PREVALENCE AND CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN *KLEBSIELLA PNEUMONIAE* AND *ENTEROCOCCUS* SPECIES FROM FARMED POULTRY EGGS, ENVIRONMENT AND FARM HANDLERS

Thesis

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

MASTER OF VETERINARY SCIENCE
in
VETERINARY PUBLIC HEALTH AND EPIDEMIOLOGY

(Minor Subject: Veterinary Microbiology)

By

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2022

CERTIFICATE – I

This is to certify that the thesis entitled, **"PREVALENCE AND CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN *KLEBSIELLA PNEUMONIAE* AND *ENTEROCOCCUS* SPECIES FROM FARMED POULTRY EGGS, ENVIRONMENT AND FARM HANDLERS"** submitted for the degree of **M.V.Sc.,** in the subject of **Veterinary Public Health and Epidemiology** (Minor subject: **Veterinary Microbiology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Arpan Khehra (L-2019-V-89-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

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

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CERTIFICATE – II

This is to certify that the thesis entitled, **"PREVALENCE AND CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN *KLEBSIELLA PNEUMONIAE* AND *ENTEROCOCCUS* SPECIES FROM FARMED POULTRY EGGS, ENVIRONMENT AND FARM HANDLERS"** submitted by **Arpan Khehra (L-2019-V-89-M)** to Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfillment of the requirements for the degree of **M.V.Sc** in the subject of **Veterinary Public Health and Epidemiology** (Minor subject: **Veterinary Microbiology**) has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

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ABSTRACT

The study suggest that egg shell is a source of drug resistance microorganisms with potential public health consequences. The risk is more from eggs coming from deep litter system of management. Freshly laid eggs are sterile, however litter droppings in the environment, quickly contaminate them after being laid. In the present study a total of 480 eggs, 72 environment samples (feed, litter and water) and 6 hand swabs were collected from 24 farms (12 of each of deep litter and cage system) for isolation and identification of *K. pneumoniae* and *Enterococcus* spp. Twenty-three *K. pneumoniae* and 136 *Enterococcus* spp. were recovered irrespective of the type of management system. Contamination of the eggs in the deep litter system (4.58%) with *K. pneumoniae* was more in comparison to the eggs of cage system (2.91%). Examination of farm environment contamination revealed that 5 (6.94%) were positive for *K. pneumoniae* and 35 (48.61%) for *Enterococcus* spp. None of the hand swabs was positive for *K. pneumoniae* whereas 6 (100%) were positive for *Enterococcus* spp. The isolates of eggs and environment from deep litter system had high resistance (78.57%) to third generation cephalosporin as compared to 44.4% from cage system. A higher resistance to vancomycin in isolates from deep litter farms (16.17%) was observed in comparison to cage system farms (10.52%). The resistance to linezolid for *Enterococcus* spp. isolates was higher from deep litter (13.23%) than cage system (7.89). Seven isolates of *K. pneumoniae* and 45 of *Enterococcus* spp. were MDR. Higher number of MDR isolates were recovered from deep litter farms than cage system. *K. pneumoniae* isolates were ESBL positive and carried genes like $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}. Fifteen isolates were found to be vancomycin resistant using broth microdilution method and out of 15 isolates, 4(26.66%) carried *van*B gene and 3(20%) carried *van*C1 gene.

Keywords: AMR, eggs, *K. pneumoniae, Enterococcus* spp., ABST, MDR

Signature of Major Advisor **Signature of the student**

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LIST OF ABBREVIATIONS

%	: Per cent
=	: Equals, the sign of equality
°C	: Degree Celcius
µl	: Micro Litre
AK	: Amikacin
AMP	: Ampicillin
AMR	: Antimicrobial Resistance
AMS	: Amoxicillin
AST	: Antibiotic Sensitivity Testing
ATCC	: American Type Culture Collection
BEA	: Bile Esculin Agar
BHI	: Brain Heart Infusion Broth
CDC	: Centre For Disease Control And Prevention
CFR	: Case Fidelity Rate
CFU	: Colony Forming Unit
CHL	: Chlorumphenicol
CIP	: Ciprofloxacin
cm	: Centimetre
COT	: Co-Trimoxazole
CTX	: Cefotaxime
DO	: Doxycycline
E	: Erythromycin
EDA	: Enterococcus Differential Agar
EMB	: Eosin Methylene Blue Agar
ESBL	: Extended Spectrum β Lactamase
et al.	: And others
fig.	: Figure
g	: Unit of Relative Centrifugal Force
GEN	: Gentamicin
gm	: Gram

hr	: Hour
i.e.	: That is
ICMR	: Indian Council Of Medical Research
IMP	: Imipenem
K	: Kanamycin
KPC	: Klebsiella pneumoniae Carbapenemase
LZ	: Linezolid
MALDITOF-MS	: Matrix Assisted Laser Desorption/Ionization
MDR	: Multi Drug Resistant
MIC	: Minimum Inhibitory Concentration
min.	: Minute
ml	: Milli Litre
MLA	: MacConkey Lactose Agar
mm	: Milli Metre
No.	: Number
OD	: Optical Density
P	: Penicillin
TBE	: Tris-Borate-Ethylene-Diamene-Tetra Acetic Acid
TET	: Tetracycline
TSI	: Triple Sugar Iron Test
V	: Volume
viz.	: Which is
VP	: Voges Proskauer
VRE	: Vancomycin Resistant Enterococci
WHO	: World Health Organization

Chapter - I

INTRODUCTION

Antibiotics have proven to be the best invention in medical sector of twentieth century, however, antimicrobial resistance (AMR) is as ancient as the invention of antibiotics with penicillin resistance being observed a year after the discovery of penicillin by Alexander Fleming. Owing to the serious threat of medical complications and mortality due to AMR, it is a public health crisis of present and future generations. The magnitude of the problem could be assessed from an estimated fact that annually around 7,00,000 people lose life to AMR and the figure is expected to bulge to more than 10 million by the year 2050 if the current situation is left unchecked (O'Neill, 2016)

Researchers studying AMR have long been concerned about the role of food-producing animals, their products, and their environment in dissemination of AMR. Among different organisms, *Klebsiella* and *Enterococcus* have made it to the WHO list of 12 families of bacteria of Antibiotic-resistant priority pathogens that are the most dangerous to human health. The bacteria are widely present in food producing animals, their products (meat, milk, eggs) and the environment around the animal sheds which helps in maintaining the antibiotic-resistance genes in the ecosystem and their dissemination to human settings.

Enterococci are gram positive, catalase negative, facultative anaerobes and non-spore forming cocci occurring singly, in pairs or short chains (Nilsson et al., 2012). *Enterococcus* can grow at a wide temperature ranging from 10-42 °C and a wide pH. They can grow in 6.5% NaCl and can hydrolyse bile in the presence of 40% bile salts. (Quinn et al., 2011)

Both humans and animals have enterococci in their natural gut microbiome. They end up in the environment as a result of faecal contamination. They can contaminate water and vegetables in a variety of ways, and then infect the intestinal tracts of domestic and wild animals. Their tolerance to harsh environmental circumstances helps them to colonise a variety of biological niches and their propagation across the food chain via contaminated animals and foods (Giraffa, 2002).

The newly heightened ambiguity about enterococci relationships with humans is due to their enteric habitat, entry into the food chain, antibiotic resistance, and potential involvement in food-borne illnesses due to the presence of virulence factors like adhesin production and aggregation substances (Giraffa, 2002).Usually, *E. faecalis* is the most common species, followed by *E. faecium* but according to the latest trends depicted by ICMR, 2020 report, *E. faecium* was observed to be the most common species among the human isolates. The development of *E. faecium* as the dominant species in most parts of India in 2020 is cause for concern, as this species is significantly more drug resistant than *E. faecalis*.

In a study conducted on 981 retail meat commodities (chicken, turkey, pork, and beef) in Iowa USA presence of enterococci was tested. The samples yielded a total of 1,357 enterococci isolates, with contamination rates ranging from 97 % in pork samples to 100 % in ground beef samples. The most common species found was *E. faecium* (61%) followed by *E. faecalis* (29%) and *E. hirae* (5.7 %). The findings show that *Enterococcus* spp. are regularly found in retail meats (Hayes et al., 2003).

Horizontal transfer of resistance genes and transmission of multidrug-resistant *E. faecalis* lineages such as sequence type ST16 were two major concerns linked with ingestion of antimicrobial-resistant enterococci from poultry. *E. faecium* lineages found in poultry meat products are unrelated to those that cause hospital-acquired infections, but they may contribute quinupristin/dalfopristin resistance and other clinically relevant resistance determinants to the human gut microbiota (Bortolaia et al., 2016).Enterococci are resistant to many antibiotics and under antimicrobial stress can become resistant to important and last resort antibiotics such as aminoglycosides and glycopeptides (Murray, 1990). The major concern in recent times is the emergence of resistance to vancomycin (vancomycin resistant enterococci; VRE) vancomycin is considered a last resort antibiotic in humans to treat gram positive bacterial infections (Khan et al., 2005). Apart from this VRE also confers resistance to other pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* by transfer of resistant genes which can also be a cause of concern (Juhász-Kaszanyitzky et al., 2007).

Enterococcus have intrinsic and acquired resistant properties making them of significant importance as nosocomial infectants. There has also been resistance to penicillinase sensitive penicillin (low level), macrolides, cephalosporins, aztreonam,

nalidixic acids & low doses of aminoglycosides & clindamycin (Murray,1990). The vancomycin resistance genes *van*A, *van*B, *van*D, *van*E, and *van*G have been discovered in various *Enterococcus* spp., while *van*CI and *van*C2 are inherent (Fines et al., 1999).

On evaluation of the presence of *Enterococcus* spp. in poultry, swine, and cattle faecal samples: *E. faecium* was the most frequently isolated (50.8%), being predominant among poultry (71.6 %) and swine (37.7%). The second most frequently-isolated species from poultry and swine, were *E. durans* (23.2%) and *E. faecalis* (21.7%) (Bustamante et al., 2003).

Foodborne enterococci have not been definitively found as direct causes of clinical infections in humans but consumption of food containing antibiotic-resistant bacterial species is a plausible route of transmission, and could result in colonisation or resistance determinant transfer to host-adapted strains. They have been shown to add to the pool of antibiotic resistance genes, facilitating the spread of antimicrobial resistance among other organisms (Nilsson, 2012).

Klebsiella, on the contrary, is a non-motile, oxidase-negative, gram-negative, facultative anaerobic rod-shaped bacteria with a unique polysaccharide capsule. Among the 8 species that come under genus *Klebsiella*; *K. pneumoniae* is the most common human pathogen and one of the most important critical multidrug resistant microorganisms. *K. pneumoniae* is a member of the *Enterobacteriaceae* family and is found throughout nature and colonises the gastrointestinal tracts of humans and animals in a benign manner. It is an opportunistic pathogen that can cause a variety of diseases in people and other animals (Davis & Price 2016) especially the ones who are hospitalised or otherwise immunocompromised (Gorrie et al., 2017).

Klebsiella can be normally found in animal settings such as cattle yards, soil, poultry litter, and sawdust. As a result, animal-derived *Klebsiella* species could be a major source of drug resistance genes. *Klebsiella* has been isolated in animals and not just from clinical diseases such as mastitis, pneumonia, and diarrhoea, but also from seemingly healthy animals (Koovapra et al., 2016).

According to the ICMR annual report, 2020 a total of 1,07,387 culture positive isolates were investigated. There were 17,108 blood isolates, 30,822 urine isolates, 15,571 lower respiratory tract isolates, 25,058 superficial infections isolate, 7,053

deep infections isolate, 688 CSF isolates, 2623 sterile areas, 1051 faecal isolates, and 7,413 other infections. The majority of the isolates (53 %) were from the *Enterobacteriaceae* family. The most prevalent isolate was *Escherichia coli* (28 %), followed by *K. pneumoniae* (17 %). The same report also depicted an increase in prevalence of *K. pneumoniae* from 13.9% in 2016 to 17.5% in 2019 which reflected a greater resistance pattern of the species.

K. pneumoniae is a contaminant of retail meats & vegetables, a colonizer of livestock, & a human pathogen that causes extraintestinal illnesses (Davis & Price., 2016). In a study of 998 food samples, 99 (9.9%) *K. pneumoniae* strains were isolated; the frequencies were 13.8% (26/188) in fresh raw chicken, 11.4% (34/297) in frozen raw food,8.2% (4/49) in fresh raw seafood, and 7.5% (35/464) in cooked food samples (Guo et al., 2016).

Antibiotics are widely utilised in the poultry and dairy industries for a variety of purposes. *Klebsiella* spp. has developed severe resistance to most antibiotic drugs as a result of the widespread use and abuse of antimicrobial agents for boosting growth and treating infections in animals. Pan-resistant strains, hypervirulent strains and multidrug-resistant strains, in particular, have emerged (MDR), and the treatment of these strains has become complicated (Sonnevend et al., 2017)

Currently, *K. pneumoniae* is resistant to a wide range of antibiotics, including aminoglycosides, fluoroquinolones & beta-lactams (Fair & Tor, 2014; Dsouza et al., 2017). *K. pneumoniae* isolates that are multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) have been documented in different parts of the world (Bi et al., 2017, Guducuoglu et al., 2018). As a result of this, there is a rising global difficulty in choosing an effective antibiotic treatment for hospital-acquired infections (Davies & Davies, 2010). According to the ICMR report 2020, five species of bacteria offer a significant risk to human health with *E. coli* being the most prevalent, followed by *K. pneumoniae* (18%).

Antibiotics are also widely employed in food-animal production, where antibiotic-resistant microorganisms are selected. Antibiotic-resistant *K. pneumoniae* has been found in a variety of retail meats, seafood, and vegetables, as well as in animals(Davis & Price, 2016). *K. pneumoniae* is a neglected pathogen in veterinary medicine and the risk of human infection concerning animal contact and food consumption is barely

investigated. Only a few reports exist on antibiotic resistance of isolates from non-human origin (Wareth & Neubauer, 2021). Hence it is of utmost importance to study the bacteria from a public health perspective.

Antibiotics are being used at an unprecedented rate in the animal health sector as growth promoters, for prophylaxis and metaphylaxis (Aarestup et al., 2005). The poultry industry is one such example where antibiotics are used for growth promotion in addition to treatment, which therefore makes it a buffer for the antimicrobial resistant organisms to grow in response to the selective pressure of antibiotics (Nisha, 2008).

Poultry meat is frequently a target of AMR research in reaction to the apparent indiscriminate use of antibiotics (Kabir et al., 2004), but the role of eggs in sheltering and propagating resistant microbes is still considerably less researched. Furthermore, the eggs produced in hatcheries on the Indian subcontinent are frequently damaged and sent to consumers without being properly cleaned or disinfected, making them a potent vector of AMR germs to people (Taneja & Sharma, 2019). Hence, we hypothesise that the eggs produced in the current management system are the source of antibiotic resistance organisms through their contaminated surfaces. Since little emphasis has been laid on the eggs although it is a powerful source of transmission of AMR, hence we have conducted the present study with prime focus on eggs.

Therefore, the current study was proposed with the following objectives:

1. Identification & isolation of *Klebsiella pneumoniae* and *Enterococcus* spp. from the eggs, environment and poultry farm workers under different management systems.
2. Characterisation of phenotypic and genotypic antimicrobial resistance profile of the isolates.

Chapter II

REVIEW OF LITERATURE

Klebsiella pneumoniae and *Enterococcus* species.

Friedlander's bacterium was the name given to *K. pneumoniae* when it was originally isolated in the late 1800s. It is a gram-negative, oxidase-negative, facultative anaerobic rod-shaped bacteria with a notable polysaccharide capsule and is non-motile. *K. pneumoniae* is a member of the *Enterobacteriaceae* family, found throughout nature and colonises the gastrointestinal tracts of humans and animals in a benign manner. It is an opportunistic pathogen that can cause a variety of diseases in animals and people (Davis &Price, 2016) especially hospitalized or otherwise immunocompromised (Gorrie et al., 2017).

As initially defined, *K. pneumoniae* was a varied species with three different phylogroups: KpI, KpII, and KpIII. *K. pneumoniae* (KpI), *K. quasipneumoniae* (KpII), and *K. varicola*(KpIII) were the three species that emerged from these three phylogroups. *K. pneumoniae* can be found in a variety of environments and hosts, *K. varicola* is commonly found in plants. *K. pneumoniae* major reservoirs are unknown. While all three species can cause extraintestinal infections, *K. pneumoniae* and *K. varicola* are the most frequent, accounting for 80 and 18 % of *Klebsiella*-related human illnesses, respectively (Davis & Price, 2016).

Enterococci on the other hand are gram-positive, facultative anaerobes, non-spore forming cocci that can be found as single bacteria, pairs, or short chains (Fisher and Phillips 2009; Schleifer and Kilpper-Bälz, 1984). They are Lactic acid bacteria (LAB) that are widely detected in the gastrointestinal tract. They differ from other gram-positive, catalase negative, oxidase negative cocci in various phenotypic characteristics, including the capacity to survive in harsh environments such as high temperatures, high salt concentrations, low pH, and a bile tolerance of 4.0 % (Franz et al. 2011).

These bacteria live in the intestines of humans, other mammals, birds, reptiles, and insects (Martin & Mundt, 1972, Sherman, 1937). Initially enterococci were considered a component of the genus *Streptococcus*. In 1984, enterococci and streptococciwere proven to be different based on DNA hybridization experiments and

subsequently the genus *Enterococcus* was introduced (Schleifer & Kilpper-Balz, 1984).

Enterococci species have ability to grow between 10 - 45°C, in 6.5 % NaCl, and at pH 9.6. They can withstand heating at 60°C for 30 minutes, and can hydrolyse esculin into esculitine (Devriese et al., 1996). They ferment glucose to L(+)-lactic acid (Murray., 1990).

Enterococci survive as an indicator of fecal contamination but nowadays they are not considered so because of increasing ubiquitous character of these bacteria in the environment. Some enterococci strains especially *E. faecalis*, *E. faecium* and *E. mundtii* are able to produce bacteriocins, which are active against potent spoilage and pathogenic micro-organism in foods, such as *Listeria monocytogenes* (Kumar &Sirvastavaa, 2011). Some enterococci are used as probiotics (Todorov et al., 2008). However, their role as probiotic is still controversial because of their increased association with multiple antibiotic resistant agents, with nosocomial infection (Montalban-Lopez et al., 2011).

Enterococci have been identified from plants and soil and are found throughout the environment (Giraffa, 2002). They are also found in mammalian intestines (Franz et al., 1999). They have been identified from the faeces of dairy cows (Batish et al., 1984). Enterococci have been demonstrated to survive longer in the stool than other faecal markers (Sinton et al., 2007). *Enterococcus* could pose risk to food safety as prospective pathogens and may act as reservoir of resistance to antibiotics and could contribute to spoilage of food (Giraffa et al., 1997, Ogier & Serror, 2008; Franz et al., 2011).

Public health associated with *K. pneumoniae* and *Enterococcus* spp.

K. pneumoniae is critical in the transmission of AMR genes from commensals microbes to clinically significant pathogens. This bacterium has a large number of sought-after AMR genes, a high plasmid load, and a wide range of G + C content, indicating a wide range of horizontal gene transfer (HGT) partners and ecological range. *K. pneumoniae* is equally likely to be discovered living in human, animal, and environmental niches. These features could make *K. pneumoniae* a significant amplifier and spreader of clinically essential AMR genes when combined. Improved

knowledge and monitoring of AMR gene transfer could help prevent the spread of AMR and extend the life of new antibiotics (Wyres & Holt, 2018).

In a study conducted in America by Jones, (2010) pathogens most likely to cause Hospital-Acquired Bacterial Pneumonia (HABP) and Ventilator-Associated Bacterial Pneumonia (VABP) were analysed. *Staphylococcus aureus* (28.0%), *Pseudomonas aeruginosa* (21.8%), *Klebsiella spp.* (9.8%), *Escherichia coli* (6.9%), *Acinetobacter species* (6.8%), and *Enterobacter spp*. (6.3%) were the organisms that caused 80% of the occurrences. Drug resistance has been rising at a rate of 1% per year among HABP and VABP bacteria.

In a study, hospitals provided a total of 189 *K. pneumoniae* isolates. The resistance rates of the 189 isolates of *K. pneumoniae* to the 15 antibiotics tested ranged from 11.6 % to 77.8%. The nosocomial germs were more resistant to the 15 drugs than the animal-source strains. The nosocomial strains had the highest resistance to ampicillin (80.8%), followed by ciprofloxacin (75.0%) and kanamycin (69.2%), and the lowest resistance to azithromycin and meropenem (both 38.5 %). The resistance rates of isolates from pigs and chickens were greater than those of isolates from cows and sheep among animal-derived strains. It is hence believed that some *K. pneumoniae* strains from many sources, including humans and animals, share common molecular types and behaviours, implying that these strains could be transmitted between humans and animals. As a result, prudent antibiotic usage in human clinical care and animal production, as well as control measures to prevent *K. pneumoniae* transmission between humans and animals, are required (Yang et al., 2019).

Antimicrobial-resistant (AMR) *K. pneumoniae* high-risk clones provide a significant global public health threat. *K. pneumoniae* ST307 and ST147 are new clones in the species family of successful clones. Due to limited detection methodologies, both clones are likely under reported. Because of their widespread distribution, capacity to cause serious infections, and relationship with AMR, including pan resistance, ST307 and ST147 have the potential to become major public health issues. The medical community as a whole, particularly those concerned with antimicrobial resistance, should be aware of the threat posed by developing AMR high-risk clones (Peirano et al., 2020)

In humans, enterococci are one of the most common causes of nosocomial infections like urinary tract infections, surgical wound infections, and endocarditis. Because of the increased prevalence of antibiotic resistance, many illnesses can be difficult to treat. Non-human reservoirs of enterococci appear to play a key role in the acquisition and spread of antibiotic resistance determinants. Antibiotic resistance has become a cause of serious concern in veterinary and human health, particularly in Southeast Asia, where many developing countries lack the legislation and regulations to regulate antimicrobial supply and use (Daniel et al., 2015).

Enterococci are naturally resistant to a wide range of antimicrobials. It has been reported that the *van*A genotype has been found in *E. faecium* from animals and animal-derived food. *E. faecium*, a vancomycin-resistant pathogen, has become a common nosocomial pathogen. When hospitalized patients are treated with broad-spectrum antibiotics, the presence of *van*A or *van*B vancomycin-resistant enterococci in the gastrointestinal tract increases, increasing the risk of invasive infections. Antibiotics are widely used in food-producing animals, which leads to an increase in drug-resistant animal pathogens that can be passed to humans. For the creation of new antibacterial medications and the design of combination therapies with traditional antibiotics, creativity is required. As a result, better knowledge of a complicated and important public health issue like high-level vancomycin and gentamicin resistance in enterococci, as well as its consequences, is required (Sparo et al., 2018).

Occurrence of *K. pneumoniae* and *Enterococcus* spp. in foods of animal origin

A Zoonosis is defined by the World Health Organization as any disease or infection that is naturally transmitted from vertebrate animals to humans and vice versa. Direct or indirect transmission from animals to humans is possible. Contaminated animal or vegetable food products are one way for indirect transmission (Giraffa, 2002). The scenario for zoonotic agents spreading down the food chain, i.e., foodborne zoonoses, is the pathogenic organisms originating from animals and contaminating human food products. Meat items could be contaminated by faecal material at slaughterhouses, while vegetables in the field could be polluted by manure or sewage water used as fertilizers for irrigation.

Many antibiotic resistance genes have zoonotic potential so any resistant bacteria found in farm animals could serve as a reservoir for resistance that could spread, harming both veterinary and human treatment (Witte, 2000).

