent) of the pools *M.domestica* tested positive for *Salmonella* Enteritidis.

Antimicrobial-resistant *E. coli* isolates from symbovine flies from a farm in the Czech Republic (2006–2007) were studied by Literak *et al.* (2009). A total of 240 flies were captured using adhesive "fly-catcher paper" and rectal swabs from 165 calves were taken for *E. coli* isolation. *E. coli* was found in 61 per cent of symbovine fly samples.

Wang *et al*. (2011) analyzed 144 *Salmonella* spp. isolated from fly and pig fecal samples from 11 pig farms in Taiwan, and 71.5 per cent of them were resistant to atleast three antibiotics.

Zhang *et al*. (2018) collected 297 flies, consisting of 252 *Musca domestica*. *E. coli* was reported to be the most frequently isolated bacterial species from the flies (41 isolates each) from all sample locations.

Poudel *et al.* (2019) trapped 493 flies from different livestock and other units and isolated *E. coli* from 43.9 per cent of the samples. The study revealed that 35.3 per cent of flies harboured antimicrobial-resistant bacteria, of which nine per cent were multi-drug resistant.

An ecological study on antimicrobial-resistant zoonotic bacteria spread by flies in cattle ranches was conducted by Wetzker *et al.* (2019). The study observed houseflies showed the presence of 23.6 per cent of bacterial isolates, predominantly *Salmonella* spp. (40 per cent).

Al Jallaa Hospital in Benghazi (Gāliņa *et al*., 2021) reported multidrug-resistant Gram-negative bacteria in house flies (*Musca domestica*). *E. coli* was isolated from 100 flies collected during the isolation process (22 per cent). The *E. coli* isolates had 70 per cent resistance to amoxicillin-clavulanic acid (AMC) but no resistance to imipenem (IPM) or ceftazidime (CAZ).

## 2.5. Antibiotics and Antibiotic resistance

The antibiotics discovery dates back to the 19th century. In 1887, Rudolf Emmerich observed that animals infected with Streptococci were immune to cholera, while in 1896, a French medical student, Ernest Duchesne, proved the antibacterial effects of the soil mold Penicillium. However, the discovery of penicillin by Nobel Prize winner Alexander Fleming in 1928 and its extensive use during World War II revolutionized antimicrobial therapy (Sheehan, 1982).

Antibiotics were categorized according to their mode of action (bactericidal or bacteriostatic), their spectrum of activity (broad or narrow), their mode of administration (oral or injectable), and their chemical structure. Major classes of antibiotics are naturally derived antibiotics (e.g., lactams, tetracyclines, aminoglycosides, and macrolides) and chemical structures of synthetic antibiotics. The lactam antibiotics, having a β lactam ring in their molecular structure, are among the most commonly used antibiotics in clinical therapy. These antibiotics have bactericidal activity against gram-positive and gram-negative bacteria by irreversibly blocking penicillin-binding proteins (PBPs) such as carboxypeptidases, endopeptidases, and transpeptidases involved in the formation of the peptidoglycan layer in their cell walls. PBPs are a family of proteins that help freshly generated peptidoglycans crosslink to the existing cell wall structure. The β -lactam antibiotics group includes penicillin, cephalosporins, carbapenems, and monobactams. They can be categorized into different generations and grouped based on their antimicrobial properties and spectrum of action (Fontana, 1990).

Antibiotic resistance to commensal and pathogenic bacteria in humans was reported for the first time by Smith in 1969 and was due to the selection pressure on bacterial populations when used for therapeutic or preventive use (Schroeder *et al.*, 2002).

Antibiotics are chemicals or compounds (secondary metabolites) produced by some microbes that might kill or impede the growth of other microorganisms. In addition, many synthetic chemicals have similar activities. More than 5000 antibiotics were discovered as of today, with roughly 100 being in routine use to treat human and animal illnesses (Khardori, 2006). Antibiotic resistance in commensal bacteria (naturally occurring host flora) indicated the selection pressure induced by antimicrobial usage and signalled the potential for future resistance in pathogens (Aarestrup *et al.*, 2010).

Antibiotics are also being used as a growth promoter in food animals though European Union prohibits it. However, the growth promoter restriction has typically resulted in increased metaphylactic and prophylactic use in animals rather than a consistent decrease in antibiotic consumption (Laxminarayan *et al.*, 2013).

The free movement of people and comodities between countries and the extensive international transportation of livestock has made AMR a global concern (Da Costa *et al.*, 2013). The environment is considered the melting pot of antimicrobial resistance as microbes acquire antibiotic resistance genes from the environmental microbiota. Furthermore, the rise of AMR correlates with a decrease in the discovery of new antimicrobial drugs. Most antibiotics currently used to treat common human and animal infections are expected to become obsolete in the next five to ten years, reverting to the pre-antibiotic era in the post-antibiotic era (Butaye *et al.*, 2015).

In human medicine, antimicrobial drugs are only used for therapy and prophylaxis. Antimicrobials in farm animals, on the other hand, have therapeutic, preventive, metaphylactic, and subtherapeutic use (Hedman *et al.*, 2020). We need to use antimicrobials more cautiously in both human and animal medicine since a complete restriction on their use in farm animals would have significant consequences for animal health, welfare, and productivity.

## 2.6. Origin and antibiotic resistance due to extended-spectrum beta-lactamase

Philippon created the phrase extended spectrum β lactamase (ESBL) in 1989. ESBLs are mostly plasmid-encoded enzymes that provide prolonged resistance to β lactam antibiotics. Nosocomial pathogens are ESBL-producing bacteria that have been increasingly recognized as a cause of infections in the community since the late 1990s. The great majority of ESBLs found in human clinical isolates until the 1990s were SHV (sulfhydryl-variable) or TEM (called after the first patient from whom the pathogen was recovered) types (Hunter *et al.*, 2010).

Resistance to β-lactam antibiotics in gram-negative organisms could be caused by three different mechanisms: mutations in penicillin-binding proteins (PBPs), reduced permeability of the cell wall (i.e., disruption of porin proteins, efflux systems), and production of β-1actamase enzymes (the most common mechanism in Enterobacteriaceae family) that hydrolyze and inactivate the β -lactam ring. More than 1000 β -lactamases have been identified to date (Bush and Fisher, 2011).

Evolution of extended spectrum β -lactamases started from the introduction of cephalosporins in the 1960s; plasmid-borne enzymes and chromosomal cephalosporinases (AmpCs) proficient in hydrolyzing all penicillins and cephalosporins became available in the 1980s, resulting in the development of second, third, and fourth-generation oxy-imino cephalosporins (cefuroxime, cefotaxime, ceftazidime, and cef (clavulanic acid, tazobactam, sulbactam, and avibactam). Due to their ability to hydrolyze and inhibit all penicillins and cephalosporins, these enzymes, known as extended-spectrum β -lactamases, became popular in the 2000s, resulting in clinical failures in antibiotic therapy and a rise in morbidity and/or mortality. Furthermore, the usage of cephalosporins causes the expression of AmpCs, which can hydrolyze all cephalosporins (except cefepime) and, to a lesser extent, carbapenemases. Carbapenems gradually displaced cephalosporins to treat infectious illnesses (Poirel *et al.*, 2011).

Antibiotics having β-lactams are the safest and most frequently used in clinical treatment. They are versatile in that they can be structurally modified to produce various powerful versions, a malleability that other antibiotic classes lack. Lactamases (or β-lactam hydrolyzing enzymes) mediate β-lactam resistance in Gram-positive bacteria, whereas β-lactamases (or β-lactam hydrolyzing enzymes) induce β-lactam resistance in Gram-negative bacteria. Paradoxically, the evolution of β -lactamases has sped up the production of new β-lactams drugs (Dolejska *et al.*, 2012).

To combat the hydrolysis induced by β-lactam enzymes, β-lactamase inhibitors had been developed, permanently binding the enzyme binding site and allowing the β -lactam to function. As a result, they were administered in combination with antibiotics susceptible to β-lactamase. Over a period, inhibitor-resistant enzymes have emerged, and some carbapenemases belonging to the Metallo-lactamase family have developed resistance to these drugs. When ESBLs emerged in the 1980s, a novel β -lactam antibiotic was produced that was stable to hydrolysis by β -lactamases. Because of their effectiveness against all resistant species, these new antibiotics, carbapenems, were the last line of antibiotics (Carattoli, 2014).

CTX-M (cefotaximase) -lactamases, evolved later are the most common ESBLs in human *Enterobacteriaceae*. The first ESBL-producing *E. coli* strain was discovered in a dog with a urinary tract infection and carried the SHV-12 gene. Later, *E. coli*, *Salmonella* spp., and *K.pneumoniae* from livestock and companion animals were found to have a variety of CTX-M, TEM, and SHV types (Dahms *et al.*, 2015).

Clavulanic acid and other class A lactamase inhibitors such as sulbactam and tazobactam inhibit an enzyme known as extended-spectrum beta-lactamase. Beta lactamase enzyme provide resistance or reduce susceptibility to narrow- and broad-spectrum cephalosporins but do not affect cephalosporins or carbapenem compounds. ESBLs have been divided into different classes based on their amino acid sequences, with most of them being first discovered in Europe. Ambler's molecular classification and BushJacobyMedeiros' functional classification are the two most widely used categorization schemes used for beta-lactamases. According to the protein homology of the enzymes, the Ambler scheme divides β-lactamases into four groups. Serine lactamases are class A, C, and D enzymes, while Metallo-lactamases are class B enzymes. The functional system of BushJacobyMedeiros was based on the functional properties of enzymes, the substrate, and inhibitor profiles (Shaikh *et al.*, 2015).

## 2.7. Origin and antibiotic resistance due to carbapenems

Carbapenems are structurally related to penems and have similar core structures. Carbapenems have a β -lactam ring fused to an unsaturated five-membered ring with carbon at position 1 of the unsaturated ring. Carbapenems have been identified from several Streptomycete fermentation products. Thienamycin, a naturally occurring carbapenem, was discovered in *Streptomyces cattleya*. Although it was unstable in aqueous solutions, the thienamycin molecule showed potent activity against gram-positive and gram-negative bacteria. The pharmaceutical sector has therefore created thienamycin compounds with adequate stability. Clinically available carbapenems are imipenem, meropenem, ertapenem, and doripenem thienamycin derivatives (EFSA, 2013).

In the 1980s, chromosomally mediated carbapenemases were discovered in the cloacae isolates of Enterobacter long before the plasmid-transmitted *K.pneumoniae* carbapenemase (KPC) was detected in 1996. Carbapenemases also hydrolyze all β-lactam antibiotics with some classes, such as, e.g., clavulanic acid showing resistance to β-lactamase inhibitors. In addition, carbapenemase-producing organisms are the most worrying of all multidrug-resistant (MDR) strains since the genetic determinants containing the carbapenemase genes almost always carry genes resistant to numerous other classes of antibiotics (Nordmann and Poirel, 2014).

Carbapenemases are divided into three categories: Ambler class A, class B, and class D. The most common clinical carbapenemase is the class A carbapenemase, specifically KPC, which is most commonly produced by *K.pneumoniae*. Their infection-related death rates are high (> 50 per cent), and their treatment options are limited. Ambler's class B carbapenemase family includes six enzymes: imipenemase (IMI), Verona integrin-encoded Metallo-lactamase (VIM), Sao Paolo Metallo-lactamase (SPM), Seoul imipinemase (SIM), Guyanese imipenemase (GIM) and New Delhi Metallo-lactamase (NDM) (Guerra *et al*., 2014).

Class B enzymes can hydrolyze all β-lactams except aztreonam, and the mortality rate for this class is estimated at 18 to 67 per cent. NDM has recently become endemic in Pakistan and India and has been detected on every continent with co-resistance to fluoroquinolones, aminoglycosides, macrolides, and all antibiotics except tigecycline colistin (polymyxin E), and polymyxin B, has raised international concern. The oxacillinases (particularly OXA-48) belong to the class D carbapenemases, first discovered in Turkey in 2003, and are currently found worldwide. It is present in *A.baumannii* and is difficult to detect using phenotypic approaches, making a collection of actual prevalence data problematic (Jager *et al*., 2014).

The clinical effectiveness of antibiotics is short-lived as bacteria adapted to them by evolving various resistance mechanisms that allow them to avoid the lethal effects. Through increased global travel and trade, antimicrobial resistance can be transported from one region of the world to another through increased global travel and trade, as was the case with NDM-expressing carbapenem-resistant bacteria discovered in Sweden in patients who had just been to India and Pakistan (Cubero *et al*., 2015). As a result, antibiotic resistance is no longer a regional problem but a global one with far-reaching economic implications due to rising healthcare spending, longer hospital stays, and costly but harmful antibiotic alternatives (Xu *et al.*, 2022).

Yamada *et al*. (2016) performed multiplex PCR to detect genes of $bla_{KPC}$, $bla_{NDM}$, $bla_{VIM}$, $bla_{IMP}$, *and* $bla_{OXA\text{-}48}$ like carbapenemases and had 100 per cent sensitivity and specificity. Because of the difficulties in interpreting MHT and MBL tests, it was recommended that molecular tests can be used for optimal detection of carbapenemase-producing *Enterobacteriaceae* (Dankittipong *et al.*, 2022).

## 2.8. Use of Antimicrobials in Animal Health

The use of antimicrobial drugs in veterinary medicine, as in human medicine, creates a selection pressure for the emergence of antimicrobial-resistant microorganisms, including animal pathogens, human pathogens with animal reservoirs, and commensal bacteria found in animals. According to available statistics, antimicrobial agents are used extensively in animals, including drugs critical to human treatment, and their use in animals may even exceed use in humans (Singer *et al*., 2003).

Agga *et al*. (2015) studied environments related to animal (cattle and pigs) and municipal (human) waste and found that antimicrobial-resistant bacterial populations were unique to the context of animal husbandry and human activities.

However, 25 of the 61 unique AMR genes found were shared by urban garbage and animal samples. AMR genes were found in external environments. Biocide and metal resistance were the key issues of these genes from external microbiomes. AMR genes were abundant and diverse in the human microbiome but with minimal taxonomic diversity.

Coyne *et al*. (2019) stated that antimicrobial use in the pig sector was influenced by a variety of factors, with multiple drivers motivating decisions. Farmers stated that good management methods, low stocking densities, and a high health status were related with low antimicrobial use, but there was no consensus on whether agricultural systems resulted in either a low or high antimicrobial requirement.

Fang *et al.* (2020) conducted a survey on antimicrobial usage in pigs and concluded that types of farms, antibiotic sources, and previous pig disease experiences were all linked to self-purchasing of antibiotics by farmers. Those who were smallholders, buying antibiotics from veterinary drug stores and village vets, and whose pigs had previously suffered diseases were more likely to self-purchase antibiotics for their pigs. In comparison to their peers, farmers who cleaned their pigsties less frequently and those whose pigs had been diagnosed with ailments used antibiotics more frequently.

Animal disease surveillance is underdeveloped, and the infrastructure to support service delivery is limited. Due to economic considerations and approachability, farmers prefer untrained "animal health workers" and para-veterinarians. Antibiotics are commonly available over-the-counter without a prescription, and medications are sold directly to farmers. Furthermore, drug withdrawal periods are uncommon, and antibiotic-contaminated milk has been documented. Antimicrobial stewardship in livestock is still being established, and there is a lack of awareness of AMR. Initiatives like the National AMR Containment Program, the National AMR Action Plan, and the National Health Policy demonstrate the government's commitment to resolving the country's AMR problem (Mutua *et al.*, 2020).

Albernaz-Gonçalves *et al.* (2022) reported that preventative measures, such as biosecurity and immunizations, are necessary yet insufficient in pig production systems to achieve excellent health standards. High-performance pigs raised in intensive systems are predisposed to disease due to restrictive, barren housing and several extensively utilised management tactics that cause pain and stress. Antibiotics are utilised as part of the infrastructure in pig farms to maintain health and high levels of production. Antimicrobial resistance (AMR) is a global health crisis impacting both humans and animals, and intensive livestock farming's uses higher amount of antibiotics is a major risk factor for the establishment and transfer of resistant bacteria from animals to humans. To address the issue of AMR, antimicrobial usage must undergo significant adjustments, including as decreasing its use for prophylaxis and eliminating their use as growth promoter altogether.

### 2.9. Housefly as potential source of AMR

The housefly (*Musca domestica*), one of the world's most widespread insects, belongs to the suborder Cyclorrhapha. It accounts for over 91 per cent of all flies in human habitation and is a major carrier and reservoir of pathogens and commensal bacteria (Rahuma *et al.*, 2005). Regularly associated with human and animal habitats, insects are a reservoir of resistance genes in the environment and a potential channel for their spread. The ability of insects to carry and transmit pathogens is well documented (Vasanthakumar *et al.*, 2008).

Adult house flies have a gray thorax and four dark longitudinal lines down their back and are about 0.3 - 0.5 inches long. Female houseflies are slightly larger than males, and their red compound eyes have a much greater spacing between them. Each female fly can lay about 500 eggs. Larvae (maggots) hatch from the eggs within a day; they live and feed on (typically dead and decaying) organic matter, such as waste or feces. The maggots crawl to a dry, cold spot towards the end of their third instar and turn into pupae, from which adult flies emerge. Complete metamorphosis refers to this whole cycle (Dahlem, 2009).

Adults can live two weeks to a month in the wild or more in controlled laboratory conditions. The flies stop growing after hatching from the pupae; small flies are not necessarily young flies but the result of malnutrition during the larval stage. Warm temperatures are necessary for houseflies to develop and reproduce; In general, the higher the temperature, the faster the flies develop and multiply (Iqbal *et al.2014*)

Adult houseflies can stray up to 20 miles from their developmental sites, and they often breed in sites associated with human and animal waste. As a result, houseflies can quickly build up large numbers on animal farms and migrate from farms to residential areas. According to the FDA, houseflies are also one of the most critical hygiene pests globally, as they make a significant contribution to the spread of food-borne infectious diseases such as cholera, shigellosis, and salmonellosis (Jonge *et al.*, 2020).

The presence of antibiotic-resistant pathogens in insects was isolated and identified specific resistant pathogens in insect hosts. Flies, cockroaches, and even bees have been reported to transmit antibiotic-resistant human infections in different habitats (Kökdener and Kiper, 2021).

## 2.10. Antimicrobial susceptibility pattern of *E. coli* and *Salmonella* species obtained from flies and faecal samples

A five gram ceftazidime disc was introduced for separating ESBL-producing and non-ESBL-producing *E. coli* and *K.pneumoniae* strains (Jacoby and Han, 1996). Rodrigues *et al*. (2004) described the Kirby-Bauer disk diffusion method, a novel antimicrobial susceptibility test designed to identify potential ESBL producers. Antibiotics such as cefoxitin (an inducer), cefotaxime, ceftazidime, ceftazidime + clavulanic acid, aztreonam, and ceftriaxone had been used in the innovative disc placement procedure.

In 2003-2004, Lim *et al*. (2007) studied antibiotic resistance in *E. coli* strains obtained from feces of cattle and pigs in Korea. They discovered 744 E. *coli* bacteria in 830 fecal samples from healthy cattle and pigs. These isolates were tested for susceptibility to 16 antimicrobial agents, including amoxicillin-clavulanic acid

(AMC) and cefotaxime (CTX). Bovine isolates showed no resistance to AMC and CTX, while porcine isolates showed resistance to both AMC and CTX.

Abraham *et al*. (2014) underlined the importance of antimicrobial stewardship programs, surveillance for antimicrobial-resistant zoonotic agents in livestock, screening for carbapenemase production and antimicrobial susceptibility testing, and MIC for chronic and recurrent diseases in livestock.

Understanding whether or not an organism produces carbapenemases (CPO) and the class of carbapenemases it produces has implications for treatment, as some drugs act selectively against certain carbapenemases. In addition, CPO spreads more quickly through patients than in non-CP-carbapenem-resistant organisms, requiring more stringent infection control measures than would be necessary for the absence of carbapenemase synthesis. The phenotypic tests are best used in clinical practice to identify CPO (Tamma and Simner 2018).

Tipisca *et al*. (2021) conducted study based on the phenotypic detection of extended-lactamase-producing *E. coli* isolated from pigs in three slaughterhouses in north-eastern Romania. The faecal samples were inoculated on to Mac Conkey medium added with cefotaxime (MC+CTX). After screening for ESBL, 51 (39.84 per cent) of the 128 samples examined grew on MC+CTX media and were identified as *E. coli*. Furthermore, 78.43 per cent of isolates with ESBL phenotype were detected.

Fournier *et al*. (2021) investigated the presence of extended spectrum β lactamase (ESBL)-producing enterobacteria in the gut of pigs from a Swiss farm and the antibiotic resistance mechanisms among them. A total of 81 faecal samples were collected and analyzed for β -lactam resistance. Sixty per cent of the samples were ESBL producers phenotypically. A total of 38 ESBL-producing *E. coli* and a single ESBL-producing *Enterobacter cloacae* were recovered from 81 pigs, indicating a 50 per cent prevalence.

## 2.11. Molecular characterization of *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ genes conferring ESBL resistance in *E. coli* and *Salmonella* species obtained from flies and faecal matter of pigs

Brinas *et al*. (2003) found that ESBLs were produced by *E. coli* strains from diseased animals in Spain. ESBL production was detected in 20 per cent of the *E. coli* strains recovered from previously treated companion animals.

Kojima *et al*. (2005) recorded a significant incidence of extended-spectrum lactamase-producing *E. coli* strains obtained from farmed animals across Japan. A total of 2,747 isolates that included 793 from pig farms, were screened over four years. Of the samples screened there is a low level of ESBL encoded genes (*bla*$_{CTX-M}$) in pigs (2/793).

The widespread use of beta-lactam antibiotics to treat gram-negative bacterial infections has led to dynamic changes in bacteria that produce beta-lactamases that increased their activity even over recently developed beta-lactam antibiotics (Paterson and Bonomo, 2005).

Antimicrobial resistance is becoming more prevalent in microorganisms. In India's, a high percentage of ESBL positive isolates was observed in Lucknow, Delhi, and Nagpur studies. Notably, only carbapenems were consistently sensitive to all ESBL-producing isolates (Jain and Mondal, 2007).

Meunier *et al*. (2006) studied CTX-M-1 and CTX-M-15 type lactams in *E. coli* clinical isolates from food producing animals in France (cattle, pigs, and poultry). They were tested for the presence of lactamase types CTX-M. The CTX-M gene was found in seven *E. coli* isolates from cattle, pigs, and poultry; PFGE was used to study these isolates further. Although two plasmids were similar in two strains of bovine, porcine, and avian, PFGE showed no epidemiological relationship between them.

Liu *et al*. (2007) characterized of β -lactamase genes from cephalosporin-resistant *E. coli* isolated from farm animals in Guangdong, China. Between 2003 and 2005, 50 (8.4 per cent) of 592 *E. coli* isolates isolated from livestock and poultry tested positive for cephalosporin resistance. Fourteen isolates (2.4 per cent) from chicken, duck, pig, and partridge tested positive for the *bla*$_{CTX\text{-}M}$ gene by PCR and sequencing studies (14 for *bla*$_{CTX\text{-}M}$). The *bla*$_{TEM}$ gene was found in all isolates. The study reported that, CTX-M producing *E. coli* in China's livestock and poultry, with the CTX-M group being the most commonly found extended spectrum lactamase.