Meat

K. pneumoniae

India is the world's 5th largest meat producer, but has one of the lowest meat consumption rates in the world per capita. Poultry farming is India's most organized and fastest-growing animal agriculture sector. Layers grow at a rate of 6-8 % per year, and broilers expand at a rate of 10-12 % per year, compared to 2.5 % for agriculture as a whole. In comparison to the world average of roughly 17 kg per year, India's per capita intake of poultry meat is projected to be around 3.1 kg per year.

Davis et al. (2015) conducted a study on retail meat which included retail turkey, chicken and pork from different stores in Arizona a total of 508 retail meat products were purchased and it was seen that *K. pneumoniae* was found in 241 (47%) of the 508 retail meat products purchased locally. The prevalence of meat product contamination varied considerably by meat type, with 58 % (65/112) for pork, 47 % (128/272) for chicken, and 38 % (48/128) for turkey. Hence the study pointed out that retail meat is a potential source of *K. pneumoniae* from food animals to humans.

Gundogan et al. (2011) conducted a study where 60 calf and chicken meat samples were purchased from various supermarkets in Ankara, Turkey; a total of 45 *Klebsiella* isolates were identified. Out of the 45 *Klebsiella* isolates, 24 (53%) were classified as *K. oxytoca*, whereas 21 (47%) were identified as *K. pneumoniae*. The detected species are frequently implicated in various illnesses in people and animals. According to these findings, raw meat and its components could be substantial sources of potential pathogenic *Klebsiella* species.

In Oklahoma, USA, multidrug-resistant enteric bacteria were recovered from turkey, cow, and chicken farm environments, as well as retail meat products by Kim et al. (2005). A total of 132 *K. pneumoniae* isolates were recovered. Most samples gathered from farm environments and retail items included *K. pneumoniae* exclusively. The species was found in bird feathers, feeds, drinking water, and feces collected on a turkey farm, as well as 22 of 30 ground turkey products. This species

was also isolated from poultry and cow farm excrement and feeds, as well as retail beef and chicken items.

***Enterococcus* spp.**

A total of 981 retail raw meat samples, including pig, beef, chicken and turkey were collected randomly from 263 grocery stores in Iowa & they were cultured for *Enterococcus* spp. in a study conducted by Hayes et al. (2003). The samples yielded a total of 1,357 enterococci isolates, with contamination rates ranging from 97 % in pork samples to 100 % in ground beef samples. The most common species found was *E. faecium* (61%) followed by *E. faecalis* (29%) & *E. hirae* (5.7 %). *E. faecium* was identified as the most common species in chicken breast (79%), ground beef (65%) and ground turkey (60%) samples, whereas *E. faecalis* was the most common species found in pork chops (54 %).

Duckova, (2007) investigated the prevalence of *Enterococcus* spp. in foods of animal origin namely; pork, beef and poultry in Slovakia. A total of 110 samples of pork (n=70), beef (n=25) and poultry (n=15) were investigated. Out of 110 samples 75 were identified as *Enterococcus* isolates. *E. faecium* (60.53 %), *E. faecalis* (14.47 %), *E. casseliflavus*, *E. mundtti* (5.26 %), *E. durans* (1.31 %) were the species identified. *E. faecium* was the most common species found in pork (72%) and poultry (39%) samples, followed by *E. faecalis* (10% pork and 23% poultry), and *E. casseliflavus* (15 % in poultry). This investigation reported dominance of *E. faecium* in pork (72%) and poultry (39%), as compared to 10% and 23% prevalence of *E. faecalis* in pork and poultry, respectively.

` Pesavento et al. (2014) examined 636 raw meat samples (including 227 beef, 238 poultry, and 171 pork), 278 cheese samples (including 110 fresh soft cheese and 168 mozzarella cheese), 214 salad samples, and 187 ham samples). *Enterococcuss* spp. was detected in 311 (23.6%) of the 1315 examined samples. The prevalence of *Enterococcus* spp. was more in raw meat (33.2%) than in retail products(14.7%). Pork whole meat (44.3%) and sausages (41.3%) were found to be more affected than beef (30.8%) and chicken (28.6%). In retail products, it was observed that cheese (27.0%) was contaminated more than other products that were examined. In raw meat, *E. faecalis* was the most common species, whereas *E. faecium* was more common in retail items.

Milk

Klebsiella pneumoniae

Osman et al., (2014) conducted a study on the prevalence of *Klebsiella* mastitis pathogens in buffalo and cow populations in Egypt. A total of 525 milk samples were obtained aseptically from seemingly healthy, subclinical and clinical mastitic animals. A total of 45 *K. pneumoniae* isolates were recovered from buffalo and cow milk (45/525; 8.6%). The prevalence of *K. pneumoniae* in cows was found to be 11.9 %, but it was much lower in buffalos (4.3 %).

Donowitz et al. (1981) investigated a nosocomial outbreak in which during a 12-day period, five patients in a Neonatal Intensive Care Unit (NICU) had primary bacteremia caused due to *Klebsiella* after being fed contaminated breast milk for 24-96 hours. All of the patients were administered milk from a single donor via a nasoduodenal tube. By staining and culturing of the donor milk obtained via electric suction pump it was found positive for *K. pneumoniae*. The safety trap and breast-pump tubing were heavily infected with *K. pneumoniae*. The outbreak was contained by ensuring that the pump equipment was properly sterilized. Hence the study concluded that hospital equipment could be a potent source of nosocomial infections.

Katsande et al. (2013) conducted a cross-sectional study in Zimbabwe to investigate the prevalence of sub-clinical and clinical mastitis in cows from small dairy farm holders. All nursing cows having composite milk samples were taken for bacterial culture and somatic cell counts. A total of 584 animals were tested from 73 farms. Mastitis affected 21.1 % (123/584) of the cows, with 16.3 % (95/584) having sub-clinical mastitis and 4.8 % (28/584) having clinical mastitis. *Klebsiella* spp. had a prevalence of 15.5 % at the herd level.

In a study conducted by Salauddin et al. (2020) 48 clinical mastitis milk samples were gathered from Bangladesh's Rangpur district. The California Mastitis Test (CMT) was used to confirm bovine mastitis. All CMT positive isolates were subjected to a battery of cultural and biochemical assays to identify bacteria. Out of a total of 48 isolates *Klebsiella* spp. showed a prevalence of 62.5 % (n = 30/48).

In yet another study conducted in Canada, collection of samples was done from 91 dairies during a period of two years. From the above samples, 53 *Klebsiella* isolates were retrieved from clinical mastitis cases. A biochemical test panel &

matrix-assisted laser desorption ionization time of-flight mass spectrometry (MALDI-TOF) were used to identify *Klebsiella* isolated from clinical mastitis cases (n = 53) to the species level.*K. pneumoniae* (n = 40), *K. oxytoca* (n = 9), *Raulotellaornithinolytica* (n = 2), and *Raulotellaplanticola* (n = 2) were identified. Hence the prevalence of *K. pneumoniae* was 75.47% (Masse et al., 2020).

***Enterococcus* spp.**

Mc Auley et al. (2015) conducted a research in three dairying locations in Victoria, Australia, and looked for the prevalence of enterococci in raw milk factory silos and pasteurized milk. Over the course of a year, 211 raw milk samples were collected from 6 milk processing factories in three dairy areas in Victoria, yielding 909 enterococci isolates (96%). *E. faecalis* was the most common *Enterococcus* spp. found in each factory. Winter counts were substantially lower than summer counts. The most frequent *Enterococcus* spp. isolated from raw milk in every facility was *E. faecalis*, which accounted for 61.5 to 83.5 % of all *Enterococcus* species. Pasteurized milk had less enterococci than raw milk and was below the detection threshold. Pasteurized milk held at 4°C for two weeks was shown to include thermoduric enterococci suggesting the ability of enterococci to survive pasteurization and contribute to milk deterioration at refrigerator temperatures.

In a study conducted from retail establishments in the northwest of Iran, presence of enterococci was detected in pasteurized milk. Out of a total of 416 milk samples *Enterococcus* spp. were recovered from 56.25 % (n = 234) samples. Using species-specific PCR, *E. faecalis* and *E. faecium* were found in 36.77% (n = 153) and 27.88% (n = 116) of the samples, respectively. Both *E. faecalis* and *E. faecium* were found in 8.41% of the samples (n = 35*).E. faecalis* and *E. faecium* accounted for more than half of the pasteurized milk samples.As a result, pasteurization did not appear to be capable of totally eliminating enterococci. Therefore the study indicated that greater temperatures or longer holding times are required.(Nasiri & Hanifian, 2021)

In a study conducted in Baghdad researchers recovered *Enterococcus* spp. from cow milk samples and vaginal swabs from aborted and sick women. A total of 100 samples of each (milk and vaginal swabs) were taken and *Enterococcus* spp. was isolated. There were 31 *Enterococcus* spp. isolates (31%) out of 100 dairy milk samples, and 20 isolates (20%) out of 100 genital swabs from aborted women

samples. *E. faecalis* was the most commonly isolated species (Hamzah & Kadim, 2018).

In a study conducted by Jamet et al. (2012), a total of 126 retail soft, semi-hard and hard French cheeses were sampled for prevalence of enterococci. Enterococci were found in 44 % of the pasteurized milk cheeses & up till 92 % of raw-milk cheeses. *E. faecalis* was the most common bacteria found (81%) followed by *E. faecium* (13%) and *E. durans* (6 %).

Eggs

K. pneumoniae

Chun & Hong et al. (2009) conducted a research to detect microbial contamination in eggs, hence the study was conducted to identify microorganisms in any infected eggs in the northern Gyeonggi area. Four different brands of eggs were collected from hypermarkets and the overall bacterial counts on the egg shells was seen, which differed substantially amongst the groups.On eggshells *K. pneumoniae, P. mendocina, A. xylosoxidans, A. faecalis,* and *E. cloacae* were found. Hence the study advocated to reduce microbial contamination and promote human health. Therefore a tight quality control and improved distribution controls are essential to reduce contamination.

In a study conducted in eggs from commercial caged layer flocks in Australia a total of 1860 eggs (60 eggs each from 31 flocks) were collected for the recovery of *Enterobacteriaceae* on the eggshell surface and in the eggshell pore. Isolates from the *Escherichia* genus were reported most frequently (60.78 %), followed by *Salmonella* (9.15 %), *Enterobacter* (8.49 %), and *Serratia* (5.22 %) among all isolates identified. All other genera had a lower frequency of identification (5%). *Cedecea*spp., *C. freundii, E. carcinogenous, Enterobacter*spp.,*E. coli, E. fergusonii, K. pneumoniae, Kluyvera*spp., *Leclercia*spp. were among the species reported at least once (Gole et al., 2013).

In another study, eggs were obtained from three US commercial egg-processing plants to recover enteric bacteria entering plants and remaining during processing. Each plant inspection yielded isolates of *E. coli* and *Enterobacter* spp. Among the other genera isolated from at least one of the three plants included*Cedecea, Citrobacter, Erwinia, Hafnia, Klebsiella, Kluyvera, Leclercia,*

Morganella, Proteus, Providencia, Rahnella, Salmonella, and *Serratia*. The study indicated that from completely processed samples, all of the genera and species were retrieved less often (Musgrove et al., 2008).

A storage study in Georgia was undertaken with unwashed and commercially washed eggs to investigate the influence of processing on the safety and quality of retail shell eggs. *Enterobacteriaceae* isolates were chosen and biochemically analysed to see which species persisted after storage on cleaned or unwashed eggs. Most of the unwashed eggs isolates belonged to *E. coli* and *Enterobacter.* Many more genera and species were found among the isolates from the unwashed control eggs like *Citrobacter, Klebsiella, Kluyvera, Pantoea, Providencia, Rahnella, Salmonella, Serratia,* and *Yersinia.* Only a small number of washed egg samples were found to be infected with any of these pathogens. These findings are useful for determining how efficient processing is at removing bacteria from commercial shell eggs. (Musgrove et al., 2004)

In a study done in Germany, a total of 799 cloacal swabs and 800 eggs were analyzed from ten organic and ten conventional laying hen farms.More than 95% (n=760) of the cloacal swabs tested positive for *Enterococcus* spp.The presence of multiple *Enterococcus* spp. in a single swab was detected in more than 30% of the cases. Enterococciwere found in 21% of the pooled egg samples (n=168), as well as 53% eggshell samples (n=424)(Schwaiger et al., 2010).

A total of 2828 *Enterococcus spp.* were collected from poultry sources in a study conducted in Poland, with 82.6 percent (2337/2828) coming from poultry, 8.8 percent (250/2828) from a poultry production environment, 2.5 percent (71/2828) from hatching eggs, and 2.5 percent (71/2828) from dead-in-shell embryos. Chicken was the source of the most numerous enterococci strains (73.7 %; 1723/2337), with 61.2 % (1431/2337) from commercial broiler chickens and 7.2 % (168/2337) from commercial layers. There were 9 most frequently isolated species in poultry ; *E. faecalis* (42.5 %) > E. *cecorum* (7.9%) >*E. faecium* (4.4%) >*E. hirae* (2.7%) >*E. gallinarum* (2.2%) > E. casseliflavus (0.6%) >*E. durans* (0.2%) >*E. avium* (0.16%) >*E. aquimarinus* (0 (0.04 %) (Dolka et al., 2017).

In an experiment conducted in Sweden; 480 hens, aged 17 to 78 weeks were housed in either furnished (with litter box, nest box and a perch) 8-hen cages or

conventional 4-hen cages. Hy-Line White and Hy-Line Brown commercial genotypes of hens were employed for the experiment. When the birds were 28 and 62 weeks old, they were tested for aerobic bacteria such as *Enterococcus* and *Enterobacteriaceae* on the shells of their eggs. It was seen that in comparison to eggs from conventional cages, eggs from furnished cages had greater concentrations of aerobic bacteria including *Enterococcus*. In addition, in the furnished cages, there was a larger percentage of eggs with *Enterococcus* on the shell than in the conventional cages. The proportion of eggs with *Enterobacteriaceae* present on the shell tended to be higher at 62 week of age than at 28 week of age, although the quantity of aerobic microorganisms was higher at 28 week of age (Wall et al., 2008).

Environment and other sources

K. pneumoniae

Wu et al. (2016) examined 90 antimicrobial-resistant *Klebsiella* bacteria found in a commercial broiler slaughterhouse in China's Shandong region. Almost majority of the isolates (89/90) were found to belong to the infectious phylogenetic group Kpl-type.

Munoz et al. (2006) conducted a study, 595 faecal samples from healthy dairy calves were examined for *Klebsiella*. More than 80% of faecal samples tested positive for *K. pneumoniae* It was found that the average prevalence of *K. pneumoniae*-positive faecal samples was above 80% in a cross-sectional survey of 100 cows from ten herds in New York and Massachusetts. They theorised that dairy cows' faecal shedding of *Klebsiella* contributed to *Klebsiella'*s prevalence in the environment.

In Germany, Podschun et al. (2001) evaluated the presence of several *Klebsiella* spp. in 208 samples of natural surface waters. It was found that 123 *Klebsiella* strains were recovered from 53 % of them with *K. pneumoniae* being the most frequent species. When these isolates were compared to a set of 207 clinical *K. pneumoniae* isolates, it was shown that water isolates also had virulence characteristics.

***Enterococcus* spp.**

Ben Said et al. (2016) conducted a study, where a total of 90 samples of food vegetables, soil, and irrigation water collected from various farms and markets in Tunisia. Slantez Bartley agar plates were inoculated with enterococci and the bacteria

was isolated from 65/90 (72.2%) samples. Food, vegetables from farms had an enterococcal recovery rate of 88.2 %, food vegetables from markets had an enterococcirecovery rate of 84.2 %, and soil and irrigation water had an enterococcal recovery rate of 51.3 %. The most common species found was *E. faecium* (52.3%), followed by *E. hirae* (35.4%), *E. faecalis* (6.15%), and *E. casseliflavus* (6.15%).

Da Silva et al. (2006) conducted research to look at the ecology of enterococci and associated bacteria in both raw and treated wastewater from a treatment facility in Portugal. A total of 148 isolates were identified as *Enterococcus* spp. based on their ability to proliferate and create blackening on Bile Esculin Azide agar. *E. hirae*, *E. faecium*, and *E. faecalis* were the most common bacteria discovered in the raw wastewater. Treatment of wastewater resulted in a decrease in *E. hirae* and an increase in *E. faecium*; the relative proportions of *E. faecalis* in the raw and treated wastewater remained the same.

Stepien-Pysniak et al. (2016) conducted a study in Poland to determine the frequency of enterococcus bacteria in different types of poultry's internal organs (heart, liver, brain, and oviduct swab). Out of a total of 2,970 samples which were examined, 911 (30.7 %) were positive for *Enterococcus* spp. broilers (88.1%), laying hens (5.3%), turkeys (3.9%), breeding hens (2.2%), & geese (2.2%) all tested positive for enterococci. *E. cecorum* (4.6%), *E. hirae* (4.6%), *E. gallinarum* (5.5%), *E. faecium* (10.1%) and *E. faecalis* (74.7%) were the most commonly recognised species.

Antibiotic resistance isolates in meat

K. pneumoniae

In an investigation, 1728 human clinical isolates and 508 locally purchased retail meat products were examined in Arizona. *Klebsiella* was found in 174 (10%) human clinical isolates, 241 (47%) of the locally purchased retail meat products. The prevalence of meat product contamination differed significantly by meat type, with 58% (65/112) for pork, 47%(128/272) for chicken, and 38%(48/128) for turkey. Out of 241 meat source isolates 44 isolates and out of 174 human isolates 38 were randomly chosen. Multidrug resistance was found in around 22% of all isolates, including 32% of meat-source isolates and 8% of clinical isolates. Meat-source isolates were resistant to significantly more individual antibiotics and antibiotic

classes (amikacin, ampicillin, ampicillin-sulbactam, cefazolin, cefoxitin, ceftriaxone, ceftazidime, cefotaxime, ciprofloxacin, gentamicin, imipenem, nalidixic acid, tetracycline, and trimethoprim-sulfamethoxazole) than human isolates.The close genetic relatedness of meat-source and clinical isolates, as well as virulence similarities, imply that transmission barriers between these two sources are low. Findings show that retail meat could be a vector for transferring virulent, antibiotic-resistant *K. pneumoniae* from food animals to people. (Davis et al., 2015)

In a study conducted in local and imported poultry meat in Ghana, a total of 200 meat samples were obtained from open markets and supermarkets across Kumasi, with 68 samples from native Ghanaian poultry and 132 samples from imported poultry meat. Out of 200 meat samples, 46 were *E. coli* and 35 were *K. pneumoniae* Extended spectrum Beta Lactamase producing isolates. $bla_{\text{CTX-M-15}}$ was the most commonly discovered ESBL genotype, with $bla_{\text{CTX-M-15}}$ found in 67 % of *E. coli* (31/46) and 86 % of *K. pneumoniae* (30/35) isolates. In *E. coli*, $bla_{\text{CTX-M-15}}$ was followed by $bla_{\text{CTX-M-1}}$(11 %; 5/46) and$bla_{\text{CTX-M-2}}$ (9 %; 4/46) genotypes, and in *K. pneumoniae*, $bla_{\text{CTX-M-4}}$(6 %; 2/35) and $bla_{\text{SHV-12}}$ (6 %; 2/35) genotypes. Concomitant ciprofloxacin resistance was found in 56 of the 81 ESBL-producing isolates (69%) and was found in 83% *K. pneumoniae* isolates & 59 % *E. coli* isolates. *K. pneumoniae* isolates had higher resistance to the antibiotic's gentamicin and trimethoprim/sulfamethoxazole than *E. coli* isolates. Surveillance along the poultry production-food-consumer chain could be a useful method for identifying developing multidrug-resistant disease origins (Eibach et al., 2018).

Gundogan et al. (2011) conducted a study where 60 calf and chicken meat samples were purchased from various supermarkets in Ankara, Turkey; that yielded *Klebsiella* isolates. Virulence characteristics such as hemolytic activity (67%), siderophore generation (44%), and serum resistance were found in a significant percentage of *Klebsiella* isolates (38%). The ESBL production was determined using the double-disk synergy test. In 29% (13/45) of the *Klebsiella* isolates, ESBL production was found. The disc diffusion method was used to assess resistance to 14 antimicrobials. All of the isolates were resistant to at least two antimicrobials. All *Klebsiella* isolates that produced ESBL were resistant to cephalosporins and monobactams. Meat and its components, hence are potentially dangerous sources of multidrug-resistant and aggressive *Klebsiella* species.

In Oklahoma, USA, multidrug-resistant enteric bacteria were retrieved from turkey, cow, & chicken farms, in addition to retail meat products. MDR *K. pneumonia* was found in the majority of the samples collected. A total of 132 *K. pneumoniae* isolates were described. All the isolates were resistant to kanamycin, gentamycin, streptomycin, tetracycline and ampicillin and were most frequently isolated from a turkey farm and ground turkey products (Kim et al., 2005).

In a study six antibiotics were tested against *Klebsiella* species isolated from free range chicken samples in South Africa; ampicillin, ciprofloxacin, gentamicin, nalidixic acid, tetracycline, and trimethoprim were the antibiotics tested. It was found that 50% were resistant to trimethoprim, 59.8% to tetracycline, 61.8 % to nalidixic acid and 66.7 % were resistant to ampicillin. Surprisingly, 40 % of the *Klebsiella* spp. identified had multiple antibiotic resistance to at least three of the six medications examined (Fielding et al., 2012).

***Enterococcus* spp.**

Krocko et al. (2011) conducted a study to find the prevalence of enterococci and antibiotic resistance in 110 samples of raw pork (n=70; 64%), raw beef (n=25; 23%), and poultry (N=15; 12%). Out of the total 110 samples, 75 were positive for enterococci. *E. faecium* was the most common bacteria found in pig (72%) and poultry (39%) samples, followed by *E. faecalis* (23% in poultry) (10% in pork). 15% of the enterococci isolates were resistant to vancomycin, 27% to ampicillin, 15% to erythromycin, 25% to gentamicin & 56% to tetracycline. It was observed that a greater incidence of intermediate resistant isolates was present in pork and poultry to ampicillin (70 and 40%, respectively), gentamicin (66 and 40%), tetracycline (only pork 54%), and erythromycin (only pork 64 %)

In a study conducted by Aslam et al. (2010), antimicrobial resistance of *Enterococcus* spp. isolated from a commercial beef processing factory was studied. Before the start of the operation, 2 hours after the start of the operation, and from ground beef, samples were taken from conveyers used to move carcasses. PCR was used to confirm the genus and species of isolates from each positive sample. The most prevalent species detected was *E. faecalis* (87%), followed by *E. faecium* (10%). Quinupristin–dalfopristin resistance was found in 42% of *E. faecium.*

Donado-godoy et al. (2015) conducted research to find antimicrobial resistance patterns of *Salmonella* serovars, *Escherichia coli*, and *Enterococcus* spp. isolates in retail poultry meat from independent stores and a main chain distribution center in Colombia. *Salmonella* serovars and *E. coli* were recovered from 51 (26%) and 165 (83%) of the meat samples, respectively, while *E./ faecalis* and *E. faecium* were found in 188 (81% and 13% respectively) of the meat samples. Over 98 % of the isolates examined were multidrug resistant. 61.5 % of *E. faecium* isolates were found to be resistant to quinupristin-dalfopristin in enterococci. This is relevant since it is used to treat nosocomial infections that are resistant to vancomycin. Resistance to vancomycin was found in 4% of the *E. faecalis* isolates.