Machado *et al*. (2008) studied antibiotic resistance integrons and ESBLs in porcine Enterobacteriaceae. At least one antibiotic resistance was found in 113 isolates. Non-susceptibility to non-lactam antibiotics was found to be more common among ESBL producers than non-ESBL producers.

Tian *et al.* (2009) studied *E. coli* from two pig farms in China and found that a total of 14 *E. coli* isolates had ESBL genes, thirteen carried *bla*$_{CTX\text{-}M,}$ and one carried *bla*$_{SHV}$. PFGE of these ESBL-producing isolates revealed that plasmids expressing ESBL genes carried additional non-lactam antibiotic resistance determinants.

High rates of ESBL-positive isolates were found in different cities, indicating that ESBL-positive strains are not limited to a single city or region (Hawser *et al.*, 2009). The high incidence of ESBL in India and its extensive transmission are a cause for concern. The prevalence of ESBL was 88 per cent among the 163 Gram-negative bacteria evaluated as part of the SARI (2004-2006) investigation (Mathai *et al*., 2009).

To assess the threat of transmission of ESBL bacteria to humans from animal sources, Horton *et al*. (2011) tested the organisms in pigs of UK farms and reported the high proportion of CTX-M positive *E. coli* organisms. They found that the per centage of CTX-M positive *E. coli* was 0.121 per cent in pigs.

Geser *et al*. (2012) evaluated 334 faecal samples from pigs. After an enrichment process, 15.3 per cent of the pig showed ESBL positives. *E. coli* was found in 89 of the 91 isolates. According to the PCR results, 78 isolates (85.7 per cent) produced CTX-M, five of the ESBLs found belonged to the SHV group (5.5 per cent), and two isolates (2.2 per cent) contained a TEM-type enzyme.

Lalzampuia *et al*. (2013) analyzed 138 gut bacteria from 53 pig fecal samples from different Mizoram regions, including 102 (73.91 per cent) *E. coli* and 26 (18.84 per cent) *Salmonella* spp. and observed eight (100 per cent) cefixime and cephalexin, seven (87.5 per cent) cefazolin and oxytetracycline, and six (75 per cent) ampicillin, ceftriaxone, cefotaxime, and enrofloxacin resistant *E. coli* isolates. The Double Discs Synergy Test proved that all of these were ESBL producers.

Olowe *et al*. (2015) collected 350 fecal samples from healthy cattle and pigs and identified ESBL. They isolated 114 *E. coli* strains, of which 72 (63.2 per cent) tested positive for ESBLs in the double disc synergism test, and 81.6 per cent tested positive in the ESBL brilliant agar. PCR specific for ESBL-producing genes was carried out, SHV in 81 *E. coli* isolates, but none were found positive. CTX-M genes were found in 51 (44.7 per cent) of the isolates, while TEM genes were found in 48 (42 per cent) of the *E. coli* isolates.

Dahms *et al.* (2015) screened inguinal swabs of 73 people from 17 pig farms having contact with animals in northeast Germany for ESBL-producing *E. coli* to find out the possibility of cross-transmission of ESBL-producing *E. coli* from animal to human. ESBL-producing *E. coli* was found in five farm labourers. CTX-M -lactamase was found in all human isolates under study, while TEM and OXA -lactamases were also found indefinite proportion.

Samanta *et al*. (2015) reported 12 (6 per cent) isolates positive for ESBL production in organized and backyard pig farms of West Bengal. Six of them turned out to be CTX-M producers, and ten of them had TEM/SHV genes.

Usui *et al*. (2015) discovered the coexistence of cephalosporin (*bla*) and colistin (*mcr*) resistance genes in Enterobacteriaceae from Thai flies. Between 2013 and 2015, 235 flies were collected from 27 locations in Thailand (18 urban areas, five pig farms, and four poultry farms). Cefotaxime-resistant Enterobacteriaceae (CtxRE) and *bla*-positive CtxRE were identified from 70 (29.8 per cent) and 48 (20.4 per cent) flies, respectively. The most abundant gene was $bla_{\text{CTX-M}}$ gene which was found positive from 93 positive isolates from 48 flies, including *E. coli*, *Enterobacteriaceae*, and *Klebsiella pneumoniae,* in case of TEM (n=62), followed by CTX-M (n=81) and SHV (n=10), with 58 isolates harboring multiple types of these genes.

In Vietnam, Nguyen *et al*. (2016) assessed the occurrence of ESBL-producing *E. coli* strains in food samples, including pork samples collected from slaughterhouses, wholesale markets, and supermarkets. ESBL-producing *E. coli* were found in 150 (45.5 per cent) of the 330 food samples examined. CTX-M (31.2 per cent) were the most common for ESBL -Activity responsible genes.

Songe *et al*. (2017) examined 56 ESBL-producing *E. coli* isolates from flies in Lusaka, and observed 42 isolates from ESBL-producing *E. coli* carried $bla_{CTX\text{-}M}$ genes. Furthermore, 18 (42.9 per cent) of the 42 $bla_{CTX\text{-}M}$ positive isolates tested positive for $bla_{CTX\text{-}M}$ genes, while 15 (35.7 per cent) tested positive for $bla_{SHV}$ genes. Some isolates (21.4 per cent) tested positive for both the $bla_{TEM}$ and $bla_{SHV}$ genes, simultaneously.

To study the faecal carriage of ESBL producing *E. coli* in piglets, a cross-sectional study was performed by Tamta *et al*. (2020) on five organized pig farms. Faecal samples from piglets (n=155) was processed for isolation and characterization of *E. coli*. A total of 124 *E. coli* isolates from piglets was recovered, with 44.4 per cent (55/124) of isolates from piglets and tested positive for ESBL production.

To identify beta-lactamase genes and develop an antibiotic resistance profile, researchers from Latvia analyzed the occurrence of ESBL-producing *E. coli* from faeces of pigs on large and small farms. Fecal samples were collected from piglets and sows on four large farms and three small farms (n=615). ESBL-producing *E. coli* were confirmed in three large farms, L1 (64.3 per cent), L2 (29.9 per cent), L3 (10.7 per cent), and one small farm, S1 (47.5 per cent). The prevalence of ESBL-producing *E. coli* differed significantly between the large and small farm groups (26.9 per cent vs. 12.7 per cent). The most common ESBL genes were $bla_{TEM}$ (94 per cent), $bla_{CTX\text{-}M}$ (86 per cent) and $bla_{SHV}$ (4 per cent). $bla_{SHV}$ was dominant on the small farms (48 per cent), while *bla*$_{CTX-M}$ was dominant on the large farms (Galina *et al*., 2021).

Throughout an 11-month investigation, Pileggi *et al.* (2021) gathered two species of filth flies from a livestock operation: house flies (*Musca domestica)*, which represent a generalist feeder, and stable flies *Stomoxys calcitrans*, which represent a specialist (blood) feeder. Culturing on antibiotic-selective media was used to determine the proportion of flies harbouring cefotaxime-resistant (CTX-R) bacteria in whole bodies and dissected guts. A total of 18 resistant isolates were obtained (including *E. coli* and *Salmonella* spp.). Cefotaxime resistant were obtained from the 14 pools of flies processed, including 81 house flies and 68 stable flies. These isolates were found resistant to a variety of antibiotics. The CTX-R isolates were mainly found in female flies that carried at least two different resistant bacterial species.

### 2.12. Molecular characterization of *bla*$_{IMP}$, *bla*$_{OXA-48}$, and *bla*$_{NDM}$ genes conferring carbapenem resistance in *E. coli* and *Salmonella* species obtained from flies and faecal matter of pigs

Liu *et al*. (2007) studied multidrug-resistant gram-negative gut bacteria found in flies collected from six different locations in China. Forty-eight antimicrobial-resistant Gram-negative gut bacteria were identified, with *E. coli* accounting for 77 per cent. In addition, phenotypic antimicrobial resistance testing revealed that no *E. coli* isolates were resistant to imipenem (IPM), meropenem (MRP), amoxicillin-clavulanic acid (AMC), piperacillin-tazobactam (PIT). Cefotaxime (CTX) resistance was found in 37 (100 per cent) isolates, and ceftazidime resistance was found in 29.72 per cent isolates.

Carbapenemase genes can be passed from bacteria to bacteria via mobile genetic elements, and flies can provide an ideal habitat for cross-bacteria transmission of resistance genes (Cornaglia *et al*., 2011).

*E. coli* producing carbapenemase and ESBL were isolated from pigs in India were screened by Nirupama *et al*. (2018). A total of 741 fecal samples and 195 piglets suffering from diarrhea were sampled. A total of 27 and 243 isolates showed carbapenem-resistance and ESBL-production, respectively. During the genotypic screening, three carbapenem-resistant isolates tested positive for the $bla_{OXA-48}$ carbapenemase gene. Out of 243 isolates 137 isolates tested positive for ESBL-genes namely $bla_{CTX-M}$. It is noteworthy to mention that the occurrence of $bla_{OXA-48}$ carrying *E. coli* recovered from pigs in India poses public health hazard.

Diaconu *et al*. (2020) discovered the first MDR, $bla_{NDM}$ positive *E. coli* isolate from a food-producing animal in Europe. In 2019, a *bla*NDM-4-positive *E. coli* strain was found in the faeces of a fattening pig.

In a study conducted by Tamta *et al*. (2020) from five organized pig farms, fecal samples from piglets (n=155) were processed for isolation and characterization of *E. coli*. Out of 124 *E. coli* isolates from piglets, a total of nine isolates revealed carbapenem- resistance. The $bla_{\mathrm{NDM}}$ gene was found in two isolates from piglets and one from farmworkers. Carbapenem resistance in *E. coli* isolated from piglets could be caused by human interaction or the shared environment and is of public health concern.

Chai *et al*. (2022) reported the occurrence of ESBL-producing *E. coli* and Carbapenem resistance in *E. coli* in pigs, and other species. A total of 400 fecal swab samples from pigs, and other species were screened from different animal farms in Malaysia and 12 ESBL-producing *E. coli* (3 per cent; 12/400) were identified. Antibiotic resistance was detected in the bacterial isolates, that include ertapenem, ampicillin, and amoxicillin-clavulanate. Multidrug-resistant *E. coli* isolates were detected in 3.3 per cent (7/212) of the *E. coli* isolates. Though, figures obtained in these study for ESBL resistance among pig was found to be higher when compared to earlier authors, the results found to be significant since there are lesser reports of ESBL producers among animals.

Gonzlez-Fandos *et al*. (2022) conducted studies on pigs to see the effect of extended spectrum β-lactamase (ESBL) and carbapenemase-producing Enterobacteriaceae. Group of 27 pigs was divided into three groups; one group received enrofloxacin treatment, another received oxytetracycline treatment, and the third received no treatment. According to this study, the administration of oxytetracycline or enrofloxacin to food-producing animals can cause ESBL and carbapenemase-producing *E. coli*. Research should be needed to validate the finding further, considering a more robust and extended experimental design.

# 3. Materials and Methods

# 3. MATERIALS AND METHOD

## 3.1. MATERIALS

### 3.1.1. Standard / Reference Strains

The standard cultures of *E. coli* MTCC 3221, *S.* Typhimurium ATCC 4842, and isolates of *E. coli* and *S.* Typhimurium confirmed of having $bla_{CTX\text{-}M}$, $bla_{TEM}$ and $bla_{SHV}$ genes for ESBL- producing ability maintained in the culture repository of the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Pookode were used in this study. The *E. coli* isolates carrying $bla_{IMP}$, $bla_{OXA}$ and $bla_{NDM}$ genes for carbapenem resistance were procured from the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Faizabad, Uttar Pradesh.

### 3.1.2. Scientific Instruments and Equipment

The scientific equipment used in the study included T100™ Thermal Cycler (Bio-Rad, USA), Gel Doc™ EZ Gel Documentation System (Bio-Rad, USA), Laboratory centrifuge (REMI equipment, India), Refrigerated centrifuge (REMI equipment, India), Cyclomixer (REMI equipment, India), Laminar airflow system (Labine, India), Shaker incubator (Labline, India), Bacteriological incubator (Labline, India), Submarine horizontal electrophoresis system (Bio-Rad, USA), Electronic weighing machine (Schimadzu, Japan) and micropipettes (Thermo Fisher Scientific, Finland; Eppendorf, Germany).

### 3.1.3. Plasticware and Glassware

Plasticware and glassware used in the study were procured from Tarsons (India) and Borosil (India) respectively. Standard protocols were followed for the washing and sterilization of glassware.

### 3.1.4. Media, Reagents, and Chemicals

The chemicals and reagents of analytical grade were procured from reputed national and international firms and used in this study.

#### 3.1.4.1. *Media used for Isolation and Identification*

Dehydrated media for the isolation of *E. coli* and *S.* Typhimurium and reagents for various biochemical tests were procured from HiMedia (India), BD-Difco (USA), Genie (India), SRL (SISCO, India), and Sigma-Aldrich (USA).

#### 3.1.4.2. *Chemicals for DNA extraction*

Reagents for DNA extraction were procured from Thermo Scientific (USA), Sigma-Aldrich (USA), and the DNA extraction kit was procured from Origin (India). The reagents for DNA extraction namely, TE buffer (10mM Tris, 1mM EDTA, pH 8.0), Proteinase-K (20mg/ml), isopropanol, and ethanol (70 per cent) were procured from Origin (India).

#### 3.1.4.3. *Oligonucleotides/Primers*

The details of the oligonucleotides/primers used for the detection of various enteric bacterial pathogens are presented in Table 1.

## Table 1. Details of primers used for detection of enteric bacterial pathogens and resistance genes

| Target organism | Gene | Gene Primer sequence | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| ***Escherichia coli*** | *uidA* | F:5'-TGGTAATTACCGACGAAAACGGC-3'<br>R: 5'-ACGCGTGGTTACAGTCTTGCG-3' | 162 | Bej *et al.*, 1991 |
| ***Salmonella* spp.** | *invA* | F:5-'GTGAAATTATCGCCACGTTCGGGCA-3'<br>R: 5'-TCATCGCACCGTCAAAGGAAC -3' | 284 | Rahn *et al.*, 1992 |
| **ESBL producing *Enterobacteriaceae*** | $bla_{CTX\text{-}M}$ | F:5'- CGCTTTGCGATGTGCAG-3'<br>R: 5'-ACCGCGATATCGTTGGT-3' | 550 | Ahmed, 2004 |
| | $bla_{SHV}$ | F:5'- GATGAACGCTTTCCCCATGATG-3'<br>R: 5'-CGCTGTTATCGCTCATGGTAA-3' | 214 | Yazdi *et al.*, 2012 |
| | $bla_{TEM}$ | F:5'- ATGAGTATTCAACATTTCCG-3'<br>R: 5'-GTCACAGTTACCAATGCTTA-3' | 847 | |
| **Carbapenemase-producing *Enterobacteriaceae*** | $bla_{NDM}$ | F: 5'-GCAGCTTGTCGGCCATGCGGGC-3'<br>R: 5'-GGTCGCGAAGCTGAGCACCGCAT-3' | 782 | Doyle *et al.*, 2012 |
| | $bla_{IMP}$ | F: 5'-GAAGGCGTTTATGTTCATAC-3'<br>R: 5'-GTACGTTTCAAGAGTGATGC-3' | 587 | |
| | $bla_{OXA\ 48}$ | F: 5'-GCGTGGTTAAGGATGAACAC-3'<br>R: 5'-CATCAAGTTCAACCCAACCG-3' | 438 | |

### 3.1.4.4. *PCR components*

The PCR reaction was performed for the detection of *E. coli* and *S.* Typhimurium in Verity 96 well Thermal Cycler System (BioRad). The details of the final reaction mixture of 20 µL volume are enlisted in Table 2.

**Table 2. Concentration of various reagents used in PCR**

| Components | Amount |
|---|---|
| Nuclease Free Water | 5.9µL |
| 2X PCR Master Mix | 10µL |
| 10µM Forward Primer | 0.8µL |
| 10µM Reverse Primer | 0.8µL |
| DNA template | 2.5µL |
| **Final reaction** | **20 µL** |

### 3.1.4.5. *Agarose gel electrophoresis components*

Reagents for agarose electrophoresis were procured from Thermo scientific (USA), Sigma-Aldrich (USA), Genei (India), and Chromous Biotech (India). The components of the agarose gel electrophoresis are detailed in Table 3.

**Table 3. Components of Agarose gel electrophoresis**

| Sl. No. | Components | Concentration |
|---|---|---|
| 1. | Agarose | 1.5 per cent (1.5 g in 100 ml of 1X TAE) |
| 2. | Tris-acetate EDTA (TAE) buffer | 1X |
| 3. | Ethidium bromide | 0.5 µg/mL |
| 4. | Gel loading dye | 1X |
| 5. | Molecular weight marker | 1kb/100bp DNA Marker ladder |

## 3.2. Methods

The study was conducted for a period of one year from December 2020 to November 2021 in five pig farms located in three taluks of Wayanad district namely, Vythiri, Sultan Bathery, and Mananthavady. A total of 210 pig fecal samples and 15 pools (10-20 flies per pool) of house flies trapped using fly traps were collected from these five farms. Similarly, another 15 pools of house flies were collected from non-farm sources (as control) like hostel premises, college canteen, etc. Details of housing and feeding system adopted, particulars of pigs reared, farming experience, and the history of antibiotic usage or treatment were collected from each farm.

### 3.2.1. Sampling

#### 3.2.1.1. *Faecal sample collection*

The faecal swabs were randomly collected from the rectum of seven pigs of farm I at a regular interval of two weeks and sampling was repeated six times from the same farm. Similarly, samples are collected from II, III, IV and V. After collection, fecal swabs were immediately transported to the laboratory using Cary Blair transport medium under a cold chain. Extra two samples were also collected from each farm for considering any damage that occurred while transportation. The samples collected from farm I and farm III belonged to pigs that are completely fed on concentrate feeds and swill feed, respectively. The fecal swabs collected from farms II, IV, and V were randomly collected from weaning piglets, mixed-age groups and breeding stocks, respectively.

#### 3.2.1.2. *Sampling of houseflies*

Traditional and commercial fly traps were used for trapping flies from the farm and non-farm settings. The traditional fly traps were made of plastic bottles filled with baits such as dried fish and chicken powder in a ratio of 2:1, along with sugar syrup. The traps were fixed at different locations in farm and non-farm settings where flies were found to be abundant. The sampling frequency was pre-determined to collect pooled samples and both traps were fixed at different locations in the pig sheds, where the flies were seen abundant. From each farm, three successful attempts were made for fly trapping to yield three pools of samples, likewise, a total of five farms which means 15 pools of samples were collected. Each pool was processed for the isolation of *E. coli* and *Salmonella* spp. provided the number of flies caught per catch was not less than 10. As a control, an additional 15 pools of samples were collected from non-farm settings (Table 4).

Flies were first washed with 1X Phosphate Buffer Saline (PBS) and examined the wing pattern under 10X magnification for the genus identification (Sen and Fletcher, 1962). Flies were then pooled and washed with 1X PBS three times to remove surface contaminants (Zhang *et al*., 2018) and put into 2ml Eppendorf tubes with 1.5ml PBS and centrifuged for 10 minutes at 12000 rpm. The flies settled down at the bottom were crushed using a sterile metal rod. Centrifugation was repeated for 10 min at 6000 rpm and the supernatant was used for bacterial isolation.

**Table 4. Details of house fly sample collection**

| **Sl. No.** | **Site** | **No. of trappings** | **Actual flies trapped per sampling** | | | **Total flies** | **Flies taken for processing** |
|---|---|---|---|---|---|---|---|
| 1. | Farm I | 3 | 28 | 22 | 18 | 68 | 60 |
| 2. | Farm II | 3 | 35 | 38 | 44 | 117 | 60 |
| 3. | Farm III | 3 | 40 | 38 | 45 | 123 | 60 |
| 4. | Farm IV | 3 | 25 | 31 | 14 | 70 | 60 |
| 5. | Farm V | 3 | 21 | 12 | 16 | 49 | 49 |
| 6. | Non-farm setting | 15 | More than 10 files from each pool | | | 178 | 178 |
| | | | | | **Total** | **605** | **467** |

### 3.2.3. Transportation of Samples

The fecal samples were collected in Cary-Blair transport medium and all the collected samples were transported under insulated chilled conditions. Samples were processed in the laboratory for further isolation and molecular detection of *Salmonella* spp. and *E. coli.*

### 3.2.4. Processing of Samples for Microbiological Analysis

The samples were enriched in the selective enrichment broth followed by streaking onto a selective agar plate. The isolation and identification of different enteric bacterial pathogens were carried out as described in section 3.2.5. by Barrow and Feltham, (2003).

### 3.2.5. Isolation, identification and molecular confirmation of *E. coli* and *Salmonella* spp. by culture method

#### 3.2.5.1. *Preparation of media*

Isolation and identification of *E. coli* from all the samples were done by methods described in the Bergey's Manual of Systemic Bacteriology (1984) and OIE Terrestrial Manual (2016), with appropriate modifications. For the isolation and identification of *Salmonella* spp. standard procedures mentioned in OIE Terrestrial Manual (2012) and FDA- Bacteriological Analytical Manual (2007) were followed. Dehydrated culture media specific for both *E. coli* and *Salmonella* spp. obtained from HiMedia Laboratories Pvt. Ltd. Mumbai, India were used for enrichment and selective plating of the organisms. The composition of each media is given in Annexure I.

#### 3.2.5.2. *Isolation of E. coli*

For pre-enrichment, fecal and fly samples were inoculated into buffered peptone water (BPW). Briefly, for enrichment, 0.5 ml of the supernatant obtained after centrifugation of the sample was inoculated into five ml of BPW and incubated at 37°C for 24 hr. On MacConkey agar, the typical pink-coloured lactose-fermenting colonies were taken for identification of *E. coli*. Further, selective plating of *E. coli* was done on EMB agar and incubated at 37°C for 24 hr. Three to five representative colonies that showed typical metallic sheen of *E. coli* on EMB agar were picked, purified, and confirmed by molecular assay. The biochemical identification of *E. coli* was carried out as per Barrow and Feltham, (2003).

### 3.2.5.3. *Isolation Salmonella* spp.

The fly samples and faecal swabs were processed by pre-enrichment, enrichment, and selective plating for the isolation of *Salmonella* spp. The fecal swab and one ml of the supernatant from the centrifuged fly sample were inoculated into nine ml each of BPW and incubated at 37°C for 24 hr. Enrichment was carried out by inoculating 0.5 ml of the pre-enriched medium in five ml of Rappaport Vassiliadis (RV) broth and incubating at 42°C for 24-36 hr in a water bath.

On Mac Conkey agar, the typical colourless or white non-lactose fermenting colonies were taken for identification of *Salmonella* spp. A loopful of the enriched media was used for selective plating on the Xylose Lysine Deoxycholate (XLD) agar plates and incubating at 37°C for 24 hr. Three to five representative black-centered colonies of *Salmonella* spp. on XLD agar were picked up, and confirmed by molecular assay. The biochemical identification of *Salmonella* spp. was carried out as per Barrow and Feltham, (2003).

**Fig. 1: Flow chart showing isolation and identification of *E. coli* and *Salmonella* spp.**

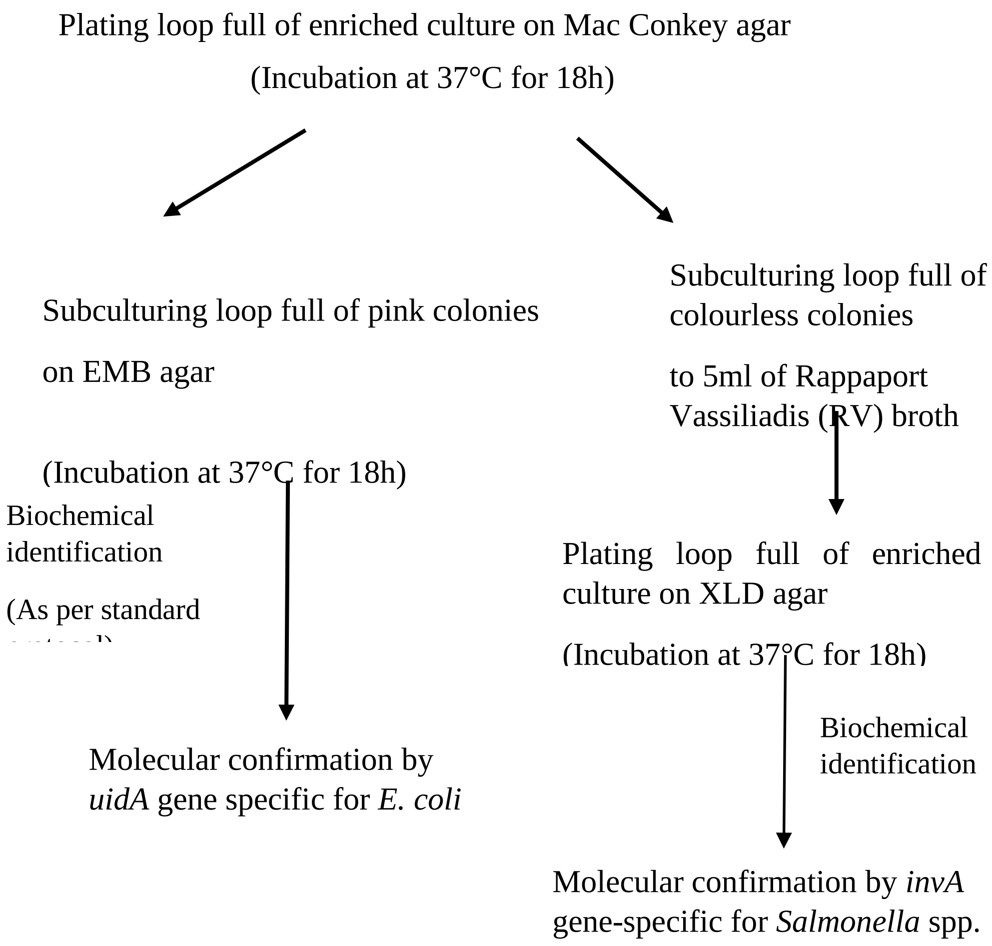
**Sample**

↓

Buffered peptone water

↓

Enrichment (1:10 dilution): Incubation at 37°C for 18h



### **3.2.5.4.** ***Molecular confirmation of isolates***

Presumptive colonies of *E. coli* and *Salmonella* spp. were confirmed using PCR targeting *uidA* gene and *invA* genes, respectively. The PCR mixture was prepared for amplification of the respective genes separately in a 0.2 mL PCR tube as mentioned in Table 6. The mixture was tapped thoroughly and spun in a microcentrifuge to settle the reagents to the bottom. PCR reaction was performed in an automated thermal cycler (Bio-Rad, USA) with a preheated lid. Reference strains, described in section 3.1.1 were used for optimizing primer pairs. Based on the trials, factors such as primer sets, annealing temperature, and PCR cycles were varied to arrive at an optimum PCR condition. The cycling conditions for various genes under study are detailed in Table 6.

### 3.2.5.4.1. *Reconstitution of primers*

The lyophilized primers were reconstituted in the required volume of Tris-EDTA buffer (TE, pH 8.0) to make a stock solution of 100 pmol/µL concentration. The reconstituted primers were stored at -20°C. A working solution with a concentration of 10 pmol/µL was prepared using sterile nuclease-free water (Invitrogen™). Reconstituted primers (stock and working) were stored at -20°C till further use.

### 3.2.5.4.2. *Extraction and quantification of genomic DNA of standard cultures*

The standard DNA of enteric bacterial pathogens mentioned in section 3.1.1 was used for the standardization of PCR. DNA was extracted using Genomic DNA Extraction kit (Origin™), and phenol-chloroform method. The isolated DNA of standard cultures was quantified by a nano-drop 2000C spectrophotometer (Thermo Scientific, USA).

Steps for isolation of genomic DNA from bacterial suspension cultures (as per manufacturer's instruction)

1- The culture was prepared by inoculating the isolate in nutrient broth (Hi-media, India) and incubated at 37°C for 24h.

2- Pipetted 1.5 ml of bacterial culture into a 2 ml microcentrifuge tube, centrifuge at 12000 rpm for 1 min and discarded the flow-through.

3- Added Buffer GA (supplied in the DNA Mini Kit) to make up the final volume to 200 µl.

4- Added 20 µl of proteinase K, mixed by vortexing, and incubated at 56°C until the cells are completely lysed. Vortexed occasionally during incubation to disperse the sample or place in a shaking water bath or on a rocking platform.

5- Centrifuged the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6- Added 200 µl of Buffer GB to the sample, mix by pulse-vortexing for 15 sec, and incubate at 70°C for 10 min to yield a homogenous solution and centrifuged the tube to remove drops from inside the lid.

7- Added 200 µl of ethanol (96-100 per cent) to the sample and mixed by pulse-vortexing for 15 sec. Centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

8- Carefully applied the mixture from step 7 (including the precipitate) to the Mini spin column (placed in a 2 ml collection tube) without wetting the rim. Closed the cap, and centrifuged at 12000 rpm for 30 sec. Placed the Mini spin column in a clean 2 ml collection tube, and discarded the tube containing the filtrate.

9- Carefully opened the Mini spin column and added 500 µl Buffer GD without wetting the rim. Closed the cap, and centrifuge at 12000 rpm for 30 sec.

10- Carefully opened the Mini spin column and added 700 µl Buffer PW without wetting the rim. Closed the cap, and centrifuged at speed (12,000 rpm) for 30 sec.

11- Added 500 µl Buffer PW to spin column and centrifuge at 12000 rpm for 30sec. Discarded the flow-through and place the spin column into the collection tube.

12- Centrifuged at 12000 rpm for 2 min additionally to eliminate the chance of possible Buffer PW carry over.

13- Placed the mini spin column in a clean 1.5 ml microcentrifuge tube, and discarded the collection tube containing the filtrate.

14- Carefully open the mini spin column and added 100 µl Buffer TE. Incubated at room temperature for 2 min, and then centrifuged at 12000 rpm for 2 min.

15- Stored the bacterial genomic DNA at -20°C till further use.

#### 3.2.5.4.3. *Submarine gel electrophoresis*

Agarose gel of 1.5 per cent concentration was prepared with 1X TAE buffer and ethidium bromide was added at a concentration of 0.5 µg/mL. The 10 µL of PCR product mixed with 2µL of 1X gel loading dye (Invitrogen™) was loaded into the agarose gel wells. Three microliters of 100 bp plus DNA marker ladder and 5 µL of 100 bp DNA marker ladders were used as molecular weight markers.

**Table 5. Components of PCR reaction mixture**

| Components | Quantity (20µL) | Final concentration |
|---|---|---|
| 2X PCR Master Mix | 10.0 µL | 1X |
| 10µL forward primer | 0.8 µL | 0.2 µM |
| 10µL reverse primer | 0.8 µL | 0.2 µM |
| Templet DNA | 2.5 µL | 1-500ng |
| Nuclease free water | **To make the final volume 20 µL** | |

**Table 6. PCR Cycling conditions used for Genus specific gene for *E. coli* and *Salmonella* spp.**

| **Organisms** | **Oligonucleotides** | **Process** | | | | | | | | Repeat 35 cycles from denaturation to extension | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Initial Denaturation** | | **Denaturation** | | **Annealing** | | **Extension** | | | **Final Extension** | |
| | | **Temp (°C)** | **Time (min)** | **Temp (°C)** | **Time (Sec)** | **Temp (°C)** | **Time (Sec)** | **Temp (°C)** | **Time (Sec)** | | **Temp (°C)** | **Time (min)** |
| *Escherichia coli* | *uidA* | 94 | 5 | 94 | 40 | 55 | 40 | 72 | 50 | | 72 | 5 |
| *Salmonella* | *invA* | 94 | 5 | 94 | 50 | 56 | 60 | 72 | 60 | | 72 | 5 |

### 3.2.6. Characterisation of ESBL- and Carbapenemase- resistant isolates

#### 3.2.6.1. Phenotypic characterisation

The antibiotic susceptibility testing for confirmed *E. coli* and *Salmonella* spp., isolates were carried out as per the guidelines provided by Clinical Laboratory Standards Institute (CLSI, 2018). *E. coli* ATCC 25922 was used as the quality control strain. The commercial antibiotic discs namely, Cefotaxime (CTX, 30 µg), Ceftazidime (CAZ, 30 µg), Ceftazidime/ Clavulanic acid (CAC, 30/10 µg), and Cefotaxime / Clavulanic acid (CEC, 30/10 µg) were used for the detection of ESBL-producing ability using double-disc synergy test (DDST), whereas Ertapenem (ETP, 10 µg), Doripenem (DOR, 10 µg), Meropenem (MRP, 10 µg) and Imipenem (IPM, 10 µg) were used for the detection of carbapenem resistance.

A loopful of pure culture was streaked onto nutrient agar and incubated at 37°C for 24 h. A single colony from the nutrient agar plate was transferred to 1 mL of phosphate-buffered saline (PBS) in a 1.5 mL microcentrifuge tube and vortexed thoroughly to obtain an optical density equivalent of 0.5 McFarland Units or 1.5 $\times 10^8$ CFU/mL, approximately for each test isolate.

A sterile cotton swab was used to spread inoculums with an optical density adjusted to 0.5 McFarland Units evenly on Mueller-Hinton (MH) agar plate for both *E. coli* and *Salmonella* spp. Antibiotic discs were placed on inoculated agar surface at about two to three centimeters apart with gentle pressure and incubated overnight at 37 °C for 16 to 18 hours. The zone of inhibition was measured for each antibiotic, initially for the quality control strain and subsequently for all test strains.

For ESBL testing, a standard double-disc diffusion synergy test was carried out for the isolates using both Cefotaxime and Ceftazidime, alone and in combination with clavulanate. An increase in the zone diameter of $\geq 5$ mm for either of the antimicrobial agents tested in combination with clavulanate versus the zone diameter of the agent when tested alone was graded as ESBL producers. For carbapenem antibiotics, the obtained zone of inhibition diameters of the isolates was compared with interpretative categories described in Table 9 to grade the test isolates as sensitive (S), intermediate (I), and resistant (R) for the respective antibiotics for phenotypic identification of carbapenem-resistant genes (CLSI, 2018).

**Table 7. Antibiotic discussed for initial screening and confirmation of ESBL production**

| **Sl.No.** | **Antibiotics** | **Abbreviation** | **Concentration (mcg/unit)** | **Kit code No.** |
|---|---|---|---|---|
| **Screening** | | | | |
| 1. | Cefotaxime | CTX | 30 | I |
| 2. | Ceftazidime | CAZ | 30 | III |
| **Confirmation** | | | | |
| 3. | Cefotaxime/clavulanicacid | CEC | 30/10 | I |
| 4. | Ceftazidime/clavulanicacid | CAC | 30/10 | III |

**Table 8. Zone diameters for interpretation of ESBL**

| **Antibiotic disc** | **Zone diameter** |
|---|---|
| **Screening** | |
| Cefotaxime | ≤27 mm |
| Ceftazidime | ≤22 mm |
| **Confirmation** | |
| Cefotaxime/clavulanicacid | A difference in the zone of inhibition of ≥ 5mm of cephalosporin discs and their cephalosporin+ CA containing discs |
| Ceftazidime/clavulanicacid | |

**Table 9. Interpretive Categories and Zone Diameter Breakpoints (mm) of carbapenem antibiotics**

| Antibiotic disc | Sensitive (S) | Intermediate (I) | Resistant (R) |
|---|---|---|---|
| Doripenem (10 µg) | ≥23 | 20-22 | ≤19 |
| Ertapenem (10 µg) | ≥22 | 19-21 | ≤18 |
| Imipenem (10 µg) | ≥23 | 20-22 | ≤19 |
| Meropenem (10 µg) | ≥23 | 20-22 | ≤19 |

### 3.2.6.2. Genotypic characterisation

The reference strains, described in section 3.1.1 were used for optimizing primer pairs. Based on the trials, factors such as primer, annealing temperature, and PCR cycles were varied to arrive at an optimum PCR condition. The combined isolates of *E. coli* and *Salmonella* spp. were characterized for the presence of ESBL-producing genes *viz*., $bla_{CTX-M}$, $bla_{SHV}$, $bla_{TEM}$. PCR conditions and oligonucleotide sequences used for the multiplex PCR for the detection of carbapenemase genes are outlined in Table 1 and 10, respectively.

Multiplex PCR has been carried out for the detection of Carbapenemase-producing genes *viz*., $bla_{NDM}$, $bla_{IMP}$, $bla_{\mathrm{OXA-48}}$ using an automated thermal cycler (Bio-Rad, USA). The multiplex PCR reaction was performed in Verity 96 well Thermal Cycler PCR System (Applied Biosystems). The multiplex PCR reaction mixture consisted of 10 µL 2x PCR Master Mix (Takara) containing TaqDNA Polymerase, buffer, $MgCl_2$ and dNTPs; 0.8 µL each of forward and reverse primers (10 µL), 3 µL DNA template and 7.2 µL nuclease-free water to get a final volume of 25µL. The PCR mixtures were subjected to the following cycling conditions: initial denaturation (94ºC/5 min - 1 cycle) followed by denaturation (95ºC/60 sec), annealing (55ºC/60 sec), extension (72ºC/60 sec), final extension (72ºC/10 min) and holding at 4ºC. Required amplification was attained through 35 cycles of PCR. Five microliters of the amplified product were subjected to electrophoresis in 1.5 per cent agarose gel dissolved in 0.5X TBE buffer, stained by ethidium bromide (0.5 mg/mL). PCR conditions and oligonucleotide sequences used for the multiplex PCR for detection of carbapenemase genes are outlined in Table 1 and 11, respectively.

### 3.2.7. Statistical analysis

The data obtained were subjected to statistical analysis following procedures described by Snedecor and Cochran (1994) using the SPSS software version 24.0.

### 3.2.7.1. Chi-square analysis

Univariate analysis for predictor categorical variables with dependent multilevel antibiotic susceptibility was performed using Pearson's chi-square analysis. Univariate analysis was also performed for predictor categorical variables and binary antibiotic susceptibility patterns. Initially, a chi-square test was performed with a three-level susceptibility pattern (0 - sensitive; 1- resistant; 2-intermediate) and variables to assess the association between them. Another chi-square analysis with a binary outcome (0 - sensitive; 1- resistant) and variables. The isolates which were showing intermediate sensitivity were clubbed together with the sensitive category for use in the binary logistic regression model to calculate the relationship with different factors.

**Table 10. Standardization of PCR for ESBL genes**

| **PCR conditions** | $\mathbf{\mathit{bla}_{CTX\text{-}M}}$ | | $\mathbf{\mathit{bla}_{SHV}}$ | | $\mathbf{\mathit{bla}_{TEM}}$ | |
|---|---|---|---|---|---|---|
| **Process** | **Temp (◦C)** | **Time (min)** | **Temp (◦C)** | **Time (min)** | **Temp (◦C)** | **Time (min)** |
| Initial denaturation | 94 | 5 | 94 | 5 | 94 | 5 |
| Denaturation | 94 | 1 | 94 | 1 | 94 | 1 |
| Annealing | 56 | 1 | 56 | 1 | 56 | 1 |
| Extension | 72 | 1 | 72 | 1 | 72 | 1 |
| The repeated cycle of denaturation to extension 35 cycles | | | | | | |
| Final extension | 72 | 5 | 72 | 5 | 72 | 5 |

**Table 11. Standardization of PCR for carbapenem-resistant genes**

| Oligonucleotides | Process | | | | | | | | Repeat from cycle of denaturation to extension 40 cycles | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial Denaturation | | Denaturation | | Annealing | | Extension | | | Final extension | |
| | **Temp** | **Time (min)** | **Temp** | **Time (Sec)** | **Temp** | **Time (Sec)** | **Temp** | **Time (Sec)** | | **Temp (°C)** | **Time (min)** |
| $bla_{NDM}$<br>$bla_{OXA-48}$ and $bla_{IMP}$ | 95 | 5 | 95 | 60 | 55 | 60 | 72 | 60 | | 72 | 10 |

# 4. RESULT

The present study was envisaged to evaluate the prevalence of major enteric bacterial pathogens from pigs and house fly (*Musca domestica*) of Wayanad district, and to characterize the carbapenem and ESBL producing ability of these isolates. Samples comprised of faecal samples/faecal swabs collected from pigs from different farm units as well as samples of houseflies (*Musca* domestica) near the farm premises of Vythiri, Sultan Bathery and Mananthavady taluks of Wayanad district. House fly samples from non-farm settings was also evaluated in order to compare possibilities of AMR gene carrying from farm settings.

## 4.1. ISOLATION AND MOLECULAR DETECTION OF *E. coli* AND *Salmonella* spp.

The study comprised of a total of 210 faecal samples from five pig farms (42 each) and 30 pools of housefly samples (15 from farm settings and 15 from non-farm settings). All the samples were screened for *E. coli* and *Salmonella* spp. using 'gold standard' cultural methods and further confirmed by using molecular methods.

### 4.1.1. Isolation of enteric bacterial pathogens by culture method

Isolation and identification of *E. coli* and *Salmonella* spp. was carried out observing standard protocol (section 3.2.5). Initially, the samples were inoculated in BPW for enrichment and incubated at 37°C for 18 h. Subsequently, the enriched cultures were streaked onto MacConkey agar plates (Fig.2) to distinguish lactose fermenting colonies (*E. coli)* and non-lactose fermenting (*Salmonella* spp.) colonies. Selective isolation of lactose fermenters (LF) and non-lactose fermenters (NLF) were carried out by streaking on to selective plates. For *E.coli*, EMB agar was used, where the organism showed blue-black colonies with greenish metallic sheen (Fig.3) and for *Salmonella*, XLD agar was used where the organism showed characteristic red coloured colonies with black center (Fig.4).

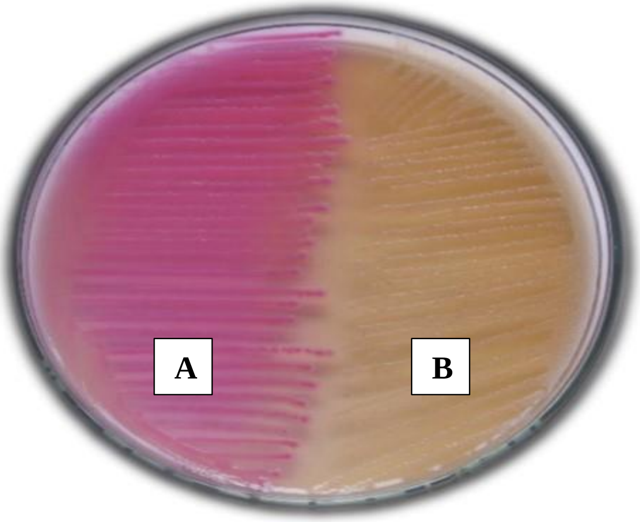


**Fig.2. Characteristic colonies of coliforms on MacConkey agar (A- Lactose fermenting colonies and B-Non-lactose fermenting colonies)**

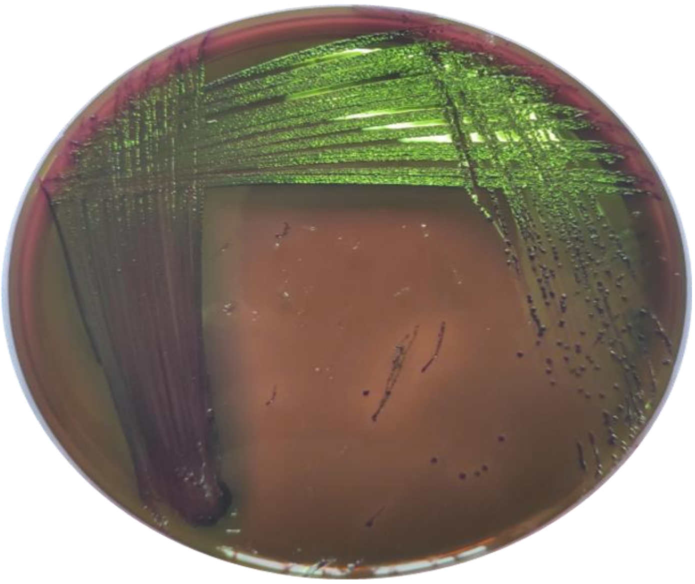


**Fig.3. Characteristic colonies of *E. coli* on EMB agar showing blue-black colonies with greenish metallic sheen**

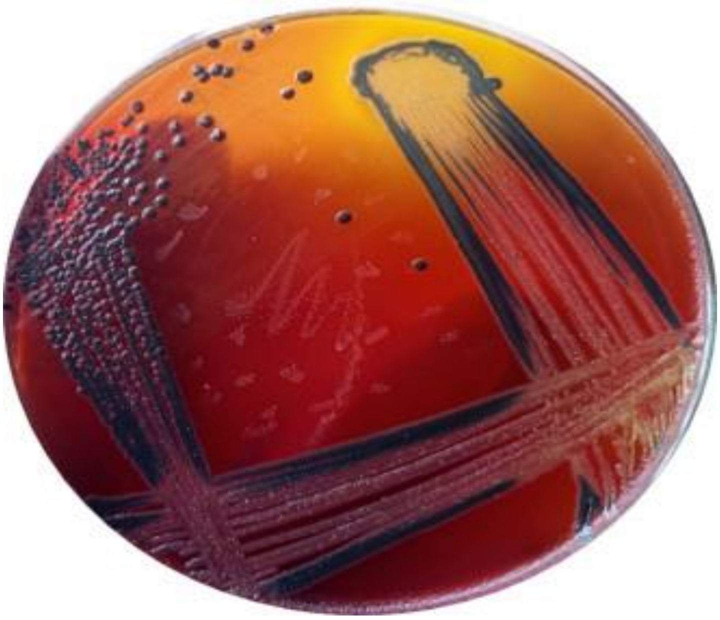


**Fig.4. Characteristic colonies of *Salmonella* spp. on XLD agar showing red colonies with black center**

### 4.1.2. Molecular confirmation of *E. coli* and *Salmonella* spp. in pig faecal sample

The standardized PCR was used for the identification of *E. coli* (*uidA*) and *Salmonella* (*invA*) from pig faecal samples and house fly samples. The positive isolates were further screened for ESBL and carbapenemase-producing genes.