In Alberta, Canada, Aslam et al. (2012) investigated antimicrobial resistance (AMR) and pathogenicity genotypes of *Enterococcus* spp., specifically *E. faecalis*, isolated from retail meats (pork, beef, turkey and chicken). There were 94, 192, 87 & 92 *E. faecalis* isolates from beef, chicken, turkey, and pork, respectively. There were 3, 9, & 2 *E. faecium* isolates from beef, chicken, and pork, respectively. Number of *E. hirae* isolates from beef, chicken and pork were 27, 3 and 8, respectively. Antimicrobial susceptibility was tested on a total of 532 enterococci, one isolate from each positive sample. *E. faecalis* was found in more than 94 % of chicken, 73 % of beef samples, and 86% of pork samples. *E. faecium* was not found in turkey meat, although it was found in 2% of beef and pork samples and 4% of chicken samples. The clinically essential antibiotics ciprofloxacin, daptomycin, linezolid and vancomycin were not resistant to any of the enterococci isolates.

Milk

Klebsiella pneumoniae

Gaffer et al. (2019), conducted a study on milk and milk products where 59 ESBLs producers colonies (24 from raw milk, 15 from kareish cheese, 13 from Damietta cheese and 7 from ice cream) were biochemically identified as *E. coli* (n = 29, 49%), *K. pneumonia* (n = 8, 13.5%), *K. oxytoca* (n = 5, 8.5%), *E.aerogenes* (n =8, 13.5%), *C. diversus* (n = 8, 13.5%) and *S. liquefaciens* . *E. coli* isolates demonstrated high resistance to cefaclor (100 %), cefoxitin (100 %), cefalexin (96.5 %), ceftazidime (93 %), penicillin (93 %), and Piperacillin/Tazobactam (79 %). Penicillin, ampicillin, cefalexin, cefaclor, cefoxitin, and ceftazidime resistance was 100 % in all *Klebsiella*

spp. isolates (n = 13). All the recovered isolates, on the other hand, were susceptible to imipenem and meropenem.The gene encoding $bla_{\text{CTX-M1}}$ was the most common among the examined genes, appearing in 13 (48%) of the dairy samples. In the case of bla_{TEM} and bla_{SHV}, they were found in 12 (44%) and 4 (14.8%) dairy sample isolates, respectively.

A study was carried to identify *Klebsiella* species found in clinical mastitis cases in Canada. All tetracycline-resistant *K. pneumoniae* and *R. ornithinolytica* strains (n = 10) carried the *tet*B and *tet*A genes, respectively. The *sul*1 gene was found in all sulfisoxazole-resistant strains (n = 7). Six of the seven isolates resistant to streptomycin and spectinomycin carried the *aad*A gene. The *tet*D gene was not observed in any of the *Klebsiella* or *Raoultella* resistant isolates. Among the 53 isolates tested, 31 (58%) were susceptible to all antimicrobials tested. The remaining 22 (42%) isolates were resistant to 1 or more of the following antimicrobials: kanamycin (2%), streptomycin (38%), spectinomycin (13%), sulfisoxazole (13%), and tetracycline (19%). Only one (2 percent) K. pneumoniae isolate was multi-drug resistant to three antibiotic classes (aminoglycosides, tetracyclines & sulfonamides) (Massé et al., 2020).

Koovapra et al. (2016) conducted a study where 291 *Klebsiella* spp. were isolated from 340 bovine milk samples. ESBL E-test identified 23 *K. pneumoniae* as ESBL producers. The study looked at 23 *K. pneumoniae* strains that produced extended spectrum -lactamases and were isolated from milk samples (n = 340) from healthy cows (n = 129) and cows with subclinical (n = 159) and clinical (n = 52) mastitis in three different Indian states: West Bengal, Jharkhand, and Mizoram. Seven of them were also β-lactamase producers of the AmpC type. Mastitic cows' milk samples had considerably more ESBL-producing *K.pneumoniae* than healthy cows' milk samples. The $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} β-lactamase genes were found in 19, 8, and 3 isolates, respectively (Koovapra et al., 2016).

In a study conducted in Iran, 416 pasteurized milk samples were acquired from retail establishments (Nasiri & Hanifian, 2021) for study on the prevalence of enterococci in pasteurized milk and their virulence features. In total, 269 isolates were recognized as *E. faecalis* or *E. faecium*, and genotypic and phenotypic analyses were performed on them. Most of the antibiotics examined were extremely resistant to *E. faecalis* and *E. faecium* isolates. Resistance to vancomycin was estimated to be 71.9

% for *E. faecalis* and 77.6 % for *E. faecium*, respectively. Furthermore, biofilm was produced by 81 % and 69 % of *E. faecalis* and *E. faecium* strains, respectively. Pasteurized milk was found to be a possible intermediary vehicle for the transmission of highly resistant enterococci strain to the humans.

Jamet et al. (2012) conducted a study in which the prevalence of enterococci and antibiotic resistance profiles of *E. faecalis* were investigated. Enterococci were found in 44 % of the pasteurized milk cheeses & up till 92 % of raw-milk cheeses. In total, 337 antibiotic-resistant enterococci were found in 29 % of pasteurized-milk cheeses & 60 % of raw-milk cheeses. *E. faecalis* was the most common antibiotic-resistant bacteria found (81%) which was followed by *E. faecium* (13%) & *E. durans* (3%). Tetracycline and minocycline resistance was the most common, followed by erythromycin,kanamycin and chloramphenicol resistance.

Antimicrobial resistance genes

K. pneumonaie

Kim et al. (2005) isolated 132 strains of *K. pneumoniae* from Korean turkeys and poultry farms. The majority (96%) had the *bla*SHV-1 gene. Both SHV-11 and TEM-1 β-lactamases were expressed in five strains. Pulsed-field gel electrophoresis was used to determine DNA restriction patterns. The same clones of multidrug-resistant K. pneumoniae were found in turkey feathers, drinking water, faeces & feed, demonstrating the spread of antibiotic-resistance genes in the ecosystem and antibiotic-resistant bacteria cross-contamination during processing and distribution.

In a study conducted by Li et al., (2012) a tertiary-care hospital in Beijing, China provided 223 *K. pneumoniae* isolates. The following drug resistance-associated genes were found at relatively high rates: $bla_{\text{CTX-M-10}}$ (35.9%), *aac*C2 (32.7%), *dhfr* (27.8%), *qnrS* (26.0%), *aac* A4 (25.6%), and *aad*A1 (25.1%).

Aminoglycoside resistance was found in seven *Klebsiella pneumoniae* isolates from dogs and cats in Spain. *bla*DHA-1 and *bla*SHV-11 B-lactamases, as well as the *qnr*B4 resistance determinant, were discovered to be coproduced by the isolates (Hidalgo et al., 2013)

K. pneumoniae was isolated from cloacal swabs, pooled faeces, boot swabs, litter, dust, and air from the broiler production chain by Daehre et al. (2018) in

Germany. All of the isolates were multidrug resistant and carried the genes for the ESBL beta-lactamase *bla*$_{SHV}$.

In research carried out in China, Cheng et al. (2018) recovered 33 *K. pneumoniae* strains from 213 cattle nasal swab samples, with ESBL genes discovered in 93.4 % of the strains, with *bla*$_{TEM}$ being the most common (93.4 %), followed by *bla*$_{CTX-M}$and *bla*$_{SHV}$ at 57.6% and 39.4%, respectively.

K. pneumoniae was isolated from milk samples (n = 340) of healthy cows (n = 129) and cows with subclinical (n = 159) and clinical (n = 52) mastitis from three different states of India viz West Bengal, Jharkhand and Mizoram. Twenty-three extended spectrum β-lactamase producing *K. pneumoniae* (strains were isolated from milk sample) (n = 340) of healthy cows (n = 129) and cows with subclinical (n = 159) and clinical (n = 52) mastitis. The β-lactamase genes *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{SHV}$ were detected in 19 (82.7%) 8(34.8%) and 3(13.04%) isolates, respectively. Ten of them carried plasmid mediated quinolone resistance gene-*qnr*S and one isolate possessed *qnr*B. Again 11(47.8%) of them were found to have sulfonamide resistance gene s*ul*1 (Koovapra et al., 2016).

In the Indian states of West Bengal & Jharkhand, 50 Gram-negative organisms (Enterobacter, Escherichia coli, Proteus, Klebsiella and Pseudomonas) were recovered from milk samples of subclinical mastitis infected cattle. ESBL-producing or tetracycline-resistant isolates were detected in 48 % (24/50) of the Gram-negative isolates. In a total of 50 Gram negative isolates, blaCTX-M was found in 18 (36%) of them, & 6 (12%) of them had blaTEM genes in PCR. blaSHV genes were not found in any of the isolates. In addition, 5 (10%) of the isolates in this investigation carried the tetA gene, while 8 (16%) carried the tet gene. The tet gene was not found in any of the samples (Das et al., 2017).

Tripathi (2017) isolated MDR strains of bacteria from poultry environment (poultry, poultry farm soil and nearby agricultural soil). Overall, *E. coli* had the highest resistance, followed by *K. pneumoniae* and *S. lentus.*

Singh, (2018) reported 3240 bacterial isolates of ESKAPE (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumanni*, *P. aeruginosa*, *Enterobacter sp*.) group from veterinary clinical cases and related sources during period from 2011 to 2017.

Metallo-ß-lactamase (MBL) and ESBL production was detected in 5% and 54 % ESKAPE isolates, respectively.

A total of 321 cloacal swabs were taken from healthy broiler, kuroiler and indigenous birds in various locations across West Bengal, India. The rate of *Klebsiella* spp. isolation varies depending on the species of poultry bird (43.8-72.3 percent). In total, 33 (10.7%) *Klebsiella spp*. isolates were detected phenotypically as $bla_{\text{CTX-M}}$ producers and all the isolates possessed $bla_{\text{CTX-M}}$ in polymerase chain reaction. Whereas 17 (51.5%) and 16 (48.5%) *Klebsiella spp.* isolates possessedbla_{SHV}, and bla_{TEM} with $bla_{\text{CTX-M}}$, respectively (Mahanti et al., 2018).

Bobbadi et al. (2020) reported that ninety nine out of 336 samples consisting of animal intestines and foods of animal origin were found to harbor *Klebsiella spp*. in Tirupati. Out of these, 89 were identified as *K. pneumoniae* and 10 as *K. oxytoca* by PCR. ESBL genes were identified in 32 isolates of *K. pneumoniae* and an isolate of *K. oxytoca*, bla_{SHV} being the predominant gene (31), followed by bla_{TEM}, $bla_{\text{CTX-M1}}$ and $bla_{\text{CTX-M9}}$ (one each). The *K. oxytoca* isolate harbored$bla_{\text{CTX-M2}}$ gene.

***Enterococcus* spp.**

Enterococci have been found to have a wide spectrum of intrinsic antibacterial resistances (Moellering 1991, Murray 1990, Leclercq & Courvalin 1997), as well as the ability to develop high levels of drug resistance to particular antibiotics (Moellering 1990, Murray 1990, Leclercq & Courvalin 1997). Resistance can be caused by poma mutations in the drug binding site, as seen in quinolones (Onodera et al., 2002) and ampicillin, or by the acquisition of resistance genes, as seen in aminoglycosides, macrolides, chloramphenicol, tetracycline, and glycopeptides, of which vancomycin resistance is the most clinically relevant (Moellering 1991, Murray 1990, Landmar & Quale 1997, Leclercq 1997 & Onodera et al., 2002).

Vancomycin is a last resort antibiotic that has been designated as critically important in human medicine for the treatment of individuals with multidrug-resistant *Enterococcus* spp. infections. The majority of gram-positive bacteria are susceptible to vancomycin, while the majority of gram-negative bacteria are resistant (French, 1998).

The presence of vancomycin resistant enterococci in chicken, swine, and cow faecal samples was investigated by Bustamante et al. (2003). The most commonly

isolated species was *E. faecium* (50.8%), which was found in the majority of poultry (71.6%) and swine (37.7%) isolates. *E. durans* (23.2 %) and *E. faecalis* (23.2 %) were the second and third most often isolated species from poultry and swine, respectively.

Purohit et al. (2017) conducted a study on characterization of vancomycin resistant enterococci in clinically significant infections in hospitalized patients. A total of 250 clinically significant isolates of enterococci were studied. *E. faecium* (162, 64.8%) was most common followed by *E. faecalis* (82, 32.84%) and *E. gallinarum* (6, 2.4%). Vancomycin MIC $\geq$ 2 μg/mL was noted in 63 (25.2%) isolates.Fifty-seven isolates showed presence of *vanA* and *vanC1* was detected in six isolates of *E. gallinarum*. Isolates with *VanB* genotype was not detected. Teicoplanin, tigecycline, linezolid, , daptomycin & quinupristin-dalfopristrin had MIC_{50} (μg/mL) values of 24, 0.064, 0.75, 2 & 0.064, respectively. Resistance to linezolid (1, 1.6%) and tigecycline (2, 3.2%) was rare. Majority (33/47, 70.2%) patients with clinically significant VRE infection showed gut colonization.

Lawpidet et al. (2021), conducted research on global prevalence of vancomycin-resistant enterococci in food of animal origin. A total of 50 publications were included among the 1352 studies that were retrieved. VRE was found to be present in 11.7 % of animal-based foods. VRE was found most frequent in aquatic food (43.4 %**)** and least frequently in dairy food (4.1 %). Secondly, according to continents, VRE was most common in Africa (18.5 %) and least common in North America (0.3 %). Since 2004, the pooled prevalence of VRE has varied between 10.5 % and 20.5 %, dropping from 79.3 % in 1998 to 13.1 % in 2003. According to the results of the meta-regression, the prevalence declined over time. Finally, the global prevalence of VRE in food of animal origin was estimated to be 12%, highlighting the burden of VRE contamination in food of animal origin.

Foka et al. (2019) carried out a study to document antibiotic resistance profiles and the presence of virulence genes in VREs isolated from feedlot cattle in South Africa's North-West Province. Six registered feedlots provided 384 faeces samples, 24 drinking trough water samples, and 24 soil samples. A total of 527 presumptive isolates were found, with 289 of these being confirmed as *Enterococcus sp*. After molecular assays, *E. faecalis* (9%), *E. faecium* (10%), *E. durans* (69%), *E. gallinarum* (6%), *E. casselifavus* (2%), *E. mundtii* (2%), and *E. avium* (2%) were screened.

Resistance genes for *van*A (62%), *van*B (17%), and *van*C (21%) were found in 176 *Enterococcus* spp., respectively.

Conclusion

Antibiotic resistance has increased among *K. pneumoniae* strains, making infection by these strains extremely difficult to cure. A number of recent investigations have been prompted by the rise of hypervirulent and antibiotic-resistant strains. (Paczosa, 2016). Antibiotic-resistant *K. pneumoniae* strains are becoming more common in hospital & community-acquired illnesses. Antibiotics are commonly used in traditional food-animal production, where antibiotic-resistant micro-organisms are selected. Antibiotic-resistant *K. pneumoniae* has been found in a variety of retail meats, milk, seafood, and vegetables, as well as in animals. Furthermore, new phylogenetic analyses reveal that *K. pneumoniae* from humans and livestock have close connections. As a result, quantifying contribution of the foodborne *K. pneumoniae* to the antibiotic-resistant human illnesses is critical (Davis & Price, 2016).

Enterococci have long been known as a prominent source of nosocomial infections, with *E. faecalis* and *E. faecium* accounting for the bulk of infections, (Facklam & Collins,1989: Malani et al., 2002) however other enterococci species can also cause infections (Cetinkaya et al., 2000).

Resistance to a variety of antibiotics, including vancomycin and aminoglycosides, is a problem in the treatment of enterococcal infections, as well as a threat for resistance spreading into the environment through the transmission of antimicrobial resistance genes and some virulence factors from enterococci to pathogenic bacteria (Murray, 1994).

The global expansion of intensive animal production systems, in which antimicrobials are routinely employed to maintain health and productivity, has aided the transition to high-protein diets in low- and middle-income nations. Antimicrobials are utilized in animals reared for food. This has been related to the emergence of antimicrobial-resistant diseases in both animals and people (Van Boeckel et al., 2019). Therefore, the impact of widespread antibiotic use in intensive animal production, as well as its implications for public health, cannot be underestimated.

Chapter-III

MATERIAL AND METHOD

3.1 Place of work

The work has been done in the Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana.

3.2 Media, Chemicals and Reagents

Media and antibiotics used in the present study were from Hi Media Labs (Mumbai). Chemicals and reagents used were of analytical and/or molecular biological grade from MBI Fermentas (USA), Sisco Research Laboratories Pvt. Ltd. (SRL, Mumbai) and other reputed National and International firms.

3.3 Bacterial strains

E. faecalis strain ATCC 29212, *E. faecium* strain ATCC 35667 and *K. pneumoniae* strain 700603 were used in the study.

HiMedia Labs in Mumbai provided all of the listed reference strains. All strains were revived according to protocol and stored at -80°C in brain heart infusion broth (BHI) supplemented with 20% v/v glycerol (HiMedia lab, Mumbai). A loopful of culture from the preserved culture (-80°C) was streaked on to a nutrient agar (NA) (HiMedia Lab, Mumbai) plate and incubated overnight at 37°C for sub-culturing of the standard strains for use in the present study. The frozen culture was returned to its normal storage temperature instantly.

3.4 Collection of samples

3.4.1. Source of sample

Two types of layer poultry farm management systems (Deep Litter and Cage System) were included in the study. Twelve farms of each type from different districts of Punjab were selected.

3.4.2 Sample types

Thirty eggs were collected from each farm and only twenty were processed for isolation purposes. The remaining eggs were used as buffer samples in order to

compensate for any breakage. Finally, 480 eggs were processed for isolation of micro-organisms. In addition to these, three types of environmental samples comprising one each of water (pooled), feed (pooled) and litter (pooled) were collected from the selected farms (Table:3.1). For pooling each sample type was randomly collected from five different sites and the collected samples were pooled as one sample of each category. Human hand swab samples were taken from farmers depending on their consent.

3.4.3 Collection and transport of sample

Freshly laid eggs were collected aseptically from each farm in labelled sterile sampling bags (Sampling bags, HiMedia, Mumbai). For collection of swab samples, they were moistened with sterile BHI broth and mopped on palm of the hand covering entire palm and the other swab was used for another palm, these swabs were put in sterile test tube having 1ml of sterile normal saline and transported on ice. Both swabs were put in one test tube and considered as one sample.

All the collected samples were transported to the laboratory in a collection box containing ice packs. The samples brought to the lab were immediately processed for the isolation of bacteria or were stored at 4°C and processed next day.

Table: 3.1 Sample collection from different districts of Punjab

		Deep litter						Cage system					
District	Farm	Eggs collected	Eggs processed	Environment			Hand swab	Egg collected	Egg processed	Environment			Hand swabs
				Feed	Water	Litter				Feed	Water	litter	
Amritsar	ASR1	-	-	-	-	-	0	30	20	1	1	1	0
	ASR2	-	-	-	-	-	0	30	20	1	1	1	0
	ASR3	-	-	-	-	-	0	30	20	1	1	1	0
Barnala	BAR1	-	-	-	-	-	0	30	20	1	1	1	0
	BAR2	-	-	-	-	-	0	30	20	1	1	1	0
	BAR3	-	-	-	-	-	0	30	20	1	1	1	0
	BAR4	-	-	-	-	-	0	30	20	1	1	1	0
	BAR5	-	-	-	-	-	0	30	20	1	1	1	0
Ludhiana	LDH1	30	20	1	1	1		30	20	1	1	1	
	LDH2	30	20	1	1	1	1	30	20	1	1	1	0
	LDH3	30	20	1	1	1		30	20	1	1	1	0
	LDH4	30	20	1	1	1	1	30	20	1	1	1	0
Mohali	MOH1	30	20	1	1	1		-	-	-	-	-	-
	MOH2	30	20	1	1	1	1	-	-	-	-	-	-
	MOH3	30	20	1	1	1		-	-	-	-	-	-
	MOH4	30	20	1	1	1	1	-	-	-	-	-	-
Rupnagar	RUP1	30	20	1	1	1		-	-	-	-	-	-
	RUP2	30	20	1	1	1	1	-	-	-	-	-	-
	RUP3	30	20	1	1	1	1	-	-	-	-	-	-
	RUP4	30	20	1	1	1		-	-	-	-	-	-
Grand total		360	240	12	12	12	6	360	240	12	12	12	0

3.5 Processing of egg content and egg shell for isolation of *K. pneumoniae and Enterococcus spp.* from egg

3.5.1 Egg content

Isolation of *K. pneumoniae* and *Enterococcus* spp. from egg was attempted as per method of Musgrove et al. (2008) after making minor modifications. Each egg was placed in a sterile plastic bag with sterile forceps. One egg was removed from the sampling bag aseptically and its inner content was removed in a 250 ml sampling bag (HiMedia, Mumbai) by breaking the egg shell using sterile glass rod. The content was homogenized for 2-5 minutes by stomacher. One ml of the homogenised content was taken and mixed with 9 ml of sterile BHI broth in a 15 ml sterile test tube (HiMedia, Mumbai) and incubated overnight at 37°C for enrichment.

The egg shell from the above after shell breaking was placed in a new 250 ml sampling bag to which 10 ml of Buffered Peptone Water was added. The egg shell was crushed in the bag using fingers for 2-5 minutes and then mixed properly for 2-5 minutes. One ml of the homogenised content was taken and mixed with 9 ml of sterile BHI broth in a 15 ml sterile tube (HiMedia, Mumbai) and incubated overnight at 37°C for enrichment.

3.5.2 Processing of environment samples

Environment samples (drinking water, feed, litter) were processed by mixing one ml or gm of the sample with 9ml BHI broth in a 15 ml sterile test tube (HiMedia, Mumbai) and incubated overnight at 37°C for enrichment.

3.5.3 Processing of human hand swabs

Cotton swab part of the swab stick was cut with a pair of sterile scissors and put in 15ml sterile test tube along with entire saline diluent to which 9ml BHI broth was added and incubated at 37°C for enrichment.