### A) Occurrence of *E. coli*

The result of Amplification of *uidA* gene of *E. coli* in various samples is shown in Fig.5. Out of 210 faecal samples analyzed, *E. coli* was confirmed in farms IV and V (100 per cent), farm III (97.60 per cent), farms I and II (92.90 per cent). The overall recovery rate of *E. coli* from pig farms is 203 out of 210 samples (96.67 per cent). The details are given in Table 12.

### B) Occurrence of *Salmonella*

The result of amplification of *invA* gene of *Salmonella* spp. is shown in Fig.6. Out of 210 faecal samples analyzed, *Salmonella* spp. was confirmed highest in farm IV (28.60 per cent), in the case of farms I, III, and V the recovery rate is six out of 42 samples (14.30 per cent). The recovery rate was lowest in samples from farm II where only three *Salmonella* spp. were isolated. The details of *Salmonella* spp. identified through PCR is given in Table 13.

### 4.1.3. Molecular confirmation of *E. coli and Salmonella* spp. from entrapped houseflies in farm setting

A total of 30 pools of houseflies were entrapped from pig farms and non-farm settings, and were screened for the occurrence of *E. coli* and *Salmonella* spp. From the fly traps, only houseflies were segregated from other insects by morphological characteristics and wing pattern structure. Wing pattern of the housefly comprised of M1+2 veins curves forward distally and the R 5 (first posterior cell/apical cell is nearly closed).

### A) Occurrence of *E. coli*

Out of 15 pools of houseflies examined from five farms, the recovery rate of *E. coli* was 100 per cent. The details are enlisted in the Table 12.

### B) Occurrence of *Salmonella* spp.

Out of the 15 pools of houseflies examined, the recovery rate of *Salmonella* spp. was comparatively low. Only two *Salmonella*-positive isolates were recovered (13.33 per cent). The details are given the Table 13.

### 4.1.4. Molecular confirmation of *E. coli* and *Salmonella* spp. from entrapped houseflies in non-farm settings

Non-farm settings are mainly comprised of areas (such as canteen, hotel etc.) where human activity was more. The area of sample collection was set in a way that the distance between the farm is comparatively higher, which means more than five kilometers. The non-farm settings include nearby college canteen, hostel, human settlements, etc. the collection point is set where the houseflies are occurring abundantly.

### A) Occurrence of *E. coli*

Out of the 15 pools of house flies collected from non-farm settings, *E. coli* was confirmed in all 15 pools. The data is given in Table 12.

### B) Occurrence of *Salmonella* spp.

The present study could detect three positive isolates of *Salmonella* spp. from 15 pools of houseflies from non-farm settings. The recovery rate of *Salmonella* spp. from non-farm settings was 20 per cent. The details are given in Table 13.

### 4.1.5. Biochemical confirmation of *E. coli* and *Salmonella* spp. isolates

All the isolates of *E. coli* and *Salmonella* spp. confirmed by PCR was also checked and confirmed by biochemical characteristics specific to organisms and the positive isolates were subcultured for further examination of AMR characterization (using phenotypic and genotypic methods).

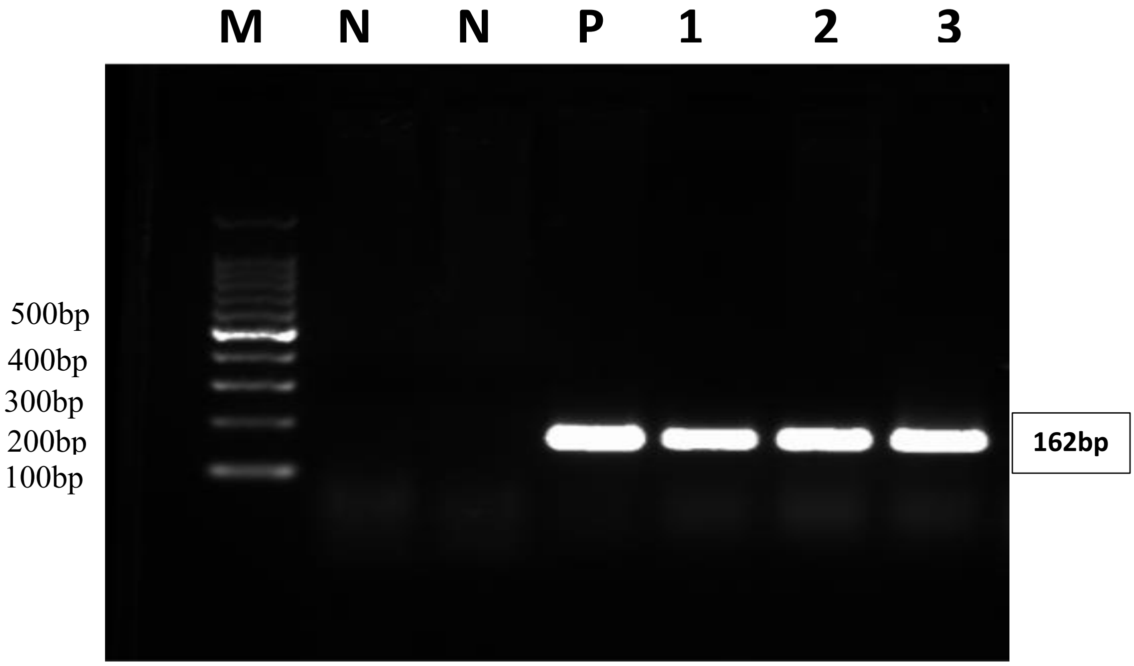


**Fig.5 : PCR amplification of *uidA* gene of *E. coli***
**Lane M** : 100 bp DNA marker
**Lane P** : Positive control of *uidA* gene
**Lane N** : Non-Template control
**Lanes 1-3** : Samples positive to *uidA* gene at 162 bp

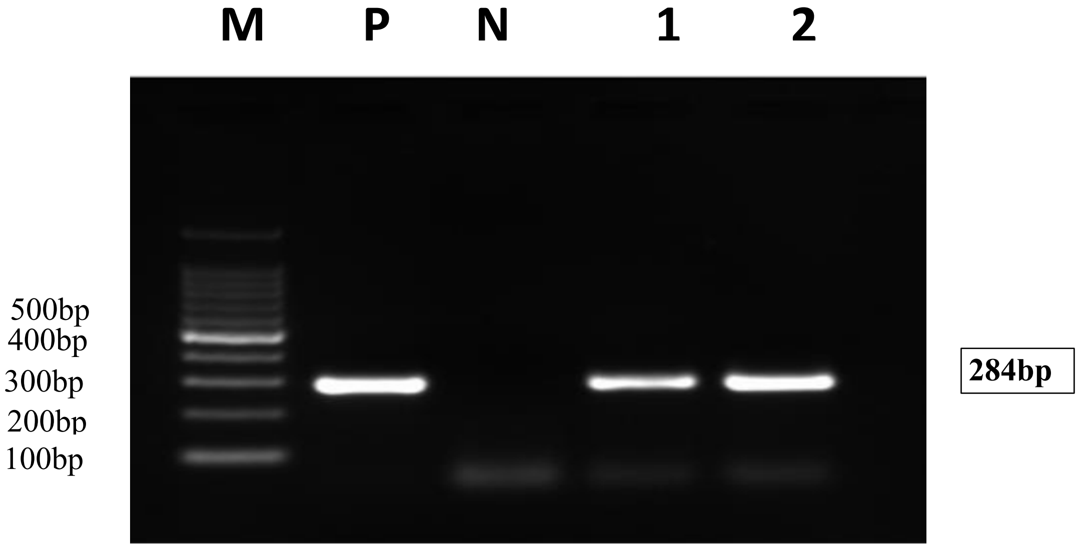


**Fig.6 : PCR amplification of *invA* gene of *Salmonella* spp.**
**Lane M** : 100 bp DNA marker
**Lane P** : Positive control of *invA* gene
**Lane N** : Non-Template control
**Lanes 1-3** : Samples positive to *invA* gene at 284 bp

**Table 12. Number and per cent of *E. coli* confirmed by PCR**

| Sl No. | Type of sample | No. of samples | No. of Positive isolates | Positive Isolates (%) |
|---|---|---|---|---|
| **1.** | Farm I | 42 | 39 | 92.90 |
| **2.** | Farm II | 42 | 39 | 92.90 |
| **3.** | Farm III | 42 | 41 | 97.60 |
| **4.** | Farm IV | 42 | 42 | 100 |
| **5.** | Farm V | 42 | 42 | 100 |
| **6** | House Fly -Farm Setting | 15 | 15 | 100 |
| **7** | Housefly Pool Non-Farm Setting | 15 | 15 | 100 |
| **TOTAL** | | 240 | 233 | 96.70 |
| **Chi-square value** | | | $6.798^{ns}$ | |
| **p-value** | | | $0.147^{ns}$ | |

ns-non significant

**Table 13. Number and per cent of *Salmonella* spp. confirmed by PCR**

| Sl No. | Type of sample | No. of samples | Positive isolates | Positive Isolates (In %) |
|---|---|---|---|---|
| **1.** | Farm I | 42 | 6 | 14.30 |
| **2.** | Farm II | 42 | 3 | 7.10 |
| **3.** | Farm III | 42 | 6 | 14.30 |
| **4.** | Farm IV | 42 | 12 | 28.60 |
| **5.** | Farm V | 42 | 6 | 14.30 |
| **6** | House Fly -Farm Setting | 15 | 2 | 13.30 |
| **7** | Housefly Pool Non-Farm Setting | 15 | 3 | 20 |
| **TOTAL** | | 240 | 38 | 15.70 |
| **Chi-square value** | | | $7.766^{ns}$ | |
| **p-value** | | | $0.101^{ns}$ | |

ns-non significant

## 4.2. ANTIBIOTIC SUSCEPTIBILITY STUDIES OF ISOLATES

The antibiotic susceptibility of the recovered isolates was carried out as per CLSI (2018) and were characterized for their ESBL production by phenotypic screening through standard disc diffusion method and genotypic screening through PCR. The resistance pattern to third-generation cephalosporins was assessed by using sterile antibiotic discs that are routinely used in human and animal therapeutics.

### 4.2.1. Phenotypic Characterization of Recovered Isolates for ESBL Production

*In vitro* antibiotic resistance pattern of the *E. coli* and *Salmonella* spp. isolates were determined by disc diffusion method of Bauer (1966). They were tested against commonly used antibiotics *viz,* ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), Ceftazidime/ Clavulanic acid (CAC 30/10 µg), and Cefotaxime / Clavulanic acid (CEC 30/10 µg). A standard double-disc diffusion synergy test was carried out for the isolates using both Cefotaxime and Ceftazidime, alone and in combination with clavulanate. An increase of ≥5 mm in zone diameter for either antimicrobial agent tested in combination with clavulanate versus the zone diameter of the agent when tested alone. The phenotypic characterization of *E. coli* and *Salmonella* spp. for ESBL production is listed in Fig.7 and Fig.8.

### 4.2.2. Phenotypic Characterization of Recovered Isolates of pig faecal sample for ESBL Production

### A) ESBL producing *E. coli*

*E. coli* isolates recovered from various pig farms were characterized phenotypically for ESBL production by standard disc diffusion method. The results of antibiotic susceptibility pattern of *E. coli* isolates are shown in Table 14. It was observed that the isolates exhibited phenotypic resistance pattern to third generation cephalosporins- cefotaxime and ceftazidime, and their clavulanic acid combinations.

Phenotypic ESBL characterization of 203 *E. coli* positive isolates recovered from pig faecal samples revealed that maximum resistance was observed for the ceftazidime and cefotaxime, in the descending order. Moreover, the isolates were sensitive to Ceftazidime/ Clavulanic acid and cefotaxime/ clavulanic acid respectively in the descending order. The maximum ESBL phenotypic resistance is exhibited by farms - III and IV and the least phenotypic resistance is shown by farm I.

#### B) ESBL producing *Salmonella* spp.

Following to CLSI (2018), the phenotypic characterization of ESBL- producing *Salmonella* spp. was evaluated, and result indicated that *Salmonella* positive isolates showed antibiotic resistance. Higher resistance towards third-generation cephalosporin is seen in farm IV. Out of 38 *Salmonella* spp. positive isolates from 210 samples screened, 15 were ESBL producers (45.50 per cent). The details are given in Table 15.

### 4.2.3. Phenotypic Characterization of Recovered Isolates for ESBL Production in house flies entrapped from farm settings

The isolates of *E. coli* (n=15) and *Salmonella* spp. (n=2) recovered from farm settings were screened for ESBL production. The total ESBL production among *E. coli* isolates was nine out of 15 isolates (60 per cent). One out of two *Salmonella* spp. isolates was showing ESBL production phenotypically by disc diffusion.

#### A) ESBL producing *E. coli*

Among the 15 *E. coli* isolates from house flies in farm settings, 60 per cent of the isolates showed phenotypic resistance to ESBL. Higher resistance was shown for cefotaxime followed by ceftazidime. The details are given in Table 16.

#### B) ESBL producing *Salmonella* spp.

From the recovered two *Salmonella* spp. isolates, one sample was found resistant to cefotaxime. The single isolate recovered was also found to be ESBL producer. The details provided in Table 17.

### 4.2.4. Phenotypic Characterization of Recovered Isolates for ESBL Production in house flies entrapped from non-farm settings

The isolates of *E. coli* (n=15) and *Salmonella* spp. (n=3) recovered from non-farm settings were screened for phenotypic assessment of ESBL production. The total ESBL producers among *E. coli* isolates was found to be six out of 15 isolates (40 per cent). One out of three *Salmonella* spp. isolates showed ESBL producing ability.

### A) ESBL producing *E. coli*

Among the 15 *E. coli* isolates from house flies in farm settings, six samples showed phenotypic resistance to ESBL (40 per cent). Higher resistance was shown for cefotaxime followed by ceftazidime. The details are given in Table 16.

### B) ESBL producing *Salmonella* spp.

From the recovered three *Salmonella* spp. isolates, the phenotypic assessment revealed one sample resistant to cefotaxime. One isolate was found as ESBL producer (33.33 per cent). The data is given in Table 17.

**Table 14. ESBL- producing *E. coli* isolates detected by disc diffusion method in pig faecal samples**

| **Farm** | **No. of *E. coli* isolates** | **CTX** | | | **CEC** | | **CAZ** | | | **CAC** | | ***ESBL*** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | S | I | R | NS | S | S | I | R | **NS** | **S** | |
| Farm I | **39** | 26 | 3 | 10 | 22 | 17 | 31 | 3 | 5 | 26 | 16 | *14* |
| Farm II | **39** | 10 | 6 | 23 | 34 | 5 | 23 | 6 | 8 | 38 | 4 | 20 |
| Farm III | **41** | 11 | 8 | 22 | 39 | 2 | 22 | 9 | 10 | 36 | 5 | 25 |
| Farm IV | **42** | 8 | 9 | 25 | 39 | 3 | 12 | 12 | 18 | 39 | 3 | 25 |
| Farm V | **42** | 17 | 2 | 23 | 40 | 2 | 19 | 4 | 19 | 39 | 3 | 24 |
| ***Total*** | **203** | 72 | 28 | 103 | 174 | 29 | 107 | 34 | 60 | 178 | 31 | 108 |
| ***Chi-square test*** | | | 35.068 | | 32.566 | | 40.178 | | | 27.21 | | $8.50^{ns}$ |
| ***P value*** | | | 0.00 | | 0.00 | | 0.00 | | | $0.001^{s}$ | | $0.07^{ns}$ |

ns-non significant

**CTX- Cefotaxime, CEC- Cefotaxime + Clavulanic acid, CAZ-Ceftazidime, CAC-Ceftazidime + Clavulanic acid combination, S- Sensitive, I- Intermediate, R-Resistant*

**Table 15. ESBL-producing *Salmonella* spp. isolates detected by disc diffusion method in pig faecal samples**

| **Farm** | **No. of isolates** | **CTX** | | | **CEC** | | **CAZ** | | | **CAC** | | **ESBL** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | S | I | R | NS | S | S | I | R | **NS** | **S** | |
| Farm I | 6 | 2 | 1 | 3 | 6 | 0 | 4 | 0 | 2 | 6 | 0 | 4 |
| Farm II | 3 | 1 | 2 | 0 | 3 | 0 | 2 | 0 | 1 | 3 | 0 | 1 |
| Farm III | 6 | 2 | 2 | 2 | 5 | 1 | 5 | 1 | 0 | 6 | 0 | 2 |
| Farm IV | 12 | 2 | 4 | 6 | 12 | 0 | 6 | 5 | 1 | 10 | 2 | 6 |
| Farm V | 6 | 3 | 1 | 2 | 3 | 3 | 5 | 1 | 0 | 4 | 2 | 2 |
| Total | 33 | 10 | 10 | 13 | 29 | 4 | 22 | 7 | 4 | 29 | 4 | 15 |
| ***Chi-square test*** | | | 12.605$^{ns}$ | | 8.665$^{ns}$ | | 19.09$^{ns}$ | | | 6.117$^{ns}$ | | 5.744$^{ns}$ |
| ***P value*** | | | 0.398$^{ns}$ | | 0.07$^{ns}$ | | 0.086$^{ns}$ | | | 0.191$^{ns}$ | | 0.219$^{ns}$ |

ns-non significant

**CTX- Cefotaxime, CEC- Cefotaxime + Clavulanic acid, CAZ-Ceftazidime, CAC-Ceftazidime + Clavulanic acid combination, S- Sensitive, I- Intermediate, R-Resistant*

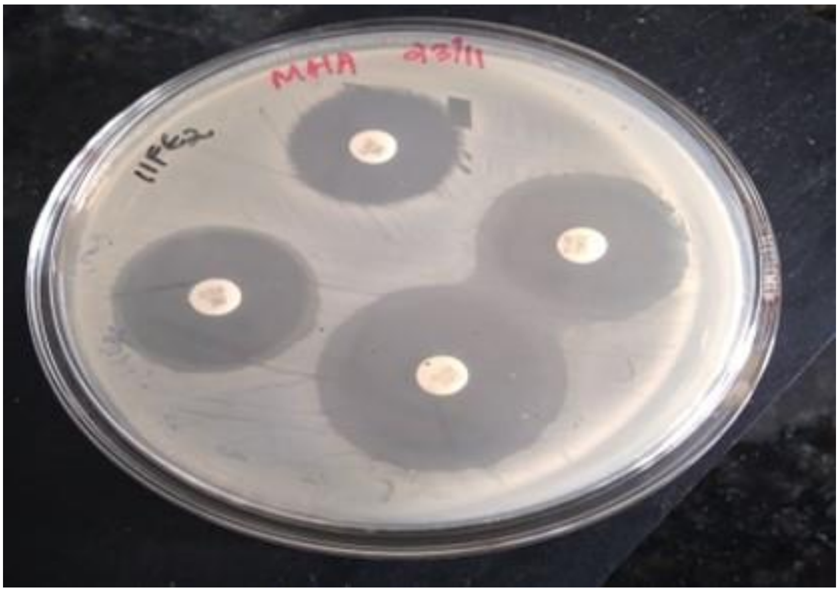


**Fig.7 : ESBL producing *E. coli* showing drug resistance**

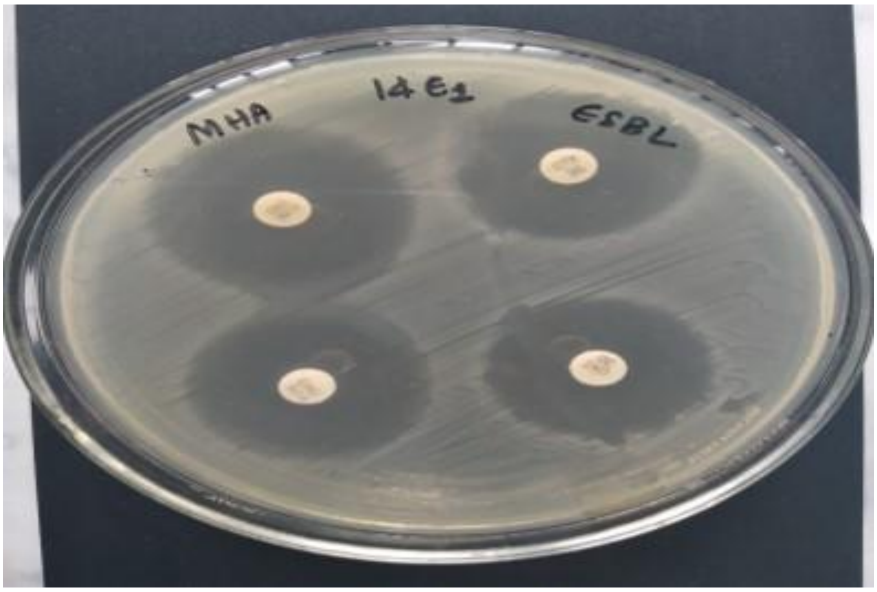


**Fig.8 : ESBL producing *Salmonella* spp. showing drug resistance**

**Table 16. ESBL-producing *E. coli* isolates detected by disc diffusion method in housefly samples**

| **Sl. No.** | **farm** | **No. of *E. coli* isolates** | **CTX** | | | **CEC** | | **CAZ** | | | **CAC** | | **ESBL** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | S | I | R | NS | S | S | I | R | **NS** | **S** | |
| **1** | Non-Farm setting | 15 | 10 | 2 | 3 | 13 | 2 | 10 | 2 | 3 | 12 | 3 | 6 |
| **2** | Farm setting | 15 | 6 | 5 | 5 | 15 | 0 | 2 | 2 | 10 | 15 | 0 | 9 |
| | Total | 30 | 16 | 7 | 7 | 28 | 2 | 13 | 4 | 13 | 27 | 3 | 15 |
| ***Chi-square value*** | | | $2.429^{ns}$ | | | $2.143^{ns}$ | | $7.538^{ns}$ | | | $3.333^{ns}$ | | $1.20^{ns}$ |
| ***p-value*** | | | $0.297^{ns}$ | | | $0.143^{ns}$ | | $0.023^{ns}$ | | | $0.068^{ns}$ | | $0.27^{ns}$ |

ns-non significant

**Table 17. ESBL-producing *Salmonella* spp. isolates detected by disc diffusion method in housefly samples**

| **Sl. No.** | **farm** | **No. of *Salmonella* isolates** | **CTX** | | | **CEC** | | **CAZ** | | | **CAC** | | **ESBL** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | S | I | R | NS | S | S | I | R | NS | S | |
| **1** | Non-Farm setting | 3 | 0 | 1 | 2 | 3 | 0 | 2 | 1 | 0 | 3 | 0 | 1 |
| **2** | Farm setting | 2 | 0 | 1 | 1 | 2 | 0 | 1 | 1 | 0 | 2 | 0 | 1 |
| | Total | 5 | 0 | 2 | 3 | 5 | 0 | 3 | 2 | 0 | 5 | 0 | 2 |
| ***Chi-square test*** | | | $2.99^{ns}$ | | | | | $0.299^{ns}$ | | | | | |
| ***P-value*** | | | $0.861^{ns}$ | | | | | $0.861^{ns}$ | | | | | |

ns-non significant

## 4.3. GENOTYPIC CHARACTERIZATION OF RECOVERED ISOLATES FOR ESBL GENES

The DNA was extracted using Origin genomic DNA kit from a total of 271 *E. coli* and *Salmonella* spp. isolates obtained from pig feacal swabs, and housefly samples in farm settings and non-farm settings. The extracted DNA from animals and pig was used as template for PCR reactions for genotypic detection and confirmation of ESBL producing genes ($bla_{CTX}$, $bla_{TEM}$, $bla_{SHV}$). All the isolates were evaluated and positive isolates showed PCR amplicon band size at 847bp for $bla_{TEM}$ (Fig.10), 550 bp for $bla_{CTX}$ (Fig.9), 214 bp for $bla_{SHV}$ (Fig.11).