3.6 Isolation of bacteria

3.6.1 Selective plating

After overnight incubation of enrichment broth, a loopful of *K. pneumoniae* inoculum was streaked onto an MLA agar for isolation of *Klebsiella* spp. and on a Bile Esculin Azide agar (BEA) for isolation of *Enterococcus* spp. The plates were incubated at 37 °C for 18-24 hours. The pink and mucoid colonies on MLA which

represented lactose fermentation were suspected to be of *Klebsiella* spp. (Fig :1) The colonies with brown black halo exhibiting brown black coloration in the surrounding BEA medium were suspected to be of *Enterococcus* spp.(Fig: 2)

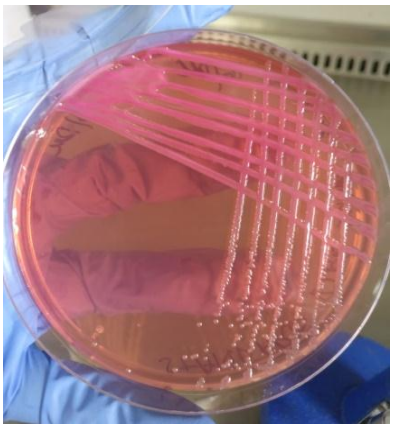


Fig 1: *K. pneumoniae* on MLA agar showing typical pink, mucoid colonies along with fermentation of the media

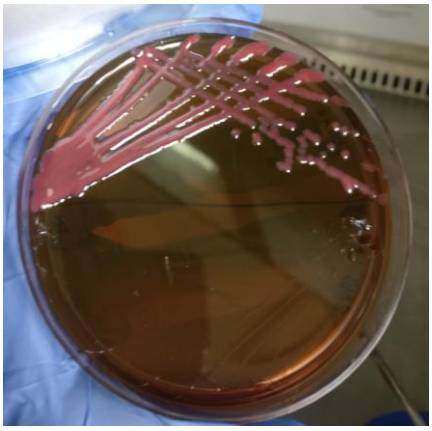


Fig 3: *K. pneumoniae* on EMB agar showing typical pink to purple mucoid colonies without metallic sheen

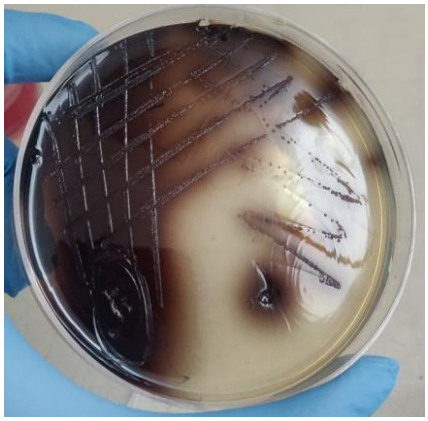


Fig 2: Suspected *Enterococcus* spp. colonies with brown black colonies with a halo exhibiting brown black coloration in the surrounding BEA agar

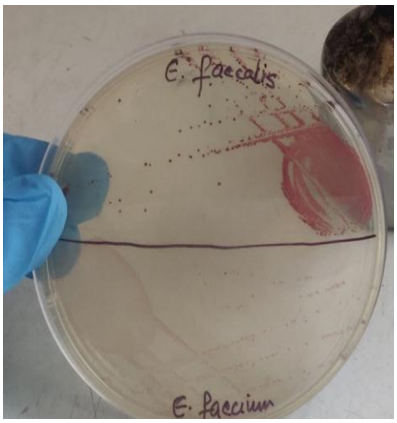


Fig 4: *E. faecium* representing pale pink colonies and *E. faecalis* representing maroon red colonies on EDA agar

Two isolates were chosen per sample. However, one isolate was chosen from those samples that only represented one typical colony either of *Klebsiella* spp. or *Enterococcus* spp. on the plates. Each typical colony was streaked onto a fresh plate i.e., MLA plate for *Klebsiella* spp. and BEA plate for *Enterococcus* spp. for purification of the primary isolate. For further confirmation *Klebsiella* isolates were also streaked on an EMB agar and mucoid pink colonies were observed which differentiated *Klebsiella* spp. from *E. coli* (distinct metallic sheen). (Fig:3) Similarly for differentiation of *Enterococcus* spp., positive isolates were streaked on an Enterococcus differential agar (HiMedia, Mumbai), pale pink colonies represented *E. faecium* while maroon red colonies represented *E. faecalis* (Fig:4). The isolates were further subjected to tests mentioned ahead.

3.6.2 Gram staining

Presumptive isolates of *Klebsiella* and *Enterococcus* spp. stored in 20% glycerol at -20°C were freshly sub-cultured on BHI agar plate and incubated overnight at 37°C.

The Gram's staining of the isolated bacterial colonies was done using Gram's staining kit (HiMedia, Mumbai) as per the kit instructions. After heat fixation, a smear of bacterial colony was placed on a fresh grease-free glass slide, and staining was done. *Enterococcus* spp. appeared as gram positive cocci in pairs, clusters or short chains whereas *K. pneumoniae* revealed as gram-negative, encapsulated rod-shaped bacillus.

3.6.3 Biochemical tests used for the preliminary identification of *Klebsiella* and *Enterococcus* spp.

3.6.3.1 Catalase test

On a clean, grease free glass slide, a drop of 3% hydrogen peroxide was placed, to which a freshly sub-cultured bacterial colony using a sterile wooden stick. The formation of gas bubbles indicated positive reaction whereas absence of gas bubbles was considered negative. Both *Enterococcus* spp. and *Klebsiella* spp. are catalase positive.

3.6.3.2 Oxidase test

A single freshly sub-cultured colony of bacteria was picked up with a sterile tooth pick and spread onto an oxidase disc (HiMedia, Mumbai). The change in the colour of the disc to dark purple within 60-90 seconds was recorded positive, whereas, no change in colour or if the reaction took more than 2 minutes to change the colour was considered negative. *Klebsiella* spp. and *Enterococcus* spp. are both oxidase negative.

3.6.3.3 Indole test

A fresh colony of presumptive *Klebsiella* spp. was inoculated into 5ml of 1% sterile peptone water and incubated overnight at 37°C. After overnight incubation 0.5ml of Kovac's reagent was added to the culture tube and development of red color was taken as positive reaction and no colour development was taken negative for the reaction. *Klebsiella spp*. gives variable reaction, however *K. pneumoniae* is indole negative. This test was not performed for *Enterococcus* spp.

3.6.3.4 Methyl red and Vogues Proskauer test

A fresh colony of presumptive *Klebsiella* spp. was inoculated into duplicate tubes containing 5 ml of MR-VP reagent (HiMedia, Mumbai) and were incubated overnight at 37°C. After overnight incubation, 5 drops of MR indicator were added in one tube whereas to another tube 0.5 ml of alpha-naphthol solution & 0.5 ml of 40% KOH solution containing 0.3% creatine was added. The tubes were vortexed for a few seconds; development of red and yellow colour in tube was considered positive and negative respectively for MR test. *Klebsiella* spp. gives variable results however *K. pneumoniae* is MR positive. This test was not conducted for *Enterococcus* spp. In the second tube which contained VP reagent, appearance of pink to red colour was considered as positive whereas absence of colour was taken as negative.

3.6.3.5 Citrate Utilization Test

A fresh colony of presumptive *Klebsiella* spp. was inoculated into simmon's citrate medium and incubated overnight at 37°C. Appearance of blue color indicated a positive reaction and. *Klebsiella* spp. is citrate positive. However, this test was not conducted for *Enterococcus* spp.

3.6.3.6 Triple sugar iron (TSI) test

A fresh colony of presumptive *Klebsiella* spp. was inoculated onto TSI medium slant and then stab inoculated in the butt. The tube was incubated overnight at 37°C and change in colour of the media and butt was recorded along with presence or absence of H_2S gas. *Klebsiella* spp. changes colour of the slant and butt indicating sugar fermentation but does not produce H_2S gas.

3.6.3.7 Salt Tolerance Test

A freshly sub-cultured colony of presumptive *Enterococcus* spp. was inoculated into 5 ml of BHI broth with 6.5% NaCl in a 15 ml sterile test tube to differentiate between *Streptococcus* and *Enterococcus* spp. Tube was incubated overnight at 37°C. Development of turbidity indicated that the isolate was tolerant to 6.5% NaCl and hence was positive for *Enterococcus* spp. and no growth was negative for *Enterococcus* spp. This test was done only for *Enterococcus* spp.

3.7 Extraction of genomic DNA

Extraction of genomic DNA from freshly grown presumptive *Klebsiella* and *Enterococcus* spp. isolates was extracted using snap chill technique. Two to three fresh grown colonies were suspended in 2ml of a micro-centrifuge tube containing 1.5ml sterile nuclease free water(NFW). The tube was vortexed for a few seconds and centrifuged at 12000 rpm for 5 minutes. After centrifugation supernatant was discarded and pellet was resuspended in 1.5 ml of NFW. The tube was vortexed again and the above step was repeated twice. After final washing, pellet was re-suspended in 1ml of NFW and vortexed. The tube with suspended pellet was put in boiling water for 10 minutes. After boiling, it was placed on ice for 10-15 minutes. Post cold shock treatment, tube was centrifuged at 12000 rpm for 5 minutes. The DNA-containing supernatant was aliquoted into a 2ml centrifuge tube & stored at -20°C until further use.

3.7.1 Optimization of PCR for the molecular confirmation of *K. pneumoniae* and *Enterococcus* spp.

PCR reaction mixture was prepared consisting of 6.5 µl mastermix (GoTaq Master Mix, Promega, USA),1 µl of 10 pmol/µl of each forward and reverse primers (Gbiosciences), 5 µl of template DNA and finally the reaction volume was made up to

20 µl using Nuclease free water (GoTaq). The primers used for the detection of *K. pneumoniae* and *Enterococcus* spp. are mentioned in table:3.2

3.7.1.1 PCR protocol for *Klebsiella pneumoniae*

PCR reaction was carried out in a thermocycler (Eppendorf, India) with the following conditions: An initial denaturation at 95°C for 5 minutes followed by 30 cycles each of denaturation at 94°C for 45 seconds, annealing at 54°C for 1 minute and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 10 minutes. *K. pneumoniae* strain ATCC 700603 was used as positive control and NFW was used as negative control.

3.7.1.2 Multiplex PCR protocol for confirmation of *Enterococcus spp*. and *E. faecalis*

A multiplex PCR was designed for molecular confirmation of *Enterococcus* spp. and *E. faecalis.* with the following conditions: An preliminary denaturation at 95°C for 5 minutes, 30 cycles each of denaturation at 94°C for 45 seconds, annealing at 54°C for 1 minute and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 10 minutes. DNA of *E. faecalis* strain ATCC 29212 was used as positive control and NFW was used as negative control.

3.7.1.2 PCR protocol for *E. faecium*

Another PCR reaction was carried out for identification of *E. faecium* with the following conditions: An initial denaturation at 95°C for 5 minutes with the following conditions: 30 cycles each of denaturation at 94°C for 45 seconds, annealing at 54°C for 1 minute and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 10 minutes. DNA of *E. faecium* strain ATCC 35667 was used as positive control and NFW was used as negative control.

Table 3.2: List of primers used for amplification of various genes:

	Gene	Oligonucleotide sequence (5`-3`)	**Amplicon size**	**Reference**
Genus specific (*Enterococcus)*	16S rRNA	F: 5'-GGATTAGATACCCTGGTAGTCC-3' R: 5'-TCGTTGCGGGACTTAACCCAAC-3'	678 bp	(Jahan et al., 2013)
Species specific	*Enterococcus faecium*	F: 5'TTGAGGCAGACCAGATTGACG-3' R: 5'TATGACAGCGACTCCGATTCC-3'	658 bp	(Dutka-Malen., et al 1995)
	Enterococcus faecalis	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' 5'-ACGATTCAAAGCTAACTGAATCAGT-3'	941 bp	
Antibiotic resistance genes				
	*van*B	F: 5'-GTGACAAACCGGAGGCGAGGA-3' R:5'-CCGCCATCCTCCTGCAAAAAA-3'	433	(Clark et al., 1993)
	*van*C1	F: 5'-GGTATCAAGGAAACCTC-3' R:5'-CTTCCGCCATCATAGCT-3'	822	(Dutka-malen et al., 1995)
Klebsiella pneumoniae	16s-23s ITSD	F:5'-ATTTGAAGAGGTTGCAAACGAT3' R:5'-TTCACTCTGAAGTTTTCTTGTGTTC-3'	160 bp	(Li et al., 2008)
Antimicrobial Genes	bla_{SHV}	F-5'- TCGCCTGTGTATTATCTCCC-3' R-5'-CGCAGATAAATCACCACAATG-3'	768 bp	(Maynard et al., 2004)
	bla_{TEM}	F: 5'-ATGAGTATTCANCATTTCCG-3' R: 5'CTGACAGTTACCAATGEITA 3'	867 bp	
	bla_{CTX-M}	F-5'-CAATOTOCAGCACCAAGTAA 3' R-5'- CGCGATATCGTTGGTGGTG-3'	540 bp	(Dutta et al., 2013)
	bla_{DHA}	5'-TGATGGCACAGCAGGATATTC-3' 5'-GCTTTGACTCTTTCGGTATTCG-3'	997 bp	

3.8 Mass Spectrometry for confirmation of *K. pneumoniae* and *Enterococcus* spp. (MALDI-TOF-MS)

All PCR confirmed isolates of *Enterococcus* spp. and *K. pneumonia* were also subjected to MALDI-TOF MS (Bruker's FLEX) mass spectrophotometric analysis for final confirmation. The pure isolates were freshly sub-cultured on BHI agar after overnight incubation at 37°C. A bacterial colony was picked up and smeared onto target metal plate. One – two µl of a matrix consisting α-Cyano-4-hydroxycinnamic acid (CHCA) was dissolved in an acetonitrile (50%) sol. and 2.5% trifluoroacetic acid was added onto it and dried on the target plate. The target plate was placed into the plating chamber of the mass spectrometer and was subjected to mass spectrometry. The isolates which gave a score value ≥ 2.00 were considered positive for genus and species and for those which showed score value ≤ 2.00, the test was repeated again.

3.9 Antibiotic Sensitivity Testing

Antibiotic spectrum of *K. pneumoniae*, *Enterococcus* spp. was examined by disk diffusion method as described by Kirby Bauer (1961). All *K. pneumoniae* isolates were subjected to AST whereas, among *Enterococcus* isolates, only *E. faecalis* and *E. faecium* isolates were subjected to AST. Antibiotic disks (HiMedia, Mumbai) used for sensitivity detection for above mentioned bacterial species are mentioned in table 3.3 All the test isolates were freshly sub-cultured on BHI agar and incubated overnight at 37°C. Two-three colonies from BHI agar were picked up with the help of sterile pointed loop and inoculated onto sterile 5ml of MH broth. The inoculated broth was incubated for 2 hours after which its turbidity was adjusted to 0.5 McFarland either using sterile MH broth if the turbidity was more than 0.5 McFarland or by putting additional colonies from BHI agar, if the turbidity was less than 0.5 McFarland. Mc Farland standard from Hi Media was used for comparison of adjusted turbidity. The broth with adjusted turbidity was used for AST. A sterile swab was put in the broth with adjusted turbidity, extra broth was removed by squeezing the swab with inside wall of test tube. The swab was then mopped on MH agar plate ensuring entire coverage of plate. The plate was allowed to dry for 3-5 minutes to allow excess moisture to be absorbed before placing antibiotic discs on the plates. After 5 minutes, discs were put on the plate with center to center distance of 24 mm between the discs. Approximately 5 discs were put on the 100 mm plate. All the discs were placed on plate within 15 min and the plates were incubated at 37°C for 18-24 hours. After incubation zone of inhibition around the discs was measured and the results were interpreted as Resistant, Intermediate or Sensitive as per manufacturer guidelines.

Table 3.3: Antibiotic discs used to study in vitro antimicrobial sensitivity for *K. pneumoniae* and *Enterococcus* spp.

S. No.	Antibiotics	Symbol	Disc Concentration	*K. pneumoniae*	*Enterococcus* spp.
1	Amikacin	AK	30 mcg	✔	✔
2	Ampicillin	AMP	10 mcg	✔	✔
3	Amoxicillin	AMS	10 mcg	✔	
4	Cefotaxime	CTX	30 mcg	✔	
5	Chloramphenicol	CHL	30 mcg		✔
6	Ciprofloxacin	CIP	5 mcg	✔	✔
7	Co-trimoxazole	COT	25 mcg	✔	
8	Doxycycline	DO	30 mcg	✔	
9	Erythromycin	E	15 mcg		✔
10	Gentamicin	GEN	15 mcg	✔	✔
11	Kanamycin	K	20 mcg		✔
12	Imipenem	IMP	10 mcg	✔	
13	Linezolid	LZ	30 mcg		✔
14	Penicillin	P	10 units	✔	✔
15	Tetracycline	TET	30 mcg		✔

3.9. Detection of Extended spectrum β-lactamase (ESBL) and Klebsiella pneumoniae carbapenemase (KPC) in *K. pneumoniae* isolates.

K. pneumoniae field isolates were inoculated on MH agar as described earlier in disc diffusion method for the detection. For the detection of ESBL, E-strips (EZY MIC, HiMedia, Mumbai) containing ceftazidime and ceftriaxone antibiotics on one side and ceftazidime, ceftriaxone and clavulanic acid antibiotics on other side were put on plate and incubated for 18-24 hours at 37°C. The zone of inhibition was noted on both sides of the strip and isolate was designated as ESBL positive or negative as per the manufacturer guidelines. Similarly for the detection of KPC, E-strips (EZY MIC, HiMedia, Mumbai) containing Ertapenem on one side and Ertapenem plus phenyl boronic acid on the other side were placed on MH agar plate which were then incubated for 18-24 hours at 37°C. The zone of inhibition was measured on both sides and isolate was designated as KPC positive or negative as per the manufacturer guidelines.

3.10 MIC for vancomycin

E. faecium and E. faecalis isolates were examined for sensitivity to antibiotic vancomycin by broth microdilution method. *E. faecalis* strain ATCC 51299 was used as positive control. The formulation of reference standard powder used was vancomycin hydrochloride. The potency for the vancomycin powder was 960 µg/mg from which working stock-I was prepared by diluting 1 mg of vancomycin hydrochloride powder in 960 µl of autoclaved distilled water giving a final concentration of 1mg/ml. From working stock-I, 512 µl of Solution was added to 488 µl of sterile MH broth to make working stock-II, yeilding a final concentration of 512 µg/ml. In a sterile 96-well micro-titre plate (HiMedia, Mumbai), 100 µl antibiotic of sterile MH broth was added from column 2 to column 11. In column 1, 100 µl of working stock-II was added. In column 2, 100 µl of working stock II was added and two twofold serial dilutions were performed from column 2 to 11 to get drug concentrations as 256 µg/ml, 64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml and 0.125 µg/ml. A total of 100 µl of culture (turbidity adjusted to 0.5 Mc FArland standard) was added in each row, making the final volume of the well as 200 µl. Second last column of the plate was inoculated with positive control. In the last row, 200 µl of sterile broth was added as negative control. Plate was incubated at 37°C for 24 hrs, and OD was taken at 625nm. MIC of vancomycin was read as the lowest concentration of vancomycin, where the OD matches to the OD of Negative control.

3.11 Identification of antibiotic resistance genes

Genomic DNA extracted as previously for identification of *K. pneumoniae*, *E. faecalis* and *E. faecium* was used for detection of antibiotic resistant genes as well. All *K. pneumoniae* isolates were subjected to detection of ESBL genes such as: bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{DHA} encoding for β-lactamase whereas, all *Enterococcus* spp. isolates were subjected to vancomycin resistant genes such as *van*B and *van*C1. Primers used for amplification of the genes were procured from GBiosciences, India and are listed in table 2 and positive controls for the ESBL genes were procured from Post Graduate Institute of Medical Education & Research, Chandigarh. Following were the controls used for *K. pneumoniae*: TEM: CRE-26; CTX-M 15: CRE-4, SHV: CRE-30 , DHA: CRE-183

Detection of ESBL genes was carried out as single plex reaction with the following conditions: an initial denaturation at 94°C for 5 minutes followed by 30 cycles each of denaturation at 94°C for 30 seconds, annealing at 47°C for 30 seconds and extension at 72°C for 1 minute followed by final extension at 72°C for 7 minutes.

PCR reaction mixture was prepared which consisted of 6.5 µl mastermix (GoTaq Master Mix) (Promega, USA),1 µl of 10 pmol/µl of each forward and reverse primers (Gbiosciences), 5 µl of template DNA and finally the reaction volume was made up to 20 µl using nuclease free water (GoTaq)

Detection of vancomycin resistance genes (*van*B, *van*C1) for *Enterococcus* spp. was also carried out in single plex reaction with following conditions:an initial denaturation at 94°C for 5 minutes followed by 30 cycles each of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute followed by final extension at 72°C for 7 minutes. Positive control used for *van*B genes was *E. faecalis* strain ATCC 51299 procured from HiMedia, Mumbai.

3.12 Agar gel electrophoresis

All the amplified PCR products were examined using agarose gel electrophoresis (1.5-2%). Electrophoresis was performed at 85 volts/cm for 45 minutes to 1 hour and a gel documentation system (Syngene, U.S.A) was used to visualize the bands in the gel.

3.13 Statistical Analysis

Data analysis was carried out with SPSS, version 22.0 Independent variable taken in the study was housing system (deep litter taken as 1 and cage system 0). Dependent variables taken were presence or absence of bacteria, presence or absence of *E. faecalis/ E. faecium*, presence or absence of resistance, presence por absence of MDR and presence or absence of Vancomycin resistance. Categorical variables were compared using the Chi-Square test. A P value <0.05 was considered to be statistically significant. Odds ratio was calculated to compare the isolates of *Enterococcus* spp. from eggs of deep litter system to that of eggs of cage system of management.

Chapter – IV

RESULTS AND DISCUSSION

4.1 Prevalence of *K. pneumoniae* and *Enterococcus* spp. in eggs

In the present study contamination of egg surface and egg content for the presence of *K. pneumoniae* and *Enterococcus* spp. was examined. The eggs were collected from two types of layer poultry housing systems *i.e*., deep litter and cage system. In our results we found that out of 480 eggs examined, 18 (3.75%) were positive for *K. pneumoniae* and 100 (20.83%) were positive for *Enterococcus* spp. irrespective of the management system from which eggs were collected.

However, 240 eggs were collected from each housing system. Out of 240 eggs, 11(4.58%) were positive for *K. pneumoniae* from deep litter housing system and 7(2.91%) from cage system. For *Enterococcus* spp. out of 240 eggs, 60 (25%) eggs from deep litter and 40 (16.66%) eggs from cage system were found to be contaminated.

Out of a total of 24 farms (12 each of deep litter and cage system) 9 farms were positive for *K. pneumoniae* (5 deep litter and 4 cage system) and 21 farms were positive for *Enterococcus* spp. (12 farms for deep litter and 9 for cage system). Contamination of the eggs in the deep litter system (4.58%) with *K. pneumoniae* was more in comparison to the eggs with *K. pneumoniae* from cage system (2.91%). Highest number of eggs (5/80, 6.25%) from Rupnagar district followed by Mohali and Ludhiana districts (3/80, 3.75%) in deep litter system were contaminated with *K. pneumoniae* (Table 4.1). Whereas, highest number of eggs from Barnala district (4/100; 4%) followed by Ludhiana (3/80; 3.75%) were contaminated with *K. pneumoniae* in cage system. However, no contamination of *K. pneumoniae* was found in inner egg content of eggs from both deep litter and cage system (Table 4.1).

Table:4.1 Occurrence of *K. pneumoniae* in Eggs

		Deep Litter				Cage System			
District	Farm	Eggs Processed	Eggs positive(%age)			Eggs Processed	Eggs positive(%age)		
			Egg Shell	District prevalence	Inner Content		Egg Shell	District prevalence	Inner Content
Amritsar	ASR1	-				20	0	0 (0%)	0
	ASR2	-				20	0		0
	ASR3	-				20	0		0
Barnala	BAR1	-				20	2 (10%)	4 (4%)	0
	BAR2	-				20	0		0
	BAR3	-				20	1 (5%)		0
	BAR4	-				20	1 (5%)		0
	BAR5	-				20	0		0
Ludhiana	LDH1	20	0	3 (3.75%)	0	20	0	3 (3.75%)	0
	LDH2	20	0		0	20	3 (15%)		0
	LDH3	20	3 (15%)		0	20	0		0
	LDH4	20	0		0	20	0		0
Mohali	MOH1	20	1 (5%)	3 (3.75%)	0	-			
	MOH2	20	0		0	-			
	MOH3	20	2 (10%)		0	-			
	MOH4	20	0		0	-			
Rupnagar	RUP1	20	3 (15%)	5 (6.25%)	0	-			
	RUP2	20	0		0	-			
	RUP3	20	0		0	-			
	RUP4	20	2 (10%)		0	-			
Grand total		240	11 (4.58%)		0	240	7 (2.91%)		0

Similarly, highest number of eggs from Rupnagar district (21/80;26.25%) followed by Mohali (20/80; 25%) and Ludhiana districts (19/80; 23.75%) were contaminated with *Enterococcus* spp. in deep litter system (Table 4.2). In cage system, highest number of eggs from Amritsar district (13/60; 21.66%) followed by Barnala (18/100; 18%) and Ludhiana (9/80; 11.25%) districts were contaminated with *Enterococcus* spp. It was also found that from the total 100 positive egg samples, 92 (92%) eggs (egg shells) and 8 (8%) eggs (inner content) content were contaminated with *Enterococcus* spp. (Table 4.2).