### 4.3.1. Genotypic characterization of *E. coli* isolates for ESBL genes

The present study screened 203 *E. coli* isolates obtained from pig faecal samples and 30 *E. coli* isolates obtained from houseflies in farm and non-farm settings for the presence of ESBL-producing genes.

#### 4.3.1.1. *E. coli isolates recovered from pig faecal sample*

From the *E. coli* isolates screened, the occurrence of $bla_{CTX-M}$ gene was 48.60 per cent (102/203 in numbers) and that of $bla_{TEM}$ gene was 43.80 per cent (92/203 in number). A lower percentage of $bla_{SHV}$ gene, that was 10.50 per cent (22/203 in numbers) was detected. Separate farm wise details of ESBL genes are given in the Table 18. A higher percentage of $bla_{CTX-M}$ gene was recorded in farm I; Whereas farm III recorded a maximum number of $bla_{SHV}$ genes. However, farm IV had a higher number of $bla_{TEM}$ genes. The presence of one or more ESBL-producing genes in the same organism is detailed in Table 23 and 24.

#### 4.3.1.2. *E. coli isolates recovered from house flies in farm settings*

The present study isolated 15 *E. coli* from 15 pools of houseflies samples entrapped from five different pig farms across Wayanad. Of this, 80 per cent (12 numbers) of isolates were found to be ESBL producers that harboured at least one ESBL producing gene. Results revealed that 60 per cent of isolates were positive for $bla_{CTX-M}$ and $bla_{SHV}$ and 46.70 per cent of isolates were $bla_{TEM}$ gene. The data is enlisted in the Table 20.

#### 4.3.1.3. *E. coli isolates recovered from house flies in non-farm settings*

*E. coli* was isolated from 15 pools of housefly samples entrapped from five different pig farms across Wayanad. Of this, 66.67 per cent (10 numbers) of isolates were found to be ESBL producers that harboured at least one ESBL-producing gene. The findings revealed that 33.33 per cent (five numbers) of isolates were positive for $bla_{CTX\text{-}M}$, 20 per cent (three numbers) of isolates were positive for $bla_{SHV}$ and 33.33 per cent (five numbers) of isolates were found positive for $bla_{TEM}$ gene. The details are summarised in Table 20.

### 4.3.2. Genotypic characterization of *Salmonella* spp. isolates for ESBL production

A total of 33 *Salmonella* spp. isolated from pig faecal samples and five isolated from houseflies in farm and non-farm settings were screened for the occurrence of gene encoding ESBL production and results are shown below.

#### 4.3.2.1. *Salmonella* spp. *recovered from pig faecal sample*

The *Salmonella* spp. isolated (n=33) from the pig faecal samples were screened for the occurrence of ESBL genes. Out of 33 samples, 48.48 per cent were positive for $bla_{CTX\text{-}M}$ (16/33 numbers). This study recorded 57.57 per cent of $bla_{TEM}$ genes (19/33 in numbers). This study also detected a lower percentage of $bla_{SHV}$ genes (12.12 per cent). Of the total samples screened, 24 samples contained at least one ESBL encoded gene (72.72 per cent). The details are mentioned in the Table 19.

#### 4.3.2.2. *Salmonella* spp. *recovered from house flies in farm settings*

The recovery rate of *Salmonella* spp. from houseflies in farm settings was comparatively lower. Out of the two *Salmonella* spp. screened for ESBL production both were found to be ESBL-producing genes. Of this, one isolate contains $bla_{CTX\text{-}M}$ and the other isolate contained $bla_{SHV}$ gene. The data is shown in the Table 21.

### 4.3.2.3. *Salmonella* spp. *recovered from house flies in non-farm settings*

Three *Salmonella* spp. isolates were obtained from houseflies in non-farm settings. All three isolates contained at least one ESBL encoded gene. The results revealed that two isolates contain both *bla*$_{TEM}$ and *bla*$_{SHV}$ genes, while one isolate was positive for *bla*$_{CTX\text{-}M}$ gene. The details are mentioned in the Table 21.

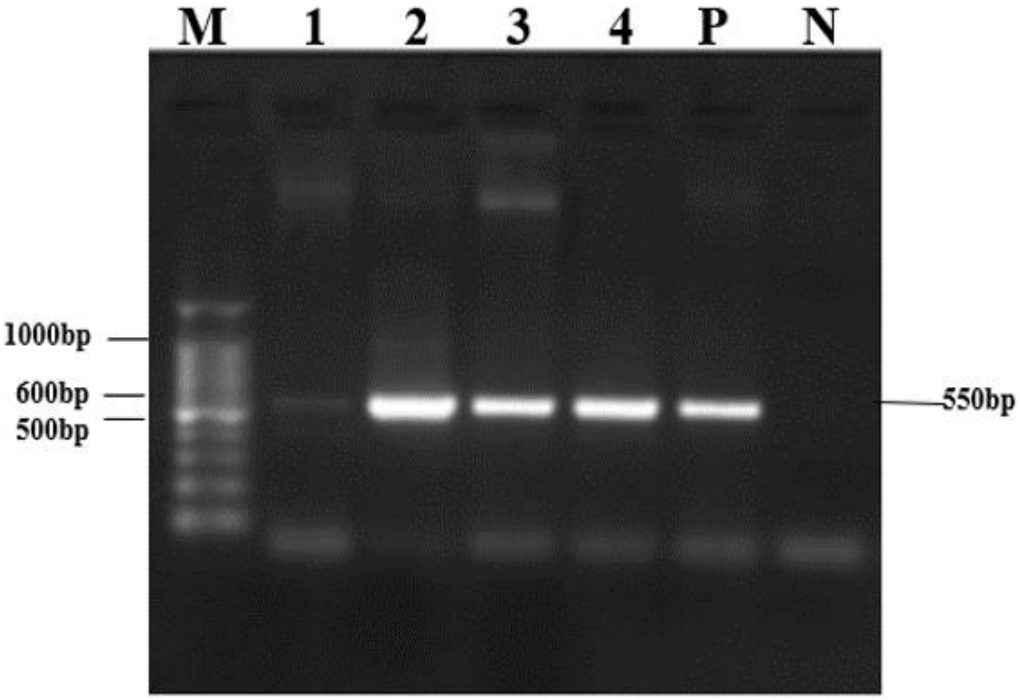


**Fig.9 : PCR amplification of *bla*$_{CTX\text{-}M}$ gene**

**Lane M** : 100 bp DNA marker

**Lane P** : Positive control of *bla*$_{CTX\text{-}M}$ gene

**Lane N** : Non-Template control

**Lanes 1-4** : samples positive to *bla*$_{CTX\text{-}M}$ gene at 550bp

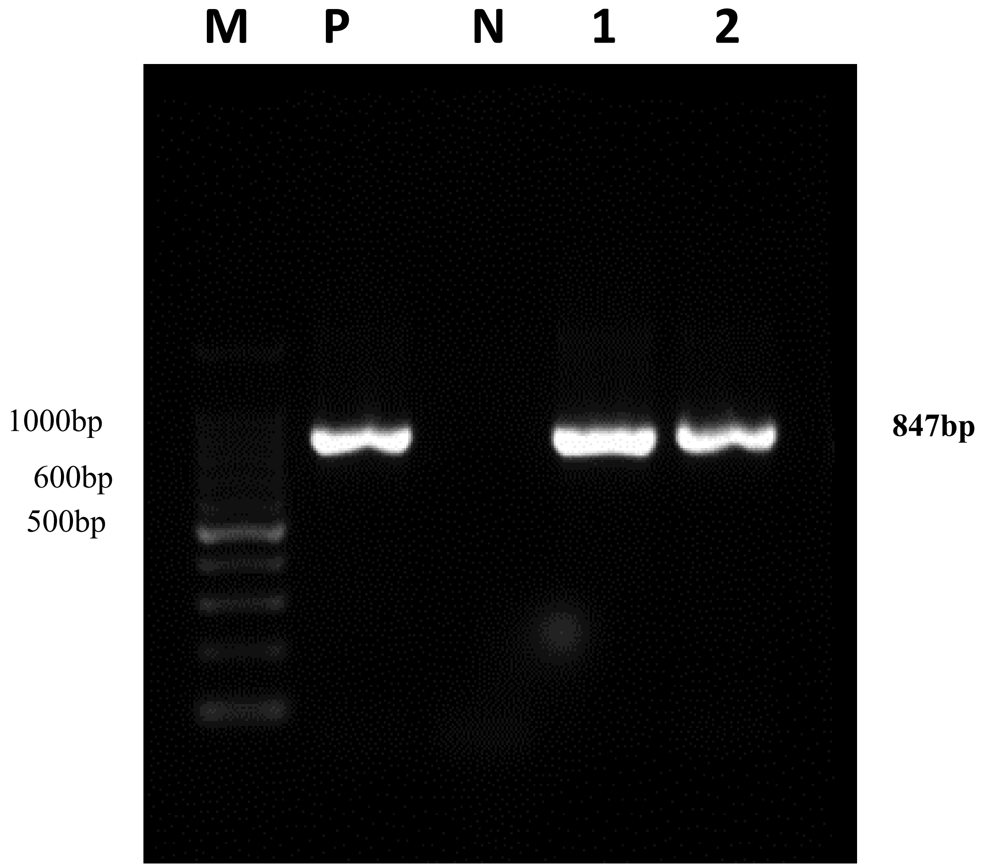


**Fig.10 : PCR amplification of $bla_{TEM}$ gene**

**Lane M** : 100 bp DNA marker

**Lane P** : Positive control of $bla_{TEM}$ gene

**Lane N** : Non-Template control

**Lanes 1-2** : Samples positive to $bla_{TEM}$ gene at 847 bp

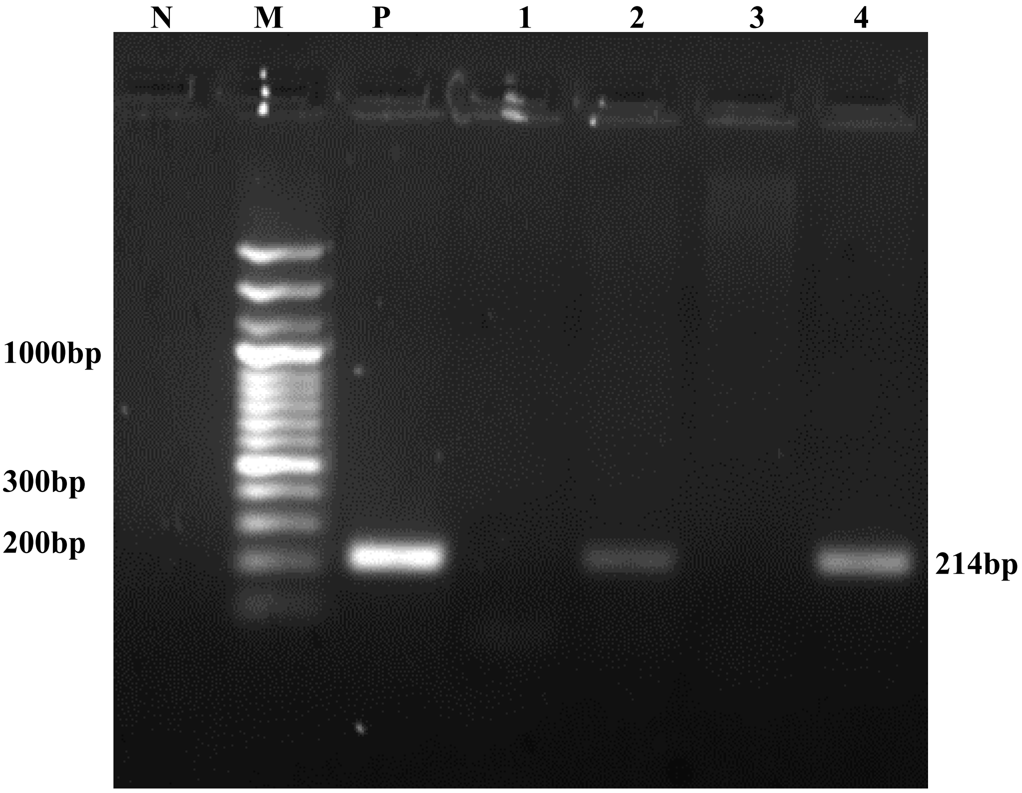


**Fig.11 : PCR amplification of *bla*$_{SHV}$ gene**

**Lane M** : 100 bp DNA marker

**Lane P** : Positive control of *bla*$_{SHV}$ gene

**Lane N** : Non-Template control

**Lanes 1-2** : Samples positive to *bla*$_{SHV}$ gene at 214 bp

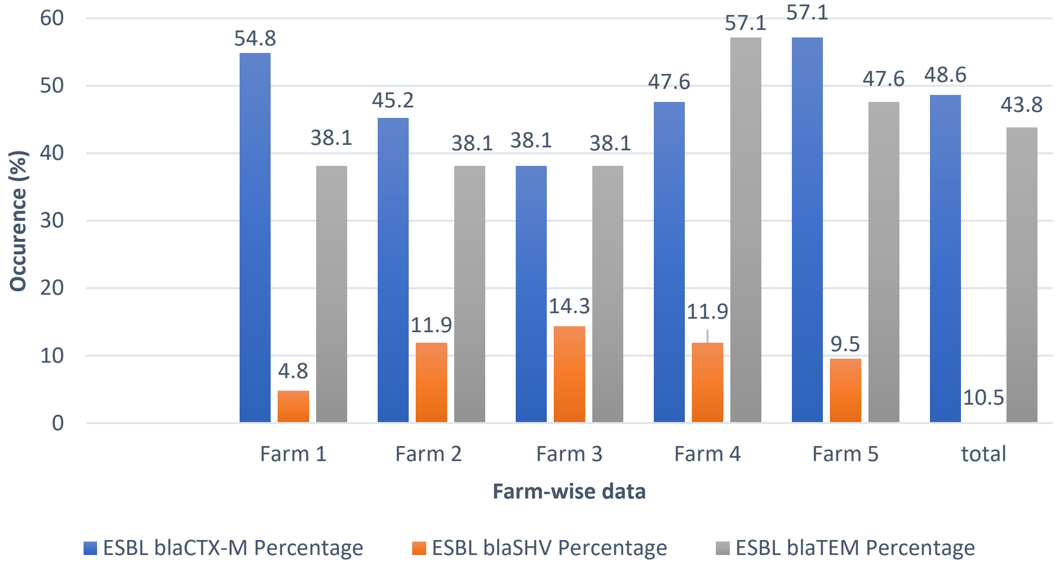


**Fig.12: ESBL-producing *E. coli* isolates (%) from pig faecal samples**

**Table 18. ESBL-producing *E. coli* isolates from pig faecal samples**

| **Sl. No.** | **Farm** | **No. of *E. coli* isolates** | ***bla*$_{CTX-M}$** | | ***bla*$_{SHV}$** | | ***bla*$_{TEM}$** | |
|---|---|---|---|---|---|---|---|---|
| | | | **Positive** | **Percentage (%)** | **Positive** | **Percentage (%)** | **Positive** | **Percentage (%)** |
| **1** | Farm I | 39 | 23 | 54.80 | 2 | 4.80 | 16 | 38.10 |
| **2** | Farm II | 39 | 19 | 45.20 | 5 | 11.90 | 16 | 38.10 |
| **3** | Farm III | 41 | 16 | 38.10 | 6 | 14.30 | 16 | 38.10 |
| 4 | Farm IV | 42 | 20 | 47.60 | 5 | 11.90 | 24 | 57.10 |
| 5 | Farm V | 42 | 24 | 57.10 | 4 | 9.50 | 20 | 47.60 |
| | Total | 203 | 102 | 48.60 | 22 | 10.50 | 92 | 43.80 |
| ***Chi-square test*** | | | $3.927^{ns}$ | | $2.336^{ns}$ | | $4.952^{ns}$ | |
| ***P-value*** | | | $0.4162^{ns}$ | | $0.674^{ns}$ | | $0.292^{ns}$ | |

ns-non significant

**Table 19. ESBL- producing *Salmonella* spp. isolates from pig faecal samples**

| **Farm** | **No. of isolate** | ***bla* $_{CTX-M}$** | | ***bla*$_{SHV}$** | | ***bla*$_{TEM}$** | |
|---|---|---|---|---|---|---|---|
| | | **Positive** | **Percentage (%)** | **Positive** | **Percentage (%)** | **Positive** | **Percentage (%)** |
| Farm I | 6 | 2 | 33.33 | 0 | 0 | 3 | 50 |
| Farm II | 3 | 2 | 66.67 | 0 | 0 | 3 | 100 |
| Farm III | 6 | 3 | 50 | 0 | 0 | 3 | 50 |
| Farm IV | 12 | 6 | 50 | 4 | 33.33 | 6 | 50 |
| Farm V | 6 | 3 | 50 | 0 | 0 | 4 | 50 |
| *total* | 33 | 16 | 48.48 | 4 | 12.12 | 19 | 57.57 |
| ***Chi-square test*** | | $3.653^{ns}$ | | 16.311* | | $1.967^{ns}$ | |
| ***P-value*** | | $0.455^{ns}$ | | $0.003^{s}$ | | $0.742^{ns}$ | |

**Table 20. ESBL -producing *E. coli* isolates from housefly samples**

| **Sl. No.** | **farm** | **No. of *E. coli* isolates** | ***bla*$_{CTX-M}$** | | ***bla*$_{SHV}$** | | ***bla*$_{TEM}$** | |
|---|---|---|---|---|---|---|---|---|
| | | | **Positive** | **Percentage (%)** | **Positive** | **Percentage (%)** | **Positive** | **Percentage (%)** |
| **1** | Non-Farm setting | 15 | 5 | 33.3 | 3 | 20 | 5 | 33.3 |
| **2** | Farm setting | 15 | 9 | 60 | 9 | 60 | 7 | 46.7 |
| | ***total*** | 30 | 14 | 46.7 | 12 | 40 | 12 | 40 |
| ***Chi-square value*** | | | $2.143^{ns}$ | | $5.000^{ns}$ | | $0.556^{ns}$ | |
| ***p-value*** | | | $0.143^{ns}$ | | $0.25^{ns}$ | | $0.456^{ns}$ | |

### Table 21. ESBL-producing *Salmonella* spp. isolates from housefly samples

| **Sl. No.** | **farm** | **No. of *Salmonella* spp.** | ***bla CTX-M*** | | ***bla$_{SHV}$*** | | ***bla$_{TEM}$*** | |
|---|---|---|---|---|---|---|---|---|
| | | | **Positive** | **Percentage (%)** | **Positive** | **Percentage (%)** | **Positive** | **Percentage ( %)** |
| **1** | Non-Farm setting | 3 | 1 | 33.3 | 2 | 66.60 | 2 | 66.60 |
| **2** | Farm setting | 2 | 1 | 13.3 | 1 | 7.1 | 0 | 00 |
| | **total** | 5 | 2 | 40 | 3 | 60 | 2 | 40 |

### Table 22. *E. coli* isolates encoding ESBL genes

| ESBL coding genes | No. of positive genes in pig sample | Positive genes in house fly sample | | Total |
|---|---|---|---|---|
| | | Farm settings | Non-farm settings | |
| *bla$_{CTX-M}$* | 102 (48.60) | 9 (60) | 5 (33.33) | 116 |
| *bla$_{TEM}$* | 92 (43.80) | 7 (46.70) | 5 (33.33) | 104 |
| *bla$_{SHV}$* | 22 (10.50) | 9 (60) | 3 (20) | 34 |

**Table 23.** ***E. coli*** **isolates encoding two ESBL genes**

| ESBL coding genes | No. of positive genes in pig sample ( per cent) | Positive genes in house fly sample ( per cent) | | Total |
|---|---|---|---|---|
| | | Farm settings | Non-farm settings | |
| $bla_{CTX\text{-}M}$ and $bla_{TEM}$ | 80 (39.40) | 7 (46.67) | 8 (53.33) | 95 |
| $bla_{TEM}$ and $bla_{SHV}$ | 18 (8.80) | 6 (40) | 3 (20) | 27 |
| $bla_{CTX\text{-}M}$ *and* $bla_{SHV}$ | 16 (7.80) | 8 (53.33) | 2 (13.33) | 26 |

**Table 24.** ***E. coli*** **isolates encoding three ESBL genes**

| ESBL coding genes | No. of positive genes in pig sample ( per cent) | Positive genes in house fly sample ( per cent) | | Total |
|---|---|---|---|---|
| | | Farm settings | Non-farm settings | |
| $bla_{CTX\text{-}M}$, $bla_{TEM}$ and $bla_{SHV}$ | 5 (2.4) | 2 (13.33) | 1 (6.67) | 8 |

**Table 25.** ***Salmonella*** **spp. isolates encoding ESBL genes**

| ESBL coding genes | No. of positive genes in pig sample ( per cent) | Positive genes in house fly sample ( per cent) | | Total |
|---|---|---|---|---|
| | | Farm settings | Non-farm settings | |
| $bla_{\text{CTX-M}}$ | 16 (48.48) | 1 (33.33) | 1(33.33) | 18 |
| $bla_{\text{TEM}}$ | 19 (57.7) | 0 | 2 (66.66) | 21 |
| $bla_{\text{SHV}}$ | 4 (12.12) | 1 (33.33) | 2(66.66) | 7 |

**Table 26. *Salmonella* spp. isolates encoding two ESBL genes**

| ESBL coding genes | No. of positive genes in pig sample (%) | Positive genes in house fly sample (%) | | Total |
|---|---|---|---|---|
| | | Farm settings | Non-farm settings | |
| $bla_{CTX\text{-}M}$ and $bla_{TEM}$ | 13 (39.39) | 0 | 1 (33.33) | 14 |
| $bla_{TEM}$ and $bla_{SHV}$ | 2 (6.06 ) | 0 | 2 (66.67) | 4 |
| $bla_{CTX\text{-}M}$ *and* $bla_{SHV}$ | 1 (3.03) | 0 | 1 (33.33) | 2 |

## 4.4. ANTIBIOTIC SUSCEPTIBILITY STUDIES FOR CARBAPENEMASE PRODUCTION

The study comprised of a total of 271 isolates of *E. coli* and *Salmonella* spp. that were screened phenotypically and genotypically for determining the occurrence of carbapenemase production. The phenotypic assay for carbapenem resistance was done using the antibiotics namely, meropenem, ertapenem, doripenem, and imipenem, as per the guidelines of CLSI (2018). The current study focuses on the genotypic detection of carbapenemase production mainly by screening three genes, which included $bla_{NDM}$, $bla_{OXA\text{-}48}$ and $bla_{IMP}$ genes.

### 4.4.1. Phenotypic Characterization of Recovered Isolates for carbapenemase production

Out of a total of 271 isolates of *E. coli* (233) and *Salmonella* spp. (38) total of 203 *E. coli* isolates were obtained from pig faecal samples and 30 isolates from house fly samples (both farm and non-farm settings). Very low level of carbapenem resistance was shown by isolates of *E. coli*. The phenotypic assessment of carbapenem resistance was performed based on the guidelines mentioned by CLSI (2018).