Contamination of farm environment (Litter, feed and water) was also examined (Table 4.3). It was found that out of 72 environment samples comprising of feed (n=24), water (n=24) and litter (n=24), 5 (6.94%) were positive for *K. pneumoniae* irrespective of the management system (Table 4.3). Out of 5 positive samples, 2 (2.77%) from litter, 2 (2.77%) each of feed and litter and 1(1.38%) of water was positive for *K. pneumoniae*. No statistically significant difference was found in the contaminants of environment of deep litter versus cage system for *K. pneumoniae* ($p>0.05$).

Contamination of these farms was also checked for *Enterococcus* spp. (Table 4.4). It was found that out of 72 environment samples, 35 (48.61%) were positive for *Enterococcus* spp. irrespective of the management system (Table 4.4). Out of 35 positive samples, 21 (29.16%) of litter, 9 (12.5%) of feed and litter and 5(6.94%) of water were positive for *Enterococcus* spp. No statistically significant difference was found in the contaminants of environment of deep litter versus cage system for *K. pneumoniae* ($p>0.05$).

Table:4.2 Occurrence of *Enterococcus* spp. in eggs

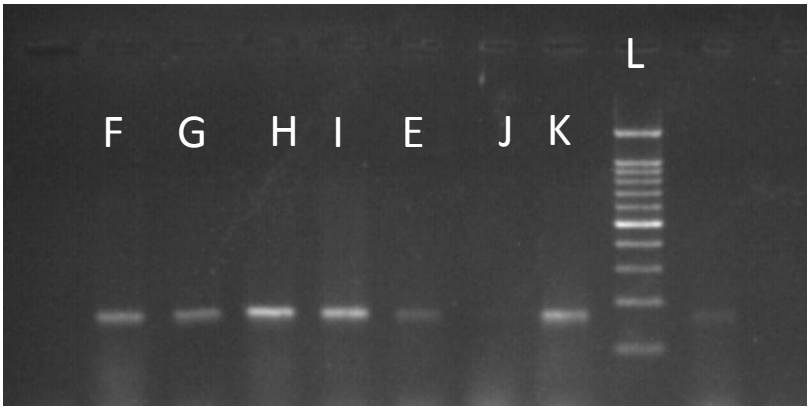
		Deep Litter				Cage System			
District	**Farm**	**Eggs Processed**	**Eggs positive (%age)**			**Eggs Processed**	**Eggs positive(%age)**		
			Egg Shell	**Inner Content**	**District prevalence**		**Egg Shell**	**Inner Content**	**District prevalence**
Amritsar	ASR1	-	-	-	-	20	0	0	13 (21.66%)
	ASR2	-	-	-	-	20	5 (25%)	1 (5%)	
	ASR3	-	-	-	-	20	7 (35%)	0	
Barnala	BAR1	-	-	-	-	20	5 (25%)	0	18 (18%)
	BAR2	-	-	-	-	20	2 (10%)	0	
	BAR3	-	-	-	-	20	7 (35%)	0	
	BAR4	-	-	-	-	20	0 (0%)	0	
	BAR5	-	-	-	-	20	4 (20%)	0	
Ludhiana	LDH1	20	4 (20%)	1 (5%)	19 (23.75%)	20	1 (5%)	1 (5%)	9 (11.25%)
	LDH2	20	4 (20%)	0		20	0 (0%)	0	
	LDH3	20	4 (20%)	1 (5%)		20	2 (10%)	0	
	LDH4	20	4 (20%)	1 (5%)		20	5 (25%)	0	
Mohali	MOH1	20	5 (25%)	0	20 (25%)	-	-	-	-
	MOH2	20	4 (20%)	1 (5%)		-	-	-	-
	MOH3	20	5 (25%)	0		-	-	-	-
	MOH4	20	5 (25%)	0		-	-	-	-
Rupnagar	RUP1	20	4 (20%)	0	21 (26.25%)	-	-	-	-
	RUP2	20	5 (25%)	0		-	-	-	-
	RUP3	20	4 (20%)	2 (10%)		-	-	-	-
	RUP4	20	6 (30%)	0		-	-	-	-
Grand total		240	54 (22.5%)	6 (2.5%)		240	38 (15.83%)	2 (0.83%)	

Table: 4.3 Occurrence of *K. pneumoniae* in environment and farm handlers

District	Farm		Deep Litter						Cage System					
		Samples Processed	Samples positive			Farm Total	District prevalence (%age)	Hand swab	Samples Positive			Farm Total	District prevalence(%age)	Hand Swabs
			Feed	Water	Litter				Feed	Water	Litter			
Amritsar	ASR1	3	-	-	-	-	-		0	0	0	0	0	0
	ASR2	3	-	-	-	-	-	-	0	0	0	0		0
	ASR3	3	-	-	-	-	-	-	0	0	0	0		0
Barnala	BAR1	3	-	-	-	-	-	-	0	0	0	0	1 (6.66%)	0
	BAR2	3	-	-	-	-	-	-	0	0	1	1		0
	BAR3	3	-	-	-	-	-	-	0	0	0	0		0
	BAR4	3	-	-	-	-	-	-	0	0	0	0		0
	BAR5	3	-	-	-	-	-	-	0	0	0	0		0
ludhiana	LDH1	3	0	0	0	0	1(8.33%)	-	0	0	0	0	1 (6.66%)	0
	LDH2	3	0	0	0	0		0	0	0	0	0		0
	LDH3	3	0	0	0	0		0	0	0	1	1		0
	LDH4	3	0	0	1	1		0	0	0	0	0		0
Rupnagar	MOH1	3	0	0	0	0	2 (16.66%)	0	0	0				
	MOH2	3	0	1	1	2		0	-	-	-			
	MOH3	3	0	0	0	0		0	-	-	-			
	MOH4	3	0	0	0	0		0	-	-	-			
Mohali	RUP1	3	0	0	0	0	0(0%)	0	-	-	-			
	RUP2	3	0	0	0	0		0	-	-	-			
	RUP3	3	0	0	0	0		0	-	-	-			
	RUP4	3	0	0	0	0		0	-	-	-			
Grand total			0	1 (1.38%)	2 (2.77%)			0	0	0	2 (2.77%)			

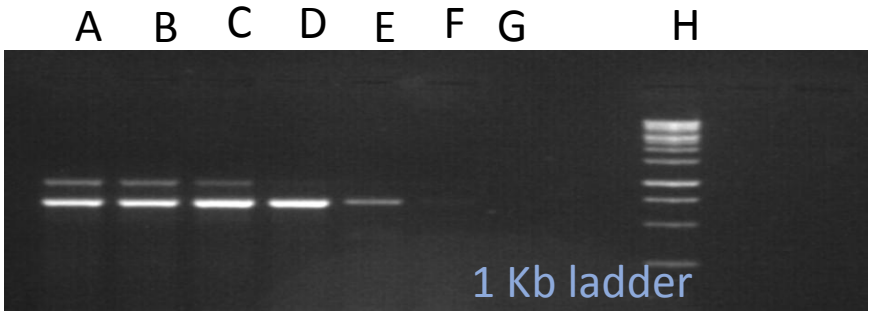
Table: 4.4 Occurrence of *Enterococcus* spp. in Environment and human hand swabs

ghg			Deep Litter						Cage System					
District	Farm	samples processed	Environment (%age)			farm total	district total	Hand swab	Environment (%age)			farm total	district total	Hand swab
			Feed	Water	Litter				Feed	Water	Litter			
Amritsar	ASR1	3	-	-	-			-	1	1	1	0	3 (33.33%)	0
	ASR2	3	-	-	-			-	1	1	1	1 (33.33%)		0
	ASR3	3	-	-	-			-	1	0	1	2 (66.66%)		0
Barnala	BAR1	3	-	-	-			-	0	0	1	1 (33.33%)	3 (20%)	0
	BAR2	3	-	-	-			-	0	0	0	0		0
	BAR3	3	-	-	-			-	1	0	0	1 (33.33%)		0
	BAR4	3	-	-	-			-	0	0	1	0		0
	BAR5	3	-	-	-			-	0	0	1	1 (33.33%)		0
Ludhiana	LDH1	3	0	0	1	1 (33.33%)	5 (41.66%)	1 (100%)	0	0	1	1 (33.33%)	4 (33.33%)	0
	LDH2	3	0	0	1	1 (33.33%)		0	0	0	0	0 (0%)		0
	LDH3	3	1	0	1	2 (66.66%)		1 (100%)	0	1	1	2 (66.66%)		0
	LDH4	3	0	0	1	1 (33.33%)		0	0	0	1	1 (33.33%)		0
Mohali	MOH1	3	0	0	1	1 (33.33%)	6 (50%)	0	-	-	-			-
	MOH2	3	0	1	1	2 (66.66%)		1 (100%)	-	-	-			-
	MOH3	3	1	0	1	2 (66.66%)		0	-	-	-			-
	MOH4	3	0	0	1	1 (33.33%)		1 (100%)	-		-			-
Rupnagar	RUP1	3	0	0	1	1 (33.33%)	8 (66.66%)	0	-	-	-			-
	RUP2	3	1	0	1	2 (66.66%)		0		-	-			-
	RUP3	3	1	0	1	2 (66.66%)		1 (100%)	-	-	-			-
	RUP4	3	1	1	1	3 (100%)		1 (100%)	-	-	-			-
Grand total		72	5 (6.94%)	2 (2.77%)	12 (16.66%)			6/6 (100%)	4 (5.55%)	3(4.16%)	9 (12.5%)			0



Lane:L ladder, K-positive control(PCT), J- Negative control(NCT) and E,F,G,H and I – positive isolates

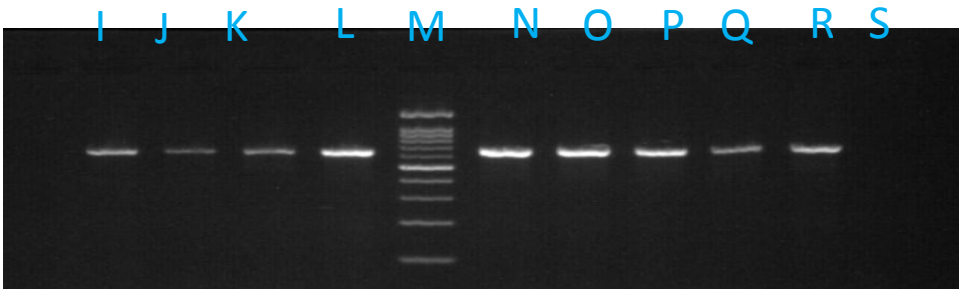
Fig: 5 PCR for identification of *K. pneumoniae* isolates



Lane A- PCT, Lane B, C,D and E – *Enterococcus* positive isolates

Lane B & C- *E. faecalis positive isolates*

Fig: 6 Multiplex PCR for identification of *Enterococcus* and *E. faecalis*



Lane M_ 100 bp ladder, Lane N- PCT, Lane S- NCT

Lane I, J, K and L and O, P, Q and R – positive isolates

Fig:7 PCR protocol for *E. faecium*

In addition to that, presence of *K. pneumoniae* and *Enterococcus* spp. was also examined on the hand of poultry farm workers employed for handling the birds. Out of a total of 6 swab samples, none was positive for *K. pneumoniae* (Table 4.2) and 6 (100%) were positive for *Enterococcus* spp. (Table 4.3).

In the study a total of 53 (9.49%) *Klebsiella* spp. isolates were obtained as confirmed by PCR and MALDI-TOF. Out of those 53 (9.49%) *Klebsiella* spp. isolates comprised of 23 isolates of *K. pneumoniae* and 30 isolates of *K. oxytoca* (Fig: 5) whereas, 136 (24.37%) *Enterococcus* spp. isolates (Fig: 6) comprising 60 (44.12%) of *E. faecalis*, 46 (33.82%) *E. faecium*, (Fig: 6 & 7) 15(11.03%) *E. gallinaru*m, 8(5.88%) *E. avium*, 4(2.94%) *E. casseliflavus* and 3(2.20%) *E. durans* were isolated from eggs obtained from both types of housing systems. (Table:4.4)

E. faecalis isolated from eggs and litter were 30(8.33%) and 3(4.16%) respectively and no *E. faecalis* isolate was recovered from feed and water of deep litter farms whereas from the cage system 20 (4.16%), 2(2.77%) and 2(2.77%) isolates of *E. faecalis*were recovered from eggs, litter and feed respectively. *E. faecium* isolates from eggs, litter, water and feed were 21, 4, 2 and1respectively from deep litter farm and 13, 5, 0, 0 respectively from cage system. *E. gallinarum* from egg, litter and feed were 2, 2, and 1 from deep litter respectively and only 5 eggs were positive for *E. gallinarum* isolates from cage system farms. *E. avium* isolates from eggs, litter, feed and water were 3, 1, 2, and 2 from deep litter farms respectively and 1, 1, 0 and 0 from cage system farms respectively. *E. casseliflavus* from eggs, feed and litter were 1, 1 and 1 from deep litter farms respectively and only one isolate from eggs from cage system farms yielded the isolates. Two isolates of *E. durans* from eggs of deep litter farms and one from cage system farms was found. (Table: 4.5)

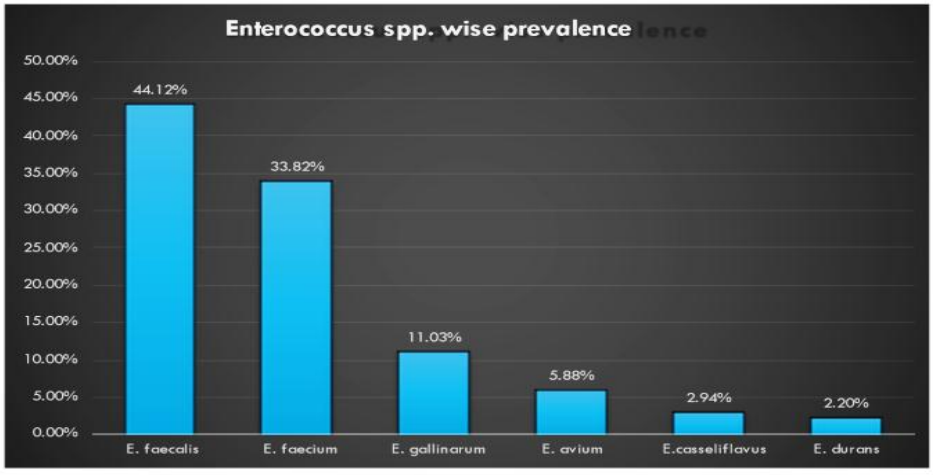


Fig: 8 Overall Prevalence of *Enterococcus* spp

Table: 4.5 Occurrence of different *Enterococcus* spp. in eggs, environment samples and hand swabs samples

District	Farm Type	Farm ID		*Enterococcus* Species					
	Deep Litter/Cage System			***E. faecalis***	***E. faecium***	***E. gallinarum***	***E. avium***	***E. cassiliflavus***	***E. durans***
Mohali	Deep Litter	MOH1	Egg	**2**	**2**	**0**	**0**	**0**	**2**
			Litter	**0**	**1**	**0**	**0**	**0**	**0**
			Water	**0**	**1**	**0**	**0**	**0**	**0**
			Feed	**0**	**0**	**0**	**0**	**0**	**0**
	Deep Litter	MOH2	Egg	**3**	**2**	**0**	**0**	**0**	**0**
			Litter	**0**	**1**	**0**	**0**	**0**	**0**
			Water	**0**	**0**	**0**	**0**	**0**	**0**
			Feed	**0**	**0**	**0**	**0**	**0**	**0**
	Deep Litter	MOH3	Egg	**2**	**3**	**0**	**0**	**0**	**0**
			Litter	**0**	**0**	**0**	**0**	**0**	**0**
			Water	**0**	**0**	**0**	**0**	**0**	**0**
			Feed	**0**	**0**	**0**	**0**	**0**	**0**
	Deep Litter	MOH4	Egg	**3**	**0**	**2**	**0**	**0**	**0**
			Litter	**0**	**0**	**1**	**0**	**0**	**0**
			Water	**0**	**0**	**0**	**0**	**0**	**0**
			Feed	**0**	**0**	**1**	**0**	**0**	**0**
Rupnagar	Deep Litter	RUP1	Egg	**2**	**2**	**0**	**0**	**0**	**0**
			Litter	**1**	**0**	**0**	**0**	**0**	**0**
			Water	**0**	**0**	**0**	**1**	**0**	**0**
			Feed	**0**	**1**	**0**	**0**	**0**	**0**
	Deep Litter	RUP2	Egg	**3**	**1**	**0**	**1**	**0**	**0**
			Litter	**0**	**0**	**0**	**1**	**0**	**0**
			Water	**0**	**0**	**0**	**0**	**0**	**0**

			Feed	**0**	**0**	**0**	**1**	**0**	**0**
	Deep Litter	RUP3	Egg	**3**	**3**	**0**	**0**	**0**	**0**
			Litter	**0**	**0**	**1**	**0**	**0**	**0**
			Water	**0**	**0**	**0**	**1**	**0**	**0**
			Feed	**0**	**0**	**0**	**0**	**1**	**0**
	Deep Litter	RUP4	Egg	**2**	**3**	**0**	**0**	**1**	**0**
			Litter	**1**	**0**	**0**	**0**	**0**	**0**
			Water	**0**	**0**	**0**	**0**	**0**	**0**
			Feed	**0**	**0**	**0**	**0**	**0**	**0**
Ludhiana	Deep Litter	LDH1	Egg	**2**	**1**	**2**	**0**	**0**	**0**
			Litter	**1**	**0**	**0**	**0**	**0**	**0**
			Water	**0**	**0**	**0**	**0**	**0**	**0**
			Feed	**0**	**0**	**0**	**0**	**0**	**0**
	Deep Litter	LDH2	Egg	**2**	**0**	**0**	**2**	**0**	**0**
			Litter	**0**	**1**	**0**	**0**	**0**	**0**
			Water	**0**	**0**	**0**	**0**	**0**	**0**
			Feed	**0**	**0**	**0**	**1**	**0**	**0**
	Deep Litter	LDH3	Egg	**3**	**2**	**0**	**0**	**0**	**0**
			Litter	**0**	**0**	**0**	**0**	**1**	**0**
			Water	**0**	**1**	**0**	**0**	**0**	**0**
			Feed	**0**	**0**	**0**	**0**	**0**	**0**
	Deep Litter	LDH4	Egg	**3**	**2**	**0**	**0**	**0**	**0**
			Litter	**0**	**1**	**0**	**0**	**0**	**0**
			Water	**0**	**0**	**0**	**0**	**0**	**0**
			Feed	**0**	**0**	**0**	**0**	**0**	**0**

District	Farm Type		Farm ID		*Enterococcus* Species					
	Deep Litter/Cage			***E. faecalis***	***E. faecium***	***E. gallinarum***	***E. avium***	***E. casseliflavus***	***E. durans***

	System								
Amritsar	**Cage System**	ASR1	Egg	0	0	0	0	0	0
			Litter	0	0	0	0	0	0
			Water	0	0	0	0	0	0
			Feed	0	0	0	0	0	0
	Cage System	ASR2	Egg	3	2	1	0	0	0
			Litter	0	1	0	0	0	0
			Water	0	0	0	0	0	0
			Feed	0	0	0	0	0	0
	Cage System	ASR3	Egg	4	2	0	0	0	0
			Litter	0	0	0	1	0	0
			Water	0	0	0	0	0	0
			Feed	1	0	0	0	0	0
Barnala	**Cage System**	BAR1	Egg	2	2	1	0	0	0
			Litter	1	0	0	0	0	0
			Water	0	0	0	0	0	0
			Feed	0	0	0	0	0	0
	Cage System	BAR2	Egg	2	0	0	0	0	0
			Litter	0	0	0	0	0	0
			Water	0	0	0	0	0	0
			Feed	0	0	0	0	0	0
	Cage System	BAR3	Egg	4	3	0	0	1	0
			Litter	0	1	0	0	0	0
			Water	0	0	0	0	0	0
			Feed	0	0	0	0	0	0
	Cage System	BAR4	Egg	0	0	0	0	0	0
			Litter	1	0	0	0	0	0

			Water	0	0	0	0	0	0
			Feed	0	0	0	0	0	0
	Cage System	BAR5	Egg	0	2	1	1	0	0
			Litter	0	0	0	0	0	0
			Water	0	0	0	0	0	0
			Feed	0	0	0	0	0	0
Ludhiana	Cage System	LDH1	Egg	**0**	0	2	0	0	0
			Litter	**0**	1	0	0	0	0
			Water	**0**	0	0	0	0	0
			Feed	**0**	0	0	0	0	0
	Cage System	LDH2	Egg	**0**	0	0	0	0	0
			Litter	**0**	0	0	0	0	0
			Water	**0**	0	0	0	0	0
			Feed	**0**	0	0	0	0	0
	Cage System	LDH3	Egg	**2**	0	0	0	0	0
			Litter	**0**	1	0	0	0	0
			Water	**0**	0	0	0	0	0
			Feed	**1**	0	0	0	0	0
	Cage System	LDH4	Egg	**3**	2	0	0	0	0
			Litter	**0**	1	0	0	0	0
			Water	**0**	0	0	0	0	0
			Feed	**0**	0	0	0	0	0

The *Klebsiella* genus includes pathogens that infect both humans and animals. In the vast majority of cases, they are opportunistic bacteria that can infect the human urinary tract or lungs. *K. pneumoniae* is a respiratory pathogen that causes a high rate of death in broiler chicks and hens (Brisse et al., 2009). Although *K. pneumoniae* is not known to cause any disease in healthy individuals but if they reach immunocompromised people through contaminated food, they can cause serious illness as *K. pneumoniae* has become alarmingly resistant to highest and high priority antibiotics reserved for human use. According to ICMR report of 2020, from a total of 65,561 culture positive isolates examined, *K. pneumoniae* was the second most commonly isolated bacteria and was found resistant to second and third generation cephalospoins.

The present study reported an overall prevalence of 4.12% of *K. pneumoniae*. There is a scarcity of data in India on the prevalence of *K. pneumoniae* in eggs. To date, no detailed investigation of the occurrence of *K. pneumoniae* in the poultry eggs has been undertaken, and no important data or reports for the same are available. In light of the foregoing, detection and characterization of *K. pneumoniae* and *Enterococcus* spp. bacteria in eggs, environment and human hand swabs were carried out.As healthy hens lay eggs, the vast majority of them are clean when they pass through the vent but may become contaminated to some extent when they come into contact with bedding or soiled birds of deep litter system for a prolonged duration of time or contaminated cages of cage system (Harry, 1963, Quarles et al., 1970, Mayes &Takeballi, 1983).The microbiological load of table eggs along with cleaning and sanitisation of the egg shell is assessed before retailing in developed nations such as the United States, Canada, and Japan to ensure the safety of consumers (Sabarinath et al., 2009). However, in developing countries like India, this practise is not very common and the eggs are sent for commercial use without any prior disinfection. Therefore, poses a risk of cross contamination to the consumer.

Hence it can be assumed that if eggs are improperly handled and come from poor management condition, they can be a source of harmful food borne bacteria. There are several reports of eggs being involved in food borne outbreaks, though majority of them are associated with *Salmonella* (vertically transmitted in birds), but there are few outbreaks which are associated with contamination of eggs with food borne organisms indicating unhygienic practices and improper handling of eggs. One

such outbreak of *Salmonella* infection occurred between late October 2020 and mid-March 2021 in Canada. According to the data, exposure to eggs was a potential cause of the outbreak. Many of the people who became sick stated they consumed, prepared, cooked, and baked eggs at home. Although no deaths were reported, nineteen people were admitted to the hospital. Hospitalized individuals ranged from age 2 to 98 years old. The most of the cases (71%) involved women.

Another multistate outbreak illness related to hard-boiled eggs was recorded in 2019 due to *Listeria monocytogenes*. The outbreak of *L. monocytogenes* affected eight individuals from five states of US. One fatality and five hospitalizations were reported. The ill individuals ranged in age from 1 to 82 years old, and 60% of the sick were men. To maintain food safety, raw eggs must be handled carefully, and egg-based products must be cooked to an internal temperature of at least 74 ° C. Although, no such outbreak is yet reported from India but the unhygienic practices at poultry farms may lead to food borne illnesses associated with eggs.

Although limited documented study is available that investigated contamination of eggs with *K. pneumoniae* and *Enterococcus* spp., however, there are studies which indirectly support our findings where contamination of poultry environment with *K. pneumonia* and *Enterococcus* spp. have been examined. The occurrence of *K. pneumoniae* and *Enterococcus* spp. has been documented at various levels. In a study conducted by Permatasari et al. (2020) in Indonesia found contamination of *K. pneumoniae* (3.87%) in cloacal swabs from layer farms. In yet another study conducted in Indonesia occurrence of *K. pneumoniae* (7.8%) from chicken cloacal swabs was recorded (Hayati et al., 2019). Aly et al. (2014) conducted a study on chick droppings and documented contamination of *K.* pneumoniae (10%) in their study. Our study is in agreement with another study where contamination of *Klebsiella* spp. (7.8%) was found from cloacal swab samples (Bhardwaj et al., 2021)

Guo et al., (2016) conducted another study where incidence of *K. pneumoniae* was found to be 9.9% which is in close association to our study with overall contamination of *Klebsiella* spp. of 9.49%. Mbah & Anyamene, (2021) recorded 4.5% occurrence of *K. oxytoca* from chicken layer farms showing similarity to our findings.

In a study conducted by Lalzampuia et al. (2014) prevalence of *K. pneumoniae* was reported at 8.21% from poultry birds in Mizoram which is higher than our

findings. Several factors such as age of the bird, season, quality of feed and water, type of bedding material, use of antibiotics and prevailing clinical diseases if any, may contribute to the differences in the level of occurrence of *K. pneumoniae* (Aly et al.,2014)

Enterococcus spp., on the other hand, are common inhabitants of the human and animal intestinal microbiome (Scupham et al., 2008). The environmental contamination with these bacteria is severe due to the emission of significant numbers of enterococci with faeces (Cetinkaya et al., 2000). Enterococci can thus be found in a variety of ecological niches, including soil, water, animals, fermented foods, and plants. As a result, *Enterococcus* spp. have emerged as a potentially high-risk zoonotic pathogen capable of causing serious public health issues and posing a huge global concern. There are many different species of these bacteria however *E. faecalis* and *E. faecium* are one of the most common species studied (Mikalsen et al., 2015). The ICMR report of 2020, reported an overall occurrence of enterococci at 7.3 % of all the isolates, with *E. faecalis* (43.7 %) and *E. faecium* (41.5 %) being the most common species.

In the present study the prevalence of enterococci in eggs, environment and human hand swabs was 24.37%. Six different species were recovered from the samples viz: *E.faecalis*>*E.faecium*> *E. gallinarum*> *E. avium*>*E.cassiliflavus*> *E. durans.* (Table:4.4)

Our study is in agreement with a study done by Nagarajan et al. (2018) wherein they reported *E. faecalis* prevalence of 44% from chicken, beef, mutton, eggs and fish. In yet another study done by Obeng et al., 2013 revealed the predominance of *E. faecalis* followed by *E. faecium* and *E. gallinarum* which was recorded in our study as well.

Osman et al. 2019 reported prevalence of *E. faecium* of 36.8% from poultry faecal samples which is in agreement with our study. Another study conducted by Dolka et al. 2017 reported higher prevalence of *Enterococcus* spp. at 41.5% from diseased birds (tissues, dead in shell embryos) and poultry environment (feed, water, litter).

Schwaiger et al. (2010) found 21% of the pooled egg samples contaminated with *Enterococcus* spp. which is in agreement with our study. Karunarathna et al.

(2017) reported an occurrence of 29.71% of *Enterococcus* spp. from broiler chicken which shows similarity to our results.

In one study by Furtula et al. (2013), enterococci were detected in 12 surface water sites from poultry farms where *E. faecalis* (27%) and *E. faecium* (26%) were the predominant species. In another study from poultry litter, feed and water, *E. faecalis* and *E. faecium* accounted for 46% and43%respectively (Sapkota et al., 2011)

Although studies on *K. pneumoniae* and *Enterococcus* spp. from eggs are limited, but there have been studies conducted in other enteric bacteria like *E. coli* and *Salmonella* which show similarity to our results. In one such study conducted by Hossain et al. (2021)in chicken eggs, the prevalence of *E. coli* was 27.78 % on egg shells. In yet another study conducted by Ferreira et al., (2020) *Salmonella* spp. were found in 3% of eggs from backyard chicken flocks in Portugal.

4.2 Antibiotic resistance spectrum of *K. pneumoniae, E. faecalis* and *E. faecium*

All *K. pneumoniae* isolates and only *E. faecalis* and *E. faecium* isolates of *Enterococcu*s spp., were subjected to antimicrobial sensitivity testing. Based on the sensitivity test, isolates were categorised into Resistant, Intermediate Resistant or Susceptible categories. However, for analysis purpose, Intermediate resistant isolates were considered as Resistant. *K. pneumoniae* isolates showed high resistance to penicillin, ampicillin and amoxicillin antibiotics and the resistance percentage was higher in *K. pneumoniae* isolates (92.7%) obtained from deep litter systemin comparison to resistance of 72.77% in isolates from cage system. (Table:4.6) The isolates from deep litter system also had high resistance (78.57%) to third generation cephalosporin (cefotaxime) as compared to 44.4% in isolates from cage system. The resistance to carbapenem antibiotic was also present and was nearly same from deep litter as well as cage system. Cefotaxime and carbapenems comes under the category of highest priority antibiotic according to WHO classification of critically important antibiotics for human use and hence are a cause of concern. Resistance to other antibiotics such as doxycycline, ciprofloxacin, amikacin and gentamycin was seen at 22.22%, 22.22%, 11.11% and 11.11%, respectively in isolates of cage system, whereas resistance to same antibiotics in deep litter system was 35.71%, 28.57%, 21.42% and 21.42%, respectively. Resistance to co-trimoxazole was also present in *K. pneumoniae* isolates with 28.57% and 22.22% resistant isolates from deep litter and

cage system, respectively (Table: 4.6). Based on the results it can be observed that *K. pneumoniae* isolates from eggs and environment are resistant to highest priority (cefotaxime) and high priority antibiotics (imipenem, ciprofloxacin, gentamycin, amikacin,) and is a cause of concern for community health (Table: 4.7)

Antibiotic-resistant bacteria pose a serious threat to veterinary and health professionals, as they degrade therapy. The overuse and misuse of antibiotics, as well as a lack of new drug research by the pharmaceutical sector due to limited economic incentives and difficult regulatory requirements, have all been implicated for the antibiotic resistance crisis (Ventola, 2015). The Center for Disease Control and Prevention (CDC), US, has identified a number of bacteria as posing urgent, serious, and serious dangers, many of which have already wreaked havoc on the health-care system, patients, and their families (Michael et al., 2014).

K. pneumoniae and *Enterococcus* spp. both require immediate attention as both these bacteria have been mentioned in the WHO global priority list of pathogens where *K. pneumoniae* is included in the critical category and *E. faecium* in high-risk category.

In the current study all classes of WHO critically reserved antibiotics (*viz*; highest priority, high priority and highly important) for human medicine have been targeted for *K. pneumoniae*. Isolates in our study have been found resistant to these categories of drugswith *K. pneumoniae* showing resistance to third generation cephalosporins and carbapenems and *Enterococcus* to vancomycin. Our study is supported by various studies conducted worldwide and in India. Bhardwaj et al. (2021) reported 90% resistance to ampicillin and 40 % resistance against tetracycline from cloacal swabs. Jones, (2010) found that all 51 (100%) *Klebsiella* isolates resistant to ampicillin, which is consistent with our findings of 92.7% resistance to ampicillin. In contrast, Fielding et al. (2012) found that only 66.7% of *Klebsiella* isolates from free range chicken were resistant to ampicillin and 59.8% to tetracycline.

In a study conducted by Aly et al. (2014) the antibiotic resistance of sixteen *Klebsiella* isolates from chicks was investigated, and the results showed that the isolates were susceptible to imipenem, amikacin and ciproflaxin, which is close to the current findings.

In another study, the AST results of *K. pneumoniae* isolates showed 100 % resistance to ampicillin and amoxicillin, and 27.3 % resistance to ciprofloxacin, showing similarity to our findings (Hayati et al., 2019). High resistance of 92% to ampicillin and 31% to tetracycline has also been reported by Guo et al., (2016) supporting our results.

An acquired resistance to at least one agent in three or more antimicrobial categories is defined as a multidrug resistant (MDR) strain of any bacteria (Magiorakos et al., 2012). In the current study out of 23 *K. pneumoniae* isolates, 7(30.53%) were resistant to three or more than three classes of antibiotics and were therefore designated as multidrug resistant. Out of these 7 MDR isolates, 5(21.73%) were from deep litter system of management and 2(8.69) from cage system of management. None of the isolates from environment of either deep litter and cage system was MDR. (Table:4.6)

Evolution in bacterial population due to persistent antibiotic treatment and selective pressure leads to the formation of multiple genetic processes (Davies &Davies, 2010). As a result of this continual development, MDR and XDR bacterial strains have emerged (Magiorakos et al., 2012), which are resistant to practically all antibiotics and have no therapeutic options (Hersh et al., 2012). This could be supported by a study conducted by Fielding et al. (2012) wherein nearly 40% of the *Klebsiella* spp. isolates from free range chicken samples were multi drug resistant which shows similarity to our findings. In another study 50% of the *K. pneumoniae* isolates of raw chicken were MDR (Hartantyo et al., 2020).

Multiple Antibiotic Resistant Index (MARI) of *K. pneumoniae* was also calculated. In our study all (100%) isolates of *K. pneumoniae* had a MAR index >0.2 and 4 (17.39%) of the isolates had a MAR index >0.5 (3 from deep litter and 1 from cage system). The range of MAR index in the current study for K. pneumoniae was 0.3-0.7. the results indicated high-risk source of contamination from poultry farms of both deep litter and cage system (Afunwa et al., 2020). Bacteria with a MAR score of greater than 0.2 come from a high-risk source of contamination when antibiotics are often utilised. Organisms with MAR indices greater than 0.2 confirm the presence of multidrug-resistant genes originating from an environment where these medications are abused, as well as the presence of one or more resistance genes, each encoding a single antibiotic resistance phenotype, in their plasmids (Afunwa et al., 2020).

This observation is in line with previous studies of Hemen et al. (2012) and Matuschek et al. (2014) that found multidrug-resistant bacteria in chicken droppings. Antibiotic-resistant illnesses are difficult to cure and frequently necessitate expensive medications, long-term therapy, and, in the worst-case scenario, death.

Table: 4.6 Antimicrobial susceptibility testing for *K. pneumoniae*

				β-Lactam			Tetracycline	Sulfonamide	Aminoglycoside		Fluoroquinolones	Cephalosporins	Carbapenems			
District	**Farm type**	**Isolate ID**	**Source Of Sample**	**PEN**	**AMX**	**AMP**	**DO**	**COT**	**AK**	**GEN**	**CIP**	**CTX**	**IMP**	**MAR**	**RESULT**	**AVG MAR**
MOHF1	Deep Litter	CGO13	Egg Shell	S	S	S	S	S	S	S	S	S	S	0/10	0	0.4
MOHF1	Deep Litter	B3O8	Environment	R	R	R	S	S	S	S	S	S	S	3/10	0.3	
MOHF1	Deep Litter	LD3O19	Environment	R	R	R	I	I	R	R	R	S	I	9/10	0.9	
MOHF3	Deep Litter	CSF1O3	Egg Shell	R	R	R	S	R	S	S	S	R	S	5/10	0.5	0.45
MOHF3	Deep Litter	B1O5	Egg Shell	R	R	R	S	S	S	S	S	I	S	4/10	0.4	
LDHF3	Deep Litter	LF1O16	Egg Shell	I	I	I	R	I	I	S	S	R	I	8/10	0.8	0.525
LDHF3	Deep Litter	B2O5	Egg Shell	R	R	R	S	S	S	S	I	I	S	5/10	0.5	
LDHF3	Deep Litter	B3O6	Egg Shell	I	I	I	S	S	S	S	S	I	S	4/10	0.4	
LDHF3	Deep Litter	B3O13	Environment	R	R	R	S	S	S	S	S	R	S	4/10	0.4	
RUPF1	Deep Litter	LDF2O15	Egg Shell	R	R	R	I	S	S	S	R	R	R	7/10	0.7	0.6
RUPF1	Deep Litter	CSF2O3	Egg Shell	R	R	R	R	S	R	I	S	I	R	7/10	0.7	
RUPF1	Deep Litter	B2O8	Egg Shell	R	R	R	S	S	S	S	S	R	S	4/10	0.4	
RUPF4	Deep Litter	B3O11	Egg Shell	I	R	I	I	R	I	R	S	I	R	9/10	0.9	0.65
RUPF4	Deep Litter	B2O6	Egg Shell	I	I	I	S	S	S	S	S	I	S	4/10	0.4	
BARF1	Cage	KURO5	Egg Shell	S	S	S	S	S	S	S	S	S	S	0/10	0	0.36

				β-Lactam			Tetracycline	Sulfonamide	Aminoglycoside		Fluoroquinolones	Cephalosporins	Carbapenems			
District	**Farm type**	**Isolate ID**	**Source Of Sample**	**PEN**	**AMX**	**AMP**	**DO**	**COT**	**AK**	**GEN**	**CIP**	**CTX**	**IMP**	**MAR**	**RESULT**	**AVG MAR**
	System															
BARF1	Cage System	CSO10	Egg Shell	R	R	R	S	S	S	S	S	I	S	4/10	0.4	
BARF1	Cage System	CSF2O14	Environment	R	R	R	I	I	S	S	R	S	R	7/10	0.7	
BARF3	Cage System	KURO19	Egg Shell	I	I	I	R	R	S	S	I	S	R	7/10	0.7	0.7
BARF4	Cage System	CSF3O12	Egg Shell	R	R	R	S	S	S	S	S	S	S	3/10	0.3	0.3
LUDF2	Cage System	CGO18	Egg Shell	S	S	S	S	S	S	S	S	S	S	0/10	0	0.3
LUDF2	Cage System	KURF2O1	Egg Shell	I	I	I	S	S	S	S	S	R	S	4/10	0.4	
LUDF2	Cage System	CSF1O5	Egg Shell	R	R	R	S	S	S	S	S	R	S	4/10	0.4	
LUDF2	Cage System	CSF1O20	Environment	I	I	I	S	S	S	S	S	R	S	4/10	0.4	

Table: 4.7 No. of *K. pneumoniae* Isolates Resistant to Antimicrobial Classes in Different Farm Management Systems

District ID	Isolate ID	Deep Litter				Cage System			
		Susceptible	One Class	Two Class	MDR	Susceptible	One Class	Two Class	MDR
MOHF1	CGO13	✓				-	-	-	-
MOHF3	CSF1O3				✓	-	-	-	-
MOHF3	B1O5		✓			-	-	-	-
MOHF1	B3O8		✓			-	-	-	-
MOHF1	LD3O19				✓	-	-	-	-
LDHF3	LF1O16			✓		-	-	-	-
LDHF3	B2O5		✓			-	-	-	-
LDHF3	B3O6	✓				-	-	-	-
LDHF3	B3O13			✓		-	-	-	-
RUPF1	LDF2O15				✓	-	-	-	-
RUPF1	CSF2O3				✓	-	-	-	-
RUPF1	B2O8			✓		-	-	-	-
RUPF4	B3O11				✓	-	-	-	-

District ID	Isolate ID	Deep Litter				Cage System			
		Susceptible	One Class	Two Class	MDR	Susceptible	One Class	Two Class	MDR
RUPF4	B2O6	✓				-	-	-	-
BARF1	KURO5	-	-	-	-	✓			
BARF1	CSO10	-	-	-	-		✓		
BARF1	CSF2O14	-	-	-	-				✓
BARF3	KURO19	-	-	-	-				✓
BARF4	CSF3O12	-	-	-	-		✓		
LUDF2	CGO18	-	-	-	-	✓			
LUDF2	KURF2O1	-	-	-	-		✓		
LUDF2	CSF1O5	-	-	-	-			✓	
LUDF2	CSF1O20	-	-	-	-		✓		
Total		3	3	3	5	2	4	1	2

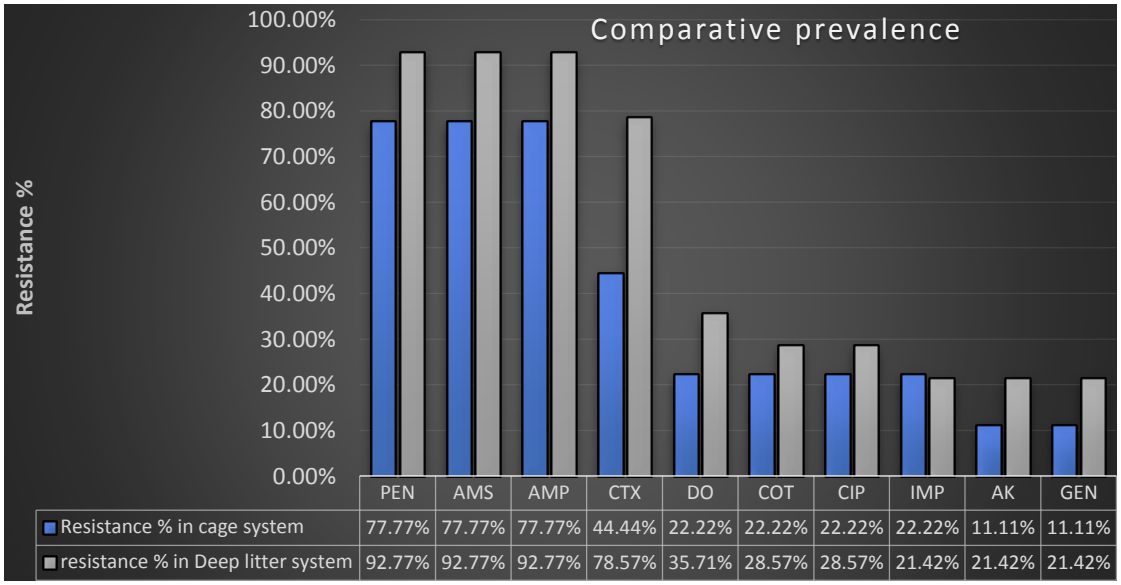


Fig. No. 9: Comparative prevalence of isolates of *K. pneumoniae* in different housing systems

Enterococcus spp. (*E. faecalis* and *E. faecium*) isolates showed high resistance to amikacin, tetracycline and erythromycin antibiotics and the resistance percentage (77.94%) was higher in isolates obtained from deep litter system than in cage system (61%). The *Enterococcus* spp. isolates from deep litter system also had higher resistance to ampicillin (47.05%) as compared to 39.47% resistance in isolates from cage system. A higher resistance to vancomycin in isolates from deep litter farms (16.17%) was observed in comparison to cage system farms (10.52%). Vancomycin is considered last resort antibiotic in *Enterococcus* infections and comes under the category of highest priority antibiotics according to WHO classification of critically important antibiotics for human use. The resistance to linezolid antibiotic was also present and was higher from deep litter (13.23%) than cage system (7.89%). Linezolid is also an important antibiotic as its used for treatment of vancomycin resistant enterococci (VRE) hence its resistance is a cause of concern (Table:4.8). Resistance to other antibiotics such as kanamycin, ciprofloxacin, chloramphenicol and gentamycin was seen at 58.28%, 57.35%, 50% and 50%, respectively in isolated from deep litter system whereas resistance to same antibiotics in isolates of cage system was observed at 44.73%, 42.10%, 42.10%, 36.84% respectively. Resistance to penicillin was also present in isolates with 61.76% and 52.63% resistance from deep litter and cage system, respectively. Based on the results we found that *Enterococcus* spp. isolates from eggs and environment were resistant to highest priority (vancomycin, ciprofloxacin and erythromycin) and high priority antibiotics (amikacin, gentamycin

and linezolid) and poses a potential risk to community health. The resistance to antibiotics in isolates of *Enterococcus* spp. from eggs of deep litter system had a higher resistance percentage than isolates from cage system and the difference was statistically significant ($p<=0.05$)

Table: 4.7(a) Antibiotic resistance patterns in *Enterococcus* spp.