### 4.4.2. Phenotypic Characterization of Recovered Isolates for carbapenemase production in pig faecal sample

Carbapenemase resistance screened among 203 *E. coli* isolates from pig faecal samples showed the emergence of less than five per cent resistance to imipenem, doripenem, ertapenem, and meropenem. When compared to *Salmonella* spp.

isolates, none of the samples showed resistance to carbapenem antibiotics. All the samples were found sensitive to all carbapenem antibiotics under study. Details are enlisted in Table 28.

#### 4.4.2.1. *Carbapenemase-producing E. coli*

A total of 203 *E. coli* isolates were evaluated for carbapenemase resistance. Resistance to carbapenem antibiotics is found in an increasing order of doripenem, meropenem (0.40 per cent), ertapenem (0.90 per cent), and highest in imipenem (1.97 per cent). There was a higher number of intermediate-resistant isolates from the present study. The details are mentioned in Table 27.

### 4.4.3. Phenotypic Characterization of Recovered Isolates for Carbapenemase production in House flies entrapped from Farm settings

Out of 15 *E. coli* isolates and two *Salmonella* spp. isolates obtained from houseflies in farm settings, the following results were obtained. Upon screening four carbapenem antibiotics in 15 *E. coli* isolates, none of the samples showed intermediate or resistance to carbapenem antibiotics. Two isolates of *Salmonella* spp. were screened phenotypically for determining carbapenem resistance, both the isolates were sensitive to the carbapenem antibiotics.

### 4.4.4. Phenotypic Characterization of Recovered Isolates for carbapenemase production in house flies entrapped from non-farm settings

Out of 15 *E. coli* isolates and two *Salmonella* spp. isolates obtained from houseflies in non-farm settings were screened for carbapenemase-producing ability. Phenotypic carbapenem resistance was screened with 15 *E. coli* isolates, none of the samples showed intermediate or resistance to carbapenem antibiotics. Three isolates of *Salmonella* spp. were screened phenotypically for determining carbapenem resistance, all three isolates were sensitive to the mentioned carbapenem antibiotics.

**Table 27. Carbapenemase-producing *E. coli* isolates detected by double-disc method in pig faecal samples**

| **Sl. No.** | **farm** | **No. of *E. coli* isolates** | **Imipenem** | | | **Meropenem** | | | **Dorapenam** | | | **Eetrapenam** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **S** | **I** | **R** | **S** | **I** | **R** | **S** | **I** | **R** | **S** | **I** | **R** |
| 1 | Farm I | 39 | 41 | 1 | 0 | 41 | 1 | 0 | 42 | 0 | 0 | 42 | 0 | 0 |
| 2 | Farm II | 39 | 32 | 6 | 1 | 32 | 6 | 1 | 37 | 2 | 0 | 34 | 4 | 1 |
| 3 | Farm III | 41 | 30 | 8 | 3 | 40 | 1 | 0 | 41 | 0 | 0 | 34 | 4 | 1 |
| 4 | Farm IV | 42 | 42 | 0 | 0 | 40 | 2 | 0 | 42 | 0 | 0 | 36 | 4 | 1 |
| 5 | Farm V | 42 | 42 | 0 | 0 | 42 | 0 | 0 | 42 | 0 | 0 | 42 | 0 | 0 |
| *Total* | | 203 | 184 | 15 | 4 | 192 | 10 | 1 | 201 | 2 | 0 | 193 | 8 | 2 |
| ***Chi-square test*** | | | 46.389$^{ns}$ | | | 25.141$^{ns}$ | | | 16.961$^{ns}$ | | | 25.051$^{ns}$ | | |
| ***P value*** | | | 0.00 | | | 0.014$^{ns}$ | | | 0.031$^{ns}$ | | | 0.015$^{ns}$ | | |

ns-non significant

**Table 28. Carbapenemase-producing *Salmonella* spp. isolates detected by double-disc method in pig faecal samples**

| **Sl. No.** | **farm** | **No. of *Salmonella* Spp. isolates** | **Imipenem** | | | **Meropenem** | | | **Dorapenem** | | | **Etrapenem** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **S** | **I** | **R** | **S** | **I** | **R** | **S** | **I** | **R** | **S** | **I** | **R** |
| 1 | Farm I | 6 | 6 | 0 | 0 | 6 | 0 | 0 | 6 | 0 | 0 | 6 | 0 | 0 |
| 2 | Farm II | 3 | 3 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 |
| 3 | Farm III | 6 | 6 | 0 | 0 | 6 | 0 | 0 | 6 | 0 | 0 | 6 | 0 | 0 |
| 4 | Farm IV | 12 | 12 | 0 | 0 | 12 | 0 | 0 | 12 | 0 | 0 | 12 | 0 | 0 |
| 5 | Farm V | 6 | 6 | 0 | 0 | 6 | 0 | 0 | 6 | 0 | 0 | 6 | 0 | 0 |
| Total | | 33 | 33 | 0 | 0 | 33 | 0 | 0 | 33 | 0 | 0 | 33 | 0 | 0 |
| ***Chi-square test*** | | | $6.585^{ns}$ | | | $6.585^{ns}$ | | | $6.585^{ns}$ | | | $6.585^{ns}$ | | |
| ***P-value*** | | | $0.160^{ns}$ | | | $0.160^{ns}$ | | | $0.160^{ns}$ | | | $0.160^{ns}$ | | |

ns-non significant

## 4.5. GENOTYPIC CHARACTERIZATION OF RECOVERED ISOLATES FOR CARBAPENEMASE PRODUCING GENES

The DNA extracted was used as the template for PCR reactions for genotypic detection and gene confirmation of carbapenemase-producing genes (*bla*$_{\text{NDM}}$, *bla*$_{\text{IMP}}$, and *bla*$_{\text{OXA-48}}$). Two (0.90 per cent) isolates showed band at 847 bp for *bla*$_{\text{OXA-48}}$ (Fig.13). The two carbapenem resistant genes of *E.coli* was obtained from the pig faecal samples collected from the farm II. Carbapenem resistant genes could not be detected in other isolates from pig faecal samples or from housefly samples (farm or non-farm settings). None of the other carbapenem resistant genes (*bla*$_{\text{NDM}}$ and *bla*$_{\text{IMP}}$) could be detected in the present study.

## 4.6. ASSESSMENT AND COMPARISON OF ESBL PRODUCTION IN DIFFERENT FARMS

### 4.6.1. Comparison of ESBL production in breeding stock and mixed stock

Farm IV was assigned as mixed stock and sample collection in farm V is done in such a way that all animals were in late pregnancy. Phenotypic assay for ESBL production in *E. coli* revealed that more number of ESBL-producing ability in breeding stock compared to the mixed stock, however the genotypic assessment confirmed there is no difference in ESBL-encoded genes in *E. coli* in both farms. A higher percentage of *Salmonella* spp. was recovered from mixed stock (farm IV). On phenotypic characterization, six out of 12 samples (50 per cent) were ESBL producers in mixed stock. The data is given in the Table 29.

**Table 29. Comparative ESBL-producing ability in mixed stock and breeding stock**

| **Sl. No.** | **Farm** | **No. of isolates obtained** | **Phenotypic assay** | **Genotypic assay** | | |
|---|---|---|---|---|---|---|
| | | | | ***bla*$_{CTX\text{-}M}$** | ***bla*$_{SHV}$** | ***bla*$_{TEM}$** |
| | | | **ESBL producers** | **No. of +ve isolates** | **No. of +ve isolates** | **No. of +ve isolates** |
| **1** | Farm IV (Mixed stock) | *E. coli* (42) | 25 | 20 | 5 | 24 |
| | | *Salmonella* spp. (12) | 6 | 6 | 4 | 6 |
| **2** | Farm V (Breeding stock) | *E. coli* (42) | 24 | 24 | 4 | 20 |
| | | *Salmonella* spp. (6) | 2 | 3 | 0 | 4 |
| Total | | *E. coli (84)* | 49 | 44 | 9 | 44 |
| | | *Salmonella* spp. (18) | 8 | 9 | 4 | 10 |

### 4.6.2. Comparison of ESBL production in animals fed with concentrate and swill feeding

In farm I, samples were collected from pigs completely fed on concentrate and in farm III samples were from animal fed on swill feeding. Phenotypic characterization showed higher number of ESBL producing *E. coli* in pigs that were fed with swill feeding than concentrate feeding. The result indicated that 20 out of 39 *E. coli* isolates were phenotypically ESBL producers in farms where swill feeding is practiced, compared to those farms where concentrate feeding was followed, 14 out of 39 *E. coli* isolates were ESBL producers. Genotypic assessment revealed higher number of $bla_{SHV}$ gene (5) in swill fed animals than concentrate fed animals (2). Three *Salmonella* isolates were recovered from pigs where swill feeding was practiced and six isolates were obtained from concentrate fed pigs. Phenotypic assessment showed four out of six isolates were ESBL producers in case of concentrate fed animals, whereas in case of swill fed animals one out three isolates was an ESBL producer. All the *Salmonella* spp. isolates from swill-fed animal carry $bla_{TEM}$ gene for ESBL production. The details are shown in Table 30.

**Table 30. Comparative ESBL-producing ability in swill fed pigs and concentrate fed pig**

| **Sl. No.** | **Farm** | **No. of isolates obtained** | **Phenotypic assay** | **Genotypic assay** | | |
|---|---|---|---|---|---|---|
| | | | | $bla_{CTX-M}$ | $bla_{SHV}$ | $bla_{TEM}$ |
| | | | **ESBL producers** | **No. of +ve isolates** | **No. of +ve isolates** | **No. of +ve isolates** |
| **1** | Farm I (Concentrate feeding) | *E. coli* (39) | 14 | 23 | 2 | 16 |
| | | *Salmonella* spp.(6) | 4 | 2 | 0 | 3 |
| **2** | Farm IV (Swill feeding) | *E. coli* (39) | 20 | 19 | 5 | 16 |
| | | *Salmonella* spp. (3) | 1 | 2 | 0 | 3 |
| Total | | *E. coli (78)* | 34 | 42 | 7 | 32 |
| | | *Salmonella* spp. (9) | 5 | 4 | 0 | 6 |

mixed stock (farm IV). The ESBL producing *E. coli* was more in mixed stock disc assay. A total of 25 out of 49 *E. coli* isolates from mixed stock are ESBL producers. The occurrence of ESBL-producing genes in *E. coli* isolates was found to be the highest in mixed stock, whereas the weaning piglet stock exhibited presence of $bla_{CTX-M}$, $bla_{TEM}$, $bla_{SHV}$ in the order of 19,16, and 5 out of 39 *E. coli* isolates, respectively. Both phenotypic and genotypic characterization revealed higher percentage of ESBL producers in mixed stock than in weaning piglets. The details are given in Table 31.

### 4.6.4. Comparison of ESBL production in weaning piglets and breeding stock

Samples collected from farm II belonged to weaning piglets, whereas those of farm V belonged to breeding stock. Phenotypic assessment revealed that 24 out of 42 *E. coli* isolates were ESBL producers in breeding stock, which is comparatively higher than weaning piglets. Similar observations were also noticed on genotypic assessment. Higher number of $bla_{CTX\text{-}M}$ and $bla_{TEM}$ genes in order of 24 and 20 out of 42 *E. coli* isolates were recovered from breeding stock, which is higher compared to the data obtained from weaned piglets. The details are given in the Table 32.

**Table 31. Comparative ESBL-producing ability in weaning piglets and mixed stock**

| **Sl. No.** | **Farm** | **No. of isolates obtained** | **Phenotypic assay** | **Genotypic assay** | | |
|---|---|---|---|---|---|---|
| | | | | $bla_{CTX\text{-}M}$ | $bla_{SHV}$ | $bla_{TEM}$ |
| | | | **ESBL producers** | **No. of +ve isolates** | **No. of +ve isolates** | **No. of +ve isolates** |
| **1** | Farm II (Weaning piglet) | *E. coli* (39) | 20 | 19 | 5 | 16 |
| | | *Salmonella* spp. (3) | 1 | 2 | 0 | 3 |
| **2** | Farm V (Breeding stock) | *E. coli* (42) | 24 | 24 | 4 | 20 |
| | | *Salmonella* spp. (6) | 2 | 3 | 0 | 4 |
| Total | | *E. coli (*81) | 44 | 43 | 9 | 36 |
| | | *Salmonella* spp. (9) | 3 | 5 | 0 | 7 |

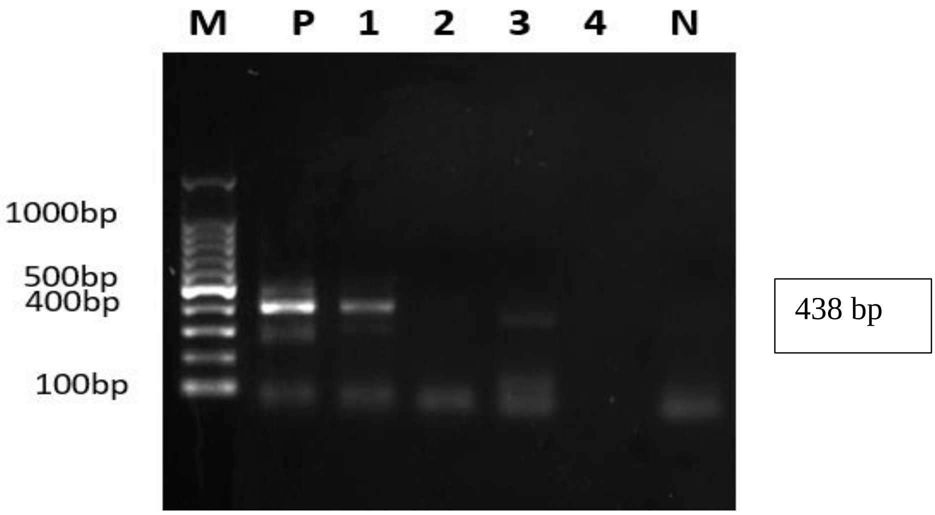


**Fig.13 : PCR amplification of *bla*$_{\textbf{OXA-48}}$ gene**

**Lane M** : 100 bp DNA marker

**Lane P** : Positive control of *bla*$_{\text{OXA-48}}$ gene

**Lane N** : Non Template Control

**Lanes 1&3** : Samples positive to *bla*$_{\text{OXA-48}}$ gene (438 bp)

**Table 32. Comparative ESBL-producing ability in weaning piglets and breeding stock**

| **Sl. No.** | **Farm** | **No. of isolates obtained** | ***Phenotypic assay*** | **Genotypic assay** | | |
|---|---|---|---|---|---|---|
| | | | | ***bla***$_{\textbf{CTX-M}}$ | ***bla***$_{\textbf{SHV}}$ | ***bla***$_{\textbf{TEM}}$ |
| | | | **ESBL producers** | **No. of +ve isolates** | **No. of +ve isolates** | **No. of +ve isolates** |
| **1** | Farm II (Weaning piglet) | *E. coli* (39) | 20 | 19 | 5 | 16 |
| | | *Salmonella* spp. (3) | 1 | 2 | 0 | 3 |
| **2** | Farm IV (Mixed stock) | *E. coli* (42) | 25 | 20 | 5 | 24 |
| | | *Salmonella* spp. (12) | 6 | 6 | 4 | 6 |
| Total | | *E. coli* (81) | 45 | 39 | 10 | 40 |
| | | *Salmonella* spp. (15) | 7 | 8 | 4 | 9 |

# 5. DISCUSSION

Antimicrobial resistance (AMR) is one of the arduous public health issues globally, with 10 million deaths and a $100 trillion economic loss predicted by 2050 (O'Neill, 2016). To fulfill the food security needs of the world's growing population, high-density farming practices as adopted, which might treble antibiotic use by livestock in developing nations by 2030 (Bellet and Rushton, 2019). Antibiotics are widely used in food animal populations, putting substantial selection pressure on pathogens of public health importance (Tiseo *et al.*, 2020). This contributes to the emergence, dissemination, and maintenance of drug resistant pathogens in other animals, people, and the environment. AMR has gained traction in human medicine, but the significance of animal husbandry techniques in combating AMR is still being debated with minimal understanding among stakeholders, especially in low-income countries. AMR has been identified as a hotspot in the country's rising intensive farming techniques, with antibiotic use in food animals expected to increase by 82 per cent by 2030 (Van Boeckel *et al.*, 2015). Many recent studies in India have highlighted the necessity for sensible antibiotic usage in the country's animal health sector, stressing the possibility of antibiotic resistance from foods of animal origin (Kumar *et al.*, 2019., Abdalla *et al.*, 2022 and Moffo *et al.*, 2022). Although a "National Action Plan on Antimicrobial Resistance" has been implemented in India to optimize antibiotic use in the country, stringent enforcement on the ground is still required.

As per the reports of FAO (2019) globally, pig meat is considered the second most favored meat for consumption due to its minimum relative prices. In total numbers, global pig meat production is expected to grow by approximately 11 Mt (9.30 per cent) by 2028 (Tzanidakis *et al.*, 2021). Intensively farmed pigs may act as a potential source of drug-resistant bacteria due to the use of antibiotics as feed growth promoters or feed additives apart from their therapeutic purposes. Such antimicrobial agents elicit 'selection pressure' on pathogenic bacteria contributing to the development of anti-microbial resistance (AMR) that can be critically important in human medicine.

Only a few confirmed occurrences of enteric bacterial pathogens of public health relevance among animals have been reported in Kerala (Mahesh, 2017). Wayanad district is located near Kerala's northern border, (on the Western Ghats) and is divided into three taluks: Vythiri, Mananthavady, and Sulthan Bathery. According to the Department of Animal Husbandry's report, Wayanad has around 250 registered pig farms. Majority of them are near the forest buffer zone. Pigs are thought to be a reservoir and amplifier for a variety of zoonotic diseases, such as Japanese encephalitis.

Flies are assumed to be the mechanical vectors for the transmission of zoonotic bacterial diseases, and there is a chance those flies that come into contact with animals and their faeces may pick up pathogens in their gut and on their skin. Flies are potential reservoirs and vectors for infections and antimicrobial resistant genes due to their feeding, breeding, and nesting activities (Rybaříková *et al.*, 2010; Usui *et al.*, 2015). A recent overview of the role of house flies in the spread of AMR was published, focusing on the medical implications of AMR in flies as well as investigations on quantitative risk assessment models including vectoral ability and colonization dose (Onwugamba *et al.*, 2018). Flies are mechanical vectors for the transmission of zoonotic bacterial diseases, (including transfer of resistant genes) and there is a chance that frequent interaction with flies would lead to transmission. House flies can proliferate in the decaying organic debris of livestock farms and have been observed living in close proximity to animals and human settlements. So, chance of house flies to harbor antimicrobial resistant genes must be explored. Thus, the goal of this research work was to investigate the incidence of specific enteric bacterial pathogens such as *E. coli* and *Salmonella* spp. and to determine their ability to produce ESBL and carbapenemase resistance. The study purposefully chose pig and houseflies as the subjects in order to represent pork meat and houseflies, both of which have a close relationship with humans.

## 5.1. ISOLATION AND MOLECULAR DETECTION OF *E. coli* and *Salmonella spp.* IN PIG FAECAL SAMPLE AND FROM HOUSEFLIES.

### **5.1.1. Isolation and identification by Culture Method**

Precise microbiological isolation of specific enteric bacterial pathogens was performed. Initially, pig faecal samples were pre-enriched for *Salmonella* spp. in BPW and incubated at 37°C for 18 hours. MacConkey agar was used to differentiate lactose fermenting *E. coli* colonies from non-lactose fermenting colonies (*Salmonella* spp.) in accordance with Nambiar *et al.* (2009). After pre-enrichment in BPW, Castagna *et al.* (2005) revealed that the optimum approach for isolation of *Salmonella* spp. is selective enrichment in RV broth followed by plating onto XLD agar with red colonies with black centres. The investigation followed the technique outlined in major reference books for pathogen isolation (Barrow and Feltham, 2003). Molecular analysis will confirmed putative colonies and provided a real picture of the pathogen of interest. All the 210 faecal samples and 30 pools of housefly samples, the presumptive *E. coli* was obtained from 100 per cent of faecal and housefly sample. Presumptive *Salmonella* spp. were obtained from 60 per cent faecal sample and 40 per cent housefly samples.

### 5.1.2. Rapid Detection of Enteric Bacterial Pathogens in a Single Reaction by PCR assay

A total of 210 faecal samples/swabs from in housed reared pigs and 30 pools of housefly samples farm and non-farm setting were analysed by using PCR. The results revealed the occurrence of high proportions of pathogens, especially *E.coli* among the three taluks of Wayanad district. The standardized PCR was used for identification of *E. coli* (*uidA*) and *Salmonella* spp. (*invA*) from pig faecal samples and house fly samples. The *invA* gene encoded for a membrane protein of bacteria, which is necessary for epithelial cells invasion. PCR targeting *invA* gene for *Salmonella* spp. is rapid and is used as a specific detection tool for *Salmonella* spp. (Lampel *et al*., 2000). The protocol that was standardized previously in the laboratory by Mahesh (2017) was employed for simultaneous detection of pathogens such as *Salmonella* spp. (*invA*), and *E. coli* (*uidA*) in the study. Similar studies for detection of these pathogens was developed by Rodrigues *et al.* (2004) and Valentin *et al.* (2014).

#### 5.1.2.1. *Occurrence of E. coli*

A total of 210 faecal swabs from five different pig farms comprising of 42 sample from each farm were analyzed across Wayanad. Molecular confirmation of *E. coli* via gene targeting *uidA* gene revealed that out of 210 samples screened, 207 isolates were found positive for *E. coli*. On farm wise observation, it was seen that all the samples from farm IV and farm V were found positive. In other three farms above 90 per cent recovery rate of *E. coli* isolates was recorded. Similar specific PCR based studies have been documented by Makhado *et al.* (2022), Abdalla *et al.* (2022), and Chance *et al.* (2021).

#### 5.1.2.2. *Occurrence of Salmonella* spp.

For isolation of *Salmonella* spp. from pig faecal swabs standard procedure mentioned in reference book are followed. Mainar-Jaime *et al.* (2008) reported molecular detection of *Salmonella* spp. using *invA* gene which had an accuracy of 91 per cent compared to conventional microbiological tests. In the study out of 210 samples screened for *Salmonella* spp., a total of 33 isolates were *Salmonella* spp. and were confirmed by amplification of *invA* gene. The highest recovery rate is recorded from the farm IV where mixed farm was practiced. In all other farms the recovery rate was below 20 per cent. This is comparatively higher when compared to the study conducted by Karabasanavar *et al.* (2022) and Borah *et al.* (2022). Recovery rate of *invA* gene in other farm animals. In studies conducted by Tiwari *et al.* (2021) recorded comparatively lower than present study. The study is in consensus with the statement of Dang-Xuan *et al.* (2019) and Ostanello and Lucia, (2020) that there is a need of screening the risk factor associated with occurrence of *Salmonella* spp. in pig farms with contaminated feed, water and unhygienic floor pens.

### 5.1.3. Molecular Detection of Enteric Bacterial Pathogens in houseflies

### A) Occurrence of *E. coli*

The study explored the prevalence of *E. coli* in house flies entrapped from farm and non-farm settings and no such reports were documented in the study area. Fifteen pools which contain not less than 10 house flies were sampled from farm and non-farm settings, separately. From both settings, the recovery rate of *E. coli* was 100 per cent. Gupta *et al.* (2012) stated that houseflies were found in moderate to high numbers in a variety of human and animal contexts, carrying clinically and veterinary important bacterial species in abundance. Sobur *et al.* (2019a) reported a lower percentage of *E. coli* recovered from house flies of non-farm settings, and has also stated that housefly trapped from different locations such as nearby farm, schools, hosiptals etc can habour a higher percentage *E. coli* with higher number of antimicrobial resistant gene, which is in agreement with present study.

The flies collected from farm settings showed 100 per cent recovery rated and is in agreement with the study conducted by Cervelin *et al.* (2018), Pohlenz *et al.* (2018), Pawade *et al.* (2019). Akter *et al.*(2020) studied the prevalence of *E. coli* in houseflies from farm setting and reported 51.40 per cent recovery rate, that is comparatively lower compared to this study. Apart of pig farm several other literature (Sobur *et al.*, 2019b; Hamilton *et al.*, 2021) stated the presence of enteric pathogens in house fly trapped from different farm settings.

### B) Occurrence of *Salmonella* spp.

Out of 15 pools of houseflies trapped from farm settings, only two (15.7 per cent) *Salmonella* spp. isolates were recovered. The present study reported lower percentage of *Salmonella* spp. compared to the study conducted by Cervelin *et al.* (2018), Xu *et al.* (2018), and Kababian *et al.* (2020). Wang *et al.* (2011) isolated *Salmonella* spp. from houseflies and from pigs and is reported to be 26.40 per cent and 19.50 per cent respectively. Furthermore,the author also stated there is linage in *Salmonella* strain that are recovered from pig and houseflies. Above literature confirms the need of phylogenetic analysis.

There are well documented reports detailing the prevalence of enteric pathogens in non-farm environment (Ommi *et al.*, 2017; Odetoyin *et al.*, 2020). The present study could obtain 20 per cent of *Salmonella* spp. from house flies entrapped from non-farm settings. Well documented reports available (Khamesipour *et al.*, 2018; Kababian *et al.*, 2020; Hassan *et al.*, 2022) showed higher occurrence of *Salmonella* spp. from houseflies of non-farm settings.

## 5.2. ANTIBIOTIC SUSCEPTIBILITY STUDIES FOR ESBL PRODUCTION

A total of 233 *E. coli* and 38 *Salmonella* spp. isolates were examined for AMR pattern by disc diffusion method as mentioned by CLSI (2018). The antimicrobials used were from the list of critical group of antimicrobials *viz*., Cephalosporins (Cefotaxime, Ceftazidime and their clavulanic acid combination). Almost all the *E. coli* isolates showed resistance to tested antibiotics. The results were analyzed as per the CLSI guidelines and manufacturer's instructions. Genotypic screening of gene encoded for ESBL production was carried out.

### 5.2.1. Phenotypic Characterisation of Recovered Isolates for ESBL Production

#### 5.2.1.1. *Escherichia coli*

The present study reported that 53.20 per cent (108/203) of *E. coli* isolated from pigs were ESBL producers which was lower when compared to earlier studies conducted by Brisola *et al.* (2019), where in the author had reported 62.10 per cent of *E. coli* isolates from pigs in southern Brazil as ESBL producers through antimicrobial susceptibility profiling. Farm III and farm V had maximum number of ESBL-producing *E. coli* and the lowest occurrence was in case of farm-1. Similarly, Kamaruzzaman *et al.* (2020) reported the occurrence of ESBL producing *E. coli* in 48 per cent of housefly sample from farm settings which was lower when compared to the present study wherein 60 per cent (9/15) of *E. coli* isolates recovered were ESBL producers. Where as 40 per cent (6/15) of *E. coli* isolates that was recovered from housefly samples (non-farm setting) is higher compared to previous studies conducted by Natalia *et al.* (2021), where author reported 35 per cent of ESBL producing *E. coli* from house fly samples entrapped from non- farm settings like hospitals, schools etc. in Poland. The present study observed higher percentage of ESBL-producing *E. coli* from housefly trapped from farm setting than non-farm settings, pointing out the chance of getting of antimicrobial resistance from farms to house fly thereby disseminating to other environment.

#### 5.2.1.2. *Salmonella* spp.

As highly sophisticated machinery and processing plant existed to ensure the maximum food hygiene across the food chain, the trends of occurrence of salmonellosis via food is increasing drastically (Shen *et al.*, 2022). Non -typhoidal *Salmonella* spp. (NTS) can harbor large scale of antimicrobial resistant gene and can be considered as public health concern due to spill over of antimicrobial gene to other pathogenic organisms (Wottlin *et al.*, 2022). The present study explored ESBL production among *Salmonella* spp. isolates from pig faecal sample (33/203), housefly sample from farm settings (2/15) and house fly samples from non-farm setting (3/15). Out of 33 *Salmonella* spp. isolates recovered from pig faecal samples, 15 isolates (45.45 per cent) were found to be positive for ESBL production. The highest number of ESBL production was recorded in farm IV where mixed farming is practiced. Phongaran *et al.* (2019) reported a lower percentage (37.5 per cent) of ESBL production in pigs from Thailand when compared to the present study.

ESBL production assay of *Salmonella* spp. was revealed, one out of two isolates (50 per cent) recovered from houseflies (farm settings). Akter *et al.* (2020) also reported the occurrence of ESBL producers among *Salmonella* spp. isolates recovered from house flies of farm settings in Bangladesh as 34.20 per cent. The author also examined samples from housefly from non-farm settings like from slaughter houses, nearby hospitals, nearby slurry etc and reported that housefly can harbour large number of Gram-negative organisms and observed a higher prevalence of resistant genes. in the current study, two out of three *Salmonella* spp. isolates from housefly sample of non-farm settings were showing ESBL production phenotypically. Onwugamba *et al.* (2020) screened filth flies from environment sources and found none of the *Salmonella* spp. isolates found to be ESBL producers. The authors also reported higher percentage of ESBL *E. coli* from non-farm settings in Nigeria.

## 5.3. GENOTYPIC CHARACTERISATION OF RECOVERED ISOLATES FOR ESBL PRODUCTION

### 5.3.1. *Escherichia coli*

Genotypic characterisation of 203 *E. coli* isolates recovered from pig faecal samples unveiled that the occurrence of ESBL genes *viz.*, $bla_{CTX\text{-}M}$, $bla_{SHV}$ and $bla_{TEM}$ as 48.60 per cent, 10.50 per cent and 43.80 per cent, respectively. Similarly, Athanasakopoulou *et al.* (2021), Hounmanou *et al.* (2021), Trongjit and Chuanchuen, (2021), Truong *et al.* (2021) had reported that CTX-M variant of ESBL was predominantly isolated from pig and this was also in accordance with the present study. Li *et al.* (2021) reported a higher percentage of $bla_{CTX\text{-}M}$ (70.50 per cent), and $bla_{TEM}$ (61.19 per cent) from pigs of East China. Of 203 *E. coli* isolates screened, 80 isolates contain both $bla_{CTX\text{-}M}$ and $bla_{TEM}$ encoded gene, whereas 18 *E. coli* isolates encoded for both $bla_{TEM}$ and $bla_{SHV}$. From all the *E. coli* isolates from pig, only five samples contain $bla_{CTX\text{-}M}$, $bla_{TEM}$ and $bla_{SHV}$. The occurence of ESBL encoded gene in larger numbers may cause wide dissemination of these genes to surrounding environment and food chain (Liu *et al.*, 2022).

Earlier literature documented the prevalence of ESBL-producing genes among houseflies trapped nearby farm premises (Gerry, 2020). Higher percentage of $bla_{CTX-M}$ and $bla_{SHV}$ were recovered from housefly samples. On screening 60 per cent (9/60) of $bla_{CTX-M}$ and $bla_{SHV}$ were isolated. In constrast, Wadaskar *et al.* (2019) reported lower percentage of prevalence ESBL encoded genes from houseflies collected from farm permises. ESBL-producing Enterobacteriaceae are spreading rapidly and have been isolated and identified all over the world, primarily from LF organisms like *E. coli* and certain NLF (Samir *et al.*, 2022). Out of 15 *E. coli* isolates screened seven (46.67 per cent) isolates contain $bla_{CTX-M}$ and $bla_{TEM}$. In case of occurrence of $bla_{TEM}$ and $bla_{SHV}$ in *E. coli* isolates, six out of 15 isolates (40 per cent) contain both genes. In addition *bla* $_{\mathrm{CTX-M}}$ *and bla* $_{SHV}$ encoded *E. coli* isolates was harboured seen in eight out of 15 isolates (53.3 per cent). Besides, two isolates contained all the above three genes.

House flies of non-farm setting were also screened to understand the prevalence of antimicrobial resistant gene especially nearby human dwellings. A prevalence of $bla_{CTX-M}$ and $bla_{TEM}$ was found to be 33.33 per cent (5 out of 15 isolates). The occurrence of $bla_{SHV}$ encoded gene from houseflies collected from non-farm setting is 20per cent (3/15). Wetzker *et al.* (2019), Tufa *et al.* (2020) and Meissner *et al.* (2022) reported a higher percentage of above mentioned ESBL-producing genes from house flies of non-farm settings. Out of 15 *E. coli* isolates screened, eight (53.33 per cent) isolates contain $bla_{CTX-M}$ and $bla_{TEM}$. In case of occurrence of $bla_{TEM}$ and $bla_{SHV}$ in *E. coli* isolates three out of 15 isolates (20 per cent) contained both genes. Two out of 15 isolates (13.3 per cent) of *E. coli* contain $bla_{CTX-M}$ *and* $bla_{SHV}$ encoded genes. The current study found two isolates contain all three genes.

### 5.3.2. *Salmonella* spp.

A total of 33 *Salmonella* spp. isolates were molecularly confirmed after screening 210 faecal samples from five pig farms across Wayanad district. The determination of ESBL-producing genes were done by PCR based amplification of $bla_{CTX-M}$, $bla_{TEM}$ and $bla_{SHV}$. The study showed a higher percentage of $bla_{TEM}$ gene from the isolates. A total of 19 out 33 isolates were having $bla_{TEM}$ gene (57.57 per cent), Prevalence of $bla_{CTX-M}$ gene was seen in 16 out of 33 isolates (48.48 per cent). The overall occurrence of $bla_{SHV}$ gene in *Salmonella* spp. isolates were lower, and only four isolates showed positive for $bla_{SHV}$. Borah *et al.* (2022) reported a lower percentage of $bla_{TEM}$ gene (34.50 per cent) from *Salmonella* spp. isolates recovered from pigs. The present study was in agreement with earlier studies reported by Wang *et al.* (2011); Dang-Xuan *et al.* (2019), Igbinosa *et al.* (2021). The presence of more than one ESBL producing gene in *Salmonella* spp. is also documented (Ainslie-Garcia *et al.*, 2018). On screening the prevalence of $bla_{CTX-M}$ and $bla_{TEM}$ among isolates, 13 isolates revealed the presence of both of these genes. The $bla_{TEM}$ and $bla_{SHV}$ was found positive among two isolates out of 33 samples examined. Only one isolate contained $bla_{CTX-M}$ and $bla_{SHV}$ genes together.

On genotypic characterisation for ESBL production in *Salmonella* spp. isolates recovered from houseflies could detected. From two *Salmonella* spp. isolates from farm, one isolate is positive for $bla_{CTX-M}$ and other showed positive for $bla_{\mathrm{SHV}}$. None of the *Salmonella* spp. isolates contained $bla_{TEM}$ gene. The above findings in the study are comparatively lower compared to the studies conducted by Förster *et al.* (2009) and Gioia *et al.* (2022).

From houseflies collected from non-farm settings, three *Salmonella* spp. isolates were recovered. Genotypic screening of ESBL-producing genes revealed the occurrence of $bla_{CTX-M}$, $bla_{TEM}$ and $bla_{SHV}$ in the order of one (33.3 per cent), two (66.66 per cent) and two (66.6 per cent), respectively. The earlier studies on occurrence of MDR-*Salmonella* spp. in house fly revealed higher recovery rate of *Salmonella* spp. which haboured many antimicrobial genes including ESBL-producing *Salmonella* spp. (Hassan *et al.*, 2022; Isam-Eldeen *et al.*, 2022). The present study could also isolate *E. coli* and *Salmonella* spp. with higher prevalence of ESBL producing genes from pig faecal samples and houseflies (farm and non-farm settings).

## 5.4. ANTIBIOTIC SUSCEPTIBILITY STUDIES FOR CARBAPENEMASE PRODUCTION

The carbapenem antibiotic class is one of the antibiotics of last resort for treating significant Gram-negative infections in human medicine. Carbapenemases are beta-lactamase enzymes produced by bacteria that have the ability to neutralize antibiotics such as penicillins, cephalosporins, monobactams, and carbapenems, rendering them ineffective when used (Meletis, 2016). New Delhi metallo-beta-lactamase (NDM), often known as $bla_{NDM}$, is considered to be the most common gene encoding carabpenemase resistance in Enterobacteriaceae in India (Pawlak *et al.*, 2022). In addition to $bla_{NDM}$, the KPC, VIM, IMP, NDM, and OXA-48 gene types are also frequently reported. Overpopulation, lack of basic sanitation and access to clean water, tropical climate, and improper antibiotic use control are factors that contribute to carbapenem resistance. These elements work together to encourage the spread of carbapenemase-producing bacteria among humans and animals (Sonnevend *et al.*, 2022).

### 5.4.1. Carbapenemase-producing *E. coli*

Carbapenemase producing *Enterobacteriaceae* is considered as an important finding where research and development is needed as the most critical category as per reports of WHO (2017). Phenotypic characterisation of carbapenem resistance using four antibiotics mentioned in the CLSI (2018) was performed in the present study (Pruthvishree *et al.*, 2017; Murugan *et al.*, 2019; Yen *et al.*, 2022). Out of the 203 *E. coli* isolates recovered from different pigs farms screened, less than five per cent of sample showed resistance to carbapenem. There are also increasing number of isolates showing intermediate resistance. This finding recalls the emergence of carbapenem resistance in pigs in Wayanad. Phenotypic emergence of carbapenem resistance was also reported by many authors earlier (Murugan *et al.*, 2019; Yen *et al.*, 2022).

None of *E. coli* isolates recovered from the houseflies in farm setting and non-farm settings showed resistance against carbapenem antibiotics. All the *E. coli* isolates recovered from houseflies showed sensitivity to carbapenem antibiotics. Sobur *et al.* (2019a), and Karami *et al.* (2021) reported the occurrence of carbapenem resistance from houseflies collected from farm settings. Similarly Kehl *et al.* (2022) and Pereira *et al.* (2022) reported the prevalence of carbapenem resistance from houseflies collected from environment settings.

### 5.4.2. Carbapenemase-producing *Salmonella* spp.

All the *Salmonella* spp. isolates recovered from pig faecal samples and housefly samples (farm and non-farm settings) showed sensitivity to carbapenem antibiotics. No isolates were found with intermediate susceptibility or resistance to carbapenem antibiotics. Li *et al.* (2022) reported a higher prevalence of carbapenem resistance in *Salmonella* spp. from pig in China. Similarly Peng *et al.* (2022) reported the occurrence of carbapenem resistance in *Salmonella* spp. from houseflies collected from farm and non-farm settings.

## 5.5. GENOTYPIC CHARACTERISATION OF RECOVERED ISOLATES FOR CARBAPENEMASE PRODUCTION

Out of 203 *E. coli* isolates screened for carbapenem resistance genes, only two isolates from farm-II (0.90 per cent) from weaning piglets were found to harbour $bla_{OXA\text{-}48}$. Nirupama *et al.* (2018) reported the occurrence of carbapenem resistance among piglets. None of the other carbapenem resistant genes-like $bla_{NDM}$ and $bla_{IMP}$ could be detected in the present study. Pruthvishree *et al.* (2017) and VinodhKumar *et al.* (2021) reported the presence of carbapenem resistance genes mainly $bla_{NDM}$ and $bla_{OXA-48}$ from diarrheic piglets across India and from wild animals, respectively. The present study could not detect any of $bla_{NDM}$ and $bla_{IMP}$ from the *E. coli* and *Salmonella* spp. isolates. Gupta *et al.* (2012), Pawlak *et al.* (2022), and Shen *et al.* (2022) reported the prevalence of carbapenem resistance in *Enterobacteriaceae* from pig and houseflies.

## 5.6. ASSESSMENT OF ESBL PRODUCTION IN DIFFERENT FARMS

The samples collected from farm I and farm III were from pigs completely fed on concentrate feeds and swill feed, respectively. The faecal swabs from farm II, IV and V were randomly collected from weaning piglets, mixed age groups and breeding stock, respectively. Farm wise comparison for ESBL production was done to understand, whether there is any different in EBSL production during different growth stages, type of feeding etc.

### 5.6.1. Comparison of ESBL production in breeding stock and mixed stock

In present study, equal number of ESBL producing *E. coli* were recovered from the pig faecal sample from mixed reared pigs and from breeding stock by both phenotypic and genotypic assay. The recovery rate of *Salmonella* spp. was higher in samples collected from mixed stock of pig. Gundran *et al.* (2020) reported higher percentage of ESBL producing *E. coli* and *Salmonella* spp. from breeder pigs of Philippines. Fournier *et al.* (2021) and Abdalla *et al.* (2022) stated that pig reared with high stocking density and mixed reared of pigs can increase the transfer of antimicrobial resistant genes.

### 5.6.2. Comparison of ESBL production in animals fed with concentrate feeding and swill feeding

Phenotypic characterisation for ESBL production in *E. coli* isolates recovered from pig where swill feeding is practiced showed higher percentage of ESBL resistance. While screening the genes ($bla_{CTX-M}$, $bla_{TEM}$, and $bla_{SHV}$) encoded for ESBL production, there is only slight difference in the occurrence of above mentioned genes recovered from *E. coli* isolates in both farms where swill feeding and concentrate feeding was practiced. There is no relevant literature regarding the comparison between the occurrence of ESBL production in animals fed with concentrate feeding and swill feeding. The present study could not assess the other risk factors associated with the emergence of AMR in food animals and it must be done in future to get the clear picture of how other factors associated with the transfer of resistant genes. Hedman *et al.* (2020) reported the emergence of AMR in low resource settings as in poultry farm.

### 5.6.3. Comparison of ESBL production in weaning piglets and mixed stock

The present study screened the ESBL producing genes from samples collected from weaning piglets as one group and from mixed group as others. The observation showed higher percentage of *E. coli* isolates from mixed stock than from weaning piglets. Several studies have shown that the gut microbiome of newborns contained more resistant bacteria than that of adults, regardless of antibiotic exposure (Pärnänen *et al.*, 2018; Yun *et al.*, 2021). The proportion of *bla<sub>TEM</sub>* gene in *E. coli* isolates from mixed stock is higher in weaning piglets. Burow *et al.* (2019) studied the AMR in *E. coli* from pigs from birth to slaughter and its relation with antibiotic treatment. The results of the study showed higher percentage of antimicrobial genes in weaning piglets regardless of their antibiotic usage. The above finding is contrary to the present study. There are other reasons to consider piglets that habour more AMR genes because nursing piglets receive more antimicrobials for growth promotion, therapy, and prophylaxis than finishing pigs. Furthermore, due to declining passive immunity, mixing of pigs from different litters or farms, and colonization by resistant organisms, weaners are younger and more susceptible to enteric diseases (VinodhKumar *et al.*, 2019). Antimicrobials are used less frequently in finishers, in part because they are so close to being ready for human consumption that antimicrobial residues in pork are a concern (Antonelli *et al.*, 2019).

### 5.6.4. Comparison of ESBL production in weaning piglets and breeding stock

Both genotypic and phenotypic characterisation revealed higher number of ESBL-producing *E. coli* in samples recovered from breeding stock. There are scarce information regarding the screening of ESBL resistance in pig during late pregnancy. Out of 39 *E. coli* sample screened from weaning piglets, there was higher occurence of ESBL producing gene, where there was a history of diarrhea and antibiotic therapy. Antimicrobial therapy was regarded to be a significant control tool for piglets diarrhoea in pig farms. High rate of AMR in bacteria has emerged as a severe concern in the pig industry as a result of the widespread use of third-generation cephalosporins as therapy (Li *et al.*, 2021). Cavaco *et al.* (2008) also reported that the introduction and spread of ESBL-positive *E. coli* in piglets has been linked to the misuse and/or overuse of third-generation cephalosporins. The overall comparison study revealed the presence of antimicrobial resistant genes in all stages of pig’s life from weaning to breeding stage, different feeding strategy, different farming practices etc. hence strict biosecurity measures including hygiene and sanitation should be followed.

# 6.SUMMARY

Antimicrobial resistance (AMR) is a global emerging issue of public health concern. Therefore, National Action Plan on Containment of AMR exist where, it is mandatory to strengthen the antimicrobial resistance surveillance. The spread of ESBL-producing bacteria has become a major public health concern due to the obvious limited treatment options. Resistance to beta-lactam antibiotics has been found to predict resistance to penicillin's, third-generation cephalosporins, and monobactam antibiotics. Resistance to β-lactams is primarily caused by the formation of ESBL enzymes, which breakdown and make β-lactam medicines useless. AMR has become more common in *E. coli* and *Salmonella* as a result of the widespread and indiscriminate use of antimicrobial drugs for therapy, prophylaxis, and growth regulators. ESBL and carbapenemase producing bacteria have recently been found in food-producing and companion animals, as well as farm settings such as waste water and manure. All reports agree that there is a major public health risk and the likelihood of human infection from animal contact or consumption of animal products.

In the Wayanad district, there are only a scare information on occurrence of enteric bacterial resistant pathogens (such as *E. coli*, *Salmonella* spp.) in pigs and houseflies. These pathogens have been found elsewhere in India, although there are few reports of them in house flies. Antimicrobial resistant bacteria, particularly ESBLs, have emerged as a severe public health hazard around the world, with their emergence and dissemination across pathogens.

In view of these facts, a research work was designed to explore the AMR in *E. coli* and *Salmonella* spp. isolated from flies trapped at farm premises and fecal samples of the in-house reared pig from December 2020 to November 2021. A total of 210 representative rectal swabs of pigs and 30 pools of flies (15 pools from farm settings and 15 pools from non-farm settings) were collected and processed for bacteriological culture. All the house flies surrounding to farm premises and non-farm areas were collected using the commercial and traditional methods. *E. coli* was recovered from all the housefly (100 per cent) and majority (96.70 per cent) of pig faecal samples. Whereas *Salmonella* spp. was isolated from two (13.33 per cent) houseflies from farm settings, three (20 per cent) flies in non-farm settings, and 33 (15.70 per cent) faecal samples.

This study revealed that the recovered *E. coli* and *Salmonella* spp. isolates to be MDR-strains and the *E. coli* isolates exhibited resistance to Ceftazidime (28.60 per cent), Cefotaxime (49 per cent), Doripenem (0), Ertapenem (1 per cent), Imipenem (1.40 per cent), and Meropenem (0.50

per cent) whereas *Salmonella* spp. isolates were resistant to Ceftazidime (12 per cent), Cefotaxime (39.90 per cent). None of them showed resistance to Doripenem, Ertapenem, Imipenem, and Meropenem. ESBL resistance was recorded more in *E. coli* (56 per cent) than *Salmonella* spp. (48 per cent) by phenotypic assays.