					β-Lactam		Amphenicols	Tetracyclin		Aminoglycosides		Fluoroquinolones	Macrolide	Oxazolidinones	Glycopeptide			
District	**Farm Type**	**Isolate ID**	**Source of Sample**	***E. Faecium/E. Fecalis***	**PEN**	**AMP**	**CHL**	**TET**	**GEN**	**AK**	**K**	**CIP**	**E**	**LZ**	**VAN**	**MAR index**	**Result**	**Average MAR**
LDH1	Deep Litter	B2O11	Egg	*E. Fecalis*	S	S	S	R	S	R	S	S	R	S	S	3/11	0.272	3.452/6=
LDH1	Deep Litter	LFO5	Egg	*E. Fecalis*	R	R	R	R	R	R	R	R	R	R	R	11/11	1	0.575
LDH1	Deep Litter	CGLO3	Egg	*E. Fecalis*	I	S	S	R	S	R	I	I	R	S	R	7/11	0.636	
LDH1	Deep Litter	B3O8	Environment	*E. Fecalis*	S	S	S	R	S	R	S	S	I	S	S	3/11	0.272	
LDH1	Deep Litter	L2O10	Environment	*E. Faecium*	I	R	I	I	S	I	S	S	R	S	I	7/11	0.636	
LDH1	Deep Litter	L5O3	Hand Swabs	*E. Fecalis*	S	S	S	I	I	I	S	S	I	S	S	4/11	0.363	
LDH2	Deep Litter	CGLO9	Egg	*E. Fecalis*	R	I	R	R	R	R	R	R	R	S	R	10/11	0.909	3.727/4=
LDH2	Deep Litter	CGLO2	Egg	*E. Fecalis*	R	R	R	R	R	R	R	R	R	S	R	10/11	0.909	0.818
LDH2	Deep Litter	B3I4	Environment	*E. Fecalis*	R	R	R	R	R	R	R	R	R	R	S	10/11	0.909	
LDH2	Deep Litter	L2O1	Environment	*E. Faecium*	R	I	I	I	I	S	R	S	S	S	S	6/11	0.545	
LDH3	Deep Litter	CGLO1	Egg	*E. Fecalis*	R	S	S	R	S	R	R	R	R	S	S	6/11	0.545	2.817/7=
LDH3	Deep Litter	CGLO5	Egg	*E. Fecalis*	R	I	I	R	R	R	R	R	R	S	R	10/11	0.909	0.402
LDH3	Deep Litter	CGLO3	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
LDH3	Deep Litter	CGLO8	Egg	*E. Faecium*	R	R	R	R	R	R	R	R	R	R	S	10/11	0.909	
LDH3	Deep Litter	KURO7	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
LDH3	Deep Litter	KURO19	Environment	*E. Fecalis*	I	S	S	R	S	R	I	I	S	S	S	5/11	0.454	
LDH3	Deep Litter	B3I3	Hand Swabs	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	00	
LDH4	Deep Litter	B2O3	Egg	*E. Fecalis*	S	S	S	I	I	I	S	S	I	S	S	4/11	0.363	2.816/5=
LDH4	Deep Litter	B3I1	Egg	*E. Fecalis*	R	R	R	R	R	R	R	R	R	S	R	10/11	0.909	0.563
LDH4	Deep Litter	KURO6	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
LDH4	Deep Litter	CGLO8	Egg	*E. Faecium*	R	S	S	R	S	I	S	S	S	S	S	3/11	0.272	
LDH4	Deep Litter	L2O4	Egg	*E. Faecium*	R	I	I	I	I	I	S	S	S	S	I	7/11	0.636	
RUP1	Deep Litter	LF1O10	Egg	*E. Fecalis*	R	S	S	R	S	R	R	R	R	S	R	7/11	0.636	1.454/3=

					β-Lactam		Amphenicols	Tetracyclin		Aminoglycosides		Fluoroquinolones	Macrolide	Oxazolidinones	Glycopeptide			
District	**Farm Type**	**Isolate ID**	**Source of Sample**	***E. Faecium/E. Fecalis***	**PEN**	**AMP**	**CHL**	**TET**	**GEN**	**AK**	**K**	**CIP**	**E**	**LZ**	**VAN**	**MAR index**	**Result**	**Average MAR**
RUP1	Deep Litter	L2O3	Egg	*E. Fecalis*	R	R	R	R	R	R	R	R	R	S	S	9/11	0.818	0.484
RUP1	Deep Litter	LF1O7	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
RUP2	Deep Litter	B2O9	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	0
MOH1	Deep Litter	CGLO18	Egg	*E. Fecalis*	R	R	R	R	R	R	R	R	R	S	S	9/11	0.818	2.999/4=
MOH1	Deep Litter	CGLO4	Egg	*E. Fecalis*	R	S	I	R	S	R	R	R	R	S	R	8/11	0.727	0.749
MOH1	Deep Litter	L3O2	Egg	*E. Faecium*	I	I	I	R	R	I	I	I	I	S	S	9/11	0.818	
MOH1	Deep Litter	L5O5	Environment	*E. Fecalis*	I	S	S	R	S	R	I	I	I	S	I	7/11	0.636	
MOH2	Deep Litter	CGF2W	Egg	*E. Fecalis*	S	S	S	I	S	I	S	S	I	S	S	3/11	0.272	3.271/7=
MOH2	Deep Litter	CGLO1	Egg	*E. Fecalis*	R	S	S	R	S	R	R	R	R	S	S	6/11	0.545	0.467
MOH2	Deep Litter	CGLO6	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
MOH2	Deep Litter	L2O1	Egg	*E. Faecium*	R	R	R	R	R	R	R	R	R	I	S	10/11	0.909	
MOH2	Deep Litter	CGLO4	Egg	*E. Faecium*	R	R	R	R	R	R	R	R	R	R	R	11/11	1	
MOH2	Deep Litter	L5O6	Environment	*E. Fecalis*	R	I	I	R	R	R	R	R	R	S	S	9/11	0.818	
MOH2	Deep Litter	L3O2	Hand Swabs	*E. Fecalis*	I	S	I	R	S	R	I	I	I	S	I	8/11	0.727	
MOH3	Deep Litter	CGLO5	Egg	*E. Fecalis*	R	R	R	R	R	R	R	R	R	R	R	11/11	1	3.906/6=
MOH3	Deep Litter	CGLO16	Egg	*E. Fecalis*	S	S	S	I	I	I	S	S	I	S	S	4/11	0.363	0.651
MOH3	Deep Litter	CGLO3	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
MOH3	Deep Litter	B3O3	Egg	*E. Faecium*	S	S	S	R	S	R	S	S	R	S	S	3/11	0.272	
MOH3	Deep Litter	L5O5	Egg	*E. Faecium*	R	R	R	R	R	R	R	R	R	R	S	10/11	0.909	
MOH3	Deep Litter	L3O1	Environment	*E. Faecium*	S	S	S	R	S	R	S	S	R	S	S	3/11	0.272	
MOH4	Deep Litter	LF1O2	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	2.999/5=
MOH4	Deep Litter	CGLO18	Egg	*E. Fecalis*	R	I	I	R	R	R	R	R	R	S	S	9/11	0.818	0.599
MOH4	Deep Litter	B3O3	Egg	*E. Fecalis*	S	S	S	I	S	I	S	S	I	S	S	3/11	0.272	
MOH4	Deep Litter	B3O12	Environment	*E. Faecium*	R	R	R	R	R	R	R	R	R	R	I	11/11	1	

					β-Lactam		Amphenicols	Tetracyclin		Aminoglycosides		Fluoroquinolones	Macrolide	Oxazolidinones	Glycopeptide			
District	**Farm Type**	**Isolate ID**	**Source of Sample**	***E. Faecium/E. Fecalis***	**PEN**	**AMP**	**CHL**	**TET**	**GEN**	**AK**	**K**	**CIP**	**E**	**LZ**	**VAN**	**MAR index**	**Result**	**Average MAR**
MOH4	Deep Litter	L2O9	Hand Swabs	*E. Fecalis*	R	R	R	R	R	R	R	R	R	R	S	10/11	0.909	
RUP1	Deep Litter	L2O5	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	0.272/2= 0.136
RUP1	Deep Litter	LF1O2	Hand Swabs	*E. Faecium*	S	S	S	I	S	I	S	S	I	S	S	3/11	0.272	
RUP2	Deep Litter	CSF1O2	Egg	*E. Faecium*	I	I	I	I	R	R	I	I	R	S	S	9/11	0.818	3.998/6= 0.666
RUP2	Deep Litter	L2O3	Egg	*E. Faecium*	S	S	S	I	S	R	S	S	R	S	I	4/11	0.363	
RUP2	Deep Litter	LF1O11	Egg	*E. Faecium*	R	R	R	R	R	R	R	R	R	S	S	9/11	0.818	
RUP2	Deep Litter	L5O8	Egg	*E. Faecium*	S	S	S	I	S	I	S	S	I	S	S	3/11	0.272	
RUP2	Deep Litter	L2O1	Enviromnet	*E. Fecalis*	R	R	R	R	R	R	R	R	R	S	S	9/11	0.818	
RUP2	Deep Litter	LF5O1	Hand Swabs	*E. Faecium*	R	R	R	R	R	R	R	R	R	R	S	10/11	0.909	
RUP3	Deep Litter	B3O4	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	2.908/7= 0.415
RUP3	Deep Litter	LF1O6	Egg	*E. Fecalis*	I	S	S	R	S	R	I	I	R	S	S	6/11	0.545	
RUP3	Deep Litter	LF1O1	Egg	*E. Fecalis*	R	S	I	R	S	R	R	R	R	S	S	7/11	0.636	
RUP3	Deep Litter	L5O6	Egg	*E. Faecium*	R	I	I	R	R	R	R	R	R	S	S	9/11	0.818	
RUP3	Deep Litter	L2O1	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
RUP3	Deep Litter	L5O1	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
RUP3	Deep Litter	L2O3	Egg	*E. Faecium*	R	R	R	R	R	R	R	R	R	R	S	10/11	0.909	
RUP4	Deep Litter	B3I10	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	3.363/6= 0.560
RUP4	Deep Litter	B3O7	Egg	*E. Fecalis*	R	R	R	R	R	R	R	R	R	S	S	9/11	0.818	
RUP4	Deep Litter	CGLO4	Egg	*E. Faecium*	R	R	R	R	R	R	R	R	R	R	S	10/11	0.909	
RUP4	Deep Litter	L5O5	Egg	*E. Faecium*	R	R	S	S	I	I	I	I	I	I	S	8/11	0.727	
RUP4	Deep Litter	B3O8	Egg	*E. Faecium*	R	R	R	R	R	R	R	R	R	R	S	10/11	0.909	
ASR2	Cage System	CSF2O12	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	1.181/6= 0.196
ASR2	Cage System	L2O19	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	

					β-Lactam		Amphenicols	Tetracyclin		Aminoglycosides		Fluoroquinolones	Macrolide	Oxazolidinones	Glycopeptide			
District	**Farm Type**	**Isolate ID**	**Source of Sample**	***E. Faecium/E. Fecalis***	**PEN**	**AMP**	**CHL**	**TET**	**GEN**	**AK**	**K**	**CIP**	**E**	**LZ**	**VAN**	**MAR index**	**Result**	**Average MAR**
ASR2	Cage System	L3O5	Egg	*E. Fecalis*	R	R	R	R	R	R	R	R	R	S	R	10/11	0.909	
ASR2	Cage System	B2I6	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
ASR2	Cage System	B2O6	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
ASR2	Cage System	KURO1	Environment	*E. Faecium*	R	R	S	S	S	S	S	S	R	S	S	3/11	0.272	
ASR3	Cage System	CSF2O11	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	1.908/7= 0.272
ASR3	Cage System	CGLO17	Egg	*E. Fecalis*	S	S	S	I	I	I	S	S	I	S	S	4/11	0.363	
ASR3	Cage System	CSF2O11	Egg	*E. Fecalis*	R	S	I	R	S	R	R	R	R	S	S	7/11	0.636	
ASR3	Cage System	B4O10	Egg	*E. Faecium*	R	I	I	R	R	R	R	R	R	S	R	10/11	0.909	
ASR3	Cage System	B1O5	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
ASR3	Cage System	B4O16	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
ASR3	Cage System	KUR02	Environment	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
BAR1	Cage System	B3O10	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	3.181/6= 0.530
BAR1	Cage	L5O4	Egg	*E. Fecalis*	I	S	S	S	R	R	I	I	R	S	S	6/11	0.545	

					β-Lactam		Amphenicols	Tetracyclin		Aminoglycosides		Fluoroquinolones	Macrolide	Oxazolidinones	Glycopeptide			
District	**Farm Type**	**Isolate ID**	**Source of Sample**	***E. Faecium/E. Fecalis***	**PEN**	**AMP**	**CHL**	**TET**	**GEN**	**AK**	**K**	**CIP**	**E**	**LZ**	**VAN**	**MAR index**	**Result**	**Average MAR**
	System																	
BAR1	Cage System	B1I14	Egg	*E. Fecalis*	R	R	R	R	R	R	R	R	R	S	R	10/11	0.909	
BAR1	Cage System	B3O16	Egg	*E. Faecium*	I	I	I	I	R	R	I	I	R	S	S	9/11	0.818	
BAR1	Cage System	KUR03	Environment	*E. Faecium*	R	R	R	R	R	R	R	R	R	R	S	10/11	0.909	
BAR2	Cage System	B3O12	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	1
BAR2	Cage System	LDHO15	Egg	*E. Faecium*	R	R	R	R	R	R	R	R	R	I	R	11/11	1	
BAR3	Cage System	B3O12	Egg	*E. Fecalis*	S	S	S	S	R	R	S	S	R	S	S	3/11	0.272	2.726/8= 0.340
BAR3	Cage System	B3O13	Egg	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
BAR3	Cage System	B3O5	Egg	*E. Fecalis*	R	I	I	I	I	I	I	S	R	S	S	8/11	0.727	
BAR3	Cage System	CSF2O3	Egg	*E. Faecium*	R	R	R	R	R	R	R	R	R	S	S	9/11	0.818	
BAR3	Cage System	LDHO19	Egg	*E. Faecium*	I	I	I	R	S	S	I	S	R	S	S	6/11	0.545	
BAR3	Cage System	CSF2O14	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
BAR3	Cage System	CGL	Environment	*E. Fecalis*	R	I	I	R	R	R	R	R	R	S	S	9/11	0.818	

					β-Lactam		Amphenicols	Tetracyclin		Aminoglycosides		Fluoroquinolones	Macrolide	Oxazolidinones	Glycopeptide			
District	**Farm Type**	**Isolate ID**	**Source of Sample**	***E. Faecium/E. Fecalis***	**PEN**	**AMP**	**CHL**	**TET**	**GEN**	**AK**	**K**	**CIP**	**E**	**LZ**	**VAN**	**MAR index**	**Result**	**Average MAR**
BAR3	Cage System	KUR04	Environment	*E. Faecium*	I	I	I	R	R	R	I	I	R	S	R	10/11	0.909	
BAR5	Cage System	B3O8	Egg	*E. Fecalis*	I	S	I	I	S	R	R	S	R	S	S	6/11	0.545	1.363/3= 0.454
BAR5	Cage System	B3I19	Egg	*E. Fecalis*	R	R	R	R	R	R	R	R	R	S	S	9/11	0.818	
BAR5	Cage System	B3O10	Environment	*E. Fecalis*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
LDH3	Cage System	B3O1	Environment	*E. Fecalis*	R	R	R	R	R	R	R	R	R	R	R	11/11	1	1
LDH3	Cage System	KUR05	Environment	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
LDH4	Cage System	B3O16	Egg	*E. Fecalis*	S	S	S	S	I	S	S	S	S	S	S	1/11	0.090	0.816/4= 0.204
LDH4	Cage System	CSO20	Egg	*E. Faecium*	R	R	I	S	S	S	S	S	R	S	S	4/11	0.363	
LDH4	Cage System	LF1O16	Egg	*E. Faecium*	S	S	S	S	S	S	S	S	S	S	S	0/11	0	
LDH4	Cage System	B3O9	Environment	*E. Fecalis*	I	S	S	R	S	I	S	S	I	S	S	4/11	0.363	

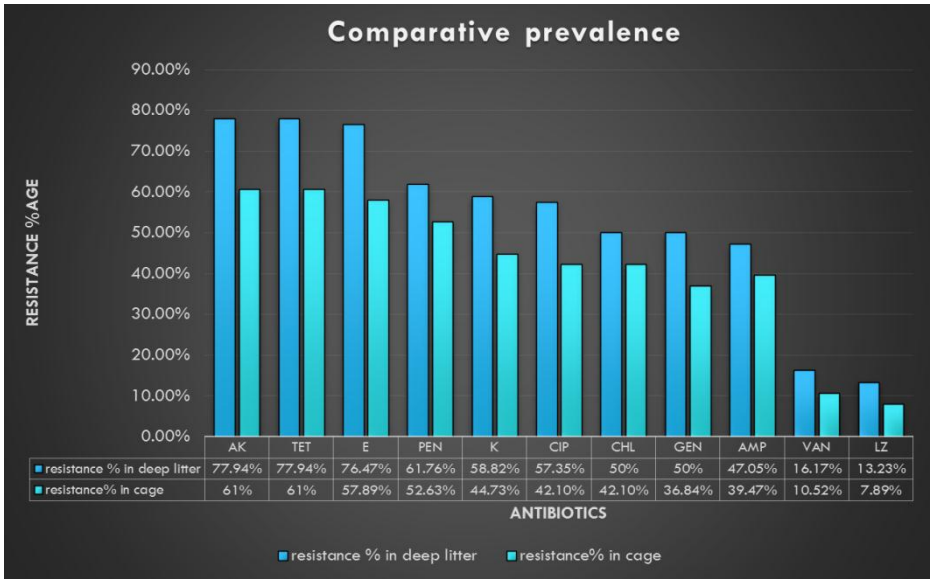


Fig: 10 Comparative prevalence of isolates of *Enterococcus* spp. in different housing systems

Antibiotic-resistant bacteria have been found often in animals, animal products, and their surroundings (Ali et al., 2014). Enterococci-related nosocomial and opportunistic infections, notably those caused by *E. faecalis* and *E. faecium* (Wassenaar & Silley, 2008) are becoming increasingly serious concerns in human medicine due to their resistance to a variety of medicines. In addition, enterococci act as a reservoir for transferable resistance genes (Sahlström et al., 2009). *Enterococcus* spp. from animal origin constitute a significant public health risk since these strains can be transmitted to humans through contaminated food, resulting in the spread and persistence of antibiotic-resistant bacteria in the general population and environment (Daniel et al., 2015). Among *Enterococcus* spp., *E. faecalis* and *E. faecium* are of nosocomial importance. They can cause urinary tract infections, wound infections, bacteraemia, and infective endocarditis in humans (Hammerum et al., 2012).

In a study conducted by Obeng et al. (2013), nearly 83% resistance was found against tetracycline which is in agreement with our finding. In another study conducted by Ngbede et al. (2017), 61% resistance to tetracycline and erythromycin was seen.

In a study, resistance of *E. faecalis* and *E. faecium* isolates from broiler chicken was recorded. The percentages of resistant *E. faecalis* and *E. faecium* isolates were 0 and 0.9 % for chloramphenicol, 0.7 and 14.5 % for ciprofloxacin, 72.6 and 80.3 % for erythromycin, 9.6 and 4.3 % for gentamicin, 25.2 and 17.1 % for

kanamycin, 0 and 0 %, respectively (Tremblay et al., 2011). In another study *Enterococcus* isolates were taken from poultry environment. Resistance to tetracycline was found to be higher in *E. faecalis* isolates (68%) than in *E. faecalis* isolates (53.7%) but resistance to erythromycin was found to be higher in *E. faecalis* isolates (69%) than in *E. faecium* isolates (34%) (Hayes et al., 2003)

In yet another study conducted in milk, beef and poultry samples,the most common species were *E. faecalis* (46.1%) and *E. faecium* (29.0%). Ampicillin resistance was seen in more than 96 and 97 % of *E. faecalis* and *E. faecium* isolates, respectively. Vancomycin resistance was prevalent. Vancomycin resistance was found in 18.8, 7.8, and 13.1 % of *E. faecalis* and in 32.8, 24.7, and 30.7 % of *E. faecium* isolates from milk, beef, and poultry samples, respectively (Chingwaru et al., 2003).

In another study conducted by Furtula et al. (2013) resistance against tetracycline, penicillin and ciprofloxacin in poultry litter isolates was found to be 65.3%, 61.1% and 49.6% respectively which is close to our findings.

Osman et al. (2019) reported resistance of 76% to chloramphenicol and 11.3% to linezolid.

In another study *E. faecalis* and *E. faecium* isolates from swine, cattle, poultry were tested for resistance. Regardless of their species, the majority of the isolates were resistant to tetracycline (60.5%) and erythromycin (54.4%), with a considerable proportion, also showing high-level resistance to kanamycin (42.1%). Resistance to ciprofloxacin (21.9%) and rifampicin (16.7%) was low, and high-level resistance to gentamicin (5.3%) was seen (Seputiene et al., 2012).

Bekele and Ashenafi (2010) reported resistance of 44% against vancomycin from poultry isolates which is higher than our study. In yet another study by Akhi et al. (2009) reported lower resistance i.e., 4.38% against vancomycin as compared to our study. The inconsistence in the resistance pattern may be attributed to difference in management practices, hygiene at the farms, biosecurity measures and effect of supplemented feed (Osman et al., 2019). According to a metanalysis done by Lawpidet et al. (2021), VRE was found in 11.7 % among all foods of animal origin, suggesting the extent of VRE contamination in animal-derived foods. Vancomycin is the last resort antibiotic for gram positive bacteria. It comes under the highest priority

category of antibiotics in WHO list of Critically Important Antimicrobials for Human Medicine, hence its use in animal sector must be avoided.

Out of a total of 106 *Enterococcus* isolates tested for AST 45(42.45%) were MDR (Table:13). Out of these 45 MDR isolates, 33(31.13%) were from deep litter system of management and 12(11.32%) from cage system of management. The isolates from eggs of deep litter system had a higher multidrug resistance percentage than isolates from cage system and the difference was statistically significant ($p<=0.05$)

According to a study done by Karunarathna et al. (2020) from broiler chicken embryo the recorded multidrug resistance was seen in 44.9% of Enterococcus isolates which is in concordance with the present study. In another study 98% of the isolates of *E. faecium* and *E. faecalis* from pigs, farmers and farm environment were MDR (Tan et al., 2018).

Multiple Antibiotic Resistant Index (MARI) of *Enterococcus* was also calculated. Bacteria with a MAR score of greater than 0.2 come from a high-risk source of contamination when antibiotics are often utilised. The range of MAR indices in the current study for *Enterococcus* spp. was 0.136- 1.00. In our study 18(75%) farms with different isolates (egg, litter, feed, water) had a MAR index score >0.2, however, 10 (41.66%) farms had a MAR index higher than 0.5. Out of these 8 (66.66%) farms were of deep litter system and only 2 (16.66%) farms from cage system. The MAR index indirectly suggests that all isolates originated in an environment where antibiotics were frequently used.

With enormous antibiotic consumption, manufacture, and misuse, India is among the countries that are the epicentre of the worldwide antimicrobial resistance problem (Broom and Doron 2020). The increased use of antimicrobials in India, combined with widespread misuse in health and agriculture, has resulted in the rise of multidrug-resistant organisms (MROS) (Laxminarayan & Chaudhury, 2016).