In the molecular study, ESBL genes could be detected in the *E. coli* that included, $bla_{CTX\text{-}M}$ (48.60 per cent), $bla_{SHV}$ (10.50 per cent), $bla_{TEM}$ (43.80 per cent), and that in *Salmonella* spp. included $bla_{CTX\text{-}M}$ (48 per cent), $bla_{SHV}$ (12.12 per cent), $bla_{TEM}$ (57.57 per cent). Only two isolates from pig faecal were positive for the carabapenemase producing $bla_{OXA\text{-}48}$ gene. The present research work is suggestive of the fact that flies harbour multi-drug resistant, ESBL producing *E. coli* and *Salmonella* spp. Unregulated use of antibiotics in piggery may result in farms acting as a hub of ESBL and carbapenem resistant genes. Further, extensive monitoring of AMR in such unconventional reservoirs of bacterial pathogens needs to be conducted to evaluate the risk associated with AMR.

In addition to the phenotypic and genotypic characterisation of isolates for ESBL production, a diversified resistance pattern was detected by PCR assay, which was consistent with previous ESBL and carbapenem studies. The study demonstrated the importance of comprehending the magnitude of incidence and resistance pattern of major entero-zoonotic infections from pigs and houseflies, which serves as a warning sign to use extreme caution when handling such animals and maintain good environmental hygiene. It is clear that these enterobacterial strains that produce ESBL and carbapenemase circulate among pigs and houseflies in a small district in rural Kerala. These findings imply that the antibiotic use in pigs should be guided by regular monitoring of carbapenemase and ESBL emergence and transmission.

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# OCCURRENCE OF CARBAPENEMASE AND EXTENDED SPECTRUM BETA LACTAMASE PRODUCING MAJOR ENTERIC BACTERIA IN FARMED PIGS AND HOUSE FLIES FROM FARM PREMISES

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## ABSTRACT

**Submitted in partial fulfilment of the requirements for the degree of**

**MASTER OF VETERINARY SCIENCE**
**(Veterinary Public Health)**

**2022**

**Faculty of Veterinary and Animal Sciences**
**Kerala Veterinary and Animal Sciences University**

**DEPARTMENT OF VETERINARY PUBLIC HEALTH**

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# ABSTRACT

Intensively farmed pigs may act as a potential source of drug resistant bacteria due to the use of antibiotics as feed additives or growth promoters apart from their therapeutic purposes. Similarly, house flies in and around farm premises make effective contact with animals, manure and the environment thereby transmitting anti-microbial resistant bacteria to the food chain finally reaching humans. Hence, a study was designed to explore the antimicrobial resistance in *E. coli* and *Salmonella* spp. isolated from fecal samples of the in-house reared pig and flies trapped at farm premises. A total of 210 representative pig rectal swabs and 30 pools of flies (15 pools from farm settings and 15 pools from non-farm settings) were collected from different farms of Wayanad district and processed for bacteriological culture. The recovery rate of *E. coli* from fly was 100 per cent and that from fecal samples was 96.7 per cent and *Salmonella* spp. were recovered from 13.33 per cent of flies from farm settings, 3 (20 per cent) flies in non-farm settings, and 33 (15.7 per cent) from fecal samples. The study also revealed that the recovered *E. coli* and *Salmonella* spp. were multi-drug resistant strains and the *E. coli* isolates exhibited resistance to Ceftazidime (28.6%), Cefotaxime (49%), Ertapenem (1%), Imipenem (1.4%), and Meropenem (0.5%) whereas, *Salmonella* spp. isolates were resistance to Ceftazidime (12%), Cefotaxime (39.9%). None of them showed resistance to following drugs Doripenem, Ertapenem, Imipenem, and Meropenem. ESBL resistance was recorded more in *E. coli* (56%) than *Salmonella* spp. (48%) by phenotypic assays. In the molecular study, ESBL genes could be detected in the *E. coli* i.e., blaCTX-M (48.6%), blaSHV (10.5%), blaTEM (43.8%), and that in Salmonella isolates included blaCTX-M (48%), blaSHV (12.12%), blaTEM (57.57%). Only two isolates from pig faecal were positive for the carabapenemase producing OXA-48 gene. The present study is suggestive of the fact that flies harbour multidrug resistant bacteria such as, ESBL producing *E. coli* and Salmonella strains. Furthermore, Pig can act as a hub of ESBL resistance and the emergence of carbapenem resistant genes in pigs cannot be neglected. The monitoring of AMR needs to be routinely conducted inorder to eliminate the risk of AMR pathogen in food source.

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**KERALA, INDIA**

## സംഗ്രഹം

ആന്റിമൈക്രോബയൽ പ്രതിരോധം (എഎംആർ) ആഗോള ആരോഗ്യ ഭീഷണിയാണ്. എഎംആർ നിയന്ത്രണത്തെക്കുറിച്ചുള്ള ദേശീയ പ്രവർത്തന പദ്ധതി പ്രകാരം, എഎംആർ നിരീക്ഷണം നടത്തേണ്ടത് നിർബന്ധമാണ്. ആയതിനാൽ, വളർത്തുപന്നിയുടെയും ഫാം പരിസരത്ത് കുടുങ്ങിയ ഈച്ചകളുടെയും മലമൂത്രവിസർജ്ജന സാമ്പിളുകളിൽ നിന്ന് വേർതിരിച്ചെടുത്ത എസ്ഷെറിച്ചിയ കോളി, സാൽമൊണല്ല എന്നീ ഇനങ്ങളിലെ ആന്റിമൈക്രോബയൽ പ്രതിരോധം പര്യവേക്ഷണം ചെയ്യാൻ ഒരു പഠനം രൂപകൽപ്പന ചെയ്തു. വയനാട് ജില്ലയിലെ വിവിധ ഫാമുകളിൽ നിന്ന് മൊത്തം 210 പന്നികളുടെ മലാശയ സ്വാബുകളും 30 പൂൾ ഈച്ചകളും (ഫാം സജ്ജീകരണങ്ങളിൽ നിന്ന് 15 പൂളും ഫാം ഇതര ക്രമീകരണങ്ങളിൽ നിന്ന് 15 പൂളങ്ങളും) ശേഖരിച്ച് ബാക്ടീരിയോളജിക്കൽ കൾച്ചറിനായി പ്രക്രിയ ചെയ്തു. വീണ്ടെടുത്ത ഇ.കോളി, സാൽമൊണെല്ല സ്പീഷീസുകൾ മൾട്ടി ഡ്രഗ് റെസിസ്റ്റന്റ് സ്ട്രെയിനുകളാണെന്നും ഇ.കോളി ഐസൊലേറ്റുകൾ സെഫ്റ്റാസിഡിം (28.6%), സെഫോടാക്സിം (49%), എർടാപെനെം (1%), ഇമിപെനെം (1.4%) മെറോപെനെം (0.5%) എന്നിവയ്ക്കെതിരായ പ്രതിരോധം പ്രകടമാക്കിയിട്ടുണ്ടെന്നും പഠനം വെളിപ്പെടുത്തി. സാൽമൊണെല്ല ഐസൊലേറ്റുകൾ സെഫ്റ്റാസിഡിം (12%), സെഫോടാക്സിം (39.9%) എന്നിവയ്ക്കെതിരായ പ്രതിരോധം കാണിച്ചു. എന്നാൽ, അവയൊന്നും ഡോറിപെനെം, എർടാപെനെം, ഇമിപെനെം, മെറോപെനെം എന്നീ മരുന്നുകളോട് പ്രതിരോധം കാണിച്ചില്ല.

സാൽമൊണെല്ല സ്പീഷീസുകളേക്കാൾ (48%) ഇ.കോളിയിൽ (56%) ഇഎസ്ബിഎൽ പ്രതിരോധം ഫിനോടൈപ്പിക് അസെസ് വഴി രേഖപ്പെടുത്തിയിട്ടുണ്ട്. തന്മാത്രാ പഠനത്തിൽ, ഇഎസ്ബിഎൽ ജീനുകൾ ഇ.കോളി, അതായത് blaCTX-M (48.6%), blaSHV (10.5%), blaTEM (43.8%), സാൽമൊണല്ല blaCTX-M (48%), blaSHV (12.12%), blaTEM (57.57%) എന്നിവയിൽ കണ്ടെത്താനാകും. കാരാബപെനെമാസ് ഉത്പാദിപ്പിക്കുന്ന OXA-48 ജീനിന് രണ്ട് പന്നികളുടെ ഫെക്കൽ ഐസൊലേറ്റുകൾ മാത്രമേ പോസിറ്റീവ് ആയിട്ടുള്ളൂ. ഇഎസ്ബിഎൽ ഉൽപ്പാദിപ്പിക്കുന്ന ഇ. കോളി, സാൽമൊണല്ല സ്ട്രെയിനുകൾ പോലുള്ള മൾട്ടിഡ്രഗ് റെസിസ്റ്റന്റ് ബാക്ടീരിയകളെ ഈച്ചകൾ ഉൾക്കൊള്ളുന്നു എന്ന വസ്തുതയാണ് ഇപ്പോഴത്തെ പഠനം സൂചിപ്പിക്കുന്നത്. കൂടാതെ, പന്നിക്ക്

ഇഎസ്ബിഎൽ പ്രതിരോധത്തിന്റെ കേന്ദ്രമായി പ്രവർത്തിക്കാൻ കഴിയും, കൂടാതെ പന്നികളിൽ കാർബപെനെം പ്രതിരോധശേഷിയുള്ള ജീനുകളുടെ ആവിർഭാവം അവഗണിക്കാനാവില്ല. ഭക്ഷ്യ സ്രോതസ്സുകളിൽ എഎംആർ രോഗകാരിയുടെ അപകടസാധ്യത ഇല്ലാതാക്കുന്നതിന് എഎംആർ നിരീക്ഷണം പതിവായി നടത്തേണ്ടതുണ്ട്.

**Acad Form No. 19**

# KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY
## Faculty of Veterinary and Animal Sciences
## PROGRAMME OF RESEARCH WORK FOR THESIS FOR MASTERS DEGREE

1. Title of thesis

Occurrence of carbapenamase and extended spectrum beta lactamase producing major enteric bacteria in farmed pigs and house flies from farm premises

2. a) Title of the departmental/KVASU research project of which this forms a part

Not Applicable

b) Code No. if any, and order by which the departmental/KVASU research project is approved:

Nil

3. a) Name of student

Manoj M.

b) Admission No.

19-MVP-18

c) Name of the Discipline

Veterinary Public Health

4. a) Name of Major Advisor

Dr. Prejit

b) Address

Assistant Professor and Head i/c,
Department of Veterinary Public Health,
College of Veterinary and Animal
Sciences, Pookode, Wayanad – 673 576

5. Objectives of the study

1. Isolation and identification of *Escherichia coli* and *Salmonella* spp. from farmed pigs and house flies (*Musca domestica*) from farm premises
2. Molecular confirmation of the isolates by Polymerase Chain Reaction (PCR)
3. Detection of extended spectrum β-lactam and carbapenem resistance by phenotypic and genotypic methods

6. Practical/ Scientific utility

Intensively farmed pigs may act as a potential source of drug resistant bacteria due to the use of antibiotics as feed additives or growth promoters apart from their therapeutic purposes. Such antimicrobial agents elicit ‘selection pressure’ on pathogenic bacteria contributing to the development of anti-microbial resistance (AMR) that can be critically important in human medicine. Flies in and around farm premises make effective contact with animals, manure and the environment thereby transmitting anti-microbial resistant bacteria to the food chain.

The present study aims to assess the prevalence of drug resistant enteric bacteria such as carbapenem resistant and Extended spectrum beta-lactamase (ESBL) -

producing *E. coli* and *Salmonella* spp. among farmed pigs. The study shall also explore the role of flies in harbouring these drug resistant bacteria.

7. Important publications on which the study is based

Wang *et al.* (2011) recovered 144 *Salmonella* isolates from flies and swine stool samples of 11 pig farms in Taiwan and 71.5 per cent of them were resistant to atleast three antibiotics.

Anju *et al.* (2014) detected seven samples positive for *Salmonella* Enteritidis from a total of 210 samples from pigs analysed.

Gao *et al*. (2015) isolated 86 ESBL-producing *E. coli* from six pig farms and *bla*TEM/*bla*CTX-M genes were detected in all the ESBL-producing isolates.

García-Cobos *et al.* (2015) evaluated 282 faecal samples from 47 pig farms in Germany and determined the prevalence of ESBL- *E. coli* in 18.8 per cent of the samples. The most prevalent ESBL gene identified was *bla*CTX-M-1 (68.9 per cent), however, no isolates exhibited carbapenem resistance.

Mollenkopf *et al.* (2017) analysed 1,500 sow faecal samples for Carbapenem-resistant Enterobacteriaceae (CRE) in the United States; however, no CRE isolate was recovered, although $bla_{IMP}$ bearing CRE could be isolated from animal contact surfaces in a farrowing barn environment.

Onwugamba *et al.* (2018) reported that flies harbour ESBL and carbapenemase-producing bacteria of clinical importance that can 'bio-enhance' the transmission of resistance.

Garboui *et al.* (2019) identified multi-drug resistant *E. coli* (22 per cent) from housefly (*Musca domestica*) in Benghazi. Of the total *E. coli* isolates, 70 per cent were resistant to amoxicillin-clavulanic acid.

Poudel *et al.* (2019) trapped 493 flies from different livestock and other units and isolated *E. coli* from 43.9 per cent samples. The study revealed that 35.3 per cent of flies harboured antimicrobial resistant bacteria, of which nine per cent were multi-drug resistant.

8. Outline of the technical programme

A total of 180 pig’s faecal sample will be collected from a minimum of five pig farms located in Wayanad district of Kerala. The samples will also include 30 pools (10-20 flies per pool) of house flies trapped using fly-traps from farm (15) and non-farm (15) sources.

During each visit, six random pig faecal samples will be collected from each farm and sampling shall be repeated six times at a regular interval from the same farm. Fly trapping will be attempted during three visits per farm to obtain a total of 15 pooled samples of flies (10-20 flies per pool). A total of 15 pools of flies shall also be collected from different non-farm sources as a control. The genus identification of the flies shall be done by microscopic examination (10X) of the wing pattern (Sen and Fletcher, 1962). The flies will then be pooled, washed and centrifuged as per the protocol described by Zhang *et al.* (2018).

All the samples (216) will be subjected to isolation and identification of *Salmonella* spp. (Andrews *et al*., 2007) and *E. coli* (Meng *et al*., 2015).

Molecular confirmation of all the isolates will be carried out by PCR amplification of *invA* gene conserved for *Salmonella* (Galan *et al*., 1992) and *uidA* gene for *E. coli* (Alqahtani *et al*., 2015).

The isolates will be screened and interpreted for ESBL and carbapenamase production by antimicrobial susceptibility test using Kirby-Bauer method (CLSI, 2018).

The isolates will also be screened for the presence of $bla_{CTX\text{-}M}$, $bla_{TEM}$ and $bla_{SHV}$ genes conferring ESBL resistance (Bhoomika *et al.*, 2016) and $bla_{IMP}$, $bla_{OXA,}$ and $bla_{NDM}$ genes conferring carbapenem resistance (Pruthvishree *et al.*, 2017).

Data will be statistically analysed using SPSS version 24.0.

9. Main items of observations to be made

1. Isolates of *E. coli* and *Salmonella* spp.
2. Amplicons of *invA* and *uidA* genes
3. Antibiogram of the isolates
4. Amplicons of $bla_{CTX\text{-}M}$, $bla_{TEM}$ and $bla_{SHV}$ genes
5. Amplicons of $bla_{IMP}$, $bla_{OXA,}$ and $bla_{NDM}$ genes

10. Facilities

a) Existing

Existing facilities available in the Department of Veterinary Public Health, other departments and Central Instruments Laboratory of College of Veterinary and Animal Sciences, Pookode will be utilised.

b) Additional facilities required

Chemicals and biologicals

11. Duration of study

Four semesters

12. Financial estimate

Cost of chemicals and biologicals : Rs. 20, 000/-
Contingencies : Rs. 5, 000/-
Total : Rs. 25,000/-

Sd/-
Signature of student

Project coordination group proposed

Animal Biotechnology-II

Pookode,
20.09.2020

Sd/-
Signature of Major Advisor

Name, address and signature of members of the Advisory Committee

1. Dr. Prejit Sd/-
   Assistant Professor and Head i/c, Dept. of VPH, CVAS, Pookode
2. Dr. Binsy Mathew Sd/-
   Assistant Professor, Dept. of VPH, CVAS, Mannuthy
3. Dr. Jess Vergis Sd/-
   Assistant Professor, Dept. of VPH, CVAS, Pookode
4. Dr. Vinu David P Sd/-
   Assistant Professor, Dept. of Veterinary Medicine, CVAS, Pookode

## 13. References

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(*Musca domestica*) and blow fly (*Protophormia terraenovae*) as vectors of bacteria carrying colistin resistance genes. *Appl. Environ. Microbiol*. 84: 01736-17.

14. Time frame of work

Semester I

1. Collection of literature
2. Planning of programme of research work
3. Preparation of Synopsis

Semester II

1. Collection of samples
2. Procurement of primers, antibiotic discs and chemicals
3. Isolation of the *E. coli* and *Salmonella* spp. from pig faecal and house fly samples
4. Molecular confirmation of the isolates

Semester III

1. Collection of samples and identification of organisms
2. Phenotypic screening of isolates for ESBL and carbapenemase production
3. Genotypic screening of isolates for ESBL (*bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{SHV}$) and carbapenemase genes (*bla*$_{IMP}$, *bla*$_{OXA,}$ and *bla*$_{NDM}$)

Semester IV

1. Analysis of data and interpretation of the results
2. Preparation and submission of thesis

## CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the Prevention of Cruelty to animals Act (Amendment, 1998).

Sd/-

Place: Pookode
Dr. Prejit

Date: 20.09.2020
Major Advisor

# ANNEXURE I

## COMPOSITION OF REAGENTS

| **Buffered Peptone Water (HiMedia)** | **g/L** |
|---|---|
| Casein enzyme hydrolysate | 10.00 |
| Sodium chloride | 5.00 |
| Disodium hydrogen phosphate $12H_2O$ | 9.00 |
| Monopotassium hydrogen phosphate | 1.50 |

Final pH (at 25°C), 7.0±0.2

| **Mueller Hinton Agar** | **g/L** |
|---|---|
| Beef infusion | 300.0 |
| Casein acid hydrolysate | 17.5 |
| Starch | 1.5 |
| Agar | 7.0 |

Final pH (at 25°C), 7.3±0.2

| **Nutrient Broth** | **g/L** |
|---|---|
| Peptic digest of animal tissue | 5.0 |
| Sodium chloride | 5.0 |
| Beef extract | 1.5 |
| Yeast extract | 1.5 |

Final pH (at 25°C), 7.4±0.2

| **Normal Saline Solution (NSS)** | **g/L** |
|---|---|
| Sodium chloride | 8.5g |
| Distilled Water | 1000 ml |

| **Cary Blair Transport Media** | **g/L** |
|---|---|
| Sodium Thioglycolate | 1.50 |
| Disodium hydrogen phosphate | 1.10 |
| Sodium chloride | 5.00 |
| Calcium chloride | 0.09 |
| Agar | 5.00 |

Final pH (at 25°C), 8.4±0.2

| **MacConkey Agar** | **g/L** |
|---|---|
| Enzymatic Digest of Gelatin | 17.00 |
| Enzymatic Digest of Casein | 1.50 |
| Enzymatic Digest of Animal tissue | 1.50 |
| Lactose | 10.00 |
| Bile salts Mixture | 1.50 |
| Sodium chloride | 5.00 |
| Neutral red | 0.03 |
| Crustal Violet | 0.001 |
| Agar | 13.50 |

| **EMB Agar** | **g / L** |
|---|---|
| Peptic digest of animal tissue | 10.000 |
| Dipotassium phosphate | 2.000 |
| Lactose | 5.000 |
| Sucrose | 5.000 |
| Eosin - Y | 0.400 |
| Methylene blue | 0.065 |
| Agar | 13.500 |

Final pH ( at 25°C) 7.2±0.2

| **XLD Agar** | **g/L** |
|---|---|
| Yeast extract | 3.000 |
| L-Lysine | 5.000 |
| Xylose | 3.750 |
| Lactose | 7.500 |
| Sucrose | 7.500 |
| Sodium deoxycholate | 2.500 |
| Ferric ammonium citrate | 0.080 |
| Sodium thiosulphate | 6.800 |
| Sodium chloride | 5.000 |
| Phenol red | 0.080 |
| Agar | 15.00 |
| Final pH ( at 25°C) | 7.4±0.2 |

| **RV Broth** | **g/L** |
|---|---|
| Soya peptone | 4.50 |
| Sodium chloride | 7.20 |
| Potassium dihydrogen phosphate | 1.26 |
| Dipotassium hydrogen phosphate | 0.18 |
| Magnesium chloride, anhydrous | 28.60 |
| Malachite green | 0.036 |
| Final pH ( at 25°C) | 5.2±0.2 |

## ANNEXURE II

### Tris Acetate-EDTA (TAE) buffer (50X stock solution)

| | |
|---|---|
| Tris Base | 24.2g |
| Glacial Acetic Acid | 5.71ml |
| EDTA (0.5M) | 10.0 ml |
| Distilled Water | 100.0 ml |

Store at room temperature

For use dilute 1: 10 - distilled water for agarose gel electrophoresis

### Gel loading dye

| | |
|---|---|
| Sucrose 40 % (w/v) | 4g |
| Bromophenol Blue 0.25 %( w/v) | 25mg |
| Xylene cynol | 25 mg |
| Distilled water | 10ml |

**Ethidium Bromide** **10mg/ml (w/v)**

Stored in dark cool place at 40°C in amber coloured bottle

### Agarose gel (1%)

One gram of agarose was dissolved by heating in 100ml 1X TAE buffer

### MCFARLAND STANDARD

| | |
|---|---|
| 1.0% Barium chloride | 0.05ml |
| 1.0% Sulphuric acid | 9.95ml |
| Approx. cell density | $1.5\times10_8$ CFU/ml |

### PCR reaction buffer (1X)

100mM Tris (pH 8.3)

500mM KCL

15mM MgCl2, Gelatin 0.1% **Storage: -20°C**

# CURRICULUM VITAE

Name : Dr. MANOJ M.

DOB : 03/03/1994

Nationality : INDIAN

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## Academic qualification

| Qualification | University/ Board | Institution | OGPA | Year of Passing |
|---|---|---|---|---|
| BVSc & AH | Kerala veterinary and animal sciences university, Pookode, Kerala | College of veterinary and animal sciences, pookode | 6.82 | 2019 |
| MVSc | Kerala veterinary and animal sciences university, Pookode, Kerala | College of veterinary and animal sciences, pookode | ongoing | |
| PGDOH | Kerala veterinary and animal sciences university, Pookode, Kerala | College of veterinary and animal sciences, pookode | 7.9 | 2022 |

**Membership of Professional Bodies :** Kerala State Veterinary Council (KSVC)
Indian veterinary association (IVA)
Pookode unit