Table:4.8 No. of *Enterococcus* spp. Isolates Resistant to Antimicrobial Classes in Different Farm Management Systems

District ID	Isolate ID	Deep Litter				Cage System			
		Susceptible	One Class	Two Class	MDR	Susceptible	One Class	Two Class	MDR
LDH1	B2O11				✓	-	-	-	-
LDH1	LFO5				✓	-	-	-	-
LDH1	CGLO3				✓	-	-	-	-
LDH1	B3O8			✓		-	-	-	-
LDH1	L2O10			✓		-	-	-	-
LDH1	L5O3		✓			-	-	-	-
LDH2	CGLO9				✓	-	-	-	-
LDH2	CGLO2				✓	-	-	-	-
LDH2	B3I4				✓	-	-	-	-
LDH2	L2O1			✓		-	-	-	-
LDH3	CGLO1				✓	-	-	-	-
LDH3	CGLO5				✓	-	-	-	-
LDH3	CGLO3		✓			-	-	-	-
LDH3	CGLO8				✓	-	-	-	-
LDH3	KURO7		✓			-	-	-	-
LDH3	KURO19			✓		-	-	-	-
LDH3	B3I3		✓			-	-	-	-

District ID	Isolate ID	Deep Litter				Cage System			
		Susceptible	One Class	Two Class	MDR	Susceptible	One Class	Two Class	MDR
LDH4	B2O3	✓				-	-	-	-
LDH4	B3I1				✓	-	-	-	-
LDH4	KURO6		✓			-	-	-	
LDH4	CGLO8			✓		---	-	-	-
LDH4	L2O4		✓			-	-	-	-
RUP1	LF1O10				✓	-	-	-	-
RUP1	L2O3				✓	-	-	-	-
RUP1	LF1O7	✓				-	-	-	-
RUP2	B2O9		✓			-	-	-	-
MOH1	CGLO18				✓	-	-	-	-
MOH1	CGLO4				✓	-	-	-	-
MOH1	L3O2			✓		-	-	-	-
MOH1	L5O5			✓		-	-	-	-
MOH2	CGF2W		✓			-	-	-	-
MOH2	CGLO1				✓	-	-	-	-
MOH2	CGLO6		✓			-	-	-	-
MOH2	L2O1				✓	-	-	-	-
MOH2	CGLO4				✓	-	-	-	-

District ID	Isolate ID	Deep Litter				Cage System			
		Susceptible	One Class	Two Class	MDR	Susceptible	One Class	Two Class	MDR
MOH2	L5O6				✓	-	-	-	-
MOH2	L3O2			✓		-	-	-	-
MOH3	CGLO5				✓	-	-	-	-
MOH3	CGLO16		✓			-	-	-	-
MOH3	CGLO3					-	-	-	-
MOH3	B3O3		✓			-	-	-	-
MOH3	L5O5				✓	-	-	-	-
MOH3	L3O1			✓		-	-	-	-
MOH4	LF1O2		✓			-	-	-	-
MOH4	CGLO18				✓	-	-	-	-
MOH4	B3O3			✓		-	-	-	-
MOH4	B3O12				✓	-	-	-	-
MOH4	L2O9				✓	-	-	-	-
RUP1	L2O5		✓			-	-	-	-
RUP1	LF1O2		✓			-	-	-	-
RUP2	CSF1O2			✓		-	-	-	-
RUP2	L2O3			✓		-	-	-	-
RUP2	LF1O11				✓	-	-	-	-

District ID	Isolate ID	Deep Litter				Cage System			
		Susceptible	One Class	Two Class	MDR	Susceptible	One Class	Two Class	MDR
RUP2	L5O8		✓			-	-	-	-
RUP2	L2O1				✓	-	-	-	-
RUP2	LF5O1				✓	-	-	-	-
RUP3	B3O4		✓			-	-	-	-
RUP3	LF1O6				✓	-	-	-	-
RUP3	LF1O1				✓	-	-	-	-
RUP3	L5O6				✓	-	-	-	-
RUP3	L2O1		✓			-	-	-	-
RUP3	L5O1			✓		-	-	-	--
RUP3	L2O3				✓	-	-	-	-
RUP4	B3I10		✓			-	-	-	-
RUP4	B3O7				✓	-	-	-	-
RUP4	CGLO4				✓	-	-	-	-
RUP4	L5O5			✓		-	-	-	-
RUP4	B3O8				✓	-	-	-	-
Total		2	18	14	33				
ASR2	CSF2O12	-	-	-	-		✓		
ASR2	L2O19	-	-	-	-	✓			

District ID	Isolate ID	Deep Litter				Cage System			
		Susceptible	One Class	Two Class	MDR	Susceptible	One Class	Two Class	MDR
ASR2	L3O5	-	-	-	-				✓
ASR2	B2I6	-	-	-	-	✓			
ASR2	B2O6	-	-	-	-		✓		
ASR2	KURO1	-	-	-	-			✓	
ASR3	CSF2O11	-	-	-	-		✓		
ASR3	CGLO17	-	-	-	-			✓	
ASR3	CSF2O11	-	-	-	-				✓
ASR3	B4O10	-	-	-	-				✓
ASR3	B1O5	-	-	-	-			✓	
ASR3	B4O16	-	-	-	-			✓	
ASR3	KUR02	-	-	-	-			✓	
BAR1	B3O10	-	-	-	-	✓			
BAR1	L5O4	-	-	-	-			✓	
BAR1	B1I14	-	-	-	-				✓
BAR1	B3O16	-	-	-	-			✓	
BAR1	KUR03	-	-	-	-				✓
BAR2	B3O12	-	-	-	-	✓			
BAR2	LDHO15	-	-	-	-				✓

District ID	Isolate ID	Deep Litter				Cage System			
		Susceptible	One Class	Two Class	MDR	Susceptible	One Class	Two Class	MDR
BAR3	B3O12	-	-	-	-			✓	
BAR3	B3O13	-	-	-	-			✓	
BAR3	B3O5	-	-	-	-			✓	
BAR3	CSF2O3	-	-	-	-				✓
BAR3	LDHO19	-	-	-	-			✓	
BAR3	CSF2O14	-	-	-	-	✓			
BAR3	CGL	-	-	-	-				✓
BAR3	KUR04	-	-	-	-				✓
BAR5	B3O8	-	-	-	-			✓	
BAR5	B3I19	-	-	-	-				✓
BAR5	B3O10	-	-	-	-	✓			
LDH3	B3O1	-	-	-	-				✓
LDH3	KUR05	-	-	-	-		✓		
LDH4	B3O16	-	-	-	-		✓		
LDH4	CSO20	-	-	-	-		✓		
LDH4	LF1O16	-	-	-	-			✓	
LDH4	B3O9	-	-	-	-			✓	
Total						6	6	14	12

The risk of *Enterococcus* spp., and its resistance in two different poultry housing systems

Risk of *Enterococcus* spp., *E.faecalis/E.faecium,* resistance, MDR and vancomycin resistance was compared for the isolates from eggs of the deep litter system to that of the isolates from cage system of management. It was found that the risk of presence of *Enterococcus* spp. isolate in eggs from deep litter was 1.70 times higher (CI-1.088-2.664) than from the eggs of cage system (Table: 4.8). The risk of presence of *E. faecium/E. faecalis* in eggs from deep litter farms was 1.684 (CI-1.040-2.727) higher than the eggs of cage system farms. Presence of *Enterococcus* isolates resistant to at least one class of antibiotic in *Enterococcus* isolates from deep litter farms was 2.3 (CI-1.23-4.46) times higher than the isolates from cage system farm management. On examining the presence of MDR it was found that risk of MDR in *Enterococcus* isolates in eggs from deep litter was 4.29 times (CI-1.833-10.069) higher than *Enterococcus* isolates from cage system farms. Presence of vancomycin resistant in isolates from the deep litter farms was 3.49 times higher than *Enterococcus* isolates from the cage system farms.

4.4 Special antibiotic susceptibility test for *K. pneumoniae*

K. pneumoniae isolates were also screened for Extended spectrum β-lactamase and *Klebsiella pneumoniae* carbapenemase (KPC) production. Out of 23 isolates 5(21.73%) were KPC producers, whereas, 9(39.13%) isolates were ESBL producers (Table: 4.10).

Extended-spectrum ß-lactamases are the most clinically important class A enzymes identified in Enterobacteriaceae, particularly *K. pneumoniae* (ESBLs). It confers resistance to a wide range of β-lactam antibiotics, including penicillin, second and third generation cephalosporins, and monobactams, but not to carbapenems or cephamycins (e.g. cefoxitin). The rapidly increasing resistance of ESBL producers to multiple antibiotic families is a severe issue that limits the therapeutic options available against ESBL producers who encode ESBLs (Pitout & Laupland, 2015).

KPC producing *K. pneumoniae* bacteria a that cause infections are associated with substantial morbidity and mortality. Penicillin, cephalosporin, broad-spectrum β-lactams and carbapenems are all degraded by KPC (Pitout et al., 2008).

In a study conducted by Abdallah et al. (2015), 11.32 % of the retail chicken meat isolates were carbapenemases producers and 65.09% were ESBL producers phenotypically. In another study by Yang et al. (2019) ESBL production by chicken isolates was 31.1% which is close to our results.

Table: 4.9 Special test for detection of ESBL and KPC in *K. pneumoniae*

KPC/ESBL	Antibiotics for screening	Antibiotics for potentiation	Interpretation	Results
KPC	Ertapenem	Ertapenem+Phenylboronic acid	MIC of KPC/ MIC of KPC+ >8 = KPC positive	**5**/23
ESBL	Ceftriaxone + Ceftazidime	Ceftriaxone +Ceftazidime+Clavulinic acid	MIC of ESBL/MIC ESBL+ >8 =ESBL positive	**9**/23

Table: 4.10 Risk of occurrence of *Enterococcus* spp., and its resistance in different poultry farm management systems'

	Total	Positive	Odd's Ratio	95% Confidence Interval	
				Lower	Upper
Presence of Enterococcus spp.					
Deep Litter	480	60	1.702	1.088	2.664
Cage System	480	40	Ref.	Ref.	Ref.
Presence of E. Fecalis/Faecium					
Deep Litter	480	50	1.684	1.040	2.727
Cage System	480	33	Ref.	Ref.	Ref.
Presence of Resistant Enterococcus					
Deep Litter	480	32	2.351	1.238	4.468
Cage System	480	15	Ref.	Ref.	Ref.
Presence of MDR Enterococcus					
Deep Litter	480	27	4.296	1.833	10.069
Cage System	480	7	Ref.	Ref.	Ref.
Presence of Vancomycin Resistance in Enterococcus					
Deep Litter	480	10	3.494	0.950	12.858
Cage System	480	3	Ref.	Ref.	Ref.

4.4 Genotypic profile of *Klebsiella pneumoniae* and *Enterococcus* spp.

Antibiotic usage has the potential to select bacteria with virulence characteristics linked to antimicrobial resistance (Lehtolainen et al., 2003). These characteristics are encoded by specific genes that can be found on the bacterial chromosome, plasmids, transposons, or gene cassettes inserted into integrons (Daka&Yihdego, 2012), making them easily transferable across isolates.

The β-lactams are a wide class of varied compounds that are considered to be the most extensively utilised therapeutic class of antibacterial recommended in human and veterinary clinical practises due to their excellent safety profile and broad antimicrobial spectrum. Unfortunately, because of the constant selective force driving diversification of the resistance mechanism, this has resulted in an increase in global resistance in health care settings as well as in the community(Ali et al., 2018). By acquiring plasmids encoding genes, *K. pneumoniae* can express a high level of resistance to third-generation cephalosporins. TEM, SHV, and CTX-M are the 3 major ESBLS. CTX-M has been found to be the most common kind among clinically infected persons all over the world (Mahanti et al., 2018).

In our study *K. pneumoniae* was screened for the presence of ESBL genes*viz;bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{DHA}}$. Out of 23 *K. pneumonaie* isolates, 11(47.82%) were positive for *bla*$_{\text{CTX-M}}$, 10(43.47%) were positive for *bla*$_{\text{TEM}}$ and 2(8.69%) were positive for *bla*$_{\text{SHV}}$ genes. None of the isolates was positive for bla$_{\text{DHA}}$.

Prakash et al. (2014) reported 38.8%, 44.4% and 27.7% prevalence of *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$,and *bla*$_{\text{SHV}}$which is in agreement to our study. In yet another study by Abdallah et al. (2015) *bla*$_{\text{TEM}}$genes was found in 61 isolates (57.55 %) and*bla*$_{\text{CTX-M}}$ genes in 49 (46.23 %) isolates of the chicken meat.

Another study revealed that 9.1% of the isolates harboured *bla*$_{\text{SHV}}$, 100% of the isolates harboured *bla*$_{\text{TEM}}$ and 90.9% of the isolates harboured *bla*$_{\text{CTX-M}}$ which is in partial concordance with our research as similar results for *bla*$_{\text{SHV}}$ (8.6%) have been recorded but *bla*$_{\text{CTX-M}}$ (47.8%) and *bla*$_{\text{TEM}}$ (43.4%) were reported lower in our study. The percentage of *K. pneumoniae* isolates that produce ESBL varies by country. These proportion were 12% in the United States, 33% in Europe, 52% in Latin America and 28% in western pacific which may be due to difference in antibiotic

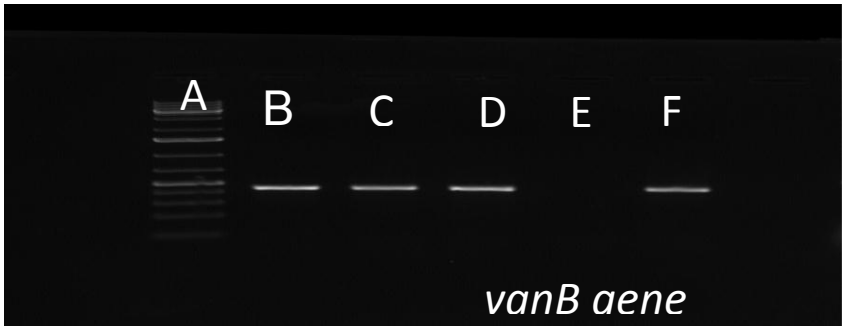
usage pattern and other behavioural differences accounting for disparity (Gharrah et al., 2017).

The number of vancomycin-resistant enterococci (VRE) isolated from cattle and food has increased dramatically (Hayes et al., 2003, Chan et al., 2008). Foodborne enterococci have not been identified as a direct cause of resistant enterococci in people, although they may offer a danger of resistance genes being transferred to human-adapted strains (Hayes et al., 2003; Werner et al., 2013). Vancomycin; a glycopeptide antimicrobial, is used to treat human infections in cases of β-lactam resistance; however, due to the emergence of VRE, vancomycin's therapeutic efficacy has been reduced (Mayhall et al., 1996; Harada et al., 2012). Therefore, in the current study resistance to vancomycin was targeted.

All phenotypically confirmed vancomycin resistant isolates were screened for *van*B and *van*C1 gene. Out of 15 isolates, 4(26.66%) carried *van*B gene and 3(20%) carried *van*C1 gene. One isolate carried both the genes.

Vancomycin resistance genes *van*A, *van*B, *van*C, *van*D, and *van*E are the most common. *van*A and *van*B are acquired gene clusters found largely in *E. faecalis* and *E. faecium*, whereas *van*C1 is intrinsically found in *E. gallinarum* (Arthur and Courvalin, 1993, Cetinkaya et al., 2000).In our study we could not standardize a protocol for *van*A gene detection, although it is predominant. The *van*A phenotype is defined by VRE that have the *van*A gene and are associated with a high level of inducible resistance to vancomycin as well as cross resistance to teicoplanin. *van*B and *van*C phenotypes, on the other hand, are frequently susceptible to teicoplanin and have variable and low levels of inducible resistance to vancomycin, respectively (Giraffa, 2002, Lee et al., 2004).

Osman et al., (2019) reported that *van*B and *van*C genes was detected in 25.5% and 33% of the enterococci isolates. In our study *van*B gene (Fig:11) was detected in 26% enterococci isolates but only 20% isolates carried *van*C1 gene.



Product size- 451 bp Lane A-1 Kb ladder Lane B- PCT Lane E- NCT
C, D and F- positive samples

Fig: 11 PCR for identification of *van*B gene

4.4 Phenotypic and genotypic correlation of ESBL

Out of the 14(60.86%) phenotypically ESBL negative isolates 5(21.73%) carried at least one of the ESBL gene (bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{DHA}). Whereas rest of the 9(39.13%) isolates were phenotypically as well as genotypically positive for ESBL. (Table 16 and 17)

Table 4.11: Positive ESBL isolates

	Phenotype	Genotype			
	ESBL	*bla*CTX-M	*bla*TEM	*bla*SHV	*bla*DHA
Positive	9	11	10	2	0
Negative	14	12	13	21	23

Table: 4.12 Phenotypic and genotypic correlation of isolates of *K. pneumoniae*

Isolate	**Housing system**	Type of sample	Phenotype	Genotype (ESBL)			
Isolate ID	Farm type	Source	ESBL	blaSHV	blaTEM	blaCTX-M	blaDHA
MOHF1	Deep Litter	Egg Shell	+ve	-ve	+ve	+ve	-ve
MOHF3	Deep Litter	Egg Shell	-ve	-ve	-ve	+ve	-ve
MOHF3	Deep Litter	Egg Shell	+ve	-ve	+ve	-ve	-ve
LDHF3	Deep Litter	Egg Shell	-ve	+ve	+ve	+ve	-ve
LDHF3	Deep Litter	Egg Shell	-ve	-ve	-ve	-ve	-ve
LDHF3	Deep Litter	Egg Shell	+ve	+ve	+ve	+ve	-ve
RUPF1	Deep Litter	Egg Shell	-ve	-ve	-ve	-ve	-ve
RUPF1	Deep Litter	Egg Shell	+ve	-ve	+ve	-ve	-ve
RUPF1	Deep Litter	Egg Shell	-ve	-ve	-ve	-ve	-ve
RUPF4	Deep Litter	Egg Shell	+ve	-ve	+ve	+ve	-ve
RUPF4	Deep Litter	Egg shell	+ve	-ve	-ve	+ve	-ve
MOHF1	Deep Litter	Enviroment	-ve	-ve	-ve	-ve	-ve
MOHF1	Deep Litter	Enviroment	-ve	-ve	+ve	+ve	-ve
LDH3	Deep Litter	Environment	-ve	-ve	-ve	-ve	-ve
KURO5	Cage System	Egg Shell	+ve	-ve	+ve	+ve	-ve
CSO10	Cage System	Egg Shell	-ve	-ve	-ve	-ve	-ve
KURO19	Cage System	Egg Shell	+ve	-ve	-ve	+ve	-ve
CSF3O12	Cage System	Egg Shell	-ve	-ve	-ve	-ve	-ve
CGO18	Cage System	Egg Shell	+ve	-ve	+ve	+ve	-ve
KURF2O1	Cage System	Egg Shell	-ve	-ve	-ve	-ve	-ve
CSF1O5	Cage System	Egg Shell	-ve	-ve	-ve	-ve	-ve
CSF2O14	Cage System	Environment	-ve	-ve	-ve	+ve	-ve
CSF1O20	Cage System	Environment	-ve	-ve	-ve	-ve	-ve

A moderate co-relation was observed between phenotypic and genotypic resistance of *K. pneumoniae* isolates from poultry origin. As there was 39.13% phenotypic resistance and 56.52% genotypic resistance towards ESBL. The observation for those isolates that were phenotypically negative (E-strip test) could be due to the lower sensitivity of phenotypic method. This may also be due to the reason that the genes were not expressed. Thus, the gene presence detected by PCR does not necessarily indicate its expression. (Bajpai et al., 2017)

We also identified isolates that were phenotypically resistant to an antibiotic but genotypically negative in this investigation. This could be due to the reason that several antibiotic resistant genes have been discovered to code for resistance to an antibiotic or set of antibiotics, but only a few were studied in this study.

Antibiotic resistance has become a threat for doctors, veterinarians, and other health care providers in their efforts to treat and prevent diseases caused by bacteria. These superbugs, are resurfacing in novel forms that are resistant to practically all antimicrobials used in clinical practise. Unfortunately, there aren't enough new treatments in the pipeline to keep up with the rise of drug-resistant bacterial illnesses (Effah et al., 2020). *K. pneumoniae* and *Enterococcus* spp. are two clinically important bacteria that have sparked widespread public health concern and therefore require immediate attention.

Chapter - V

SUMMARY AND CONCLUSION

Antibiotics have proven to be the best invention in animal and human health sector however, simultaneous emergenceof antimicrobial resistance (AMR) has somewhat undervalued this importance.Penicillin resistance was observed a year after its discovery by Alexander Fleming. AMR is a public health crisis of present and future generations,owing to the serious threat of medical complications and mortality. Researchers studying AMR have long been concerned about the role of food-producing animals, their products, and their environment in dissemination of AMR. Antibiotics are being used at an unprecedented rate in the animal health sector as growth promoters, for therapeutics, prophylaxis and for metaphylixis (Aarestup et al., 2005). The poultry industry is one such example where antibiotics are used regularily, which therefore makes poultry production system potential reservoir of antimicrobial resistant organism (Nisha, 2008).

K. pneumoniae is associated with majority of the human illnesses and is documented to be multidrug resistant (MDR) bacteria worldwide (Santajit & Indrawattana, 2016). *Enterococcus* are the second most common cause of nosocomial infections after *Staphylococcus* worldwide (Sakka et al., 2008).

K. pneumoniae and *Enterococcus*spp. are organisms of zoonotic potential. Risk of AMR in *K. pneumoniae* and *Enterococcus* spp. associated with eggs and poultry environment has not been investigated well.Keeping in mind the potential role of eggs in dissemination of AMR organisms, the current study was designed to study the occurrence of *K. pneumoniae* and *Enterococcus* spp. in eggs and poultry environment.

Hence,the isolation and identification of *K.pneumoniae* and *Enterococcus* spp. from eggs and poultry environment along with their antimicrobial resistance evaluation and antibiotic resistance genes was carried out. A total of 558 samples comprising of eggs (480), environment samples (72) and farm handler's hand swabs (6) from different districts of Punjab were collected. The samples were further processed for isolation and identification of *K. pneumoniae* and *Enterococcus* spp. Out of these 558, 23 (4.12%) samples were contaminated with*K. pneumoniae* and 136

(24.13%) samples were contaminated with*Enterococcus* spp. as confirmed by biochemical tests, PCR and MALDI-TOF analysis.

Antibiotic sensitivity test of the isolates was also performed.*K. pneumoniae* isolates showed high resistance to penicillin, ampicillin and amoxicillin antibiotics and the resistance %age was higher in *K. pneumoniae* isolates (92.7%) obtained from deep litter systemin comparison to the resistance of 72.77% in isolates from cage system. (Table:4.3) The isolates from system also had high resistance (78.57%) to third generation cephalosporin (cefotaxime) as compared to 44.4% resistance in isolates from cage system. The resistance to carbapenem antibiotic was also present and was nearly same in isolates both from deep litter as well as cage system. Resistance to other antibiotics such as doxycycline, ciprofloxacin, amikacin and gentamycin was recorded at 22.22%, 22.22%, 11.11% and 11.11%, respectively in isolates of cage system whereas, resistance to same antibiotics in deep litter system was 35.71%, 28.57%, 21.42% and 21.42% respectively. Resistance to co-trimoxazole was also present in *K. pneumoniae*isolates with 28.57% and 22.22% resistant isolates from deep litter and cage system, respectively.

Enterococcus spp. (*E. faecalis* and *E. faecium*) isolates showed high resistance to amikacin, tetracycline and erythromycin antibiotics and the resistance %age (77.94%)was higher in isolates obtained from deep litter system than in cage system (61%). The *Enterococcus* spp. isolates from deep litter system also had higher resistance to ampicillin (47.05%) as compared to 39.47% resistance in isolates from cage system. A higher resistance to vancomycin in isolates from deep litter farms (16.17%) was observed in comparison to the isolates from cage system farms (10.52%).The resistance to linezolid antibiotic was also present and was higher in isolates from deep litter (13.23%) than in cage system (7.89%).Resistance to other antibiotics such as kanamycin, ciprofloxacin, chloramphenicol and gentamycin was also seen at 58.28%, 57.35%, 50% and 50%, respectively in isolates of deep litter system whereas resistance to same antibiotics in isolates of cage system was observed at 44.73%, 42.10%, 42.10%, 36.84% respectively. Resistance to penicillin was also present in isolates with 61.76% and 52.63% resistance from deep litter and cage system, respectively.

Out of total 23 isolates of *K. pneumoniae,* 7(30.43%) and out of total 136 isolates of *Enterococcus* spp., 45(42.45%) were discovered to be multi-drug resistant.

Nine(39.13%) and 5(21.73%) of *K. pneumoniae*were positive for ESBL and KPC, respectively. Among *Enterococcus,*15 (11.02%) isolates were found to be resistant to vancomycin by broth dilution method.

Genotypically, 11(47.82%)*K. pneumoniae* isolates were positive for $bla_{\text{CTX-M}}$, 10(43.47%) for bla_{TEM} and 2(8.69%)for bla_{SHV} genes. None of the isolates was positive for bla_{DHA}.

Four (26.66%) *Enterococcus* isolates carried *van*B gene and 3(20%) carried *van*C1 gene. One isolate carried both the genes.

As a result of the current research, it can be inferred that:

- The overall occurrenceof *K. pneumoniae* and *Enterococcus* spp. in poultry farm (eggs, environment and hand swab samples) was 4.12% and 24.37% respectively.
- The eggs from deep litter system were found to becontaminated more with *K. pneumoniae*(4.58%) and *Enterococcus* spp. (25%) than the eggs from cage system.
- Seven (30.43%) isolates of *K. pneumoniae* and 45 (42.45%) isolates of *Enterococcus* spp. were discovered to be resistant to a variety of drugs.
- Presence of MDR organisms was higher in eggs and environment of deep littersystem.
- The odds of occurrence of *Enterococcus* spp. was 1.702 times higher in eggs of deep litter system in comparison to cage system.
- The odds of occurrence of *E. faecalis/E. faecium* was 1.684 times higher in eggs of deep litter system in comparison to cage system.
- The odds of occurrence of MDR *Enterococcus* was 4.296 times higher in eggs of deep litter system in comparison to cage system.
- The odds of occurrence of vancomycin resistance in *Enterococcus* spp. was 3.494 times higher in eggs of deep litter system in comparison to cage system.
- More number of isolates from eggs of deep litter system were found resistant to antibiotics reserved for human use from WHO critically important category.
- More number of *Enterococcus*isolates from deep litter system were found to be vancomycin resistant.

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